Biochimica et Biophysica Acta, 1111 (1992) 211–220

BBAMEM 75792

Influence of staphylococcal δ-toxin on the phosphatidylcholine headgroup as observed using ^{2}H-NMR

John R. Rydall and Peter M. Macdonald

Department of Chemistry and Erindale College, University of Toronto, Toronto, Ontario (Canada)

(Received 6 April 1992)

Key words: δ-Toxin; Phosphatidylcholine; NMR, ^{2}H-; NMR, ^{31}P-; Protein–lipid interaction

The interaction of the δ-toxin peptide isolated from *Staphylococcus aureus* with the headgroup region of lipid bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated using deuterium (^{2}H) and phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy. At relatively low peptide/lipid ratios (P/L < 0.10), all ^{2}H- and ^{31}P-NMR spectral lineshapes at 25°C were indicative of a single population of liquid-crystalline lipids in a bilayer arrangement. At these P/L ratios, δ-toxin had only marginal effects on the size of the quadrupole splitting measured from POPC labelled at either the α-methylene (POPC-α-d_2) or the β-methylene segment (POPC-β-d_2) of the choline headgroup and, similarly small effects on the magnitude of the chemical shift anisotropy (CSA) of the ^{31}P-NMR spectrum. With increasing amounts of δ-toxin (0.10 < P/L < 0.15) the size of the ^{2}H quadrupole splitting from POPC-α-d_2, as well as the magnitude of the ^{31}P-CSA, decreased progressively and rapidly. The quadrupole splitting from POPC-β-d_2, however, remained relatively unaffected. At yet higher levels of δ-toxin (P/L > 0.15), all ^{2}H- and ^{31}P-NMR spectra indicated the presence of multiple lipid populations experiencing varying degrees of increased conformational disordering. The spectral lineshapes of these apparently nonbilayer spectral components reverted to bilayer-type lineshapes upon lowering the measuring temperature to 5°C. At the utmost highest level of δ-toxin measured here (P/L = 0.20), all ^{2}H- and ^{31}P-NMR spectra consisted of a single, broad, apparently nonbilayer-type component, indicative of hindered but virtual isotropic motional averaging of the POPC headgroups. In this case no reversion to bilayer-type spectra could be obtained by decreasing the temperature. We could obtain no evidence that the conformation of the choline headgroup of POPC was responding to any specific influence of δ-toxin on bilayer surface electrostatics.

Introduction

Many peptides and proteins that interact with lipid bilayers adopt amphipathic α-helical secondary structures, in which the distribution of hydrophobic and hydrophilic amino-acid side-chains on the outer surface of the helix is polarized [1]. Many interesting 'surface-seeking' proteins have been identified as possessing amphipathic α-helical regions (including certain bacterial toxins, hormones, apolipoproteins) and particular interest has begun to focus on understanding more precisely the nature of their interactions with lipid bilayer surfaces.

The cytolytic peptide δ-toxin is one such 'surface-seeking' amphipathic peptide and is an ideal candidate for study. It is secreted from the gram positive bacterium *Staphylococcus aureus* and is highly surface active; lysing a wide variety of cells and organelles and binding with high affinity to phospholipids (for reviews, see Refs. 2–4). When bound to lipid micelles [5] or membranes [6], this 26-residue peptide adopts a secondary structure which approaches 80% α-helix content. From a practical perspective, this peptide can be produced and purified easily and rapidly in high yields [7–9].

^{31}P- and ^{2}H-nuclear magnetic resonance (NMR) spectroscopy are especially valuable tools for studying membrane-protein interactions. ^{31}P-NMR, for instance, offers the ability to detect changes in the overall macroscopic architecture of lipid aggregates, such as might be induced by the addition of peptides and other agents [10,11]. ^{2}H-NMR likewise offers certain distinct advantages. In particular, the ^{2}H-NMR spec-

Correspondence to: P.M. Macdonald, Department of Chemistry, University of Toronto, 80 St. George St., Toronto, Ontario, Canada, M5S 1A1.

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance; ^{2}H-NMR, deuterium nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TLC, thin-layer chromatography; CSA, chemical shift anisotropy; TPS, 2, 4, 6 triisopropylbenezenesulfonylchloride; P/L, peptide to lipid molar ratio.

trum of partially deuterated molecules is readily assigned, and the replacement of a proton by deuterium at specific sites in a molecule provides an essentially non-perturbing probe. More importantly, the ^{2}H-NMR spectrum contains information about molecular ordering, conformation, and dynamics. In addition ^{2}H-NMR of the phosphatidylcholine headgroup allows one to examine electrostatic interactions between charged species and a membrane surface [12]. The choline headgroup has been shown to respond to the presence of any charged species, including peptides and proteins, which binds to the membrane surface. Several peptides have been shown to influence surface electrostatics when examined using this technique, including melittin [13], M13 coat protein [14] and the synthetic bilayer-spanning peptide $K_2GL_nK_2A$ [15]. However, other peptides/proteins known to associate with lipid bilayers, such as the intrinsic membrane protein cytochrome-c oxidase, elicit no such electrostatic response, and can be shown to affect primarily lipid mobility [16].

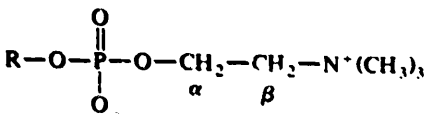
Although extensive studies have been carried out on δ-toxin/lipid systems, to date none have focused on the influence of δ-toxin on the polar headgroup region of phospholipids. Specific investigation of the effects of δ-toxin on the dynamics of lipid alkyl chains, using ^{2}H-NMR, and phospholipid headgroups, using ^{31}P-NMR, [17] reveal that in fluid membranes the lipid acyl chains become more disordered upon interaction with δ-toxin, while at temperatures near (but slightly above) the gel-liquid transition temperature the peptide had the opposite effect, i.e., promoting acyl-chain order.

In this report, we examine in detail the response of the choline headgroup in phosphatidylcholine membranes to the presence of δ-toxin by using ^{2}H-NMR and ^{31}P-NMR and we address specifically the question of whether or not δ-toxin influences surface electrostatics in a lipid bilayer.

Materials and Methods

Synthesis of headgroup-deuterated lipids

The following nomenclature is employed to indicate deuteron positions in the phosphocholine headgroup:



Non-deuterated lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Choline was selectively deuterated at the α- and β-segments by a combination of the methods described by Harbisson and Griffin [18] and Aloy and Rabaut [19]. POPC-α-d_2 and POPC-β-d_2 were prepared by coupling 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) with choline tetraphenylboron salt, selectively deuterated at either the α or β position, using 2,4,6-triisopropylbenzenesulfonylchloride (TPS) as the condensing agent [20]. The choline-deuterated phosphatidylcholines were purified by column chromatography on silica gel and CM-52 as described by Comfurius and Zwaal [21]. The purity of the synthesized lipids was evaluated by thin layer chromatography (TLC) and ^{1}H-NMR. All final products resulted in a single spot on an overloaded TLC plate, migrating with an R_f identical to authentic POPC. The ^{1}H-NMR spectra were identical to that of non-deuterated POPC, except for the changes expected in the presence of α- or β-deuterons.

δ-Toxin preparation and purification

Staphylococcal δ-toxin was prepared from *Staphylococcus aureus* strain NCTC 10345 grown on yeast extract diffusate medium [22]. The δ-toxin was purified by the solvent transfer method [9] followed by precipitation and washing with 1 M TCA and lyophilization [23]. The purity of the δ-toxin preparation was assessed by a variety of methods. SDS-PAGE [24,25] on 12.5% gels containing 7 M urea revealed a single broad band with an apparent molecular mass of slightly less than approx. 3 kDa (δ-toxin, 2977 Da). On thin-layer chromatography (TLC) the peptide migrated as a single spot (R_f 0.72), using a solvent system composed of n-butanol/water/acetic acid (45:30:25 v/v/v) [26]. Amino-acid composition and primary sequence analyses yielded results consistent with authentic δ-toxin [27]. Fluorescence measurements (25°C) using an SLM model 4800 fluorometer (excitation wavelength 280 nm; emission maximum 333 nm) and ultraviolet (UV) absorption spectra ($E_{280}^{0.1\%} = 1.14$) were both characteristic of δ-toxin. Finally, the biological activity of the peptide was confirmed using a haemolytic assay on human red blood cells [28].

NMR measurements

^{2}H-NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer (equipped with a temperature control unit) operating at 45.98 MHz by employing the quadrupole echo technique [29] and by using quadrature detection with complete phase cycling of the pulse pairs [30]. Specifics regarding the 90° pulse length (2 μs), the interpulse delays (40 μs), the spectral width (100 kHz), the recycling delay (250 ms), the data size (2K), and the total number of acquisitions (12000) are those noted in parentheses. ^{31}P-NMR spectra were recorded at 121.25 MHz by using a Hahn echo-pulse sequence with phase cycling of the pulses and proton decoupling as described by Rance and Byrd [31]. Specifics regarding the 90° pulse length (4.75 μs), the interpulse delay (40 μs), the spectral width (100 kHz), the recycling delay (1 s), and the number of acquisitions (6400) are again noted in parentheses. The

temperature was controlled to within 0.1°C. In variable temperature studies, between each interval the temperature was varied at a rate of about 0.25°C per min, and the samples were equilibrated at each temperature for at least 30 min.

Sample preparation

Samples were prepared for NMR spectroscopy as follows. A volume of chloroform containing 13 μmol of deuterated POPC was dried under a stream of nitrogen. A volume of chloroform : methanol (2 : 1 v/v) containing the desired amount of δ-toxin was added to the dried POPC and the mixed POPC/δ-toxin sample was dried under a stream of nitrogen, with any remaining solvents being removed under high vacuum. The lipid/δ-toxin mixtures were dispersed in 400 μl of aqueous buffer (10 mM Hepes, 150 mM NaCl, (pH 7.4)). In order to ensure complete equilibration of the peptide and lipids, the resulting multilamellar vesicles were subjected to repeated cycles of freeze-thawing as described by Macdonald and Seelig [32]. The equilibrated suspension was then centrifuged at $13000 \times g$ for 30 min. The resulting pellet was taken for NMR measurement, while the supernatant was assayed for δ-toxin content using UV and fluorescence measurements.

Results

In order to determine the extent to which the added δ-toxin is incorporated into a lipid bilayer structure, we quantified the level of free peptide remaining in solution after an extensive period of equilibration with POPC. Both fluorescence and UV absorption spectroscopy were employed for this purpose and yielded essentially identical results.

By measuring peak heights and areas, and comparing with a standard curve, the concentration of unbound peptide was calculated for various peptide to lipid (P/L) molar ratios. The results are plotted in Fig. 1 in the form of a binding isotherm. They demonstrate that over the majority of the concentration region examined in our studies, virtually all the δ-toxin is associated with membrane lipids. Only at extremely high P/L ratios (i.e., P/L > 0.15) are significant levels of free peptide detected. Even so, the amount of δ-toxin remaining in solution never exceeds 5% of the initial concentration. We can be reasonably certain, therefore, that essentially all the peptide added is incorporated into the resulting multilamellar vesicles.

Fig. 2 shows the effects of increasing δ-toxin concentration on the ^{31}P- and ^{2}H-NMR spectra from POPC membranes at 25°C. The ^{31}P-NMR spectrum at the top of Fig. 2A was obtained in the absence of δ-toxin and is typical of fluid phospholipids in a bilayer arrangement [10,11]. The other ^{31}P-NMR spectra in

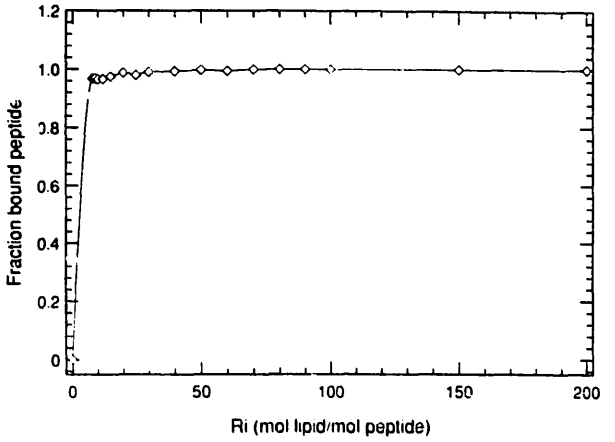


Fig. 1. δ-Toxin binding isotherm as measured by the variation of the fraction bound peptide with increasing lipid-to-peptide ratio. The fraction of bound peptide was determined by both fluorescence and ultraviolet absorption spectroscopy.

Fig. 2A demonstrate that, except at the highest levels of peptide, the membrane lipids retain an overall bilayer configuration. At extremely high levels of peptide (i.e., P/L > 0.15), the ^{31}P-NMR spectra indicate the presence of several lipid populations, one bilayer and others experiencing varying degrees of conformational disordering. By progressively increasing the peptide levels (above P/L 0.15) the relative ratio of the apparently nonbilayer to bilayer type components in the spectra shifts until (at P/L 0.2) only a single, broad isotropic peak ($\Delta\nu_{1/2}$ 10 ppm) is evident. The overall effect of increasing peptide concentration on the ^{31}P chemical-shift anisotropy (CSA) is to decrease the absolute magnitude of the CSA envelope relative to pure POPC.

Fig. 2B and C show the influence of δ-toxin on the ^{2}H-NMR spectra from POPC-α-d_2 and POPC-β-d_2, respectively. Except for extremely high peptide concentrations (P/L > 0.15), all ^{2}H-NMR spectra are typical Pake doublets, characteristic of fluid lipids in a bilayer arrangement [10]. The quadrupole splitting ($\Delta\nu_Q$) corresponds to the separation (in Hz) between the two maxima in the ^{2}H-NMR spectrum. The ^{2}H-NMR spectra at high peptide concentrations show effects analogous to those observed in the ^{31}P-NMR spectra, in that both isotropically averaging and bilayer type components are evident. At extremely high levels of peptide complete collapse of the quadrupole splitting results, culminating in a broad isotropic peak with widths-at-half-height of 0.54 kHz and 2.71 kHz for POPC-α-d_2 and POPC-β-d_2, respectively (at P/L 0.2). Addition of δ-toxin progressively decreases the quadrupole splittings arising from both POPC-α-d_2 and POPC-β-d_2.

Fig. 3 illustrates in detail the influence of δ-toxin on the ^{31}P-NMR CSA and on the quadrupole splittings from POPC-α-d_2 and POPC-β-d_2 at 25°C. The addition of δ-toxin causes a general decrease in the abso-

lute magnitude of the ^{31}P-NMR CSA relative to pure POPC. Specifically this response can be divided into two distinct linear regions, corresponding to low- (P/L < 0.10) and high- (P/L > 0.10) peptide concentrations. In the low-concentration region, δ-toxin has only a marginal effect on the magnitude of the CSA. However, at high concentrations, a progressive and pronounced decrease in the magnitude of the CSA is observed. As noted above, at extreme peptide concentrations, the ^{31}P-NMR spectrum collapses to a broad,

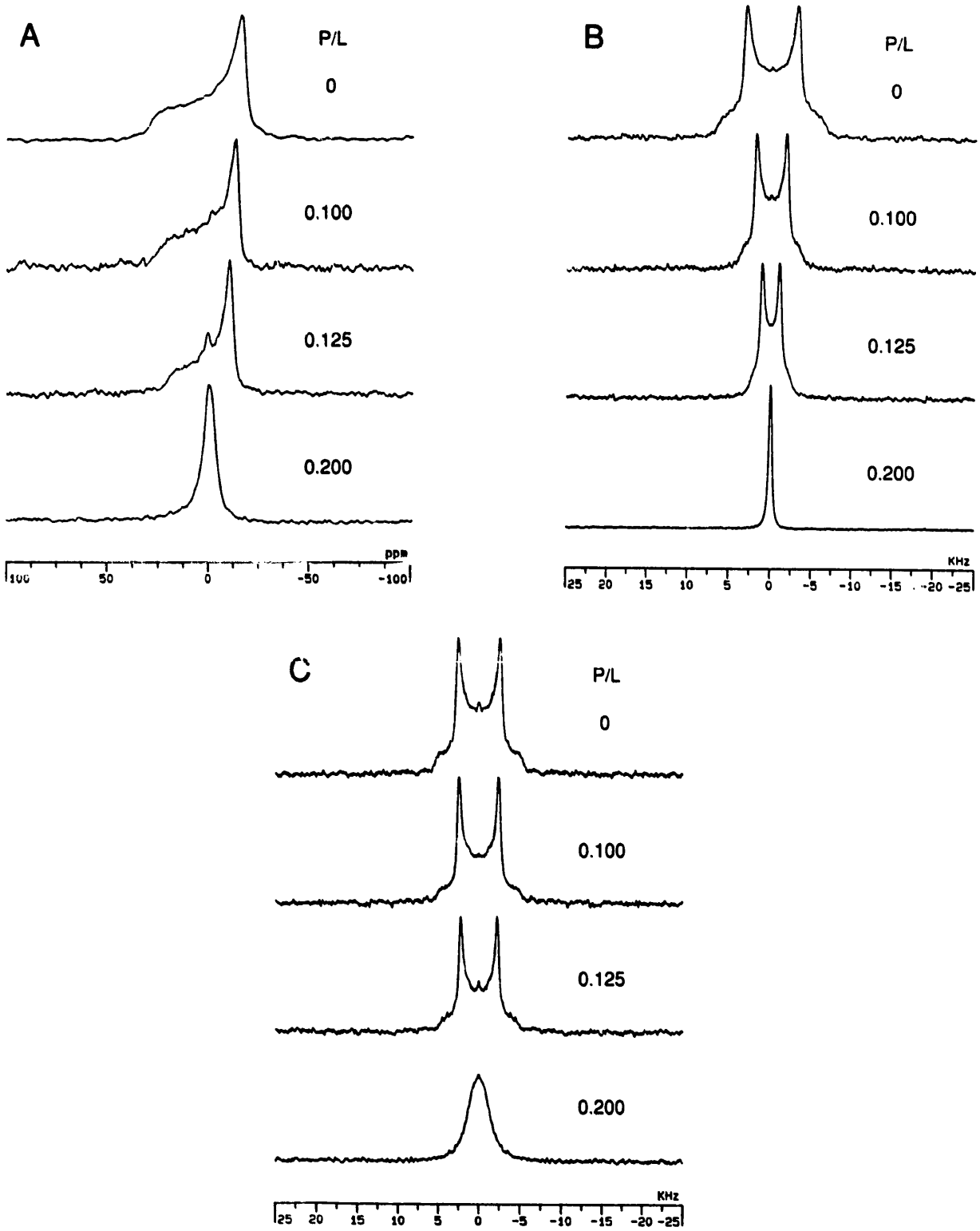


Fig. 2. Representative ^{31}P (A) and ^{2}H (B and C) NMR spectra in the presence of various concentrations of δ-toxin at 25°C. In (A), ^{31}P-NMR spectra are shown as obtained from POPC membranes containing the indicated peptide/lipid molar ratios. In (B), ^{2}H-NMR spectra from POPC-α-d_2 are shown and in (C), ^{2}H-NMR spectra for POPC-β-d_2 are shown, again, for membranes containing the indicated P/L ratios.

isotropic resonance line. The ^{2}H-NMR quadrupole splitting from POPC-β-d_2 decreases gradually as peptide levels are increased. The response of the quadrupole splitting from POPC-β-d_2 to δ-toxin binding is very weak and approximately linear over the entire concentration range examined. In general, the effect of δ-toxin on POPC-α-d_2 is to decrease the ^{2}H-NMR quadrupole splitting. However, quantitatively, several significant differences are seen. POPC-α-d_2 is much more sensitive to the presence of peptide and shows substantially larger changes in quadrupole splitting relative to POPC-β-d_2. Also, the overall dependence of the POPC-α-d_2 quadrupole splitting on peptide concentration is more complex than that observed for the POPC-β-d_2. As the peptide concentration increases, the quadrupole splitting exhibits three different regions of linear response. At low levels of peptide, a fairly steep drop in the quadrupole splitting occurs. The response then levels off, leading to a plateau region where higher amounts of peptide have small effects on the quadrupole splitting. Further addition of peptide causes the quadrupole splitting to decrease rapidly once more, in a manner similar to that seen at low concentrations. The ^{31}P-NMR and ^{2}H-NMR spectra and the values of the ^{31}P CSA and the ^{2}H quadrupole splittings measured here demonstrate that δ-toxin has an overall disordering effect on the entire headgroup region of phosphatidylcholine bilayers.

A specific response of the phosphatidylcholine headgroup to surface charges is characterized by a counter-directional change in the size of the ^{2}H-NMR quadrupole splittings measured from deuterons located at the α- vs. the β-methylene segment of the choline headgroup. We can obtain no NMR evidence indicating any such effect of δ-toxin on surface electrostatics

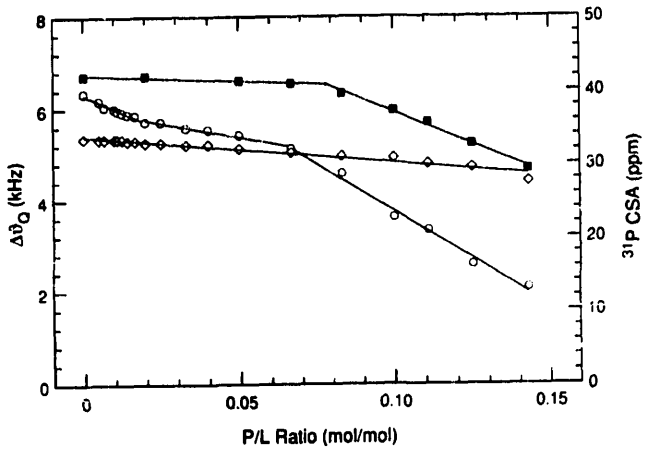


Fig. 3. Variation of the ^{31}P-NMR chemical shift anisotropy (CSA) (squares) and ^{2}H-NMR quadrupole splittings from POPC-α-d_2 (circles) and POPC-β-d_2 (diamonds) at 25°C as a function of the peptide-to-lipid molar ratio.

at 25°C. It is possible that the large disordering effect of δ-toxin is masking a smaller response of the ^{2}H-NMR quadrupole splittings to changes in surface electrostatics due to the presence of the peptide. Therefore, in an attempt to reduce the disordering effect and thereby unmask any electrostatic response to δ-toxin, we investigated the effect of decreasing temperature on the ^{31}P-NMR and the ^{2}H-NMR spectra. The ^{31}P-NMR spectra in Fig. 4A (P/L 0.167) appear to consist of multiple components, each of which corresponds to a different degree of conformational ordering. As the temperature is decreased, the ^{31}P-NMR spectral lineshape alters and approaches that characteristic of a bilayer arrangement of lipids. However, the magnitude of the CSA at 5°C is still far smaller than that of 100% POPC (i.e., see Fig. 2A). Fig. 4B and C show typical ^{2}H-NMR spectra of POPC-α-d_2 for both low (P/L 0.025) and high (P/L 0.167) peptide concentrations, respectively. In Fig. 4B, it is clear that, at low levels of peptide, decreasing temperature has no major impact on the overall shape of the ^{2}H-NMR spectrum. At high peptide concentration (Fig. 4C) at 25°C the ^{2}H-NMR spectrum of POPC-α-d_2 is quite narrow and appears to consist of multiple components, each of which corresponds to a different degree of conformational ordering. With decreasing temperature the overall contribution of the narrow spectral components decreases, such that a lineshape characteristic of bilayer lipids emerges at 5°C. When the temperature dependence of the ^{31}P and ^{2}H spectral lineshapes is investigated for the highest peptide concentration (P/L 0.20), it is not possible to convert the broad, featureless lineshape back to a bilayer type lineshape by lowering the temperature.

As the temperature decreases, the quadrupole splittings from both POPC-α-d_2 and POPC-β-d_2 increase. Fig. 5A and B show the temperature dependence for various peptide/lipid ratios. For pure POPC, decreasing the temperature causes the quadrupole splittings from both POPC-β-d_2 and POPC-α-d_2 to increase. However, the quadrupole splitting from POPC-β-d_2 exhibits a much greater temperature dependence than POPC-α-d_2, as has been reported by others [33]. In general, for POPC-α-d_2 (Fig. 5A), increasing the amount of peptide has a minimal effect on the weak temperature-dependence of the quadrupole splitting. This is obvious from the lines plotted in Fig. 5A, where the slopes are all very small and roughly equal. Decreasing the temperature has a greater effect on the POPC-β-d_2 quadrupole splitting. Fig. 5B shows that as peptide levels rise, temperature dependence of the POPC-β-d_2 quadrupole splitting is reduced. From these studies of the temperature-dependence of the quadrupole splittings it is clear that, while decreasing temperature can moderate the overall disordering effect of δ-toxin on the POPC headgroup, no response of the headgroup which can be attributed to changes in

the surface electrostatics is revealed by reducing the temperature, for any level of δ-toxin.

Shown in Fig. 6 is the relationship between the α- and β-^{2}H-NMR quadrupole splittings. In all cases investigated to date, the slopes of such α-β correlation plots are negative for situations in which an agent under investigation influences bilayer surface electrostatics. The essential features of this correlation plot for the case of δ-toxin are that the quadrupole splittings vary, at best, in an approximately linear fashion across the entire concentration range, but that the slope of the line is positive and quite small.

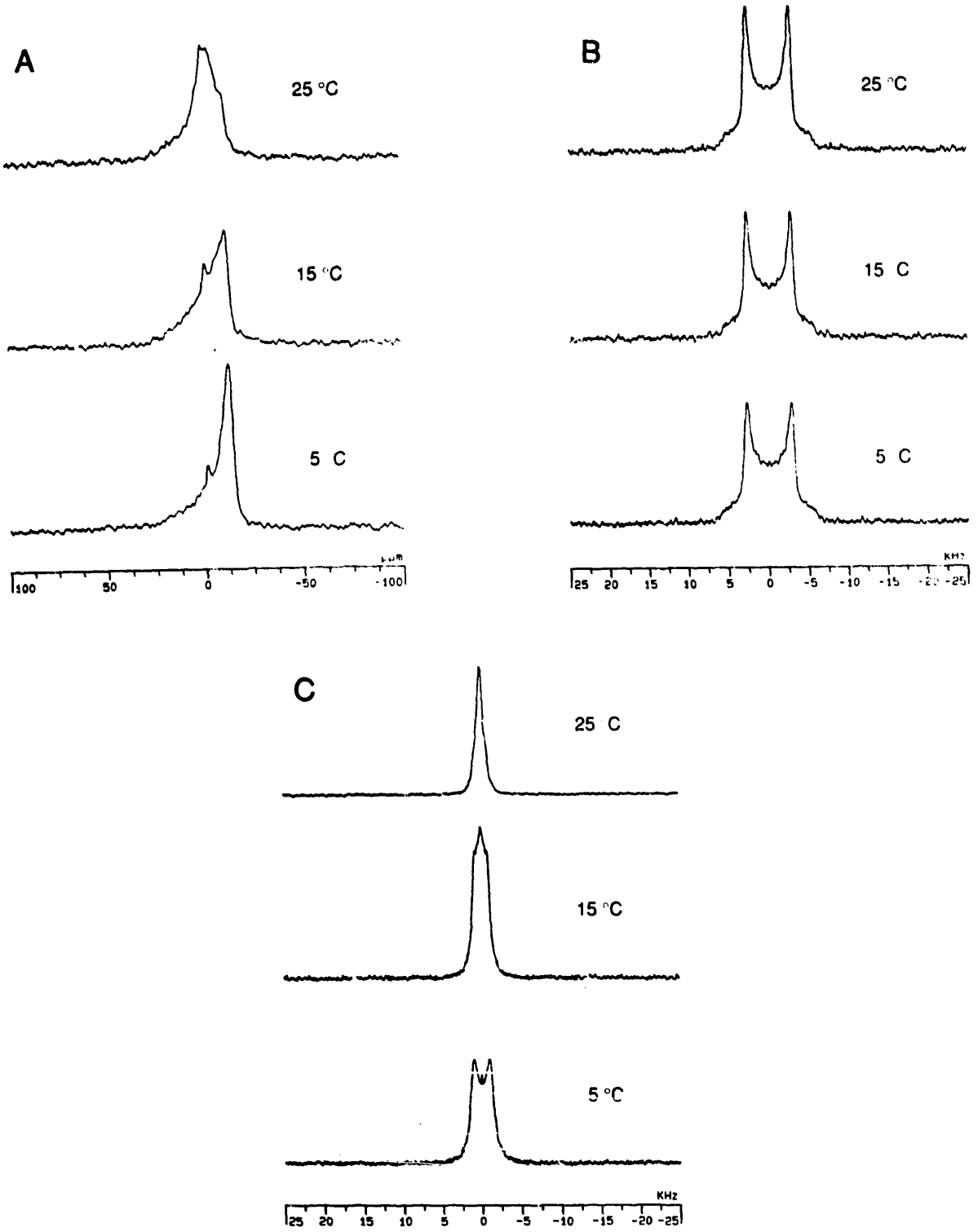


Fig. 4 Temperature-dependence of the ^{31}P-NMR (A) and ^{2}H-NMR (B and C) spectra of POPC in the presence of δ-toxin. In (A), ^{31}P-NMR spectra of POPC membranes in the presence of δ-toxin (P/L 0.167) are shown. In (B and C), ^{2}H-NMR spectra from POPC-α-d_2 at low (B) (P/L 0.04) and high (C) (P/L 0.167) peptide concentrations.

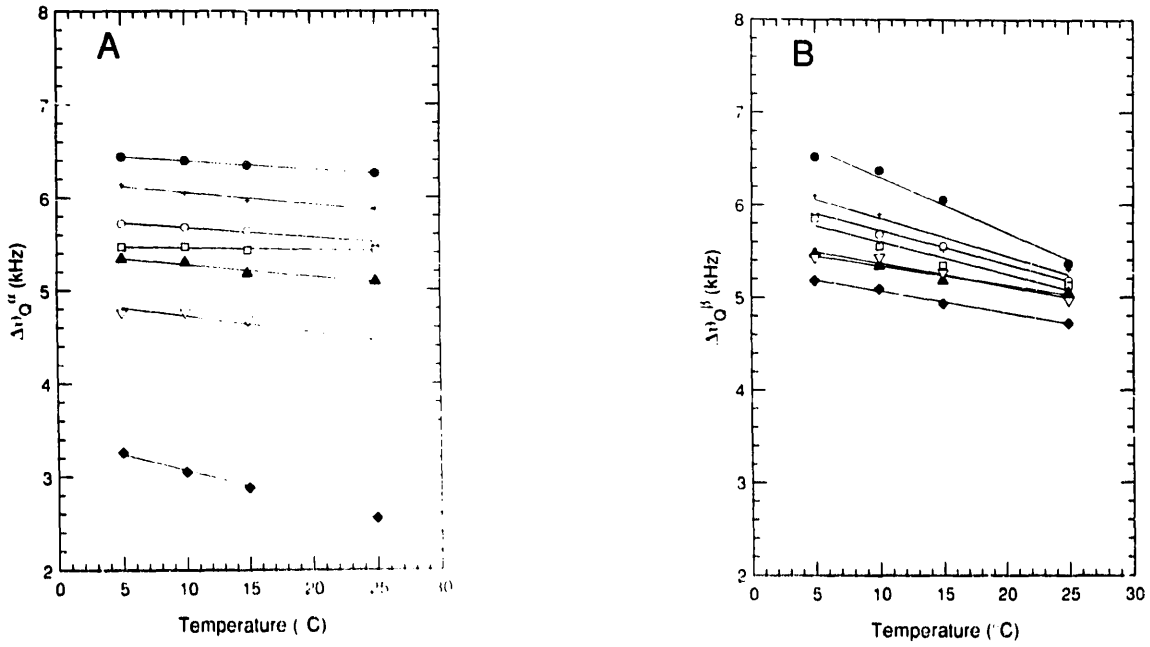


Fig. 5. Relationship between the ^{2}H-NMR quadrupole splitting and temperature at various peptide concentrations. In (A), temperature-dependence of the POPC-α-d_2 quadrupole splittings for increasing peptide/lipid ratios; 0 (●), 0.017 (+), 0.04 (○), 0.05 (□), 0.067 (▲), 0.083 (△), and 0.125 (◆) are shown. In (B), temperature-dependence of the POPC-β-d_2 quadrupole splittings for increasing peptide/lipid ratios; 0 (●), 0.017 (+), 0.04 (○), 0.05 (□), 0.067 (▲), 0.083 (△), and 0.125 (◆).

Discussion

The amino-acid sequence of *Staphylococcus aureus* δ-toxin is shown in Fig. 7. Since the amino-terminus is N-formylated, at physiological pH the peptide carries an overall neutral charge. Note that in this regard δ-toxin differs markedly from other surface-seeking peptides such as, for example, melittin which bears a net +5 positive charges at physiological pH. It appears that δ-toxin adopts a predominantly α-helical secondary structure upon associating with membrane lipids [34]. In addition, it is evident that an α-helical structure adopted by δ-toxin would be highly amphipathic. An amphipathic helix has a polarized distribution of hydrophilic and hydrophobic amino-acid side chains on its outer surface. A quantitative comparison of the degree of amphipathicity of different helical segments is obtained by calculating the so-called hydrophobic moment introduced by Eisenberg et al. [35]. The greater the hydrophobic moment, the greater the amphipathicity or polarization of hydrophobic and hydrophilic side-chain distribution about the helical axis. Using the procedure of Eisenberg et al. [35], a mean hydrophobic moment of 9.87 is obtained for δ-toxin, which may be compared with the value of 5.84 calculated for melittin [34]. Thus, δ-toxin may be considered to possess a highly polar helical face and a highly nonpolar helical face. Upon closer inspection of the distribution of charged residues, one notes that the amino-terminal half of the peptide is predominantly negatively-charged

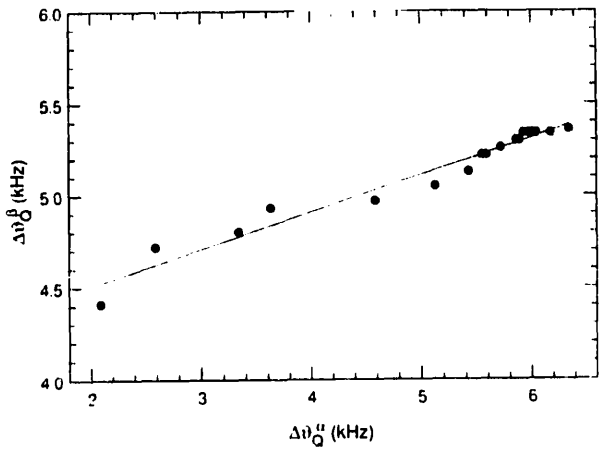


Fig. 6. Correlation plot of the quadrupole splittings from POPC-α-d_2 and POPC-β-d_2. The quadrupole splittings from the two deuteron positions, obtained under identical conditions of peptide concentration, are plotted with respect to one another. The line of best fit is described by the equation $\Delta\nu_Q^{\beta} = 0.202\Delta\nu_Q^{\alpha} + 4.1$ (r^2 0.96).

1 10 15 26
fMet-Ala-Gln-Asp-Ile-Ile-Ser-Thr-Ile-Gly-Asp-Leu-Val-Lys-Trp-Ile-Ile-Asp-Thr-Val-Asn-Lys-Phe Thr Lys-Lys

Fig. 7. Amino-acid sequence of Staphylococcal δ-toxin.

while the carboxy-terminal half is predominantly positively-charged.

Although δ-toxin has been studied extensively using a variety of physical techniques, controversy still surrounds the fundamental question of how the peptide disrupts the integrity of lipid bilayers and cell membranes. Two models have been proposed to account for its cytolytic effects. In one case, it has been proposed that δ-toxin remains localized at the surface of the lipid bilayer, but penetrates to some degree into the bilayer proper where it acts somewhat like a wedge, creating defects in the lipid bilayer structure [36]. This model is similar to that proposed for the mechanism of action of melittin. Others have proposed that δ-toxin inserts into and spans the lipid bilayer [2,37–39]. Aggregation of such membrane spanning units could then produce a transmembrane pore which leaks the cell contents.

^{2}H-NMR and ^{31}P-NMR of phospholipid headgroups can provide insight into the nature of associations occurring at the membrane surface and their effects on lipid headgroup conformation and dynamics. For membrane proteins and peptides these usually fall into one of two categories: effects on lipid ordering and dynamics, or electrostatic effects on headgroup conformation. ^{2}H-NMR is particularly useful for detecting electrostatic effects at the membrane surface, since it is well-established that the headgroup of phosphatidylcholine senses and responds to the presence of membrane surface charges [12]. In this respect phosphatidylcholine is said to behave like a 'molecular voltmeter'. The major feature of this response is a counter-directional change in the size of the ^{2}H-NMR quadrupole splittings from deuterons located at the α- vs. the β-methylene segment of the choline group. This has been taken as indicating that the choline headgroup undergoes a concerted conformational change in response to surface charges. Roux et al. [15] have presented a model of this conformational change wherein the headgroup experiences a torque as the dipole moment of the phosphocholine moiety seeks to align itself with an electrical field emanating outwards from the bilayer surface. Macdonald et al. [40] have shown that such a 'choline tilt' model can reproduce all of the essential features of the dependence of the ^{2}H-NMR quadrupole splittings on both positive and negative surface charges.

The 'molecular voltmeter' response is produced by a number of membrane-associating proteins and peptides. These include melittin [13,41–43], which is a 26 amino-acid peptide bearing 5 positive charges located near its amino-terminus and which is believed to remain located at the membrane surface. Another example is the synthetic cationic membrane-spanning peptide studied by Roux et al. [15] which, by virtue of the two lysine residues located at both ends of a hydrophobic central sequence of 20 leucines, induces a 'molecular voltmeter' response indicative of a positive surface charge density. Yet another example is the case of cyclic somatostatin [44] which is a predominantly hydrophobic, cyclical peptide of only 8 amino acids in length, one of which is lysine. Cyclic somatostatin intercalates readily into lipid bilayers and induces a cationic 'molecular voltmeter' response, indicating that the lysine residue is located at the membrane surface.

The ^{2}H-NMR results obtained here clearly indicate that δ-toxin fails to induce any such 'molecular voltmeter', response. In particular, the counter directional change in the value of the quadrupole splittings from POPC-α-d_2 vs. POPC-β-d_2 characteristic of the unique conformational change undergone by the choline headgroup in response to surface charge is absent. Rather, δ-toxin induces a decrease in the quadrupole splittings from both POPC-α-d_2 and POPC-β-d_2, as well as the CSA measured from ^{31}P-NMR spectra. These effects can only be interpreted in terms of a generalized disordering of the phospholipid headgroup region in the presence of δ-toxin. There are several possible reasons for this lack of electrostatic response to δ-toxin. First, the peptide itself bears no net charge. This does not in itself preclude an electrostatic response because, as noted above, the peptide contains many positive and negative charges and their distribution along the length of the helix is highly asymmetric. Second, aggregation of the peptide into membrane-spanning dimers, or higher oligomers, could occur in a head-to-tail fashion in order to maximize formation of salt bridges. While it is not known whether an anti-parallel or a parallel bundling of helices is favoured by δ-toxin, anti-parallel aggregation would lead to elimination even of the asymmetry of charge distribution. Third, any membrane spanning oligomer must segregate polar residues towards the interior of the aggregate, away from exposure to the hydrophobic acyl chains of the membrane lipids. Hence, it is unlikely that in such a state any uncancelled charges would ever lie near the membrane surface, proximal to the phospholipid headgroups.

The response of the ^{2}H- and ^{31}P-NMR from the phospholipid headgroup of POPC upon interaction with δ-toxin indicates a disordering of the choline headgroup, at all temperatures examined. We are by no means the first to observe this type of disordering by δ-toxin, Dufourc and co-workers [6] reported similar results for acyl-chain deuterated DMPC systems that were well above the lipid gel to liquid-crystalline phase transition temperature (T_c). However, at temperatures near, but sightly above the T_c, they report that δ-toxin preferentially orders the acyl chains. We have found no evidence for any preferential ordering of the choline headgroup by δ-toxin at any temperature above the lipid T_c (−5°C for POPC). Our ^{31}P- NMR results were similar to those of Dufourc et al. [6], indicating lipid

headgroup perturbations at all temperatures. Therefore, it would appear that δ-toxin has differential ordering effects upon the acyl chain vs. the headgroup region at temperatures near the lipid T_c.

It is interesting to consider the possible origins of the distinctly smaller effect of δ-toxin on the quadrupole splitting from POPC-β-d_2 vs. the quadrupole splitting from POPC-α-d_2 or the ^{31}P CSA. One reasonable interpretation is that this difference reflects the details of the orientation of δ-toxin with respect to the plane of the membrane. A surface orientation, with the long helical axis of the peptide arranged parallel to the membrane surface, would be expected to perturb all positions in the phosphatidylcholine headgroup more or less equally. A transmembrane orientation, with the long helical axis of the peptide arranged perpendicular to the membrane surface, might not extend far enough beyond the hydrophobic core of the membrane to perturb all lipid headgroup positions equally. Thus, this line of argument appears to support a transbilayer orientation for δ-toxin. Another equally cogent interpretation is that this difference in the response of POPC-β-d_2 vs. POPC-α-d_2 merely reflects a relative insensitivity of the β-methylene segment to conformational perturbations. For instance, the conformational freedom of the β-methylene segment is expected to be greater than that of the α-methylene segment, hence, the far greater temperature-dependence of the POPC-β-d_2 quadrupole splitting. Since δ-toxin has an overall disordering effect, its impact will be felt most at those positions having greater conformational order, i.e., the α-methylene segment. We can only conclude that the response of the choline headgroup to δ-toxin is not definitive with respect to the orientation of the peptide in or on the membrane.

Numerous membrane proteins have been shown to disorder and immobilize bilayer lipids, including gramicidin A [45] and cytochrome oxidase [46]. Pink et al. [47] have proposed a model to account for the observed disordering induced by these proteins. The model is based upon evaluating the number and type of lipid-protein interactions in the system. The essential assumptions are that a lipid is only slightly disordered when in contact with only one protein molecule, but is highly disordered when trapped between two or three protein molecules.

At low P/L ratios, two populations of lipids will predominate, bulk lipids and lipids in contact with one peptide molecule. The equilibrium exchange rate between these two populations will be fast on the NMR time-scale (i.e., less than 10^{-5}–10^{-6} s). This is consistent with our observation of only a single ^{2}H-NMR quadrupole splitting or ^{31}P-NMR CSA in the corresponding spectra for low amounts of δ-toxin. We observe only a gradual increase in the disorder at low P/L ratios, since on average the lipids are in contact with only a single peptide molecule. A lipid in contact with only a single peptide likely exhibits only a small disordering effect because it remains in contact, simultaneously, with other lipids, which tends to maintain the conformational order.

At higher P/L ratios, there are no longer enough lipids to completely surround all the peptides and an increasing number of lipids are trapped between two or three peptide molecules. Again, essentially only two lipid populations exist, consisting of lipids adjacent to one peptide, and those adjacent to two or three peptides. The lipid populations in this regime are still in fast exchange and the NMR spectra consist of a single spectral component. The rapid decrease in the quadrupole splittings and CSA arises due to the increasing population of lipids which are trapped between two or three peptide molecules. A lipid in contact with more than one peptide is more highly disordered, because there are fewer lipid-to-lipid contacts available to alleviate the disordering effects of the close proximity to a peptide molecule.

At extremely high peptide concentrations (i.e., P/L > 0.167), all the lipids are in contact with two or three peptide molecules and are essentially devoid of lipid-to-lipid contacts. At this point, the peptide spacing dictates the packing in the bilayer, resulting in defects and gaps which are large relative to the size of a lipid molecule. The resulting conformational disorder gives rise to spectra characteristic of non-bilayer structures. The exchange rate between different populations of lipids is slow on the NMR time-scale and explains the multiple components seen in the ^{2}H- and ^{31}P-NMR spectra in Fig. 4 A and C (where P/L = 0.167). Overall the effect of decreasing temperature is to order the local environment of the lipid. By decreasing the temperature, the difference in the degree of disorder between the lipid populations is reduced. Hence, the trend towards a common, more-ordered environment with decreasing temperature which yields an overall bilayer type ^{2}H- and ^{31}P-NMR spectrum. Note that this observation allows us to exclude the formation of small vesicles as a cause for the collapse of the spectrum at high peptide concentrations, since lowering the temperature should merely broaden the isotropic resonance line from a small vesicle, rather than causing a return to a bilayer-type spectrum. No such firm statement can be made, however, regarding the highest peptide concentration (P/L 0.20).

The model of Pink et al. [47] allows for a quantitative fit to experimental quadrupole splittings using an evaluation of the statistical variation of lipid populations accompanying changes in the P/L ratio. An additional complication for the case of δ-toxin, not specifically considered by Pink et al. [47], is that of possible variations in the oligomeric state assumed by a particular protein. For example, it is likely that there exists a

distribution of states of δ-toxin aggregation ranging from dimers through hexamers to, possibly, even higher aggregation states and this distribution will change as a function of the P/L ratio [48]. The details are presently unknown. While it is tempting to endeavour to gain information concerning such questions by employing simulation models which incorporate the possibility of a distribution of oligomeric states, the uncertainty surrounding any interpretations would be prohibitory without independent evaluation of the oligomeric states of the peptide. Clearly, what is required to address the outstanding issues of the orientation of δ-toxin with respect to the plane of the membrane and its state of aggregation upon association with the membrane, is direct examination of the peptide itself using, for example, solid-state NMR techniques.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada. We wish to thank Dr. M. Blum of the Department of Biochemistry, University of Toronto, for providing the amino-acid composition and sequence analyses.

References

1 Kaiser, E.T. and Kézdy, F.J. (1983) Proc. Natl. Acad. Sci. USA 80, 1137–1143.
2 Bernheimer, A.W. and Rudy, B. (1986) Biochim. Biophys. Acta 864, 123–141.
3 Freer, J.H. and Arbuthnott, J.P. (1982) Pharm. Ther. 19, 55–106.
4 Thelestam, M. (1983) in Staphylococci and Staphylococcal Infections (Easman, C.S. and Easman, C.A. eds.), Vol. 2, pp. 705–744, Academic Press, London.
5 Lee, K.H., Fitton, J.E. and Wüthrich, K. (1987) Biochim. Biophys. Acta 911, 143–153.
6 Dufourc, E.J., Dufourcq, J., Birkbeck, T.H. and Freer, J.H. (1990) Eur. J. Biochem. 187, 581–587.
7 Kreger, A.S., Kwang-Shin, K., Zaboretsky, F. and Bernheimer, A.W. (1971) Infect. Immunol. 3, 449–465.
8 Kantor, H.S., Temples, B. and Shaw, W.V. (1972) Arch. Biochem. Biophys. 151, 142–156.
9 Heatley, N.G. (1972) J. Gen. Microbiol. 69, 269–278.
10 Seelig, J. (1977) Q. Rev. Biophys. 10, 353–418.
11 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 599, 3399–3406.
12 Seelig, J., Macdonald, P.M. and Scherer, P.G. (1987) Biochemistry 26, 7535–7541.
13 Kuchinka, E. and Seelig, J. (1989) Biochemistry 28, 4216–4221.
14 Sanders, J.C., Poile, T.W. Spruijt, R.B., Van Nuland, N.A.V., Watts, A. and Hemminga, M.A.(1991) Biochim. Biophys. Acta 1066, 102–108.
15 Roux, M., Neumann, J., Hodges, R.S., Devaux, P.F. and Bloom, M. (1989) Biochemistry 28, 2313–2321.
16 Tamm, L.K. and Seelig, J. (1983) Biochemistry 22, 1474–1483.
17 Dufourc, E.J., Bonmatin, J. and Dufourcq, J. (1989) Biochimie 71, 117–123.
18 Harbison, G.S. and Griffin, R.G. (1984) J. Lipid Res. 25, 1140–1142.
19 Aloy, M.M. and Rabaut, C. (1913) Bull. Soc. Chim. France 13, 457–460.
20 Aneja, R., Chada, J.S. and Davies, A.P. (1970) Biochim. Biophys. Acta 218, 102–111
21 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
22 Bernheimer, A.W. and Schwartz, L.L. (1963) J. Gen. Microbiol. 30, 455–468.
23 Bhakoo, M., Birkbeck, T.H. and Freer, J.H. (1982) Biochemistry 21, 6819–6883.
24 Laemmli, U.K. (1970) Nature 227, 680–685.
25 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477.
26 Smith, G.M. and Shaw, W.V. (1981) J. Gen. Microbiol. 124, 365–374.
27 Fitton, J.E., Dell, A. and Shaw, W.V. (1980) FEBS Lett. 115, 209–212.
28 Birkbeck, T.H., and Freer, J.H. (1986) Methods Enzymol. 165, 16–22.
29 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390–394.
30 Griffin, R.G. (1981) Methods Enzymol. 72, 108–174.
31 Rance, M. and Byrd, R.A. (1983) J. Magn. Reson. 52, 221–240.
32 Macdonald, P.M. and Seelig, J. (1988) Biochemistry 27, 6769–6775.
33 Brown, M.F. and Seelig, J. (1978) Biochemistry 17, 381–384.
34 Thaudiere, E., Siffert, O., Talbot, J., Bolard, J., Alouf, J.E. and Dufourcq, J. (1991) Eur. J. Biochem. 195, 203–213.
35 Eisenberg, D., Weiss, R.M. and Terwilliger, T.C. (1982) Nature 299, 371–374.
36 Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361.
37 Habermann, E. (1980) in Natural Toxins (Eaker, D. and Wadstrom, T., eds.) pp. 173–181, Pergamon Press, Oxford.
38 Freer, J.H. and Birkbeck, T.H. (1982) J. Theor. Biol. 94, 535–540.
39 Vogel, H., Jahnig, F., Hoffman, V. and Stumpel, J. (1983) Biochim. Biophys. Acta 733; 201–209.
40 Macdonald, P.M., Leisen, J. and Marassi, F.M. (1991) Biochemistry 30, 3556–3558.
41 Dempsey, C.E. and Watts, A. (1987) Biochemistry 26, 5803–5811.
42 Dempsey, C. Bitbol, M. and Watts, A. (1989) Biochemistry 28, 6590–6596.
43 Beschiaschvili, G. and Seelig, J. (1990) Biochemistry 29, 52–58.
44 Beschiaschvili, G. and Seelig, J. (1990) Biochim. Biophys. Acta 1061, 78–84.
45 Rice, D. and Oldefield, E. (1979) Biochemistry 18, 3272–3279.
46 Kang, S.Y., Gutowsky, H.S., Hsung, J.C., Jacobs, R., King, T.E., Rice, D. and Oldefield, E. (1979) Biochemistry 18, 3257–3267.
47 Pink, D.A., Georgeallas, A. and Chapman, D. (1980) Biochemistry 20, 7152–7157.
48 Sansom, M.S.P. (1991) Prog. Biophys. Mol. Biol. 55, 139–235.