Lipid Chain-Length Dependence for Incorporation of Alamethicin in Membranes: Electron Paramagnetic Resonance Studies on TOAC-Spin Labeled Analogs

Derek Marsh,* Micha Jost,† Cristina Peggion,‡ and Claudio Toniolo†
*Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, 37070 Göttingen, Germany; and †Institute of Biomolecular Chemistry, CNR Padua Section, Department of Chemistry, University of Padova, 35131 Padova, Italy

ABSTRACT Alamethicin is a 19-residue hydrophobic peptide, which is extended by a C-terminal phenylalaninol but lacks residues that might anchor the ends of the peptide at the lipid-water interface. Voltage-dependent ion channels formed by alamethicin depend strongly in their characteristics on chain length of the host lipid membranes. EPR spectroscopy is used to investigate the dependence on lipid chain length of the incorporation of spin-labeled alamethicin in phosphatidylcholine bilayer membranes. The spin-label amino acid TOAC is substituted at residue positions n = 1, 8, or 16 in the sequence of alamethicin F50/5 [TOACn, Glu(OMe)7,18,19]. Polarity-dependent isotropic hyperfine couplings of the three TOAC derivatives indicate that alamethicin assumes approximately the same location, relative to the membrane midplane, in fluid diC10PtdCho bilayers with chain lengths ranging from N = 10–18. Residue TOAC16 is situated closest to the bilayer midplane, whereas TOAC8 is located farther from the midplane in the hydrophobic core of the opposing lipid leaflet, and TOAC1 is remains in the lipid polar headgroup region. Orientational order parameters indicate that the tilt of alamethicin relative to the membrane normal is relatively small, even at high temperatures in the fluid phase, and increases rather slowly with decreasing chain length (from 13° to 23° for N = 18 and 10, respectively, at 75°C). This is insufficient for alamethicin to achieve hydrophobic matching. Alamethicin differs in its mode of incorporation from other helical peptides for which transmembrane orientation has been determined as a function of lipid chain length.

INTRODUCTION

Alamethicin is a predominantly α-helical, 19-amino acid residue peptide from Trichoderma viride, which has N-terminal and C-terminal residues that are blocked by an acetyl and a phenylalaninol group, respectively (1,2). This hydrophobic peptide is able to induce voltage-activated ion conduction across lipid membranes, the channel characteristics of which depend strongly on lipid chain length (3). The slope of the I/V curves and the effective number of monomers per alamethicin channel increase steeply with increasing chain length of the lipids that make up the host bilayer membrane. Spectroscopic studies reveal that the peptide is monomeric in fluid phospholipid membranes in the absence of a membrane potential (4,5), and electrophysiological experiments suggest that consecutive channel conductance levels are generated by incorporation of alamethicin monomers into existing pore aggregates (6).

Studies on the effects of hydrophobic matching between transmembrane peptides/proteins and the membrane lipids have revealed that, whereas single bitopic helices tend to tilt or oligomerize in response to a positive mismatch, larger integral proteins tend to respond by inducing distortions in conformation of the surrounding lipids (7,8). A negative mismatch, on the other hand, would tend to induce lipid distortions in both cases. In addition, it has been found that the indole side chains of interfacial tryptophan residues are able to adapt their orientation such as to compensate, at least partially, for hydrophobic mismatch with the adjacent lipids (9,10).

Alamethicin, although hydrophobic, is devoid of tryptophan residues and the length of the peptide, even for a straight helix, is on the border of being sufficient to span the hydrophobic thickness of a typical membrane bilayer (4,11) and http://mosberglab.phar.umich.edu/projects/proj11.php). The orientation and/or aggregation state of alamethicin may therefore depend strongly on the chain length of the host lipid. Recently, we have shown that EPR of TOAC spin labels incorporated in the peptide backbone can be used to determine the orientation and mode of incorporation of alamethicin analogs in lipid membranes (5). Here we use this method to investigate the dependence on lipid chain length by using diacyl phosphatidylcholines with chain lengths from

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Address reprint requests to Derek Marsh, E-mail: dmarsh@gwdg.de.
Abbreviations: Aib, α-aminoisobutyric acid; diC10PtdCho, 1,2-didecanoyl-sn-glycero-3-phosphocholine; diC12PtdCho, 1,2-dilauroyl-sn-glycero-3-phosphocholine; diC14PtdCho, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; diC16PtdCho, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; diC18PtdCho, 1,2-distearoyl-sn-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; n-PSCL, 1-acyl-2-{n(4,4-dimethyl-oxazolidin-5-oxyl)[stearyl-sn-glycero-3-phosphocholine; OEt, ethoxy; OMe, methoxy; Phol, phenylalaninol; ST-EPR, saturation transfer EPR; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid; V1, first harmonic absorption EPR spectrum detected in phase with respect to the static magnetic field modulation; V2, second harmonic absorption EPR spectrum detected 90° out-of-phase with respect to the static magnetic field modulation.
C_{10} to C_{18}, the fluid-state bilayers of which encompass the likely hydrophobic thicknesses of natural membranes (12).

**MATERIALS AND METHODS**

**Materials**

Synthetic phosphatidylcholines with symmetrical saturated chains (diC10PtdCho, diC12PtdCho, diC14PtdCho, and diC16PtdCho) were obtained from Avanti Polar Lipids (Alabaster, AL). Spin-labeled analogs of alamethicin F505 [TOAC, Glu(OMe)]^2-18,19, were synthesized according to Peggion et al. (13,14). The complete amino-acid sequences of the three analogs are

Ac-TOAC-Pro-Ala-Ala-Glu(OMe)-Ala-Val-Ala-Gly-Leu-Ala-Pro-Val-Ala-Ala-Glu(OMe)-Glu(OMe)-Phol [TOAC,Glut(OMe)]^2-18,19; Ac-Ala-Pro-Ala-Ala-Ala-Glu(OMe)-Glu(OMe)-Phol [TOAC,Glut(OMe)]^2-18,19; Ac-Ala-Pro-Ala-Ala-Ala-Glu(OMe)-Glu(OMe)-Phol [TOAC,Glut(Ome)]^2-18,19.

In each case, TOAC is substituted for an Aib residue, which is a conservative replacement because both are C^{NMe3}-disubstituted amino acids that favor helix formation, as is confirmed by x-ray diffraction on the [TOAC, Glut(OMe)]^2-18,19 alamethicin analog (15). Circular dichroism in MeOH additionally shows that all three analogs are folded in similar, highly helical conformations (16). Electrophysiological experiments demonstrate ion channel activity for these synthetic alamethicin analogs in membranes (15).

**Sample preparation**

Diacyl phosphatidylcholine, diC5PtdCho (~1 mg), and 1 mol % of TOAC spin-labeled alamethicin (in MeOH) were codissolved in CH2Cl2, and the latters were flame sealed.

**EPR spectroscopy**

EPR spectra were recorded on a Varian (Palo Alto, CA) Century-Line 9-GHz spectrometer with 100-kHz field modulation (50 kHz for second-harmonic detection). Sample capillaries were accommodated in standard quartz EPR tubes that contained light silicone oil for thermal stability. Temperature was regulated by thermostated nitrogen gas flow through a quartz Dewar and was measured with a fine-wire thermocouple situated in the silicone oil at the top of the microwave cavity. Samples of ~5-mm height were centered in the rectangular TE_{102} resonator to minimize microwave- and modulation-field inhomogeneities (17). The microwave H_1-field at the sample was measured as described in the latter reference. Conventional EPR spectra were recorded in the in-phase first-harmonic absorption mode (V_1-display) and ST-EPR spectra in the out-of-phase second-harmonic absorption mode (V_2-display) under standard conditions for microwave and modulation field amplitudes (18).

Conventional EPR spectra were analyzed in terms of the outer and inner hyperfine splittings, 2A_{\text{max}} and 2A_{\text{min}} respectively. In the motional narrowing regime, at high temperature, \( A_{\text{max}} \) is equal to the parallel element, \( A_{\parallel} \), of the partially motionally averaged, axial hyperfine tensor. The perpendicular element, \( A_{\perp} \), is obtained from the separation, 2A_{\text{min}}, of the inner extremum, according to Schorn and Marsh (19):

\[
A_{\parallel} (\text{gauss}) = A_{\text{min}} + 1.32 + 1.86 \times \log_{10}(1 - S_{\text{app}})
\]

for \( S_{\text{app}} \geq 0.45 \) (1)

and

\[
A_{\perp} (\text{gauss}) = A_{\text{min}} + 0.85 \quad \text{for} \quad S_{\text{app}} < 0.45,
\]

where \( S_{\text{app}} = (A_{\text{max}} - A_{\text{min}})/[A_{\parallel} - (1/2)(A_{\text{xx}} + A_{\text{yy}})] \) for a spin-label hyperfine tensor with Cartesian elements (\( A_{\text{xx}}, A_{\text{yy}}, A_{\text{zz}} \)). The environmental polarity was characterized by means of the isotropic \(^1^H\)-hyperfine coupling, \( a_0 \) (20), which is given by

\[
a_0 = \frac{1}{3} (A_{\parallel} + 2A_{\perp}).
\]

Near constancy, with respect to temperature, of the value of \( a_0 \) obtained from Eq. 3 was used as a criterion to identify the fast motional regime (5,21,22).

The orientational order parameter of the spin-label \( z \) axis, in the fast motional regime, is given by

\[
S_{zz} = \frac{A_{\parallel} - A_{\perp}}{A_{\parallel} - \frac{1}{2}(A_{\text{xx}} + A_{\text{yy}})} \times \frac{A_{\text{xx}} + A_{\text{yy}} + A_{\text{zz}}}{3a_0},
\]

where the final factor on the right is a polarity correction to the hyperfine tensor elements. Values taken for the hyperfine tensor elements are (\( A_{\text{xx}}, A_{\text{yy}}, A_{\text{zz}} \)) = (6.0 G, 7.3 G, 34.5 G) from 2,2,6,6-tetramethylpiperidine-1-oxyl in a toluene glass (23). For uniaxial motional averaging, the EPR order parameter of TOAC is given by the addition theorem for spherical harmonics:

\[
S_{zz} = \langle P_2(\cos \gamma) \rangle \times P_2(\cos \theta_{zz}),
\]

where \( y \) is the angle that the principal rotational diffusion axis, \( R \), of the TOAC-labeled alamethicin molecule makes with the membrane normal, \( N \), and \( \theta_{zz} \) is the inclination of the spin-label \( z \) axis to \( R \) (Fig. 1). \( P_2(\cos \gamma) = (3\cos^2 \gamma - 1)/2 \) is a second-order Legendre polynomial, and the angular brackets indicate a time average over the rotational motion.

Saturation transfer EPR spectra were analyzed in terms of the diagnostic line-height ratios, \( L^+L^- \), \( ^{13}C^+C^- \), and \( ^{15}N^+N^- \), defined in the low-field, central, and high-field regions of the spectra, respectively (24), and by the normalized integrated intensity, \( I_{22} \) (25). Effective rotational correlation times, \( \tau_{\text{eff}} \), were obtained from ST-EPR line-height ratios, \( R \), by using calibrations with spin-labeled hemoglobin in solutions of known viscosity from Horváth and Marsh (25,26).

**RESULTS**

**Conventional spin-label EPR spectra**

Fig. 2 shows the EPR spectra of the three different TOAC\(^1\), TOAC\(^8\), and TOAC\(^16\) analogs of [Glu(OMe)]^2-18,19 alamethicin in fluid-phase bilayer membranes of phosphatidylcholines with different chain lengths. At this relatively high temperature (75°C) in the fluid phase, all spectra are approximately in the fast motional regime, with the possible exception of those of the TOAC\(^16\) analog. This conclusion is substantiated by the constancy with temperature of the effective isotropic hyperfine coupling (see later and (5)). Spin-spin broadening is absent from the EPR spectra, which indicates that the spin-labeled alamethicins are dispersed as monomers in the lipid membrane. The spectra from all three TOAC derivatives display axial motional averaging, as indicated by the well-defined outer and inner hyperfine splittings, 2\( A_{\text{max}} \) and 2\( A_{\text{min}} \), respectively (27).
Fig. 3 shows the temperature dependences of the outer hyperfine splitting, $2A_{\text{max}}$, for alamethicin with the different positions of TOAC labeling in phosphatidylcholine membranes with different lipid chain lengths, $N$. The chain-melting transition of diCNPtdCho bilayers takes place at temperatures of $-6^\circ\text{C}$, $-2^\circ\text{C}$, $24^\circ\text{C}$, $41^\circ\text{C}$, and $55^\circ\text{C}$ for $N = 10$, $12$, $14$, $16$, and $18$, respectively (12). For the most part, there are small discontinuities in the temperature dependence of $A_{\text{max}}$ at these chain-melting transitions. However, the values of $A_{\text{max}}$ at temperatures above the lipid transition are still indicative of a high degree of order, or limited amplitude of angular motion, of the spin-labeled alamethicin. There are, nonetheless, clear differences between the temperature dependences for the three label positions. These differences are similar in the five host lipids and arise from the different intramolecular orientations, $\theta_{\text{zr}}$, of the TOAC spin-label group. The values of $A_{\text{max}}$ decrease steadily in response to the increased extent of lipid chain motion with increasing temperature, to comparable extents for each lipid host. At a given temperature in the fluid phase, the values of $A_{\text{max}}$ increase with increasing lipid chain length.

**Isotropic hyperfine couplings**

Fig. 4 gives the temperature dependence of the effective isotropic hyperfine couplings, $a_{\text{eff}}$, defined by Eq. 3, for alamethicin with the three positions of TOAC labeling in diCNPtdCho membranes of different lipid chain lengths, $N$. With the exception of the diC10PtdCho host lipid, the values of $a_{\text{eff}}$ for the TOAC1 and TOAC8 analogs are practically constant at temperatures of $50^\circ\text{C}$–$55^\circ\text{C}$ or higher. This finding means that the EPR spectra are in the fast motional regime at these temperatures. Consequently, the values of $a_{\text{eff}}$ derived from Eq. 3 over this temperature range are a true reflection of the polarity of the environment in which the spin label is situated (28,29), uncontaminated by artifactual contributions from slow motion. Similarly for the TOAC16 derivative in membranes of diC16PtdCho and diC18PtdCho, the values of $a_{\text{eff}}$ tend to a constant limiting value, characteristic of fast motion, but at higher temperatures (above $70^\circ\text{C}$). Only for the TOAC16 derivative in diC12PtdCho and diC14PtdCho, and all derivatives in diC10PtdCho, is the temperature dependence of $a_{\text{eff}}$ apparently anomalous throughout the entire range of measurement.

The left-hand panel of Fig. 5 shows the dependence on lipid chain length, $N$, of the isotropic hyperfine coupling
increments, \( \Delta a_o \), for the different positions of alamethicin TOAC labeling. Because these values are obtained from measurements at high temperature, in the region where \( a_o \) is approximately constant (Fig. 4), they should depend only on the polarity of the spin-label environment. To correct for intrinsic differences between the three TOAC positions, the values are referred to the isotropic splittings of the respective analogs in methanol. From isotropic spectra at 20\( ^\circ \)C, the hyperfine splitting constants in MeOH are \( a_o = 15.24 \pm 0.03, 15.37 \pm 0.02, \) and \( 15.11 \pm 0.04 \) G for TOAC\( ^1 \), TOAC\( ^8 \), and TOAC\( ^{16} \) alamethicin, respectively.

The uncertainty ranges in \( \Delta a_o \) that are given by the vertical bars in Fig. 5 correspond to the standard deviation over the temperature range chosen for evaluation. For lipid chain lengths \( N < 16 \) with the TOAC\( ^{16} \) analog, the uncertainty is relatively large because the effective value of \( a_o \) that is derived from Eq. 3 continues to decrease with increasing temperature up to the highest temperatures of measurement. This result could indicate either that the spectra of this derivative are still not entirely in the fast motional regime at the highest temperatures or that the position of the spin label is changing with temperature. As found already for TOAC analogs of [Glu(OMe)\( ^7,18,19 \)] alamethicin in diC\( ^{14} \)PtdCho bilayers (5), the lower values of \( \Delta a_o \) for TOAC\( ^8 \) and TOAC\( ^{16} \) reveal that these residues are situated deeper in the hydrophobic core of the membrane than is the TOAC\( ^1 \) residue, at least at the high temperatures for which \( a_o \) can be measured reliably. The corrected values also show that TOAC\( ^8 \) is located in a region of lower polarity than TOAC\( ^1 \) and TOAC\( ^{16} \), indicating that the latter two residues are situated in opposite leaflets of the bilayer. For lipid chain lengths \( N > 10 \), the values of \( a_o \) decrease with increasing chain length, corresponding to locations of decreasing polarity for all three residue positions.

Comparable data for \( \Delta a_o \) of spin-labeled phospholipid chains from Marsh (20) are also included in Fig. 5 (right panel). These will be used in the later Discussion.
Orientational order parameters

Fig. 6 shows the dependence on lipid chain length, \( N \), of the effective isotropic hyperfine couplings, \( a_o \), for [Glu(OMe)_7,18,19] alamethicin TOAC\(^1\) (squares), TOAC\(^8\) (circles), and TOAC\(^16\) (triangles) analogs in fluid diC\(_{14}\)PtdCho bilayers. Right-hand panel: positional dependence (\( n \)) of the isotropic hyperfine couplings for nitroxides at position C-\( n \) in the sn-2 chain of \( n \)-PCSL phosphatidylcholine spin probes in diC\(_{14}\)PtdCho (circles) or diC\(_{16}\)PtdCho (squares) fluid bilayers (20). Values of \( a_o \) are given relative to those in methanol: \( \Delta a_o = a_o(\text{PtdCho}) - a_o(\text{MeOH}) \). Horizontal dotted lines are the values of \( \Delta a_o \) in MeOH and in EtOH, as indicated.

Saturation transfer spin-label EPR spectra

Fig. 8 shows the saturation transfer EPR spectra of the TOAC\(^1\), TOAC\(^8\), and TOAC\(^16\) analogs of [Glu(OMe)_7,18,19] alamethicin in bilayer membranes of diC\(_{14}\)PtdCho with different chain lengths, \( N \). Spectra for diC\(_{14}\), diC\(_{16}\), and diC\(_{18}\)PtdCho are shown mostly at temperatures both above (45°C, or
60°C for diC18) and below (5°C) the respective bilayer chain-melting transitions. For diC10 and diC12PtdCho, both low and high temperatures (5°C and 45°C) correspond to the fluid phase of these bilayers. (In a few cases, where the ST-intensity was too low at the higher temperature, the second spectrum is at a somewhat lower temperature.)

In the gel-phase, all the ST-EPR spectra (for N = 14–18) have appreciable intensity in the diagnostic regions at intermediate positions in the low-, high-, and center-field manifolds of the 14N-hyperfine structure that are sensitive to slow rotational motion. These nonvanishing ST-EPR line heights reflect the extremely slow rotational diffusion of the gel-phase lipids, with effective correlation times beyond the microsecond regime (30,31). At the fixed temperature of 5°C, the ST-EPR spectra indicate increasingly slow motion with increasing chain length, because the bilayers lie deeper in the gel phase as the lipid chain length increases. Above the chain-melting transition, the spectral line shape changes dramatically because of preferential reduction in the line heights at the intermediate diagnostic spectral positions relative to the stationary turning points for each of the three hyperfine manifolds at low-, central-, and high-field, respectively (24). For all chain lengths of the lipid host, the spectra are characteristic of very fast motion, no longer in the microsecond regime, and reflect the rapid lipid chain motions in the fluid membrane phase (30,32). Note that the V2-EPR line shapes in diC10 and diC12PtdCho are rather similar at 5°C and 45°C because the bilayers are in the fluid phase at both temperatures.

The ST-EPR spectra in Fig. 8 are scaled to line height, rather than to the absolute intensity, which decreases with increasing temperature. Fig. 9 gives the temperature dependence of the normalized ST-intensity, I_ST, for the TOAC8 alamethicin analog in diC8PtdCho membranes. The ST-intensities are appreciable in the gel phase (I_ST ~ 10^{-3} - 10^{-5}) although they display a considerable temperature dependence that demonstrates mobility of the peptide on the microsecond timescale. At the chain-melting transition, the ST-intensities drop to almost zero (I_ST ~ 10^{-5}) for all chain lengths of the lipid host (or throughout the entire temperature range for diC10 and diC12PtdCho). These latter values of I_ST correspond to effective rotational correlation times of <2.9 × 10^{-8} s in the fluid phase (26). Such rapid rotational re-orientation indicates that the peptide is not aggregated in fluid phosphatidylcholine membranes, even at the extreme chain lengths of N = 10 or 18. This conclusion is in agreement with the lack of spin-spin broadening in the conventional EPR spectra from fluid-phase bilayers.

**DISCUSSION**

The central results obtained from this study are the dependences of the isotropic hyperfine coupling, a_o, and of the
order parameter, \( \langle P_2(\cos \gamma) \rangle \), of the alamethicin diffusion axis on the chain length of the bilayer membrane lipid host (Figs. 5 and 7). Data on overall rotational diffusion from ST-EPR suggest that alamethicin is present predominantly as a monomer in the fluid-phase membranes at all lipid chain lengths studied.

**Vertical positioning from \( a_0 \)**

The location of the different TOAC residues in the bilayer membrane can be determined by comparing the isotropic hyperfine couplings with those determined for phosphatidylcholines (\( n \)-PCSL) that are spin labeled at different positions in the \( sn-2 \) lipid chain (20). For comparison with the TOAC-values that are given in the left-hand panel of Fig. 5, the incremental isotropic hyperfine couplings of the \( n \)-PCSL spin labels in fluid diC\(_{14}\)PtdCho and diC\(_{16}\)PtdCho bilayers are shown in the right-hand panel of the figure. These vary from a high plateau value of \( \Delta a_0 = 0.05\text{–}0.1 \) G for \( n = 4\text{–}6 \) toward the polar-apolar interface to a low plateau value of \( \Delta a_0 = -0.45 \text{ to } -0.55 \) G for \( n = 10\text{–}16 \) toward the middle of the membrane. These values, like those for TOAC\(^n\), are expressed relative to \( a_0 \) in methanol, i.e., \( \Delta a_0 = a_0(\text{PC}) - a_0(\text{MeOH}) \), to correct for the intrinsic difference between the isotropic couplings of doxyl nitroxides (in \( n \)-PCSL) and TOAC nitroxides.

For all three TOAC positions, the effective isotropic hyperfine splitting constants decrease with increasing lipid chain length, from \( N = 14 \) onward. This result implies that alamethicin is anchored in the hydrophobic core of the membrane, rather than at some specific interfacial location of an anchoring residue that is fixed at one end of the peptide. In diC\(_{12}\)PtdCho and diC\(_{14}\)PtdCho, the TOAC\(^1\) residue of alamethicin is situated in a more polar location than the 4-C atom of the phosphatidylcholine \( sn-2 \) chain. However, the TOAC\(^{16}\) residue is by no means fully exposed to water in these lipids because the isotropic hyperfine coupling increment in that case would be much higher (\( \Delta a_0 = 1.0\text{–}1.3 \) G, depending on the pH/charge state of the peptide) (33). For longer chain lengths, TOAC\(^1\) is located at the polar-apolar interface or in the plateau region of the 4-C atom. In lipids with chain lengths \( N = 10\text{–}14 \), the TOAC\(^8\) residue of alamethicin is located at a depth similar to that of the 8–9 C-atom of the lipid chains. In lipids with longer chain lengths, the TOAC\(^8\) residue is situated toward the center of the bilayer, in the region of the 10 C-atom of the lipid chains or beyond. The TOAC\(^{16}\) residue of alamethicin is located close to the 8 C-atom of the lipid chains in diC\(_{16}\)PtdCho and diC\(_{18}\)PtdCho, and in the region of the 7 C-atom or higher in diC\(_{14}\)PtdCho. Clearly, TOAC\(^{16}\) resides in the opposite bilayer leaflet from that of TOAC\(^8\) and TOAC\(^1\), and is closer to the polar-apolar interface than is TOAC\(^8\), which lies closer to the bilayer midplane. Note that x-ray diffraction studies exclude the possibility of extensively bent or looped structures for the peptide backbone. In [TOAC\(^{16}\), Glu(OMe)\(^{17,18,19}\)] alamethicin, replacement of Aib by TOAC does not significantly affect the backbone conformation (15). In particular, the overall fold of molecules A and B of the TOAC analog (15) is quite similar to that reported for the three independent molecules of alamethicin itself (1), although molecule A of the analog is bent slightly more sharply near Pro\(^{19}\) than is molecule B.

A positioning of alamethicin consistent with the isotropic hyperfine couplings in diC\(_{16}\)PtdCho is obtained if the TOAC\(^{16}\) residue is located close to the polar-apolar interface of the bilayer. A similar location relative to the bilayer midplane is also consistent with the isotropic hyperfine couplings in diC\(_{14}\)PtdCho. Fig. 10 shows the most recent x-ray refinements of the bilayer dimensions for diC\(_{12}\)PtdCho and diC\(_{14}\)PtdCho (34) and for diC\(_{18}\)PtdCho (35) in the fluid phase at 50°C. The approximate position of the residues in alamethicin is indicated by the heavy dumbbells. For the purposes of illustration, a straight \( \alpha \)-helix is assumed. If the position relative to the bilayer midplane is retained for all chain lengths, the N-terminal of alamethicin lies within the lipid polar group region for chain lengths \( N < 18 \). Similarly, the C-terminal Phol resides in the polar group region of the opposite leaflet for the same lipid chain lengths. Such a location of the C-terminal is essentially consistent with the proposals in Barranger-Mathys and Cafiso (4), although perhaps closer to that of the leucine analog, which is compared with native alamethicin in this latter reference. (Note that diC\(_{18}\)PtdCho bilayers are still in the gel phase at 50°C, and the extrapolated values of \( d_c \) and \( d_0 \) must be reduced by 5% to reach 65°C in the fluid phase.) With the disposition indicated in Fig. 10, the TOAC\(^8\) and TOAC\(^{16}\) residues are situated within the hydrocarbon core of the membrane for all lipid chain lengths, consistent with the isotropic hyperfine splittings in Fig. 5.
for TOAC1, TOAC8, and TOAC16, respectively.

Alamethicin and Lipid Chain Length

Measurements of the hydrocarbon core, bilayers for

\[ d_{st} = \frac{d_{c}}{C_0} \]

consistent with Fig. 5, is indicated by the heavy vertical line and horizontal dotted lines (C). Solid squares: Thickness

\[ d_{c} = \frac{1}{C_0} \]

(34). Sloping lines are linear regressions. The approximate position of [Glu(OMe)\textsuperscript{7,18,19}] alamethicin in diC\textsubscript{14}PtdCho and diC\textsubscript{16}PtdCho bilayers, consistent with Fig. 5, is indicated by the heavy vertical line and horizontal dotted lines (C\textsuperscript{a}-atoms), which are located at ~ +1.45, +0.4, and -0.8 nm for TOAC\textsuperscript{1}, TOAC\textsuperscript{8}, and TOAC\textsuperscript{16}, respectively.

Peptide tilt from order parameters

The distance between the C\textsuperscript{a} atoms of residues Aib\textsuperscript{1} and Phol\textsuperscript{20} in the crystal structure of [TOAC\textsuperscript{16},Glu(OMe)\textsuperscript{7,18,19}] alamethicin is 2.8 nm (15), close to the repeat distance for a straight α-helix of this length. The entire length of the molecule is ~3.3–3.4 nm and contains no polar anchoring groups. Hydrophobic matching will therefore be achieved approximately with diC\textsubscript{16}PtdCho bilayers, for which the thickness of the hydrocarbon core is d\textsubscript{c} = 2.79 nm in the fluid phase at 50°C (35). This conclusion is essentially consistent with the deductions made above from isotropic hyperfine splittings that TOAC\textsuperscript{1} is situated only 0.15 nm outside the hydrocarbon core of diC\textsubscript{16}PtdCho bilayers (Fig. 10). On the other hand, the total length of alamethicin corresponds to the total anhydrous thickness of diC\textsubscript{14}PtdCho bilayers (d\textsubscript{a} = 3.4 nm at 50°C) and is less than the estimated total steric extent of diC\textsubscript{12}PtdCho bilayers (d\textsubscript{a} = 3.76 nm).

The order parameters, \( \langle P_2(\cos \gamma) \rangle \), of the long axis of alamethicin are relatively large, even at the high tempera-

tures for which they can be measured reliably (Fig. 7). Most significantly, the order parameters decrease rather slowly with decreasing chain length of the host lipid. The latter result is not consistent with a tight matching of the peptide tilt to the thickness of the hydrophobic core of the membrane. Fig. 11 shows model calculations for tightly coupled hydrophobic matching. The order parameter is calculated assuming either a fixed tilt \( \gamma \)

\[ \langle P_2(\cos \gamma) \rangle = \frac{1}{2} (3\cos^2 \gamma - 1) \] (6)

or a random wobble within a cone of maximum amplitude \( \gamma \)

\[ \langle P_2(\cos \gamma) \rangle = \frac{1}{2} \cos \gamma (1 + \cos \gamma). \] (7)

The geometric condition for hydrophobic matching is given by

\[ \cos \gamma = \left( d_{c}^2 + N \Delta d_{c} \right) / d_{p}. \] (8)

where d\textsubscript{c} is the hydrophobic thickness of alamethicin and the parameters d\textsubscript{c} and \( \Delta d_{c} \) governing the thickness of the hydrophobic lipid core, d\textsubscript{c}, are obtained from the linear regressions in Fig. 10. Clearly, geometrical hydrophobic matching would require a far greater dependence of order parameter on lipid chain length than is observed. Attempts to fit Eqs. 7 and 8 (or equivalently Eqs. 6 and 8) to the experimental data give rise to effective thicknesses of the lipid bilayer that vary far less with chain length (and correspondingly far larger end contributions) than is found experimentally. The chain-length dependence of the hydrocarbon core thickness for phosphatidylcholines that is given in Fig. 10 is characterized by values

![Image](78x452 to 269x724)

FIGURE 10 Chain-length dependences of the thickness of diC\textsubscript{N}PtdCho bilayers for N = 12, 14 (34), and 16 (35) at 50°C. Solid squares: thickness of the hydrocarbon core, d\textsubscript{c}; open squares: anhydrous bilayer thickness, d\textsubscript{a}; open circles: steric thickness of the bilayer, d\textsubscript{p} = d\textsubscript{c} + 1.8 nm. Measurements of d\textsubscript{c} and d\textsubscript{p} for N = 12 and 14 at 30°C are corrected to 50°C by using an expansion coefficient \( \alpha_d = (1/d)(\partial d/\partial T) = -0.0032 K^{-1} \) (34). Sloping lines are linear regressions. The approximate position of [Glu(OMe)\textsuperscript{7,18,19}] alamethicin in diC\textsubscript{14}PtdCho and diC\textsubscript{16}PtdCho bilayers, consistent with Fig. 5, is indicated by the heavy vertical line and horizontal dotted lines (C\textsuperscript{a}-atoms), which are located at ~ +1.45, +0.4, and -0.8 nm for TOAC\textsuperscript{1}, TOAC\textsuperscript{8}, and TOAC\textsuperscript{16}, respectively.

![Image](323x161 to 538x339)

FIGURE 11 Predicted dependence of the order parameter of the long axis of alamethicin on chain length of host diC\textsubscript{N}PtdCho bilayers according to Eq. 6 (solid lines) or Eq. 7 (dashed lines) and Eq. 8 for hydrophobic matching. Dimensions of the lipid hydrocarbon core, d\textsubscript{c}, are taken from Fig. 10, and the hydrophobic span of alamethicin is d\textsubscript{c} = 2.8 nm or 3.4 nm, as indicated. Squares (circles) are order parameter measurements at 75°C (85°C); dotted lines are nonlinear fits of Eqs. 7 and 8 with d\textsubscript{c} = 2.8 nm or 3.4 nm, as indicated. Squares (circles) are order parameter measurements at 75°C (85°C); dotted lines are nonlinear fits of Eqs. 7 and 8 with d\textsubscript{c} = 2.8 nm or 3.4 nm, as indicated.
of $\Delta d_C/d_p = 0.0741 \pm 0.0005 (0.0610 \pm 0.0004)$ and $d_C^0/d_p = -0.189 \pm 0.007 (-0.155 \pm 0.006)$, assuming that $d_p = 2.8 (3.4)$ nm. These parameters are very different from those required to describe the chain-length dependence of the order parameters in Fig. 11.

At 75°C, the effective angle of tilt deduced from Eq. 6 varies from $\gamma = 13^\circ$ for diC10PtdCho to $\gamma = 23^\circ$ for diC16PtdCho. For comparison, the geometrical criterion for hydrophobic matching would require that $\gamma = 56^\circ$ for diC10PtdCho, taking $d_p = 2.8$ nm in Eq. 8. Clearly, alamethicin remains oriented relatively close to the membrane normal, even in thin phosphatidylcholine bilayers. The mode of incorporation is that indicated schematically in Fig. 10 and deduced from the isotropic hyperfine couplings. The peptide is disposed in a similar way relative to the bilayer midplane for all lipid chain lengths and remains relatively untitled.

**Comparison with other data**

Extensive experiments on hydrophobic matching have been performed with alanine-leucine model peptides that, unlike [Glu(OMe)]$_7$[18,19] alamethicin F50/5, have well-defined terminal anchoring residues: either tryptophans in the WALP peptides or lysines in the KALP peptides (36–39). Of these, GW$_2$(AL)$_8$LW$_2$A (WALP23) and GK$_2$(AL)$_8$LK$_2$A (KALP23) are likely to have a hydrophobic span similar to that of alamethicin, although they are of longer total length. Both peptides are found to have very small tilts, with only small systematic variations, in phosphatidylcholine bilayers of different thicknesses (39). At 40°C, WALP23 is found to have tilts of 5.2° and 8.1° in bilayers of diC$_{14}$PtdCho and diC$_{12}$PtdCho, respectively, and the corresponding tilt angles for KALP23 are 7.6° and 11.2°, respectively. Qualitatively similar results are obtained with a shorter peptide WALP19, which has the same anchoring sequences as WALP23 but two fewer AL dipeptide units (37). Similarly, the transmembrane segment of the EGF receptor, which like the more symmetrical KALP peptides is also anchored by charged groups, exhibits only small tilt that is insensitive to hydrophobic matching (40). For the WALP and KALP peptides, adaptation to hydrophobic membrane thickness is thought to be achieved by adjustment of the position of the side chains of the anchoring residues (8,10,41). Significantly, appreciable tilts of $\sim 30^\circ$ are registered by longer peptides, WALP27 and WALP31, in diC$_{14}$PtdCho (36). This degree of freedom by side-chain adjustment is not available, however, to the terminal residues of alamethicin (note that Phol does not possess the indole anchoring propensity of tryptophan). It is therefore likely that alamethicin is anchored rather differently from the WALP and KALP peptides solely by embedding in the hydrophobic region of lipid membranes, as is indicated in Fig. 10. This situation may well require distortion in the polar region of the membrane and also contribute to the potential of alamethicin to form pore assemblies under the influence of a transmembrane potential. It could also account for the observed dependence of the ion channel properties on lipid chain length (3) because propensity to form channels will increase with increasing distortion of the polar-group region.

The mode of membrane anchoring of alamethicin differs much more from certain other peptides, for instance the transmembrane domain of the Vpu protein from the HIV-1 virus (42), than it does from the WALP and KALP model peptides. The transmembrane helix of Vpu responds almost quantitatively to membrane thickness by tilting by up to 51° in the thinnest bilayers (diC$_{10}$PtdCho). The Vpu helix construct has a strongly positive C-terminal domain but no obvious anchoring residues at the N-terminal. Relatively large tilts have also been obtained with the M2 transmembrane peptide from influenza A virus (43) that are more than sufficient to achieve hydrophobic matching with the host lipid. In the latter case, both charged residues at the N- and C-termini and a tryptophan may be involved in the transmembrane anchoring. Appreciable tilts are also achieved with the M13 coat protein (44) although the dependence on lipid chain length is insufficient alone to achieve simple hydrophobic matching. This latter peptide is also expected to be anchored by highly charged domains at the N- and C-termini.

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