Communication

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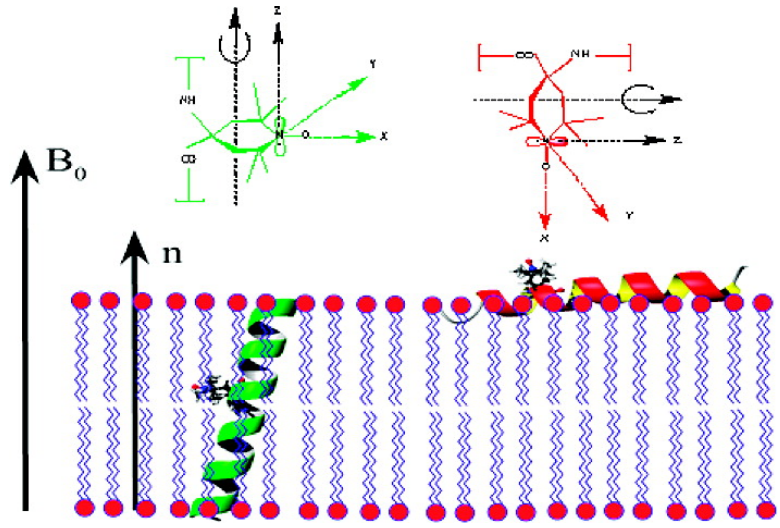

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Comparing the Structural Topology of Integral and Peripheral Membrane Proteins Utilizing Electron Paramagnetic Resonance Spectroscopy

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The alignment of membrane proteins in lipid bilayers can provide pertinent structural and dynamic information. Structural topology data gleaned from such studies can be used to help determine the functional mechanisms associated with a wide variety of integral membrane proteins. Significant progress in alignment studies has been made utilizing solid-state NMR (SSNMR) and more recently electron paramagnetic resonance (EPR) spectroscopy.[1−5] In this communication, we successfully demonstrate, for the first time, the determination of the structural topology and helical tilt of an antimicrobial peptide (magainin 2) that lies on the membrane surface using aligned X-band spin-label EPR spectroscopic techniques. The corresponding EPR hyperfine splitting and line shapes are in sharp contrast to a hydrophobic integral membrane protein such as the M2δ acetylcholine receptor. This new spectroscopic approach allows researchers to easily compare and distinguish between the two major types of membrane-associated proteins (integral and peripheral) utilizing routine X-band EPR spectroscopic techniques. This novel comparison unlocks many possibilities utilizing EPR spectroscopy to probe antimicrobial peptide topologies with increased sensitivity and may also give further clues to elucidate their corresponding mechanisms.

Integral membrane proteins have been explored predominantly with SSNMR and have provided a wealth of structural information.[6] While this technique is extremely powerful, it is not very sensitive. Typically, large quantities of protein (milligram scale) and long data acquisition sets are needed for these NMR experiments. These experiments may cause the sample to be studied under nonphysiologically relevant conditions (high P/L ratio) and potentially overheat the sample. EPR spectroscopy allows us to align samples at a lower magnetic field and probe dynamics at a different time scale regime. The M2δ domain has also been shown in previous work using SSNMR and more recently EPR to lie nearly parallel to the lipid bilayer normal with a helical tilt of $14 \pm 4°$.[7,8] EPR spectroscopy has proven to be extremely practical for studying integral membrane proteins and requires only 50 μg of peptide due to the approximated 1000-fold increase in signal sensitivity when compared to SSNMR.[9]

Antimicrobial peptides are an important class of peripheral peptides that have received a lot of attention of late due to an increased prevalence of drug-resistant species.[10] In this study, we investigate one of the more potent surface antimicrobial peptides magainin 2. It has been previously well-characterized with SSNMR and serves as an excellent model system for a structural topology comparison utilizing EPR spectroscopy. Magainin 2 is known to lie on the membrane surface, perpendicular to the bilayer normal, and serves a critical role for pore formation in bacterial membranes.[11]

To probe backbone motion in peptides, 2,2,6,6-tetramethyl piperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) has predominately been used as the EPR spin label of choice as it does not dramatically alter secondary structure or overall function, yielding a high level of accuracy.[9,12] Additionally, TOAC has been shown to exhibit minimal motion, which can be used to measure the structural topology and the helical tilt angle.[4,5] This approach takes advantage of the anisotropic hyperfine tensor of the TOAC spin label. In this work, new site-specific substitutions have been synthesized utilizing the TOAC spin label. The novel peptides include substitutions on both M2δ (EKMSTAISVLLAQAV-CLLLTSQR) and magainin 2 (GIGKFKHSAKKFGKAFVGE-IMNS) at positions 15 and 8, respectively.[7,11] The procedure for synthesizing M2δ and magainin 2 has been well-established.[7,13] Both peptides were prepared using Fmoc solid-phase synthesis and purified using reverse-phase HPLC. A C4 preparative column was applied for magainin 2, while a C18 preparative column was used with M2δ. The peptides were purified separately. A two-solvent system was used for each peptide. Solvent A was composed of water + 0.1% TFA, while solvent B comprised 90% acetonitrile + 10% water + 0.1% TFA. MALDI-TOF data revealed a purity of >95% for both peptides. Both peptides were then inserted into a dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) lipid bilayer as described below.

Alignment methods optimal for investigating peptides in artificially constructed lipid bilayers have been shown to increase spectral resolution.[14,15] Several techniques have been used in the past for both SSNMR and EPR spectroscopic techniques including bicelles, glass plates, and nanotube arrays.[4,16,17] Glass plates have proven in the past to be a powerful approach for accurate representations of aligned samples for both SSNMR and EPR spectroscopy using a variety of different types of phospholipid bilayers.[1,8]

Lipid−peptide samples were first prepared by adding 50 μg of peptide in 50 μL of TFE, 50 μL of chloroform, to 6 mg of DMPC in 300 μL of chloroform. The solution was reduced to 100 μL and was then delivered onto five glass coverslips (6 × 10 mm). The glass plates were allowed to dry and were then subjected to vacuum desiccation overnight. Samples where then hydrated by adding 4 μL of deuterium-depleted water. The samples were placed in a chamber with a relative humidity of 93% for 24 h at 42 °C. Each sample was then mounted on a quartz rod and inserted into the EPR cavity. All experiments were preformed on a Bruker EMX X-band CW-EPR spectrometer using the following experimental parameters: 100 G sweep width, 42 s sweep time, 9.434 GHz microwave frequency, 100 kHz modulation frequency, 1.0 G modulation amplitude, microwave power of 10 mW, and at a temperature of 318 K.[8]

Figure 1 shows EPR spectra collected from aligned glass plate samples and unoriented multilamellar vesicles (MLVs) powder pattern spectra of M2δ TOAC15 (Figure 1A,C) and magainin 2 TOAC8 (Figure 1B,D) and the corresponding microscopic order macroscopic disorder (MOMD) simulated spectra developed by Freed and co-workers.[19] In Figure 1A,B, the lipid bilayers are

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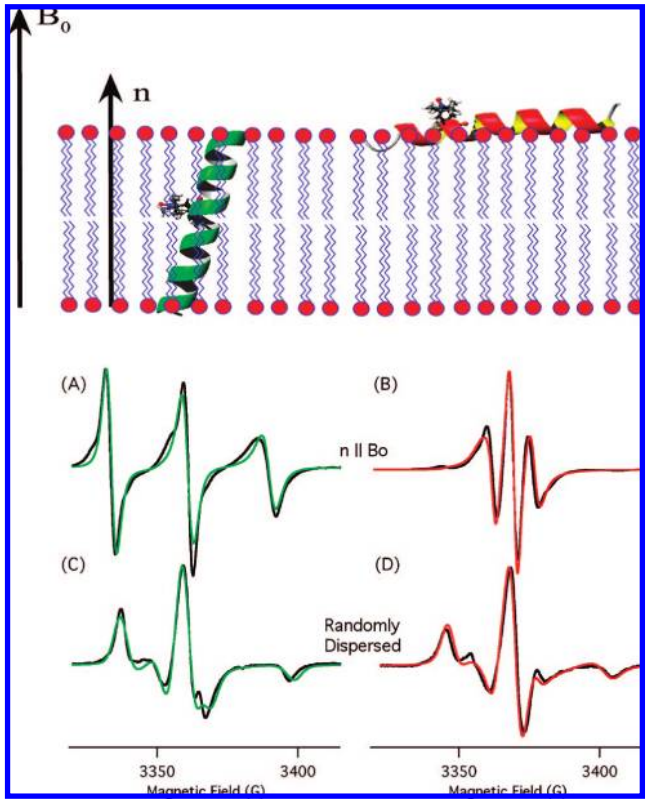

Figure 1. (Left, green) X-Band EPR spectra of TOAC15 M2δ incorporated into (A) mechanically aligned DMPC bilayers parallel to both the lipid bilayer normal and static magnetic field and (C) randomly dispersed. (Right, red) EPR spectra of TOAC8 magainin 2 incorporated into (B) mechanically aligned DMPC bilayers parallel to the lipid bilayer normal and (D) randomly dispersed samples. The solid black lines are experimental EPR data, while the red and green lines are the corresponding MOMD simulations.

aligned such that the membrane normal is parallel with B_o, whereas panels C and D of Figure 1 are the corresponding powder spectra. The aligned EPR spectra shown in Figure 1 clearly indicate major differences between the interactions of the two peptides with respect to the lipid bilayer. Significant differences are observed in both the hyperfine splitting and the corresponding line shapes, indicating different structural topologies. The A_{exp} values were determined to be 7.55 and 26.67 G for magainin 2 and M2δ, respectively. The z-axis of TOAC makes an angle of 21° with respect to the helical axis, and this angle is taken into account for the helical tilt angle calculation.[8,9] Previously, we have shown that the anisotropic hyperfine splitting observed for a transmembrane peptide such as M2δ aligned parallel to B_o reveals a large A_{exp} splitting when compared to the powder pattern; due to the orientation of A_{zz} with B_o. For the first time, in Figure 1B, we show the aligned EPR spectrum of an antimicrobial peptide. The hyperfine splitting is in sharp contrast to the aligned M2δ TOAC peptide.

A detailed procedure for obtaining the helical tilt of membrane proteins with respect to the bilayer normal (n) has already been established for transmembrane peptides.[8] Equation 1 can be used when the magnetic field makes an angle (ψ) with the director tilt vector (Z_D).

$$A_{exp} = (A_{||}^2 \cos^2 \psi + A_{\perp}^2 \sin^2 \psi)^{1/2} \quad (1)$$

The hyperfine splitting values for magainin 2 were measured in Figure 1D and determined to be $A_{||} = 29.53$ G and $A_{\perp} = 6.03$ G from the randomly dispersed spectrum. The angle between the director axis and the magnetic field, using previous work by the Lorigan Laboratory, according to eq 1 can be calculated as 81 ± 4° from the parallel orientation (i.e., $\angle(B_0, n) = 0°$). This result indicates that the helical tilt angle is 81 ± 4° with respect to the bilayer normal. The helical tilt angle implies that magainin 2 lies on the membrane surface, which agrees well with SSNMR studies which have indicated an 80° tilt.[18] Furthermore, the helical tilt was confirmed with the best-fit MOMD simulations (82 ± 5°).

To the best of our knowledge, we are the first to report these significant anisotropic hyperfine differences that elegantly contrast surface and transmembrane topologies for aligned membrane protein EPR TOAC spectra. The large hyperfine differences observed in the EPR spectra provide strong evidence that EPR spectroscopy can be a powerful tool to probe the structural topology and helical tilt of a wide variety of membrane-associated peptides. This new powerful technique is very advantageous when compared to other biophysical techniques such as NMR due to its high sensitivity, fast EPR data collection (1 EPR spectrum, 42 s), and small sample requirement (only 50 μg of peptide) to derive structural information. The aligned anisotropic EPR spectra and the corresponding helical tilt calculations developed in this communication provide a new approach for probing the structural topology of antimicrobial peptides.

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Supporting Information Available: The MOMD parameters used for simulating the EPR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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