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Determining the Helical Tilt Angle of a Transmembrane Helix in Mechanically Aligned Lipid Bilayers Using EPR Spectroscopy

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The utilization of aligned phospholipid bilayers in both NMR and electron paramagnetic resonance (EPR) spectroscopy has provided a wealth of structural and dynamic information about membrane-associated macromolecules.[1−4] The orientational dependent behavior of various nitroxide spin labels attached to lipids incorporated into aligned membrane systems has been investigated by several researchers, including our recent investigations with bicelles using EPR spectroscopy.[5,6] For the first time, a spin label rigidly coupled to a peptide backbone was mechanically aligned in planner-supported lipid bilayers to measure the helical tilt angle of a transmembrane helix with EPR spectroscopy. The rigid coupling of a 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) spin label to the backbone of a protein provides more accurate information about the helical tilt angle because there is minimal motion between the label and the peptide. A recent study showed that the incorporation of the TOAC spin label onto the membrane protein, phospholamban, did not alter the secondary structure or function.[7] Thus, the TOAC spin label provides some unique advantages for probing the topology and helicity of integral membrane proteins over the more flexible 1-oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-methylmethane thiosulfonate spin-label (MTSSL) approach. Additionally, the use of mechanically aligned glass plates coupled with highly sensitive EPR spectroscopy is very advantageous when compared to other methods (vide infra).

Previously, only a few studies have shown anisotropic angular dependent EPR spectra using the flexible MTSSL spin label attached to proteins on planner glass plate-supported lipid bilayers.[8,9] Because the TOAC is rigidly coupled to the peptide backbone, analysis of angular dependent EPR spectra of this spin label provides direct information on the orientation and the helical tilt of the transmembrane helix with respect to the membrane. We have used the M2δ domain of the nicotinic acetylcholine receptor (AChR), a 23-residue peptide with a transmembrane helix, as a model system with known helical tilt information based on PISEMA NMR experiments and molecular dynamic simulations to establish this new method.[10]

Recently, we have successfully demonstrated the incorporation of a TOAC spin-labeled AChR M2δ (TOAC substituted at amino acid 18) into parallel aligned bicelles and perpendicular aligned nanotube arrays.[3,4] Also, we determined the helical tilt angle of the M2δ domain from the experimentally measured hyperfine splitting values of the aligned spectrum. In this study, a TOAC spin-labeled AChR M2δ peptide was incorporated into 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) lipid bilayers interfaced between glass plate substrates.

The procedure for synthesizing and purifying spin-labeled amino acid 18 substituted TOAC of the M2δ transmembrane segment of AChR has been established.[3] Similarly, amino acid (Gln) at position 13 substituted TOAC was synthesized using Fmoc solid phase synthesis and purified using reverse-phase HPLC for this study. The mechanically aligned lipid−peptide sample was prepared by dissolving 50 μg of TOAC13AChR M2δ into 50 μL of trifluoroethanol (TFE) before it was added to 5 mg of DMPC dissolved in chloroform (200 μL). The sample was reduced to 1/3 volume slowly with nitrogen gas. Then the solution (1 μL) was spread on to microscope glass cover slips (6 × 10 mm) and allowed to air-dry for 30 min before vacuum drying overnight in a vacuum desiccator. Deuterium-depleted water was added onto the peptide/lipid mixture, and the glass plates were stacked on top of each other. The stacked glass plates were then allowed to equilibrate at a relative humidity of about 93% by annealing for 12 h at 42 °C in a humidity chamber consisting of a saturated ammonium monophosphate solution. The sample was then mounted with double-sided tape to a glass rod with a flat quartz end and introduced into the EPR cavity.

All EPR experiments were carried out on a Bruker EMX X-band CW-EPR spectrometer and acquired by taking a 42 s field-swept scan with experimental parameters: 3370 G center field, 100 G sweep width, 9.434 GHz microwave frequency, 100 kHz modulation frequency, 1.0 G modulation amplitude, and a microwave power of 10 mW. Simulations of the EPR spectra were carried out using the MOMD program.[11,12] The principal values of A and g were taken from Inbaraj et al.[3] The optimal fits for 0−90° spectra were obtained with a minimal set of parameters: parallel and perpendicular rotational diffusion coefficients ($R_{\|}$ and $R_{\perp}$), director tilt angle (ψ), and the coefficient of ordering potential. A global fitting approach was carried out for all the spectra. The dynamic parameters were obtained from the vesicle solution spectrum of TOAC-AChR. A global fit was performed to a series of angular dependent EPR spectra to extract a consistent set of dynamic parameters.

Figure 1A shows the EPR spectra of the TOAC13 spin label attached to AChR M2δ observed in oriented DMPC lipids as a function of θ (from 0 to 90°), the angle between the static magnetic field and the bilayer normal. The line shape and hyperfine splittings in the aligned spectra are different than the powder pattern spectrum shown in Figure 1B. Inspection of the spectra clearly shows a continuous significant variation in the low-field and high-field peaks as the angle is varied from 0 to 90° (Figure 1A). The minimum and maximum hyperfine splittings are observed at 90 and 0°, respectively.

In previous studies, we have reported a detailed description of the matrix algebra used to calculate the angle between the helical axis (h) and the bilayer normal, n (angle ϕ), from the experimentally determined angle between the director tilt vector (Z_D) and the bilayer normal (angle ζ) of the parallel aligned bicelle[3] and perpendicularly aligned nanotube array.[4] Briefly, the following equation can be used when the magnetic field makes an angle (ψ) with Z_D:[3]

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

For the parallel aligned sample ($\angle \mathbf{B}_0,n = 0°$), the director tilt angle (ψ) with respect to $\mathbf{B}_0$ is equal to the director tilt angle (ζ)

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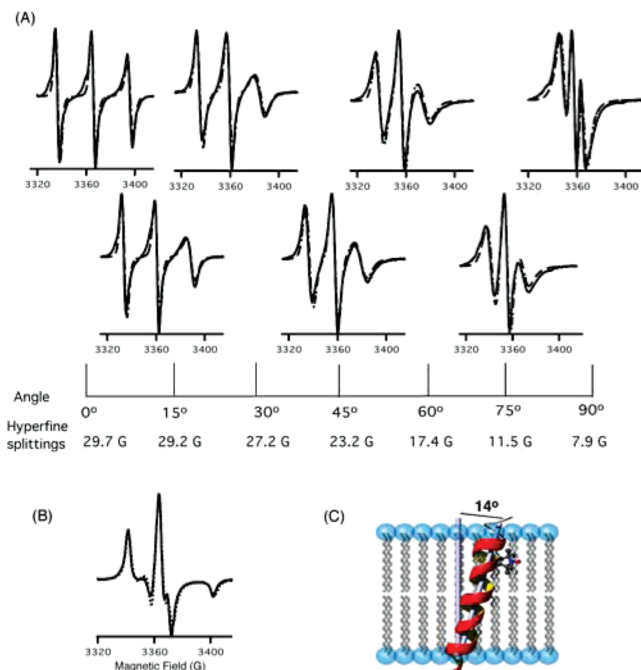

Figure 1. (A) EPR spectra of TOAC13AChR M2δ incorporated into mechanically aligned DMPC bilayers as a function of ϑ (from $\angle(\mathbf{B}_0,n) = 0-90°$) with respect to the static magnetic field ($\mathbf{B}_0$). (B) Randomly dispersed sample. The dashed lines represent spectral simulations of the spectra. (C) Helical tilt gleaned from EPR data.

with respect to n. However, for perpendicularly aligned bilayers ($\angle\mathbf{B}_0,n = 90°$), a distribution of the director angles ψ with respect to $\mathbf{B}_0$ from $\pi/2-\zeta$ to $\pi/2$ exists and the corresponding ζ value is calculated according to the literature.[4] In the case of an intermediate angle ($\zeta \leq \angle(\mathbf{B}_0,n) \leq 90-\zeta$), the spatial distribution of all directors causes the averaged director axis to be along the normal axis of the bilayer. So, the angle ψ derived from eq 1 or from the MOMD simulation is expected to be equal to $\angle(\mathbf{B}_0,n)$.

The hyperfine splitting values measured in Figure 1A were $A_{\text{exp}} = 29.7$ and 7.9 G for the sample aligned at 0 and 90°, respectively. The hyperfine splitting values of $A_{\|} = 30.5$ and $A_{\perp} = 7.0$ G were obtained from the randomly dispersed spectrum (Figure 1B). According to eq 1, the angle between the director axis and the magnetic field can be measured as 13° from parallel aligned (i.e., $\angle(\mathbf{B}_0,n) = 0°$), whereas, for the perpendicularly aligned (i.e., $\angle(\mathbf{B}_0,n) = 90°$) sample, integration from $\pi/2-\zeta$ to $\pi/2$ according to the expression given in ref 4 yielded a director tilt angle of 14°. This agrees well with the angle (15 ± 5°) obtained from the best-fit spectral simulations using the MOMD program. On the basis of the previous crystal structure of TOAC-attached α-helical peptides, it is known that there is a deviation of 21° in the orientation of the nitroxide p-orbital with respect to the helix axis.[14] Taking into account the average director tilt angle (13.5°) obtained from the EPR spectra, 21° deviation of Z_D with respect to helical axis based upon the crystal structure,[14,15] and unitary transformations involving three Euler angles (α,β,γ; see Supporting Information of Inbaraj et al.[3] for more details) results in a helix tilt angle of 14 ± 4° with respect to the bilayer normal. This is in good agreement with the helical tilt of the helix determined by PISEMA NMR experiments, and the 14−15° tilt angle predicted from molecular dynamic simulations, bicelles, and nanotube array studies.[3,4,10,13]

The glass plate alignment technique has several advantages when compared to other alignment techniques, such as magnetically aligned bicelles or bilayers lining the inside of the pores of nanotube arrays. The main advantage is that it is well-known to easily reconstitute larger membrane proteins.[1] Second, the bicelle technique is limited by the choice of lipids (DMPC) for optimal alignment. Some proteins may not properly align in bicelles due to hydrophobic mismatch conditions. The hydrophobic mismatch of membrane proteins due to the different acyl chain length of lipids can be directly related to the function of the membrane proteins. Recently, Park and Opella have demonstrated the effect of bilayer thickness on the tilt angle of a transmembrane helix with solid-state NMR experiments in mechanically aligned lipid bilayers.[16] Thus, mechanically aligned bilayers are more suitable for probing a wider range of membrane proteins with different types of phospholipids (charge and length). In a similar fashion, our new EPR methodology can now be easily used to probe helical tilt angles with glass plates due to hydrophobic mismatch. Finally, the direct advantage of this technique is that a series of EPR spectra can be obtained for a number of different angles of orientated lipid−protein samples in the magnetic field. Thus, providing a much larger data set to more accurately determine the helical tilt angle.

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. However, the incorporation of the spin label is bulkier than a ^{15}N NMR label. The insertion of TOAC is limited to peptides and smaller proteins (<60 amino acids) using Fmoc solid-phase peptide synthesis. In future studies, we will explore the use of alternative spin labels and/or site-specific insertion of spin-labeled amino acids in proteins using tRNA suppressor techniques that will enable larger membrane proteins to be studied with this powerful new method.

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