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Electron Paramagnetic Resonance Studies of an Integral Membrane Peptide Inserted into Aligned Phospholipid Bilayer Nanotube Arrays

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Nanoporous aluminum oxide disks have recently been used as a new substrate upon which lipid bilayers have been aligned for magnetic resonance spectroscopic studies.[1−3] The highly uniform nature and pore size adjustability provide a unique environment into which lipids may form one or many rings of bilayers lining the inside of the pore such that all bilayer normals may be positioned perpendicular to the static magnetic field (Figure 1). The use of anodized aluminum oxide nanodisks (AAO substrates) was first studied by Smirnov et al. who used EPR spectroscopy to probe the alignment of spin labeled lipids.[1] Lorigan et al. then used NMR to study isotopically labeled proteins incorporated in lipid bilayers aligned in AAO substrates.[2] There are many advantages for using nanoporous aluminum oxide to align membrane protein samples when compared to other commonly used methods such as mechanically aligned glass plates or bicelles. Those advantages include the ability to maintain sample stability and integrity after months of analysis (bicelles and glass plates are typically only stable for a few days). Also, nanotube arrays are quick and easy to prepare (minutes instead of days). Finally, these samples can be easily rehydrated. Additionally, the high pore density and large volume of exposed water to the bilayer surface in the AAO substrate is ideal to facilitate protein bilayer incorporation. For the first time, the current study demonstrates the ability to use EPR spectroscopy to study spin-labeled peptides inside aligned lipid bilayer nanotube arrays.

Recently, the Lorigan lab successfully incorporated a TOAC spin-labeled AChR M2δ peptide (TOAC substituted at amino acid 18) into parallel aligned bicelles and determined the corresponding helical tilt.[4] In this study, a rigid TOAC spin-labeled peptide was incorporated into 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) phospholipid bilayer nanotube arrays. In addition to the increased sensitivity gained with EPR spectroscopy over NMR spectroscopy, we demonstrate herein how to calculate the helical tilt angle from a perpendicularly aligned EPR sample formed in a nanoporous AAO substrate. The sensitivity of this new method is comparable to our previous bicelle study.[4]

The procedure for synthesizing and purifying spin-labeled amino acid 18 substituted TOAC acetylcholine receptor (AChR) using the channel-forming transmembrane domain (AChR M2 domain, δ subunit) has been established in the literature.[4] Sample preparation was achieved by dissolving 50 μg of TOAC18 into 75 μL of triflouroethanol (TFE) before it was added to 5 mg of DMPC dissolved in chloroform. The sample was dried slowly with N_2 gas and put into a vacuum desiccator overnight. A tris[hydroxymethyl]-aminomethane (TRIS) buffer (pH = 7, 10 mM) was prepared and 50 μL was added to the dried sample and solubilized by vortexing while freeze/thaw cycles were added to minimize sample frothing. 3.5 μL of this solubilized mixture was added to a 5 mm × 20 mm piece of 20 nm diameter pore size AAO substrate (Whatman) and then placed in a 40 °C oven for 1 min. The AAO substrate was then wiped gently to remove any excess lipid on the surface before

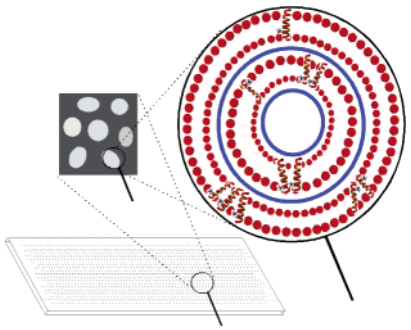

Figure 1. Illustration of aligned lipid bilayers in AAO substrates. Beginning from the AAO plate and then magnifying to a model electron microscopy drawing of the pores, magnifying to a model of the lipid bilayers lining the pores. See Gaede et al. for a complete structural characterization of the lipid bilayers in nanopores.[3]

5 μL of water was added. The sample was again heated in the oven for 1 min before gently wiping the excess water and placing the AAO substrate with hydrated sample in a 5 mm diameter EPR tube.

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 (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 approach in which all bilayer membrane normals are randomly or perpendicularly aligned with respect to the magnetic field in the NLSL program.[6] The **A** and **g** tensoral values were taken from Inbaraj et al.[4] Standard variables and procedures involved in the mathematical calculations were taken from the literature.[6−8]

The EPR spectrum of TOAC18 labeled AChR M2δ domain aligned perpendicular to the static magnetic field inside the pores of nanoporous aluminum oxide is shown in Figure 2A. The line shape and hyperfine splittings in the aligned EPR spectrum is dramatically different than the powder spectrum shown in Figure 2B. The lipid bilayers in the AAO substrates cannot be aligned such that the bilayer normal is parallel with $\mathbf{B}_o$.[2] To authenticate alignment of the oriented EPR spectrum, ^{31}P NMR spectra of aligned and powder spectra are shown in the insets of parts A and B of Figure 2, respectively. The sharp single ^{31}P peak in the Figure 2A inset at 15.8 ppm corresponds to the $\sigma_\perp$ perpendicular turning point of the powder spectrum (inset of Figure 2B). The ^{31}P NMR spectra clearly indicate that the bilayer normal of Figure 2A is aligned perpendicular to $\mathbf{B}_o$.

A detailed description of the matrix algebra needed to calculate the angle between the long helical axis vector (**h**) and the bilayer normal **n** (angle ϕ) from the experimentally determined angle between the director tilt vector ($\mathbf{Z}_D$) (axis of motional averaging) and the bilayer normal (angle ζ) of a parallel aligned bicelle sample is found in Inbaraj et al.[4] Briefly, the following expression can be

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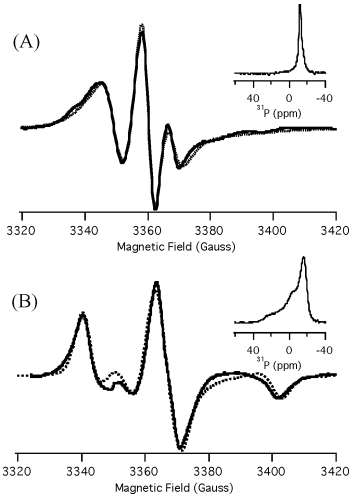

Figure 2. EPR and ^{31}P NMR spectra of TOAC18 spin-labeled M2δ AChR incorporated into phospholipid bilayer nanotube arrays from AAO substrates: (A) perpendicularly aligned TOAC18 M2δ AChR EPR spectrum; inset shows perpendicular aligned ^{31}P NMR spectrum; (B) randomly oriented TOAC18 EPR spectrum (obtained by rotating the sample in (A) by 90°); inset shows randomly dispersed ^{31}P NMR spectrum. The solid lines are experimental EPR data and the dotted lines are MOMD simulations. NMR spectra were acquired on a 500 MHz Bruker NMR spectrometer; 1 K scans were averaged at 37 °C.

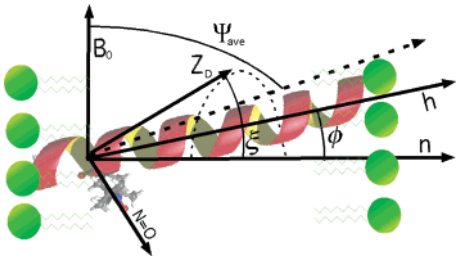

Figure 3. Illustration of the angles used to determine the helical tilt of a perpendicularly aligned spin-labeled peptide sample.

used when $\mathbf{B}_0$ makes an angle (ψ) with $\mathbf{Z}_D$:[4]

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

For the parallel-aligned sample, the director tilt angle (ψ) with respect to $\mathbf{B}_0$ is equal to the director tilt angle (ζ) with respect to **n**. However, to calculate ζ for perpendicularly aligned bilayers, the algorithms for the parallel case need to be modified (Figure 3). When $\mathbf{B}_0$ is perpendicular to the bilayer normal, the azimuthal axes of the bilayers are randomly averaged within the sample on the macroscopic scale. Thus, a distribution of the director angles ψ with respect to $\mathbf{B}_0$ from $\pi/2 - \zeta$ to $\pi/2$ exists. The averaged angle ψ_{exp} can be written as

$$\langle \cos \psi_{ave} \rangle = \int_{\pi/2-\zeta}^{\pi/2} \cos \psi \, d\psi / \zeta \quad (2)$$

where the integration was taken among all the azimuthal orientations in the first quarter due to symmetry constraints.

The hyperfine splitting value measured in Figure 2A was $\mathbf{A}_{exp}$=10.2 G for the perpendicularly aligned spectrum, and $\mathbf{A}_{||}$ = 30.8 G and $\mathbf{A}_{\perp}$ = 7.2 G was obtained from the randomly dispersed spectrum (Figure 2B). Using eq 1, the angle ψ between the director axis and the magnetic field can be calculated as 76 ± 2° which agrees well with the angle (75 ± 5°) obtained from the best fit of the simulation using the MOMD program. Numerical calculations using eq 2 from ψ yield a director tilt angle ζ = 28 ± 4° with respect to the bilayer normal **n**. By taking into account this director tilt angle (28°) gleaned from the EPR spectrum of the perpendicular-aligned samples, 21° deviation of $\mathbf{Z}_D$ with respect to the helical axis based upon crystal structures, and unitary transformations involving three Euler angles (α, β, γ), a helix tilt angle of 15 ± 4° (error estimated from EPR hyperfine splittings) is calculated with respect to the membrane normal.[4] This predicted angle agrees well with the 12° tilt predicted from PISEMA NMR experiments, and the 14° tilt predicted from molecular dynamic simulations and bicelle studies.[4,9,10]

A novel EPR spectroscopic method has been developed for the first time to align spin-labeled peptides in nanotube bilayer arrays to determine the helical tilt of the peptide with respect to the membrane. Previously, CW-EPR and power saturation immersion depth measurements have been used to estimate the topology of membrane proteins using the flexible MTSSL spin label.[5,7] In this communication, the rigidly coupled TOAC spin label is used to more accurately determine the helical tilt of the peptide with respect to the membrane. The anisotropic hyperfine splitting gleaned from the perpendicularly aligned EPR spectrum and a straightforward calculation were used to determine the helical tilt of the peptide. The efficiency and simplicity of the sample preparation (30 min), quick EPR data collection (1 EPR spectrum, 42 s), and minimal amount of sample used (only 50 μg of peptide) indicate that this new powerful method is very advantageous when compared to other biophysical techniques. For example, single-site aligned ^{15}N NMR experiments require approximately 1 day of experimental time and approximately 2 mg of ^{15}N-labeled peptide. However, the incorporation of the spin label is bulkier than a ^{15}N NMR label. Currently, this method relies upon the incorporation of the TOAC spin label via Fmoc peptide chemical synthesis. Future studies will explore the use of alternative spin labels and biochemical methods such as tRNA suppressor techniques that will enable larger integral membrane proteins to be studied.[11]

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