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Geometry of Hydrogen Bonds Formed by Lipid Bilayer Nitroxide Probes: A High-Frequency Pulsed ENDOR/EPR Study

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Electrostatic interactions and formation of hydrogen bonds are known to play the governing role in protein folding and function as well as in efficient charge separation and stabilization in enzymes, where hydrogen-bonding networks provide pathways for proton-coupled electron-transfer processes. The same interactions also define insertion of membrane proteins into lipid bilayers.[1] Detailed analysis of the thermodynamics of the insertion of small model peptides demonstrated that both favorable hydrophobic interactions and formation of hydrogen bonds between amino acid residues are required to lower the thermodynamic penalty of dehydrating the peptide bonds when placed inside the bilayer hydrocarbon core.[2] Other sources of hydrogen-bond donors are water molecules that have significant penetration into the lipid bilayer hydrocarbon core.[3−5]

Initial evidence of water penetration into lipid bilayers has been obtained by spin-labeling EPR.[3] Consequently, the bilayer hydration profile was derived from Gaussian fits of the X-ray diffraction data.[4,5] Further details on the bilayer water penetration were provided by electron spin−echo envelope modulation spectroscopy (ESEEM) that detected two distinct populations of ^{2}H.[6] On the basis of the DFT calculations, these components were assigned to water (^2H_2O) molecules that are (i) hydrogen-bonded to spin-labeled lipids and (ii) free (unbound) water. The ESEEM water penetration profile was found to be similar to the polarity profiles derived earlier from measuring magnetic parameters, principal axis components A_{zz} and g_{xx}, of spin-labeled stearic acids and lipids from rigid-limit continuous wave EPR spectra.[3,7,8]

Magnetic parameters of nitroxide spin labels are known to be sensitive to intermolecular interactions and, in particular, to hydrogen bonding and local solvent polarity.[3] The solvent effects on nitroxide magnetic parameters can be easily detected by EPR at high magnetic fields from accurate measurements of both A_{zz} and g_{xx}, providing better means to elucidate hydrogen bonding and electrostatic effects.[7,8] Experimental and theoretical calibrations of electric field effects on nitroxide EPR spectra also were reported,[9−11] indicating that formation of the hydrogen bond between the nitroxide moiety and water should have the major effect on g_{xx}, while the dielectric constant provides a minor contribution affecting A_{zz} and g_{xx} in a correlated way.[11]

It should be noted that, while formation of hydrogen bonds between the nitroxide moiety and water or alcohols has been implicated and discussed in many spin-labeling polarity studies,[3,6−8,11] none of these experiments provided detailed determination of parameters of the bond formed. We are interested in understanding the fundamental nature of the hydrogen bonds between the nitroxides and the likely donors and exploring these data for studying proteins in a lipid bilayer environment.

Here we report on high-frequency (HF) ENDOR studies of a lipid bilayer spin probe—5-doxyl stearic acid (5DSA). This probe is routinely used in biophysical studies of phospholipid bilayers,[3] is soluble in a variety of protic and aprotic solvents, and is structurally similar to spin-labeled lipids. A series of polar hydrogen-bond donor alcohols (such as deuterated 2-propanol-d_1, butanol-d_1, ethanol-d_1, and methanol-d_1) and nonpolar deuterated toluene-d_8 were used as solvents to determine (i) the magnitude of magnetic interactions between the 5DSA electronic spin and deuterium of the hydroxyl group, (ii) the existence of any correlations between the hydrogen bond length and the solvent polarity and (iii) the hydrogen bond geometry.

HF D-band (130 GHz) EPR and ENDOR measurements were carried out with a spectrometer described previously.[12] Field-swept echo-detected EPR spectra of 5DSA frozen solutions were recorded at $T = 25$ K using a sequence of two pulses of 50 and 100 ns in length separated by a 350 ns delay and repeated at a 500 Hz rate. Typical spectra are shown in Figure 1. Notably, while a single g_{xx} component was observed for toluene (Figure 1C) and THF (not shown) solutions, a partial splitting was detected for all alcohols studied (e.g., Figure 1B). The low-field component characterized by g^0_{xx} was assigned to nitroxides that are not engaged in hydrogen bonds with solvent molecules, and the high-field component g^1_{xx} was attributed to nitroxide forming a single hydrogen bond. While some correlation between g_{xx} components and A_{zz} has been observed (not shown), the variations in g^0_{xx} and g^1_{xx} with the type of alcohol did not exceed the experimental error. Previously, a partial splitting in the g_{xx} component of spin-labeled lipid bilayers was also observed; it was explained by the presence of free- and hydrogen-bonded nitroxides in the bilayer.[8]

In order to directly probe the structure of 5DSA hydrogen bonds to the likely donors, pulsed Mims-type ^{2}H-ENDOR spectra were recorded from this protonated spin label at three magnetic positions corresponding to principal axis orientations of the g-matrix, g_{xx}, g_{yy}, and g_{zz} (approximate positions are marked by dashed lines in Figure 1). Selected experimental spectra are shown in Figure 2. All experimental ^{2}H-ENDOR spectra recorded in four alcohol solvents were found to be similar: a central peak observed at the ^{2}H-Larmor frequency was superimposed with a doublet of symmetric lines (Figure 2B). The relative intensity of the central peak with respect to the doublet varied with the solvent, indicating that the former originated from the "matrix" deuterons. Intensity of the central line was found to correlate with the number of deuterons in the remote coordination spheres of the nitroxide moiety. Furthermore, for nonpolar solvent toluene-d_8, only the central matrix ENDOR line was detected, thus, reassuring the initial assignment. Therefore, it was concluded that the doublet ENDOR line should arise from the splitting on the ^{2}H of the hydrogen bond. This splitting was found to be different for the x-, y-, and z-principal axis orientations (Figure 2B). For all four alcohols, the magnitudes

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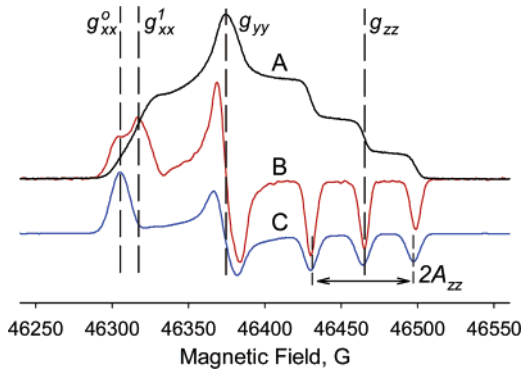

Figure 1. Echo-detected 130 GHz EPR spectra from 5DSA in deuterated 2-propanol-d_1 (A) and its first derivative (B), and in deuterated toluene-d_8 (C, derivative), $T = 25$ K. Dashed lines mark positions of principal axis components. Note partial splitting of g_{xx} for 2-propanol-d_1 (B).

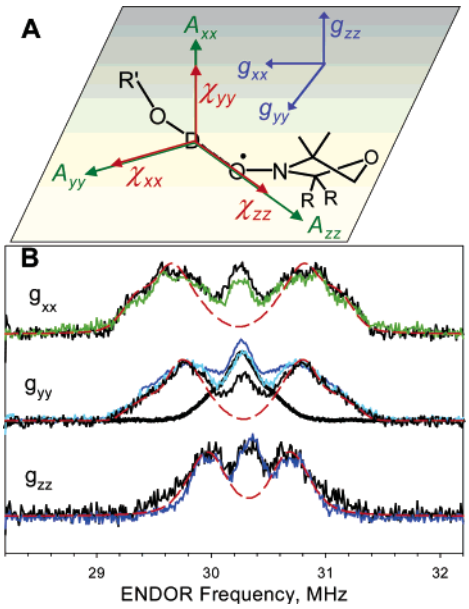

Figure 2. (A) Orientation of the 5DSA g-matrix and the bridging ^{2}H hyperfine (A_{xx}, A_{yy}, A_{zz}) and nuclear quadrupole (χ_{xx}, χ_{yy}, χ_{zz}) tensors in the molecular frame of 5DSA coordinated with an alcohol molecule D−O−R′. Note that vectors g_{xx}, g_{yy}, A_{yy}, and A_{zz} lie in the same plane, and that tensors A and χ are collinear. (B) Superimposed Mims-type HF ENDOR spectra of 5DSA in alcohols (black, 2-propanol-d_1; green, butanol-d_1; cyan, methanol-d_1; blue, ethanol-d_1) and toluene-d_8 (bold line for g_{yy} orientation). Dashed red lines: least-squares simulations.

of the splittings as well as line shapes were nearly identical. This indicates that both the strength of the hydrogen bond (i.e., spin to ^{2}H distance) and the bond geometry remain essentially the same for all four alcohol solvents studied. Simulations of the ENDOR spectra were carried out with a package "SimBud" provided by Dr. Astashkin (University of Arizona).[13] In order to decrease the number of adjustable parameters, several assumptions were made. First, it was assumed that the deuteron of the hydrogen bond lies in the plane of the nitroxide ring. Second, the ^{2}H hyperfine and the nuclear quadrupole axes are collinear and determined by the direction of the hydrogen bond (Figure 2A). Third, the main contribution to the anisotropic hyperfine interaction arises from the unpaired electron spin density of the p_Z orbitals of oxygen and nitrogen, set to 47.5% for both. Finally, direct calculations of the hyperfine tensor[14] were carried out instead of an oversimplifying point dipole approximation. Under these assumptions, we have carried out simultaneous least-squares fitting of the ENDOR spectra for all three principal axis orientations of the 5DSA g-matrix.

Table 1. Best-Fit Parameters for ENDOR Spectra Simulations[a]

A_{xx}	A_{yy}	A_{zz}	χ_{xx}	χ_{yy}	χ_{zz}	$R_{O\cdots D}$[b]	φ_{NOH}
0.72	1.60	−1.72	−0.19	−0.08	0.27	1.74 ± 0.06	120 ± 10

[a] Components of ^{2}H hyperfine coupling A tensor, A_{ii}, and nuclear quadrupole χ tensor, χ_{ii}, are given in megahertz; hydrogen bond length, $R_{O\cdots D}$, is given in angstroms, and angle, φ_{NOH}, between the hydrogen and the N···O bond is in degrees. [b] A distribution of hyperfine coupling parameters of $\delta A_{ii} = 0.1$ MHz was assumed.

The best-fit results summarized in Table 1 lead to the following conclusions: (i) the hydrogen-bonded deuteron makes an angle of ∼120° with the N−O bond, (ii) the bond length is ∼1.74 Å, and (iii) the best fit is obtained for a distribution of hyperfine coupling parameters, $\delta A_{ii} = 0.1$ MHz. While these distances and geometries agree well with theoretical studies[11] and ENDOR data on hydrogen bonds formed by organic radical cofactors,[15,16] molecular modeling of the nitroxide−ethanol complex indicates small potential van der Waals contacts of the alcohol with the nitroxide methyl groups.

Overall, we have provided direct experimental demonstration of hydrogen bonds formed by a lipid bilayer spin probe in hydrogen-bond donor solvents and have determined the bond geometry. A correlation between the appearance of the high-field g^1_{xx} component (Figure 1B) in the rigid limit HF EPR spectra and the characteristic doublet component in the ENDOR spectra has been observed. The length of the hydrogen bond and its geometry were found to be essentially the same for all alcohols studied, indicating that nearly identical hydrogen bonds have been formed regardless of the solvent dielectric constant. This strengthens a hypothesis that HF EPR spectra are exclusively sensitive to formation of hydrogen bonds and could be used to probe the hydrogen-bond network in complex biomolecular assemblies and lipid bilayers by site-directed spin-labeling.

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Supporting Information Available: Experimental procedures, details of simulations, and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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