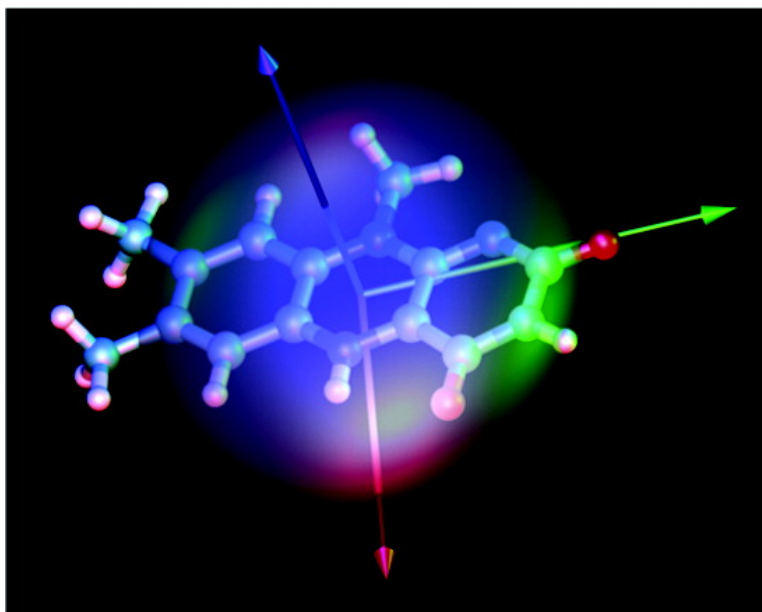Communication

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Unambiguous Determination of the g-Matrix Orientation in a Neutral Flavin Radical by Pulsed Electron−Nuclear Double Resonance at 94 GHz

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Photoinduced radical pairs comprising a flavin radical and an oxidized amino acid residue (tyrosine or tryptophan) have recently been observed in various blue-light-sensitive proteins including the DNA-repair enzymes (6−4) photolyase[1] and DNA photolyase,[2] and the cryptochromes which are involved in setting the circadian clock in humans, animals, and plants.[3] Furthermore, a radical-pair mechanism involving flavin-radical intermediates has been suggested[4,5] as the magnetic-field dependent process underlying navigation of migratory birds. Strong support for flavin-radical intermediates has also been garnered for the primary events of blue-light directed plant growth, chloroplast relocalization, and the opening of stomata mediated by the phototropin family of flavin−chromophore photoreceptors.[6−8]

Electron paramagnetic resonance (EPR) has the unique capacity to identify radicals in intermediate radical-pair states, as has been demonstrated in studies on photosynthetic reaction centers,[9,10] provided the anisotropies and orientations of the **g**-matrices of the individual radicals are known. From high-frequency/high magnetic field EPR, such information is available for tyrosine[11] and tryptophan[12] radicals which are also involved in the reactions mentioned above. However, respective information on the **g**-matrix of flavins is incomplete.

In a recent study of the flavin−adenine dinucleotide radical cofactor, FADH•, in *Escherichia coli* DNA photolyase, we have measured the principal values of the **g**-matrix using EPR at 360 GHz and 12.8 T:[13] $g_X = 2.00429(2)$, $g_Y = 2.00359(2)$, and $g_Z = 2.00218(2)$.[13,14] X, Y, and Z denote the directions of the principal axes. For aromatic systems, Z is usually aligned perpendicular to the π-plane. As the isoalloxazine moiety in FADH• does not significantly deviate from planarity, the Z direction of **g** is well defined. However, in these experiments, the orientation of the X and Y axes could not be unambiguously established.

In this contribution, we determine the orientation of the **g**-matrix of FADH• by electron−nuclear double resonance (ENDOR) at 95 GHz (W-band) and 3.5 T. At such high values of the microwave frequency and the magnetic field, the **g**-anisotropy provides improved EPR spectral resolution (see Figure 1A) compared to experiments performed at conventional 9.5 GHz (X-band) and 0.35 mT.[15] This enables one to utilize Zeeman magnetoselection to obtain single-crystal-like ENDOR data from disordered samples in frozen solution. Experiments exploiting this orientation selection have allowed us to use the hyperfine coupling (hfc) of the methyl protons H(8α) of FADH• to determine the angle between the molecular frame and the principal axes of **g**.

Figure 1A depicts the 2-pulse electron-spin−echo detected W-band EPR signal of FADH• of *E. coli* DNA photolyase recorded at 80 K. The signal shape is asymmetric due to the anisotropy of
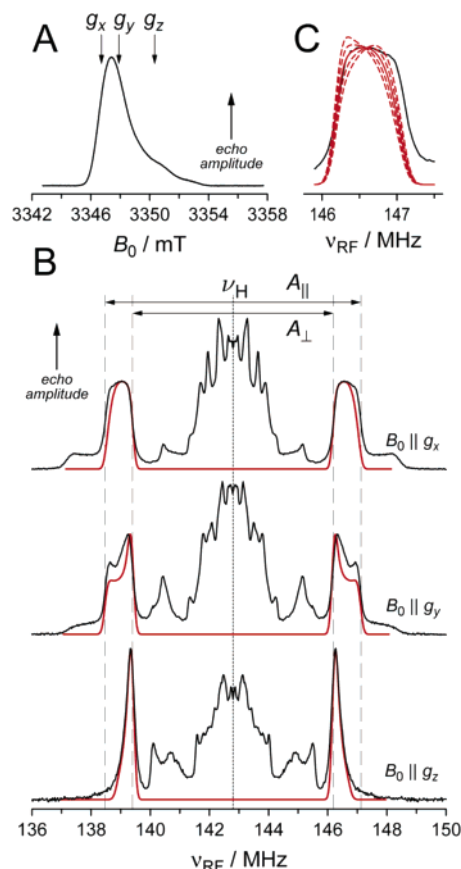
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Figure 1. W-band 2-pulse electron-spin−echo detected EPR spectrum of FADH• in *E. coli* DNA photolyase in deuterated buffer (A). A $\pi/2-\tau-\pi$ pulse sequence with 240-ns $\pi/2$ pulses and $\tau = 400$ ns was used. (B) Symmetrized W-band pulsed ENDOR spectra recorded at magnetic-field values corresponding to the resonant positions of the principal components of the **g**-matrix as described in (A). The spectra were obtained using a standard Davies-type ENDOR sequence with 120-ns $\pi/2$ pulses and a radio frequency pulse of 20 μs. The red curves show simulations of the H(8α)-proton ENDOR signals using $\varphi = +46°$ and $\delta = -14°$ (for details, see text and Figure 2). In (C), the variation of the simulated ENDOR spectra for the g_X orientation is shown as the angle φ is changed from 42° to 50° in 2° steps. All spectra have been obtained with EPR instrumentation described previously.[14]

the Zeeman interaction, which under solid-state conditions is dominant at W-band. By recording ENDOR spectra at different magnetic fields within the FADH• EPR spectrum, hyperfine spectra are obtained that contain only signals arising from the subset of those molecules that are on resonance at the respective position in the EPR spectrum. Such orientation-selective spectra in the proton-ENDOR range are depicted in Figure 1B. In general, the ENDOR spectrum for a nucleus n with spin $1/2$ is composed of paired transitions that, to first order, are given by $\nu^{\pm} = |\nu_n \pm A/2|$, where
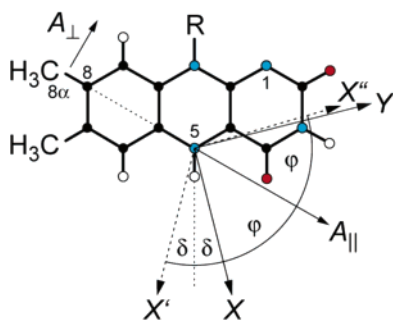
Figure 2. 7,8-Dimethyl isoalloxazine moiety of FADH•. R denotes the ribityl side chain. $|\varphi|$ represents the angle between the $A_{\parallel}$ component of $\mathbf{A}(H(8\alpha))$ and the X axis of $\mathbf{g}$ (giving either X or X''), and $|\delta|$ is the angle between the $N(5)-H$ bond and the X axis of $\mathbf{g}$ (giving either X or X'). The consistent geometrical solution gives the directions of the X and Y principal axes of the $\mathbf{g}$-matrix with respect to the molecular plane. The Z-axis of $\mathbf{g}$ is oriented perpendicular to the molecular plane of FADH•.

ν_n is the nuclear Larmor frequency, and A is the orientation-dependent hfc constant. As the main features of the proton ENDOR spectrum have already been discussed in detail for experiments at 9.5 GHz,[15] we will restrict this discussion to the prominent features arising from hfcs in the 6−9-MHz range of the W-band ENDOR spectra. These have been assigned to the protons of the methyl group at C(8) of the isoalloxazine ring.

Typically, methyl groups rotate freely about their C−C bond at temperatures >30 K. Hence, at 80 K, the three hfc tensors of the individual methyl group protons H(8α) in FADH• merge to only one hyperfine tensor, $\mathbf{A}(H(8\alpha))$ with axial symmetry; the component $A_{\parallel} = (8.66 \pm 0.1)$ MHz is aligned along the C(8α)−C(8) bond, and the second component, $A_{\perp} = (6.8 \pm 0.1)$ MHz, is aligned perpendicular to this direction. In the pulsed ENDOR spectrum that was taken at a magnetic-field position at which the molecules with their Z axis parallel to the external magnetic field are on resonance, $B_0 \parallel g_Z$, only the $A_{\perp}$ component is expected to be visible, as is indeed observed. For the ENDOR spectra taken at the g_X and g_Y orientations in Figure 1B, both components, $A_{\parallel}$ and $A_{\perp}$, are observed: the relative intensity depending on the angle between the hyperfine and $\mathbf{g}$ principal axes, and the degree of orientation selection. At the g_X (and g_Z) position of the EPR signal of a nonoriented sample, single-crystal-like spectra are expected, while in the center of the signal at g_Y, a more isotropic spectrum is anticipated, and is indeed observed.

Hence, to determine the angle φ between $A_{\parallel}$ and the X-axis of $\mathbf{g}$, Figure 2, we have varied φ in ENDOR simulations until the best agreement between experimental and calculated spectra was obtained. In Figure 1C, simulations of the ENDOR signal of the methyl group protons at C(8α) are shown with angles in the range between 42 and 50°, clearly demonstrating the dependency of the line shape on the angle, and giving an optimal agreement with $|\varphi| = (46 \pm 2)°$. Note that we have concentrated on the inner shoulder of the line, as the outer part also contains contributions from the protons at C(1′) on the ribityl side chain of FADH•.

Using the principal values for the $\mathbf{A}(H(5))$ hfc tensor from pulsed X-band ENDOR,[14] we have recently estimated the angle $|\delta|$ between the $N(5)-H$ bond and the X axis of $\mathbf{g}$ from the splitting of the g_Y line in 360-GHz EPR spectra to be $|\delta| \approx 17°$.[13] Thus, taking the information on the orientation of $\mathbf{g}$ with respect to both hfc tensors, $\mathbf{A}(H(8\alpha))$ and $\mathbf{A}(H(5))$, there is only one consistent geometric

solution: With the principal axes system of $\mathbf{g}$ as the reference coordinate frame, the angle between the X axis and the C(8α)−C(8) bond is $\varphi = (+46 \pm 2)°$, and the angle between X and the $N(5)-H$ bond is $\delta = (-14 \pm 2)°$. It would be interesting to compare this finding with theory; however, predictions of the $\mathbf{g}$-matrix orientation of flavins using various quantum-chemical methods have thus far yielded inconsistent results.[13]

Quite surprisingly, the $\mathbf{g}$-matrix in FADH• is not oriented as one would have expected for a 1,3-semibenzoquinone radical.[13] For the latter, the X-axis of $\mathbf{g}$ commonly bisects the smaller angle between the two axes along the C=O bonds. In FADH•, the large spin density on N(5) and C(4a) apparently contributes to a significant (44°) reorientation of the $\mathbf{g}$-matrix principal axes. In the assumption that the sign obtained here is generally applicable for protein-bound neutral flavin radicals, then taking published data for Na+-translocating NADH:quinone oxidoreductase,[16] a consistent but slightly larger deviation (56°) is obtained.

To conclude, we have for the first time precisely determined the orientation of the $\mathbf{g}$-matrix with respect to the molecular frame of a protein-bound flavin radical. By exploiting Zeeman magnetoselection at high magnetic fields on nonoriented samples, this information has been obtained without the use of single crystals, which considerably reduces the experimental effort. We expect that these results will form the foundation for future analyses of radical-pair intermediates in flavoproteins.[10]

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