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Determination of the Distance between the Two Neutral Flavin Radicals in Augmenter of Liver Regeneration by Pulsed ELDOR

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Proteins that form weak intermolecular complexes with other proteins or DNA/RNA play important roles in cell biology, yet obtaining structural information is often difficult, as crystallography cannot always solve the structures of intermolecular complexes.[1−3] Docking calculations to assess the likely disposition of interacting partners provide an emerging alternative,[4] but these studies often generate multiple orientations that ultimately require experimental validation.

A powerful method for obtaining accurate spatial information in macromolecular structures is electron paramagnetic resonance (EPR).[5−7] Owing to the large magnetic moment of the electron (compared, for example, with that of the proton), EPR can determine distances up to 80 Å. Distance measurements by magnetic resonance techniques depend on the magnitude of the dipole−dipole coupling, ν_{DD}, between the two spins, which is given in the EPR case by eq 1:

$$\nu_{DD}(\theta, r) = \frac{g_1 g_2 \mu_0 \mu_B^2}{4\pi h} \frac{1}{r^3}(3\cos^2\theta - 1) \qquad (1)$$

where g_1 and g_2 are the g values of the two spins, r is the interspin distance, and θ is the angle between the spin−spin vector and the external magnetic field.

Distances up to 15 Å may be readily determined by line-shape analysis of continuous-wave EPR spectra,[8−10] while for larger distances, pulsed techniques are required to separate the relatively small dipolar coupling from the other magnetic interactions.[11−13] Here we use pulsed electron−electron double resonance (ELDOR), whose potential for the determination of distances between pairs of nitroxide spin labels has been illustrated by studies on rigid model systems,[12,14] peptides,[15] and RNA.[16]

Several groups have applied the same technique to naturally occurring radicals and paramagnetic centers, an approach which allows the native protein to be used. Thus, distances have been determined between the manganese cluster and the redox-active tyrosine residue, Y_D, in Photosystem II,[17] between the two tyrosyl radicals in *Escherichia coli* ribonucleotide reductase[18] and between the catalytic [NiFe] center and a $[3Fe-4S]^+$ cluster in hydrogenase.[19] To date, however, there is no report of an ELDOR experiment involving a flavin radical (see Figure 1), although this is one of the most ubiquitous biological cofactors.

In this contribution, we employ pulsed ELDOR to obtain structural information from Augmenter of Liver Regeneration (ALR), which is a homodimeric FAD-dependent sulfhydryl oxidase. Sulfhydryl oxidases catalyze the formation of disulfide bonds using molecular oxygen as the electron acceptor. However, it has recently been shown that ALR can also utilize cytochrome *c* as an alternative

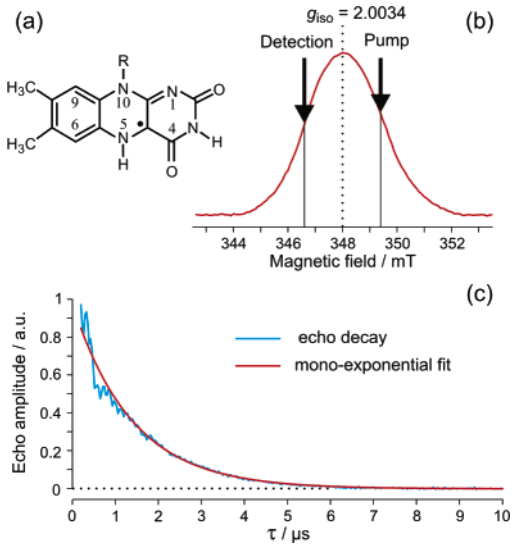

Figure 1. (a) Molecular structure of the redox-active isoalloxazine moiety of the flavin cofactor in its neutral radical form, where R indicates the ribityl side chain. The center of gravity of the electron spin density is close to C4a, approximately where the ● is depicted. (b) Field-swept electron spin−echo spectrum of the radical form of ALR recorded at 80 K with an X-band Bruker ELEXSYS E580 spectrometer. A two-pulse echo sequence ($\pi/2-\tau-\pi$) was used with a 32 ns π-pulse and τ = 200 ns. The arrows indicate the positions of the detection and pump frequencies ($\Delta\nu$ = 78.4 MHz ≡ 2.8 mT) in the three- and four-pulse ELDOR experiments shown in Figure 2. They are placed symmetrically around the maximum of the spectrum. (c) Decay and monoexponential fit of the echo amplitude with increasing τ, recorded at the maximum of the spectrum in (b). Traces (b) and (c) were both recorded with a repetition rate of 200 Hz due to the long T_1 of the neutral flavin radical.

electron acceptor,[20,21] thereby coupling the formation of protein disulfide bonds to the respiratory chain. It has also been demonstrated that, under aerobic turnover with dithiothreitol (DTT), large, possibly stoichiometric, amounts of neutral flavin radical (i.e., two per homodimer) are formed in ALR.[20,22] To explain this observation, it was suggested that DTT (a two-electron reductant) reduces the flavin in one subunit to $FADH_2$, and that subsequently a comproportionation reaction between the reduced $FADH_2$ and the oxidized FAD in the other subunit results in the formation of two neutral flavin radicals.

The crystal structure of recombinant rat ALR, which has been determined to 1.8 Å, shows that the closest distance between isoalloxazine rings is about 19 Å.[22] Hence, the comproportionation reaction could be facilitated by electron tunneling between the subunits. Here we have used the short form of human ALR expressed in *E. coli*,[20] allowing us to test whether the distance between the flavins in the crystal structure of the rat enzyme may be used as a basis for mechanistic discussions of the human enzyme.

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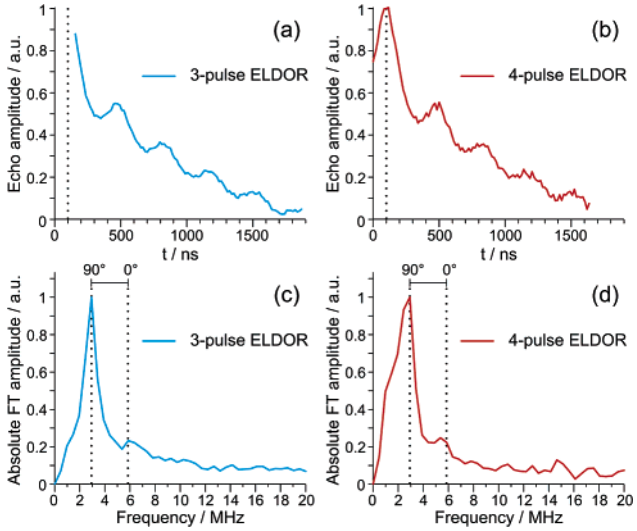

Figure 2. (a) Three-pulse and (b) four-pulse ELDOR time traces from the radical form of ALR at 80 K. Both traces were recorded with a repetition rate of 200 Hz. The three-pulse experiment was based on a two-pulse echo sequence ($\pi/2-\tau-\pi$) with $\tau = 1800$ ns. The four-pulse experiment was based on a refocused echo sequence ($\pi/2-T-\pi-\tau-\pi$) with $T = 200$ ns and $\tau = 1600$ ns. A 32 ns π-pulse was used in both experiments. (c and d) Fourier transforms of the traces in (a) and (b) following subtraction of the decay (fitted by a third-order polynomial), Hamming filtering, and zero-filling.

In Figure 1b, the field-swept electron spin−echo spectrum of the blue radical form of human ALR recorded at 80 K is presented. The spectrum is centered at $g_{iso} = 2.0034$, which is typical for neutral flavin radicals.[23]

It is important for the success of distance determinations that the phase memory relaxation time, T_M, is not too short. Hence, we have also recorded the variation of the echo amplitude as a function of pulse separation, τ (Figure 1c). The deviations at early time are due to electron spin−echo envelope modulation (ESEEM), but this decays after approximately 1 μs, and the latter part of the trace can be well-fitted with a monoexponential function, giving a $t_{1/e} \approx 1.4 \pm 0.1$ μs. Hence, the relaxation rate of the flavin radical is slow enough to allow the use of a reasonably long pulse separation, τ, in the ELDOR experiments, without undue loss of echo intensity.

The time-domain traces for three- and four-pulse ELDOR experiments are depicted in Figure 2a,b. Both show the typical features of pulsed ELDOR spectra: an oscillation superimposed on a decay function. The frequency-domain spectra (Figure 2c,d) both show maxima at 2.9 ± 0.3 MHz. Equation 1, which only takes the dipole−dipole interaction into account, is valid here since the radical separation is so large that exchange interactions are expected to be negligible.[11,12] Furthermore, from eq 1, we would expect to observe the full dipolar tensor because the *g*-anisotropy of the neutral flavin radical[23] is so small that it is unresolved at X-band and, therefore, all orientations are excited. This assumption is supported by the observation of a Pake pattern, with a singularity at 2.9 MHz ($\theta = 90°$) and a shoulder around 6 MHz ($\theta = 0°$). Hence, from eq 1, we obtain a dipole−dipole distance of 26.1 ± 0.8 Å for the two FAD radicals bound in human ALR. This distance should, however, be related to the center of gravity of the electron spin density[24] of the flavins, which may be estimated from density functional theory calculations.[25] The center is very close to the maximum electron spin density at C4a, and in the structure of rat ALR, a very similar distance of 26.9 Å can be estimated.[22]

To conclude, we have shown that the crystal structure of rat ALR may be used as a basis for discussions of FAD separations in the human enzyme in solution. The experiments also give independent confirmation of optical data suggesting that both FADs are in the neutral radical form following aerobic treatment of ALR with DTT. Finally, the relaxation behavior, the depth, and slow decay of the ELDOR modulations suggest that flavins have excellent properties to be used as natural spin labels for distance determinations by pulsed ELDOR. This approach is likely to be useful for investigating inter-cofactor distances and electron-transfer reactions in other flavoprotein complexes, such as that formed between trimethylamine dehydrogenase and electron transferring flavoprotein.[3]

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