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High-Frequency 180 GHz PELDOR

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Abstract. For aromatic organic radicals, pulsed electron–electron double resonance (PELDOR) experiments at high magnetic fields offer the possibility to achieve orientation-selective pumping and detection that could allow one not only to determine the distance between paramagnetic species but also their relative orientation with respect to the interconnecting dipolar axis. We present a PELDOR two-frequency setup that was introduced into our homebuilt 180 GHz pulsed electron paramagnetic resonance (EPR) spectrometer and we discuss its technical and experimental features. The capability of 180 GHz PELDOR has been tested using the three-pulse ELDOR sequence on the protein RNR-R2 (ribonucleotide reductase) from *Escherichia coli*, which contains two tyrosyl radicals at a distance of 3.3 nm. At 180 GHz, orientation selectivity is observed and the modulation frequency was found in good agreement with theoretical predictions, which take into account the relative orientation of the radicals from X-ray data.

1 Introduction

Pulsed electron–electron double resonance (PELDOR) is a method that monitors weak dipole–dipole interactions between the electron spins of radicals that span distances between approximately 1.5 and 8 nm [1, 2]. In structural biology, this method has been successfully applied at X-band frequencies to measure distances between native cofactors in proteins such as those in photosystem II [3], in hydrogenase [4], ribonucleotide reductase [5], or using nitroxide spin labels for distances in ribonucleic acid (RNA) [6], desoxyribonucleic acid (DNA) [7] and membrane proteins [8]. In the high-field approximation, the coupling strength of the electron–electron interaction ν_{AB} can be expressed by the following equations as the sum of the orientation-dependent dipole–dipole coupling constant and the isotropic exchange coupling constant J

$$\nu_{AB} = \nu_{Dip}(3\cos^2\theta - 1) + J\,, \tag{1}$$

where

$$\nu_{\mathrm{Dip}} = \frac{\mu_B^2 g_A g_B \mu_0}{4\pi h} \frac{1}{r_{AB}^3}, \tag{2}$$

θ is the angle between the direction of the external magnetic field and the inter-spin axis, r_{AB} is the distance between the paramagnetic species A and B, μ_B is the Bohr magneton, μ_0 is the permeability of vacuum, g_A and g_B are the effective g-values of the unpaired electrons A and B. For distances larger than 1.5 nm, J can be neglected.

The PELDOR experiment described previously [1, 2] in detail is based on the electron spin echo method and consists of a two-pulse Hahn echo with a fixed delay τ at the observer frequency ν_A and an additional pump pulse of a flip angle π at a different frequency ν_B with a variable delay time T with respect to the first pulse at the observer sequence (Fig. 1). Thus, for a spin pair AB, the detection occurs on spin A, whereas pumping is achieved at the second frequency ν_B on spin B. The PELDOR effect is recorded as a modulation of the amplitude in the spin echo signal $V(2\tau)$ as a function of the time position of the pump pulse.

The PELDOR experiment at high magnetic fields is more difficult to implement and perform than that at standard X-band frequency for various reasons. First, the implementation of a second frequency channel in the high-frequency microwave bridge without degrading the pulse performance of the spectrometer is much more demanding. Further, the limited available microwave power renders the pumping pulse more selective. This, combined with the fact that electron paramagnetic resonance (EPR) lines at higher fields usually become much broader because of g-anisotropy, leads to the expectation that the modulation effect will be much weaker than that observed at X-band. On the other hand, the experimental conditions at high fields allow one to perform an orientation-selective experiment, which can additionally provide the relative molecular orientation of the two radicals with respect to each other. Recently, Bruker BioSpin reported the first PELDOR modulation trace between nitroxide biradicals at 94 GHz [9].

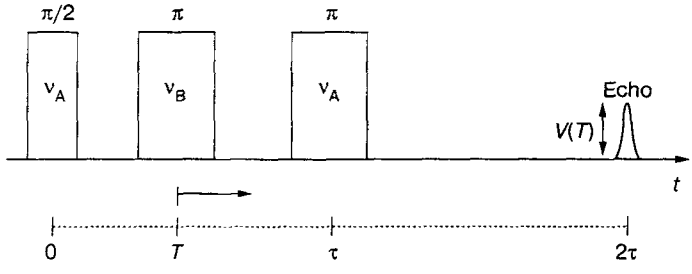

Fig. 1. Three-pulse ELDOR sequence. ν_A, detection frequency; ν_B, pump frequency; τ, time distance between preparation and detection pulses; T, current position of pump pulse in time.

In this paper we report the first PELDOR experiment recorded at 180 GHz after modification of our previously reported homebuilt EPR spectrometer [10]. The PELDOR setup has been tested with a sample of ribonucleotide reductase RNR-R2 from *Escherichia coli*, which contains two tyrosyl radicals at a distance of 3.3 nm. Experimental results were compared with calculated frequencies predicted from the relative orientations of the radicals obtained from X-ray data.

2 Experimental

2.1 Microwave Bridge

The 180 GHz pulsed EPR microwave bridge [10] has been extended for a two-frequency experiment by inserting an additional frequency channel into the transmitter at the 45 GHz stage, before the frequency multiplication chain, to avoid the introduction of additional less efficient G-band components. The new oscillator can be tuned manually in the range of 44–46 GHz to adjust the desired pumping position. The pulses of both frequencies are formed at the 45 GHz stage by fast switches (5 ns rise/fall time) in the two separate channels that are combined via a magic-*T* and then fed into the multiplication/amplification stages of the transmitter. The exact frequency at the pump and probe pulses is monitored with an HP8563 spectrum analyzer which is connected to the magic-*T*.

2.2 Experimental Conditions

The time traces were recorded with the three-pulse sequence displayed in Fig. 1, by pumping at a fixed resonance position in the EPR powder spectrum corresponding to the maximal signal amplitude ($B \parallel g_2$) and varying the resonance position of detection (Fig. 2). Since the detection frequency of the microwave bridge is fixed (ν_A = 180 GHz), the four different resonances of detection were set by adjusting the external magnetic field values. This led to the following experimental settings: position 1, B = 64185 G, ν_B = 180.1 GHz; position 2, B = 64168 G, ν_B = 180.05 GHz; position 3, B = 64115 G, ν_B = 179.9 GHz; position 4, B = 64080 G, ν_B = 179.8 GHz.

The conventional cylindrical TE_{011} cavity with a loaded quality factor $Q \approx$ 1000 critically coupled to the bridge was used for the experiment. The cavity was tuned to the observer frequency ν_A of 180 GHz to provide the highest possible detection sensitivity. The microwave pulse power was about 20 mW at both pump and observer frequencies, which translates into an optimal π pulse length at ν_A of about 100 ns and corresponds to a π pulse bandwidth of approximately 10 MHz. The two-pulse echo spacing τ between preparation and detection pulses was set to 2.5 μs. The pulse sequence repetition time was 40 ms. All measurements were done at 3.3 K.

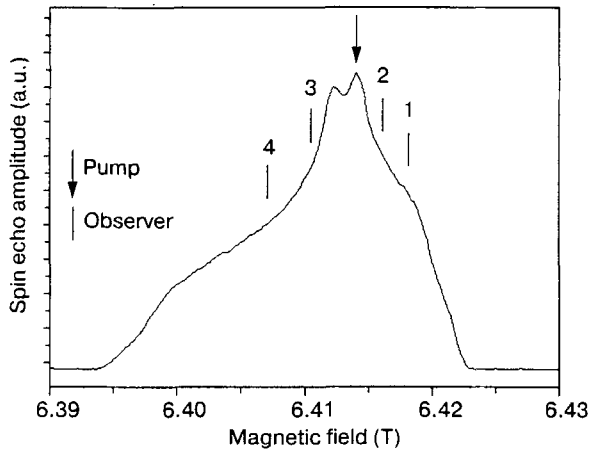

Fig. 2. 180 GHz echo-detected EPR spectrum of the tyrosyl radicals in RNR-R2 with marked pump and detection positions of the PELDOR experiments.

2.3 Sample

As a prototype sample for the first PELDOR experiment at 180 GHz we used a solution (approximately 1 mM) of the protein subunit R2 of *E. coli* ribonucleotide reductase (RNR). R2 is a homodimer that contains two tyrosyl radicals ($Y^{\bullet}$) at a distance of 3.3 nm [5, 11]. Generally, the great advantage of performing PELDOR with endogenous protein biradicals with respect to spin-labeled proteins is that radical cofactors are located at a very precise distance and orientation as determined by their role in the protein function. In this case, the modulation effect does not suffer from damping of the oscillation due to orientation disorder. Secondly, the knowledge of the X-ray crystal structure of this protein enables us to model the expected PELDOR response for the frozen solution sample. Both aspects were essential to explore the feasibility of the PELDOR experiment at 180 GHz.

In the wild-type RNR-R2 protein from *E. coli*, about 1.1–1.3 radicals per R2 dimer have been found in preparations in vitro. Recent X-band PELDOR experiments [5] suggested that at least 25% of these radicals are paired. Since the sensitivity of the PELDOR experiment depends on the number of available radical pairs, we employed the C268S mutant of R2, which exhibited a larger fraction of radicals per dimers (approximately 1.6). Preliminary X-band PELDOR experiments on this mutant had revealed that the distance between the two radicals is not affected by the mutation. The sample was placed into a quartz capillary tube with an inner diameter of 0.4 mm.

3 Calculations

The effect of orientation selectivity in a PELDOR experiment at high fields adds a layer of complexity in the analysis of the modulation traces with respect to the X-band case, since at high fields the EPR line shape is dominated by g-anisotropy and the observed modulation frequency and depth become a function of the spectral positions of pumping and detection pulses [12]. For a spin pair AB, the detection pulse with frequency ν_A spectrally selects orientations for the spins A and a similar orientation selection is achieved by the pumping pulse with frequency ν_B for the spins B. A PELDOR response can only be found if the detected spins A are indeed the partner of the pumped spins B depending on the relative orientation of the radical pair. To predict in our sample the detection positions that give a nonzero PELDOR response as a function of the pumping pulse position, we first determined the relative orientation of the g-tensor principal axes in the radical pair (Fig. 3).

The orientation of the g-tensor with respect to the molecular axes of the tyrosine is well known [13], with the direction of g_1 pointing along the C–O bond and g_3 perpendicular to the ring plane. This information together with the coordinates of the crystal structure of R2 [11] allowed us to determine the rotation matrix $\mathbf{R}_g(g_A \rightarrow g_B)$, which consists of the direction cosines between the g-tensor principal axes of spin A and B. Subsequently, if the experimentally selected g-tensor for the spin A is $g_{eff}(\theta,\varphi)$, the resonance of the partner spin B is calculated as $h\nu_B = B\mu_B \mathbf{R}_g g_{eff}(\theta,\varphi)\mathbf{R}_g^T$, with the constants as defined in Sect. 1. The small effect of the hyperfine coupling was neglected for simplicity.

The calculation was carried out for the tyrosyl radical pair in R2 with pumping at $B \| g_2$ and for an excitation and detection bandwidths given by 100 ns π-pulses. The result is displayed in Fig. 4 and is superimposed to the EPR line shape for comparison. The intensities are normalized and we note that the area under the response curve is given by the fraction of spins excited by the pump

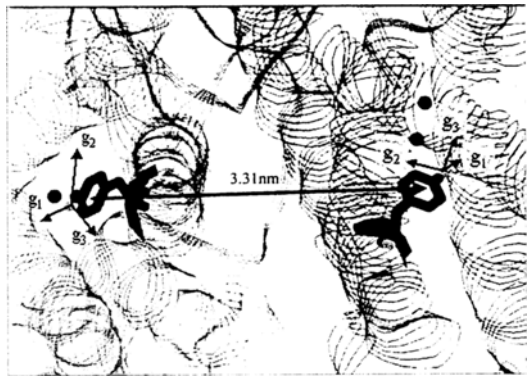

Fig. 3. Relative orientation of the two tyrosyl radicals in the *E. coli* R2 homodimer as depicted from the crystal structure [11].

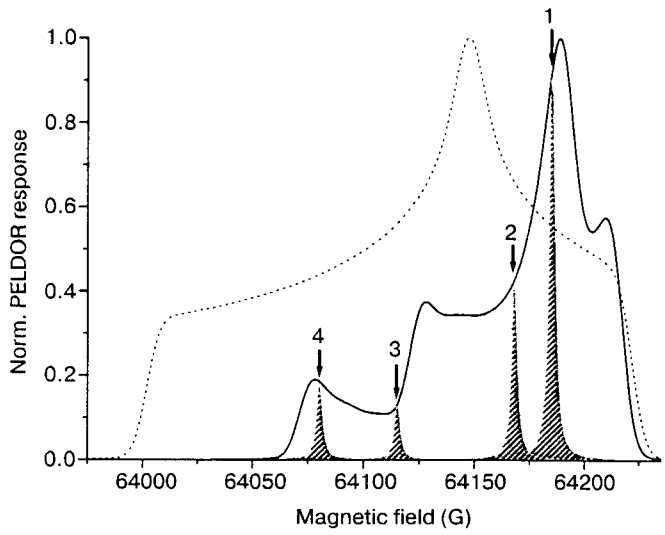

Fig. 4. Computed resonances of the radical pair contribution to the PELDOR signal (PELDOR response) – solid line, for pumping at maximum of the powder spectrum ($B \| g_2$). The EPR powder line shape is displayed as a dashed line for reference. Marked by arrows are the detection positions set in the experiments. The areas marked represent the excitation profiles for 100 ns detection pulses.

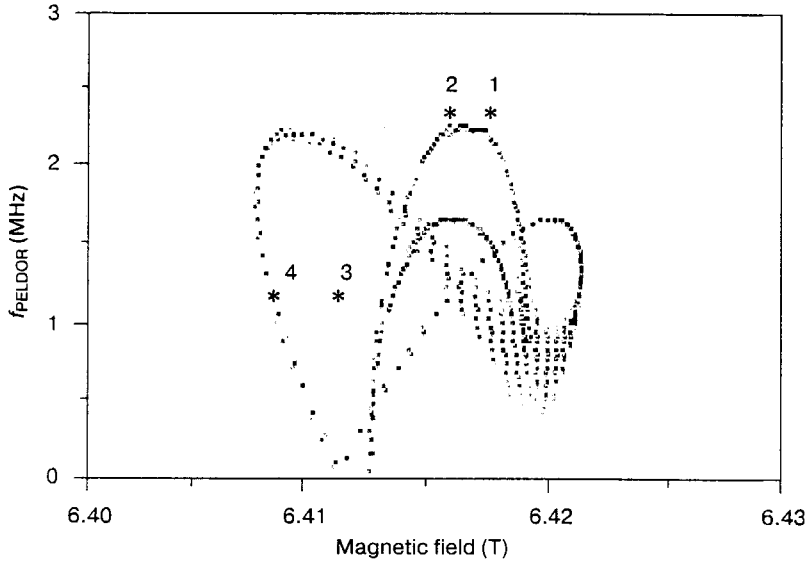

Fig. 5. Calculated PELDOR frequencies with respect to the magnetic field values at detection when pumping at maximum of the powder pattern ($B \| g_2$). Calculated negative frequencies were flipped into the positive range since only positive frequencies are detected. Asterisks represent positions of the PELDOR signal found experimentally.

pulse. The result shows that the largest PELDOR response should be observed when detecting between $B \| g_2$ and $B \| g_3$. The fraction of detected partner spins should ideally correspond to the expected modulation depth [2] if all spins are paired. This number can be calculated by comparing the area under the pulse excitation profile at each observation field with the area under the response curve (Fig. 4). We obtained that at detection $B = 64185$ G (point 1) only 7% of the partner spins are observed and 3.5, 1, and 1.4% at points 2, 3, and 4, respectively. The results indicate that the largest expected modulation effect is rather weak even at detection close to the maximum of the response curve (point 1).

The observed dipolar frequencies are also a function of the orientations selected by the pump and detection pulses. To predict the dipolar frequencies, we determined the orientation of the g-tensor with respect to the dipolar tensor (D) from the coordinates of the X-ray structure and setting the principal axis of D ($D_{\|}$) coincident with the vector connecting the center of mass of spin density distribution, located close to the C–4 atom. Our previous X-band data showed that this last assumption agrees well with the observed distance of 3.31 nm [5].

With knowledge of the principal axis values of the dipolar tensor from the X-band PELDOR data ($D_{\|} = 2D_{\perp} = 2.88$ MHz [5]) the dipolar frequencies were computed as a function of the detecting field position as illustrated in Fig. 5. This result is discussed and compared with the experimental data in Sect. 4. We point out that the whole calculations did not contain free parameters since all structural parameters as well as the magnetic resonance parameters were known. The only assumption concerns the fact that the crystal structure of the R2C286S mutant used in the experiments is identical to the structure of the wild-type protein employed in the calculation.

4 Results and Discussion

Time-domain PELDOR traces for detection at four different positions in the EPR line and pumping at $B \| g_2$ are presented in Fig. 6a. The echo decays display an unusual nonexponential behavior whose origin is currently not understood. We assume that the high concentration of the sample leads to deviations from a random distribution of spins and that intermolecular effects likely play a role. Nevertheless, some traces contain visible oscillations superimposed on the echo decay. The echo decays were fitted with second-order polynomials, subtracted from the time-domain traces and the frequency-domain spectra were obtained after direct Fourier transform (FT) without further manipulation (Fig. 6b). The largest PELDOR response (modulation effect) is observed when detecting at $B = 64185$ G, in agreement with the theoretical prediction (point 1) of Fig. 4. The FT spectrum shows a modulation frequency peak at 2.3 MHz that is close to the $D_{\|}$ value of 2.88 MHz [5]. A similar modulation is observed when detecting at $B = 64168$ G (point 2). Changing the detection position below $B \| g_2$ (points 3 and 4), the modulation effect decreases and a new peak in the FT spectrum appears at 1.17 MHz close to the expected $D_{\perp}$ value of the dipolar Pake pattern.

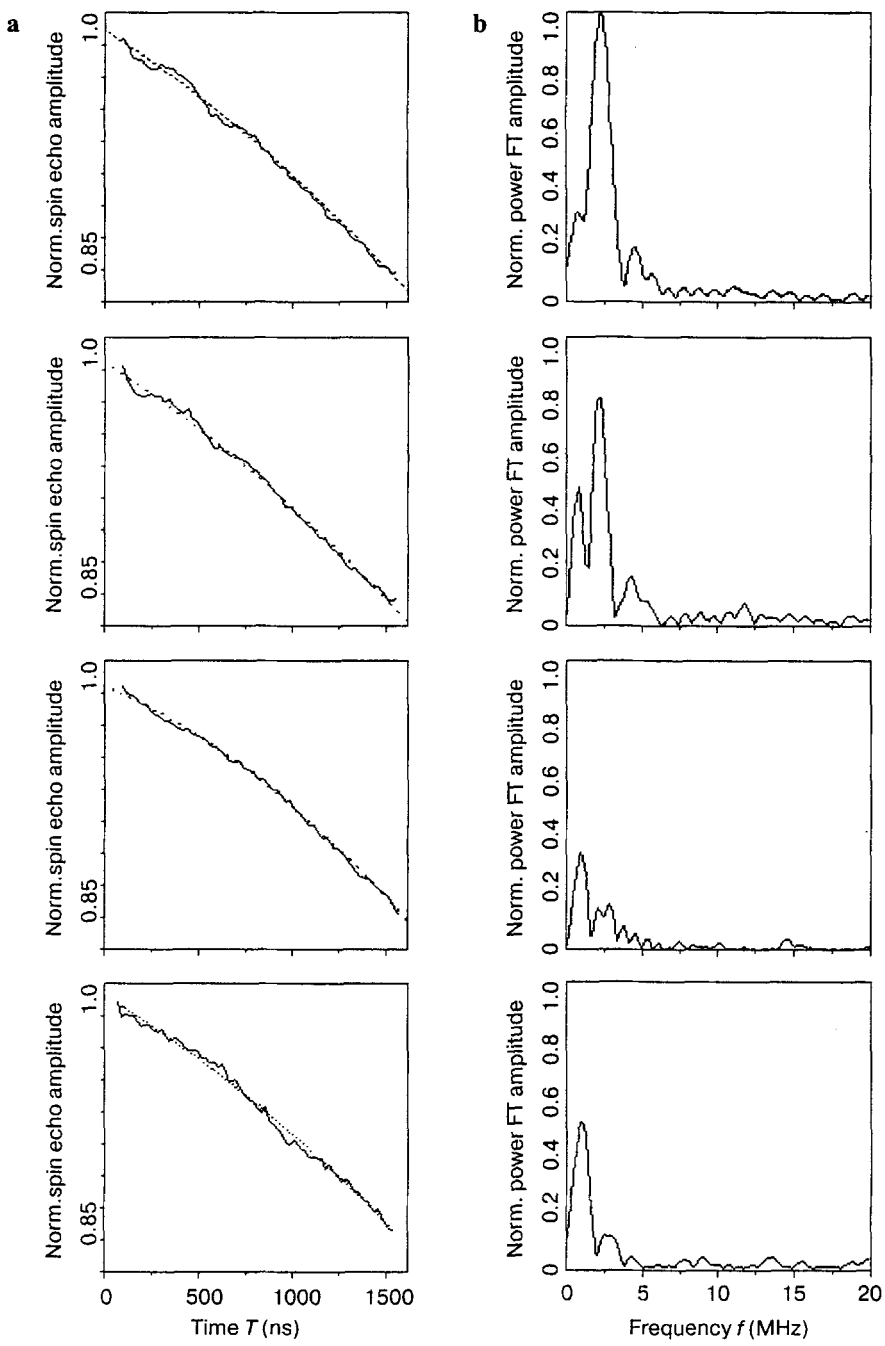

Fig. 6. **a** PELDOR time traces recorded at the field positions as explained in the text. The zero point corresponds to the total overlap of the pumping pulse with the excitation $\pi/2$ pulse. The missing initial points represent the experimental dead time. The time increment amounts to 25 ns; **b** FT of the frequency domain PELDOR signal after subtraction of the decay.

An evaluation of the modulation depths λ shows values that decrease from point 1 to 4 according to $\lambda = 0.01$, 0.008, 0.003, 0.005 (± 0.001), respectively. The field dependence of λ correlates reasonably with the computed fraction of spin pairs at each detection position in Sect. 3; however, the absolute observed values are much smaller. Indeed, the calculation does not take into account imperfections in the pump pulse which is not located at the cavity resonance dip, cross-relaxation due to the high concentration of RNR, and the fact that in our sample not all tyrosyl radicals are paired could also attenuate the modulation depth.

In Fig. 5 we compare the modulation frequencies obtained experimentally (marked by asterisks) with the dipolar frequencies predicted as a function of the resonance field position of the detected spins. The width of the asterisk contains an error bar of ± 5 G that approximately accounts for hysteresis effects. Due to orientation selectivity, the calculation predicts a complex pattern of dipolar frequencies at each detection position. The experiment at each detection point shows at least one frequency with values in satisfactory agreement with the calculation for cases 1, 2, and 4. The modulation frequency observed in case 3 deviates from the calculated value and the origin of this discrepancy is still not well understood. We have already noted that the modulation effect at points 3 and 4 is very weak and the error is likely to be very large[1] [14]. A more precise quantitative evaluation of the error requires a series of systematic studies of all experimental features, which are in progress.

5 Conclusions

A 180 GHz PELDOR setup was implemented and its performance tested on a well known protein sample, RNR-R2 from *E. coli*, containing two tyrosyl radicals at a fixed distance and orientation. The PELDOR response at high magnetic fields and frequencies revealed substantial dependence on the resonance position of the detection with respect to the pumping pulse, as expected for orientation selectivity. Most of the experimental observation showed reasonable agreement with calculations, which contained all information about the relative orientation of the radicals from the crystal structure and the magnetic parameters (g- and D-tensors) from previous measurements. The experiments demonstrate that PELDOR at high frequencies and fields is feasible and should allow determination of the relative orientation of the two radicals when the structure is unknown.

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[1] According to the referee's comment, part of the problem may arise from limited excitation bandwith of the pulses, which tends to distort patterns for intermediate frequencies. The observed frequencies may be intermediate with respect to the excitation bandwidth of 100 ns pulses.

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