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DEER Sensitivity between Iron Centers and Nitroxides in Heme-Containing Proteins Improves Dramatically Using Broadband, High-Field EPR

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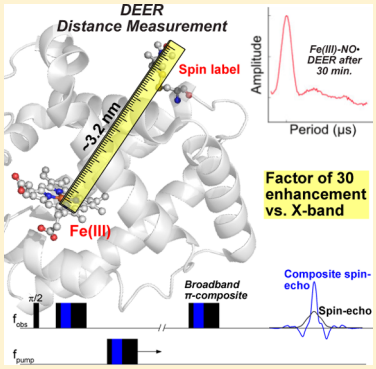
Supporting Information

ABSTRACT: This work demonstrates the feasibility of making sensitive nanometer distance measurements between Fe(III) heme centers and nitroxide spin labels in proteins using the double electron−electron resonance (DEER) pulsed EPR technique at 94 GHz. Techniques to measure accurately long distances in many classes of heme proteins using DEER are currently strongly limited by sensitivity. In this paper we demonstrate sensitivity gains of more than 30 times compared with previous lower frequency (X-band) DEER measurements on both human neuroglobin and sperm whale myoglobin. This is achieved by taking advantage of recent instrumental advances, employing wideband excitation techniques based on composite pulses and exploiting more favorable relaxation properties of low-spin Fe(III) in high magnetic fields. This gain in sensitivity potentially allows the DEER technique to be routinely used as a sensitive probe of structure and conformation in the large number of heme and many other metalloproteins.



Heme proteins contain a metalloporphyrin chelating an iron atom. They are found in all kingdoms of nature, and their functions are diverse and include enzymatic, ligand-uptake, and electron-transfer roles. They are studied widely, often to understand conformational changes upon ligand binding with the aim of designing synthetic analogues and therapeutics against disease.[1,2]

One very useful method for investigating elements of the structure of proteins, conformational changes, or protein−protein binding is double electron−electron resonance (DEER, also known as PELDOR).[3−6] This is a pulsed electron paramagnetic resonance (EPR) spectroscopic method, which measures the dipolar interaction between two paramagnetic centers. The dipolar interaction is distance dependent and DEER can measure distances in the nanometer range.[7−12] Often the paramagnetic centers have been engineered into the protein of interest by using site-directed mutagenesis to give a protein with the desired number of cysteine amino acids. The thiol of the cysteine is then reacted with a small molecule containing an NO radical called a nitroxide spin label.[13] In these systems, the practical distance limits of DEER are from 1.8 to ∼6 nm in protonated proteins and beyond 10 nm in deuterated proteins.[14−16] If a protein already contains a paramagnetic center, then it is generally advantageous to use it directly in DEER distance measurements.[7,17−23] Often proteins will contain just one paramagnetic center and then it can be utilized along with spin labels. This is conceptually similar to orthogonal labeling, where one label has different spectroscopic properties to others and therefore its dipolar interaction to the others can be measured in isolation. Gd^{3+} chelates are proving useful for this methodology.[17,24,25] Using a combination of extrinsic and intrinsic paramagnetic centers, fewer mutants of the protein need to be made and the distance obtained directly relates to the fixed center. The exogenous label has a typical length of 1 nm and may adopt many conformations, which leads to decreased accuracy in the distance measurement.

However, bringing the DEER paradigm to heme proteins is challenging because they exhibit extremely broad spectra (in EPR experiments) and rapid electronic relaxation, strongly reducing sensitivity required for Fe(III)-nitroxide DEER. Despite these problems there have been recent reports of DEER experiments between a low-spin ferric heme and a nitroxide label giving distances of 2.1[18] and 2.7 nm.[19] These measurements were made at X- and Q-band frequencies (9.5 and 34 GHz, respectively) with human neuroglobin (NGB) and cytochrome P450cam. The low-spin ferric heme signals at X-band were 280 and 80 mT wide, respectively. These experiments required long measurement times to provide moderate signal-to-noise, which degrades rapidly with spectral width. It has been demonstrated on the spectrally narrower

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P450cam, prepared with the heme in a single paramagnetic state, that an alternative dipolar method, RIDME,[19] gives a factor of 7 improvement in sensitivity compared with X-band measurements. Previous studies on spin-labeled metmyoglobin using saturation recovery EPR for distance measurements in 1.5 to 2.5 nm have also been reported.[26,27]

Higher frequency, high-power EPR offers strongly increased concentration sensitivity for standard DEER measurements between nitroxides, but this gain is offset by the increased spectral broadening of the EPR spectrum of the low-spin ferric heme.[30] This can be partially compensated for by using broadband excitation techniques and exploiting the fact that the longitudinal relaxation becomes much more favorable at high frequencies and lower temperatures. Many pulsed techniques have been developed to compensate for excitation bandwidth to improve signal-to-noise and modulation depth in DEER experiments.[31−35] These techniques involve the use of modulated pulses that use phase or amplitude changes within a pulse. Such techniques have been used in NMR experiments since 1979,[36] and their adoption into EPR experiments has increased in the past few years since the development and availability of fast arbitrary waveform generators (AWG); however, their use at higher frequency becomes more challenging as the performance of components in this range becomes more critical. Here we show the implementation of fixed-amplitude phase-modulated composite pulses[37,38] for our home-built 1 kW pulsed W-band (94 GHz) spectrometer, HiPER.[39] Composite pulses are composed of a number of contiguous subpulses of varying phase and length that produce an overall excitation (90°) or inversion/refocusing (180°) of spin packets over a larger bandwidth than an equivalent standard pulse. Composite pulses were used both as a replacement for the pump pulse that inverts the partner spins in the experiment and as a replacement for refocusing π pulses in the detection sequence.

Two heme-containing proteins, human neuroglobin (NGB) and sperm whale myoglobin (Mb), were used in this study to test the method and to demonstrate its utility. The proteins were both prepared to have low-spin Fe(III) heme centers, although in the Mb case some high-spin Fe(III) was detectable (see Supporting Information (SI)). The EPR spectral width for the low-spin ferric heme is ∼3 T for NGB and 1.6 T for Mb at W-band. Mutagenesis was used to provide free cysteine amino acids for attachment of the (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate (MTS) spin label, R1. Four different samples were prepared: NGB-C120R1, Mb-S3R1, Mb-S117R1, and the doubly spin-labeled Mb-S3R1-S117R1. Estimates of expected distances were derived from appropriate crystal structures using MMM[40] and MtsslWizard;[28] see Figure 1 (top panel). All samples were measured at 6 K in frozen water/glycerol mixtures.

The DEER results for NGB-C120R1 are shown in Figure 2. This was carried out with the pump and probe frequencies separated by 314 MHz, as shown in the bottom panel of Figure 1. This set-up results in the predominant excitation being in the g_y part of the spectrum of both the iron and the nitroxide. This leads to a broad range of dipolar angles being excited, which largely eliminates any orientation-selection effects.[41,42] The composite pulse used was $90_0180_{180}270_0$,[37] where the subscript denotes the phase of each subpulse. DEER sequences using a combination of composite and normal pulses were compared. The composite and standard sequences used are shown in Figure 3. Full combinations are shown in Figure S2. The

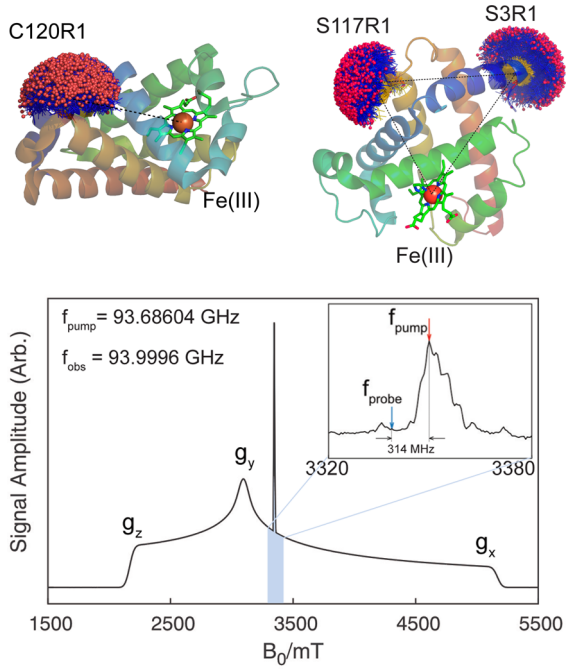

Figure 1. (Top panel, left) Human neuroglobin (PDB 1OJ6) showing the position of G19-R1 (Cys 120) spin label. (Top panel, right) Myoglobin (PDB 1MBI) with R1 at positions 3 and 117 shown, both generated using MtsslWizard.[28] (Bottom panel) NGB C120R1 field-swept echo experiment data inset, with simulation of the spectra generated using EasySpin[29] at W-band (94 GHz). Blue shaded part indicates the part of spectrum that is viewed inset. The full experimental spectrum cannot be viewed due to limitations of the magnet's sweep coil (±200 mT).

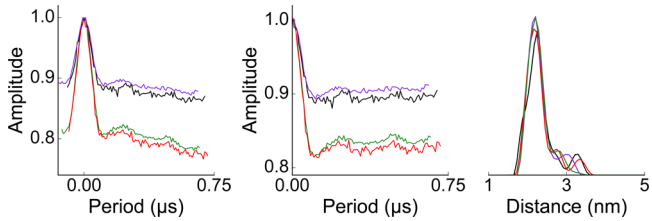

Figure 2. NGB-C120R1 DEER results showing traces obtained at W-band using all normal π pulses (black), pump composite (red), observer (or probe) composite pulses (purple), and all π-composite pulses (green). The all-π-composite trace (green) has better signal-to-noise and modulation depth over the trace using conventional normal pulses (black) for the same number of scans.

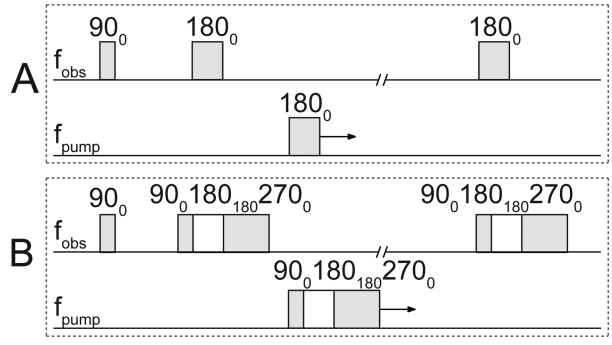

Figure 3. Comparison of the standard DEER sequence (A) with the composite-pulse DEER (B) sequence used.

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combinations were: all standard (Figure 3A), all composite (Figure 3B), standard observer pulses with a composite pump, and composite observer with a standard pump. (See SI, Figure S4C,D.)

A composite observer sequence (green and purple, Figure 2) improved the signal-to-noise of the traces by a factor of 1.8 in comparison with the standard observer sequences (black and red, Figure 2). The modulation depth was improved by 80% by replacing f_{pump} with a composite pulse (green and red traces, Figure 2). Thus, by combining both composite pulse sequences (green, Figure 2), the overall signal-to-noise ratio for the DEER experiment is improved by a factor of 3. All DEER experiments were conducted at 6 K, with traces obtained in 30 min. The distance measured was 2.25 nm, in agreement with the previous X-band result of 2.15 nm,[18] which is also ~0.3 to 0.5 nm shorter than modeled. This can be attributed to a shortcoming of the rotamer-library-based approach or freezing-induced changes to the spin label.[43] Further discussion can be found in the SI.

DEER experiments were carried out on the Mb-S3R1, Mb-S117R1, and Mb-S3R1-S117R1 (Figure 4). In these experiments, similar offset between pump and probe (350 MHz) and g_y/g_y orientations was used to avoid orientation effects. Similar trends in terms of improvement in modulation depth and signal-to-noise using the same composite pulse combinations as for the NGB-C120R1 were observed. Figure 4 shows the DEER traces obtained and their respective distance distributions using all-composite π-pulse DEER sequence (Figure 3B).

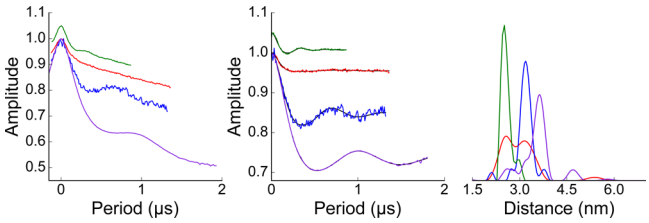

Figure 4. DEER results obtained at W-band for Mb-S3R1 (blue), Mb-S117R1 (green), Mb-S3R1-S117R1 taken at 6 K with observer Mb, pump nitroxide (red), and Mb-S3R1-S117R1 taken at 58 K with observer nitroxide g_z and pump nitroxide g_y (purple). Left panel shows normalized raw data, center shows data post-background subtraction with black line showing the fit obtained using DeerAnalysis2015,[44] and right showing the resulting distance distribution for each of the traces.

The data were analyzed using DeerAnalysis2015.[44] For Mb-S117R1 and Mb-S3R1, mean distances of 2.57 and 3.17 nm were obtained, respectively. The distance distribution obtained for the heme doubly nitroxide labeled Mb-S3R1-S117R1 measured at 6 K, shows approximate peaks that overlay the distances obtained for the singly labeled mutants, although it would not be recommended for use as a primary measurement of the two distances. The cause behind the differences in modulation depth of the mutants shown is unknown, as there is no evidence of incomplete spin-labeling.

Additionally, a DEER measurement was carried out on the doubly labeled Mb-S3R1-S117R1 at 58 K on the nitroxides to extract the distance distribution between the two nitroxide labels, shown in Figure 4 (purple). At 58 K, the Fe(III) signal is negligible due to its much faster relaxation at this temperature. The experiment was carried out using normal pulses, with observer on g_z and pump on g_y. Composite pulses were not used for this measurement due to spectral overlap of the pump and observer pulses, instantaneous diffusion, and the extremely large signal already obtained using standard π pulses. The mean distance obtained was 3.67 nm. Full details of the pulse sequences and distance analysis can be found in the Supporting Information.

The distance measurements on NGB-C120R1 correspond to an increase in sensitivity of over 30 compared with previous X-band measurements on the same system.[18] It should be noted that in the previous X-band study the experiments were carried out at 15 K, whereas they have been carried out at 6 K in this study. This is possible, partly due to the much shorter T_1 at low temperatures due to the direct spin−lattice relaxation process becoming dominant over the Raman process in high fields.[45,46] Relaxation due to the direct process for this system is expected to scale with $T^{-1}B_0^4$ and thus has a very strong field (B_0) dependence, which becomes highly significant at W-band. Thus, T_1 is significantly shorter below 10 K at W-band than at X-band or Q-band. For example, Mb-S3R1S117R1 at 5K has a T_1 of 1.1 ms at Q-band and 30 μs at W-band (see SI). This much shorter T_1 allows measurements with faster repetition rates at lower temperatures (where there is also an increase in signal due to the Boltzmann distribution). It should be noted that although at these temperatures the nitroxide magnetization is expected to be saturated, this does not matter as the DEER effect is independent of the pumped spins polarization.

DEER measurements were also made on the Mb-S117R1 mutant with a Bruker high-power (150 W) TWT Q-band system (see SI). The signal-to-noise ratio obtained was one-fifth of that obtained using HiPER with composite pulses, requiring 25 times longer averaging to compensate.

Thus, the very substantial gain in sensitivity obtained in these measurements at W-band is attributed to measuring at high frequencies using the high-power broadband system, the use of broadband composite pulses for both pump and probe spins (factor of 3), lower temperatures (factor of 3), and higher averaging rates.

This level of increase in sensitivity makes the DEER methodology practical for measuring long-range distances (several nanometers) in heme (and other low-spin iron proteins), using the heme as an effective point dipole. This potentially allows DEER to be used in these broadband systems as a tool to investigate and quantify subtle conformational changes that occur on protein or molecular binding.

EXPERIMENTAL METHODS

All W-band 94 GHz experiments were conducted using a home-built high-power (1 kW) pulsed spectrometer, with integrated vector modulator phase box. Full experimental details and sample preparation can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b00456.

Descriptions of sample preparation, including UV−vis experiments; EPR experiment parameters and pulsed EPR data; DEER fitting parameters; T_1 and T_m results; signal-to-noise analysis; and Q-band DEER. (PDF)

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Notes

The authors declare no competing financial interest.

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