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A pulsed EPR method to determine distances between paramagnetic centers with strong spectral anisotropy and radicals: The dead-time free RIDME sequence

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ABSTRACT

Methods to determine distances between paramagnetic metal centers and radicals are scarce. This is unfortunate because paramagnetic metal centers are frequent in biological systems and so far have not been employed much as distance markers. Successful pulse sequences that directly target the dipolar interactions cannot be applied to paramagnetic metal centers with fast relaxation rates and large *g*-anisotropy, if no echos can be detected and the excitation bandwidth is not sufficient to cover a sufficiently large part of the spectrum. The RIDME method Kulik et al. (2002) [20] circumvents this problem by making use of the T_1-induced spin-flip of the transition-metal ion. Designed to measure distance between such a fast relaxing metal center and a radical, it suffers from a dead time problem. We show that this is severe because the anisotropy of the metal center broadens the dipolar curves, which therefore, only can be analyzed if the full curve is known. Here, we introduce five-pulse RIDME (5p-RIDME) that is intrinsically dead-time free. Proper functioning of the sequence is demonstrated on a nitroxide biradical. The distance between a low-spin Fe(III) center and a spin label in spin-labeled cytochrome *f* shows the complete dipolar trace of a transition-metal ion center and a spin label, yielding the distance expected from the structure.

1. Introduction

Pulsed EPR has taken a leap forward as a method for structure determination in disordered chemical and biological systems ever since pulsed EPR methods had been developed that directly and selectively probe the dipolar interaction between electron spins [1–3]. Amongst them are 2 + 1 methods and DEER [4–6], solid-echo type single-frequency techniques for refocusing dipolar couplings (SIFTER) [7], and double quantum coherence methods (DQM) [5,6,8]. These techniques are optimized for systems with low spectral anisotropy, such as nitroxide-type spin labels and organic radicals, and require the excitation of a significant part of the spectrum. The spectral widths of the EPR transitions of nitroxides and organic radicals are in the order of several mT at the conventional operating frequency of 9 GHz (X-band EPR), which compares well with presently available excitation bandwidths of a few mT (e.g. a pulse length of 24 ns results in 1.5 mT bandwidth). For transition-metal ions, the spectral width is usually larger. Examples for transition-metal DEER refer to those transition-metal ions that have moderate spectral anisotropies, such as Cu(II), where *g*-values between 2.37 and 2.08 give rise to spectral widths of 70 mT at X-band EPR, [9–12] or iron–sulfur centers [13]. Most other transition-metal ions have larger *g*-anisotropies and faster relaxation times even at cryogenic temperatures. For those metal ions, fractional excitation of the spectrum or short relaxation times will either severely limit the sensitivity or make the application of the method impossible. Therefore, novel approaches to address such paramagnetic centers are sought. The present account describes a method tailored to determine the interaction between a low *g*-anisotropy center and a center of large *g*-anisotropy and is ideally suited to address the distance between a nitroxide spin label and a paramagnetic transition-metal ion. For structure determination this combination is highly relevant, because transition-metal centers are often present in proteins. Another advantage of such centers is that they are firmly anchored in the protein and therefore, are not fraught with the problem of flexible linkers as the commonly used spin labels. Previously, most approaches to measure such distances made use of the change in relaxation properties of the small *g*-anisotropy center caused by the transition-metal ion, as pioneered by the group of G. Eaton and S.S. Eaton [14–16]. The approach was used in several recent applications [17,18] and its implementation to obtain long-range distance determination using rare-earth metal ions was described [19]. Nevertheless, the complexity of relaxation-based approaches from the point of view of experiment, but most of all interpretation has so far limited the applications.

The method proposed here directly probes the dipolar interaction between the metal center and a nitroxide or an organic radical. It is based on the relaxation induced dipolar modulation (RIDME)

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method suggested by Kulik et al. [20], in which the change is detected in the resonance frequency of the observed spin, i.e. the nitroxide (*A*-spin), by the spontaneous flip of the electron spin on the partner paramagnetic center (*B*-spin). Here, the flip of the *B*-spins is not induced by a pump pulse as in traditional sequences, but is left to the longitudinal relaxation of the *B*-spin. As a consequence, there is no need to flip the *B*-spin by a pump pulse, avoiding the problem of the limited excitation bandwidth. The RIDME sequence as proposed originally [20] (Fig. 1a) and the four-pulse version suggested subsequently [21], suffer from a dead time problem that severely limits the usefulness of these methods for distance determination involving systems with high *g*-anisotropy, as we will demonstrate.

We propose a five-pulse version of the RIDME sequence that completely eliminates the dead time. We demonstrate that the sequence works and that it yields the expected distances on a nitroxide biradical (PH2, see Fig. 2) by comparing the results of the new RIDME sequence and a conventional DEER experiment. Subsequently, we apply the new sequence to measure the distance between the low-spin heme iron(III) center, a paramagnetic center with large *g*-anisotropy, and a nitroxide spin label in cytochrome *f* (cyt *f*). This protein is part of the electron transfer chain in photosynthesis and contains an intrinsic low-spin heme Fe(III) center. By site-directed mutagenesis, a cystein was introduced at position 104 and a spin label was attached, resulting in a system with a nitroxide–Fe(III) distance in the order of 1.43 nm. We show that in spite of the large *g*-anisotropy of the Fe(III) center, the distance between the two paramagnetic centers can be determined. A Gaussian distance distribution centered at 1.81 nm with a width of 0.27 nm is obtained. Presently, there is no other method to determine distances in such cases with similar accuracy.

2. Experimental

2.1. Sample preparation

Mutagenesis. The pEAF-wt [22] expression plasmid containing the sequence encoding the soluble domain of cyt *f* from *Nostoc* sp. PCC7119 has been kindly provided by the group of Prof. Miguel

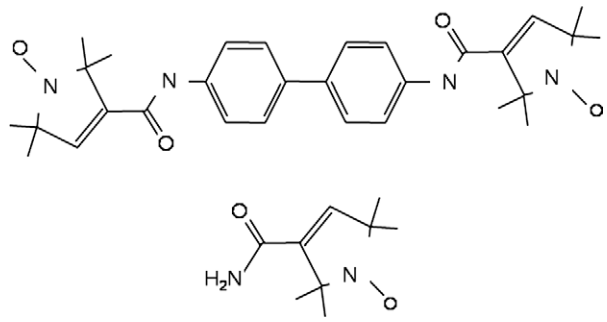

Fig. 2. Chemical structures of the biradical PH2 and the monoradical PH0.

De la Rosa, Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla, Spain. In order to prepare the single-cysteine cyt *f* variants Q104C and N71C, mutations were introduced by site-directed mutagenesis using the Quik Change™ polymerase chain reaction protocol (Stragene, La Jolla, CA) with the plasmid pEAF-wt as a template. To introduce a cysteine instead of the asparagines at the position 71 the direct primer GGCTC***C***AAGGTCGGCTTA ***TG***CGTCGGTGCTG (31 bases) was designed from the nucleotide sequence, inserting at the same time the Sty I restriction site next to the 5′ end of the leader of this primer. Analogously, to introduce a cysteine instead of the glutamine at the position 104 the direct primer CGGCGATGTTTACTTC***TGC***CCCTACGGCGAAG (32 bases) was designed, inserting an extra Bgl I restriction site respect to the *wild type*. Both constructs were verified by DNA sequencing.

2.2. Expression and purification of the cyt f mutants

To improve the maturation and correct insertion of the heme group, *Escherichia coli* strain MV1190 cells (Bio-Rad) were co-transformed with plasmids pEC86 [22] and the cyt *f* expression plasmids. The cells were incubated on Luria–Bertani (LB) medium plates (added by 20 mg/L ampicillin, 20 mg/L chloramphenicol) at 37 °C for 24 h. Several pre-cultures were incubated in 100 mL flasks with 20 mL of LB medium supplemented with 20 mg/L ampicillin (amp) and 20 mg/L chloramphenicol (cam) at 37 °C and

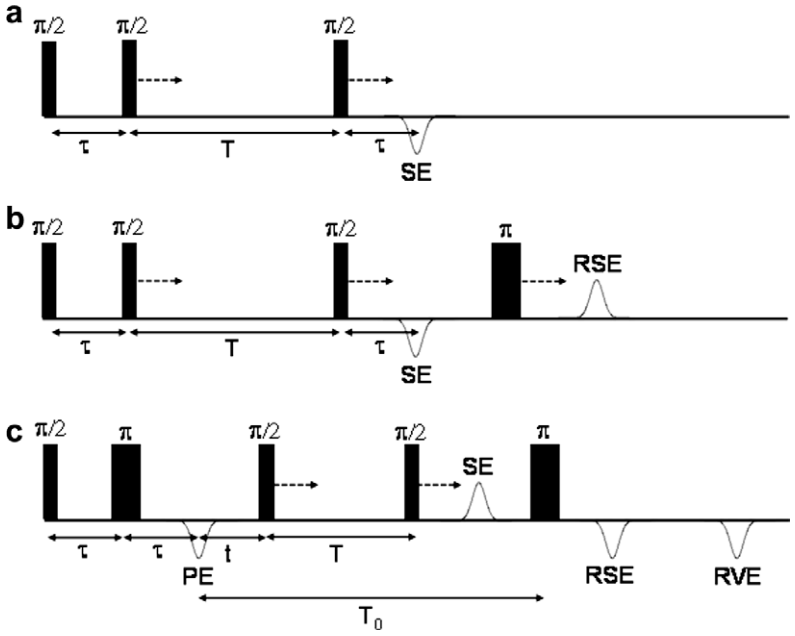

Fig. 1. Pulse sequences for RIDME experiment: (a) three-pulse RIDME and (b) four-pulse RIDME, τ is incremented; (c) five-pulse RIDME, time t is incremented and echo is detected as a function of t. Positions of primary (PE), stimulated (SE), refocused stimulated (RSE) and refocused virtual (RVE) echoes are shown.