Treatment of spin-coupled metal-centres in pulsed electron–electron double-resonance experiments

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Abstract
In applications of ELDOR (electron–electron double-resonance) spectroscopy to metal centres, significant g-anisotropy and spin-coupling within multinuclear clusters have to be considered. We show the difficulties and the advantages arising from these effects.

Introduction
Pulsed ELDOR (electron–electron double resonance), also called DEER (double electron-electron resonance), has become a technique widely used in structural biology. The basic concept and some applications of this experiment have been reviewed recently [1,2]. The main use of pulsed ELDOR in biophysics is the determination of distances between paramagnetic cofactors and/or chemically introduced nitroxid-based paramagnetic species [3,4]. Here, we focus on the application of pulsed ELDOR on systems containing two intrinsic metal-based paramagnetic species. The consequences of spin-coupling within a multi-nuclear metal cluster on the interpretation of ELDOR results will be discussed. As a model system, we use a hydrogenase in the as-isolated state with the paramagnetic catalytic [NiFe] centre and the paramagnetic [3Fe-4S]⁺ cluster in the electron transfer chain [5–7].

Materials and methods
The pulsed ELDOR experiment [8a,8b] is designed to separate the spin–spin coupling of two electrons from the other terms of the spin Hamiltonian. This is achieved by applying a constant time two-pulse echo (Hahn echo) experiment on one spin system (A spins), thereby refocusing all spin packets experiencing different precession frequencies due to static inhomogeneities. In between the pulses of the Hahn echo sequence, a π–pulse of different microwave frequency is used to invert the second spin system (B spins). This π–pulse is shifted in time within the primary echo sequence, causing a modulation of the echo intensity with the frequency of the spin–spin coupling. For well-localized $S=1/2$ systems, the distance between the two spin centres can be derived by using the point-dipole approximation

$$
ν_d = \frac{g_{\text{det}} g_{\text{pump}} β^2 \mu_2 3 \cos^2 θ - 1}{4π \hbar r_{AB}^3}
$$

(1)

where $g_{\text{det}}$ and $g_{\text{pump}}$ refer to the g-values which are excited by the detection and the pump frequency respectively, $ν$ is the angle between the interspin vector $r_{AB}$ and the external magnetic field $B$, and the $(3 \cos^2 θ - 1)$ term describes the orientation-dependence. For two spins at fixed distance with respect to each other in a sample with random orientation distribution, a distribution of frequencies $ν_d$ of the axial dipolar spin–spin coupling, with a maximum at $ν_d(θ = 0°) = ν_∥$, and an edge at $ν_d(θ = 90°) = ν_⊥$, commonly referred to as Pake pattern, is obtained.

Two specific problems arise in the application of ELDOR on metal centres: (i) the usually significant anisotropy of the g-matrix of a metal cluster, and (ii) the spin-coupling within a multi-nuclear centre.

Orientation selection
An ELDOR experiment on a two-spin system where both spin centres can be completely excited by the respective microwave pulses results in the full Pake pattern of the dipolar coupling.

If the spectral width of a species due to its g-anisotropy is larger than the excitation bandwidth of a microwave pulse (typical pulse-length 8–64 ns), only a certain range of g-values is excited. Only those spin systems that are oriented such that their effective g-values fall within this range contribute to the signal. Since the orientation of the g-matrix is well defined in the molecular frame, this results in an orientational selection of molecules. In an ELDOR experiment on a sample where one spin system (e.g. the A spins) can be completely excited, but the second spin system (B spins) shows this orientation selection effect, a distortion of the Pake pattern for the dipolar frequencies is observed [9]. For pump frequencies exciting either of the two extreme g-components, ‘single-crystal’-like spectral patterns are obtained. If both (A- and B-spin) experience orientation selection, the fixed structural relation between the two spin centres has to be considered. Since both spin centres in the same molecule have to be in resonance simultaneously, only very restricted combinations of pump and detection frequencies result in an ELDOR modulation.

To demonstrate this effect, we have chosen a hydrogenase from Desulfovibrio vulgaris Miyazaki F, which has
in its as-isolated oxidized state two paramagnetic centres, an [NiFe] centre and a [3Fe-4S]$^{+}$ cluster [10]. The positions of the metal ions are well known from the X-ray structure at 1.8 Å (1 Å ≈ 0.1 nm) resolution [5]. In Figure 1, the EPR spectrum of these two metal centres in the hydrogenase is shown. The $S=1/2$ signal of the [NiFe] centre stretches over approx. 60 mT. The signal arises from two states called Ni-A and Ni-B (Ni-A: $g_x = 2.317; g_y = 2.229; g_z = 2.014$; and Ni-B: $g_x = 2.333; g_y = 2.163; g_z = 2.010$). In the as-isolated hydrogenase, 70% of the [NiFe] centres are in the Ni-B state; Ni-A as a minority species is therefore neglected in the following calculations. The [3Fe-4S]$^{+}$ cluster gives rise to an EPR signal of an $S=1/2$ ground state, which is well separated from the first excited state [11] with $g_1 = 2.032; g_2 = 2.024$ and $g_3 = 2.016$ [12]. The orientation of both $g$-tensor systems within the molecular structure are known from EPR experiments on single crystals [10,12]; the orientation of the dipolar axis can be directly extracted from the crystallographic data. This complete structural and EPR data set makes this hydrogenase useful to demonstrate the discussed orientation selection effects in ELDOR.

ELDOR spectra have been calculated for different detection frequencies at a fixed pump frequency (Figure 1). In this model calculation, both spin centres were treated as point-dipoles, i.e. eqn (1) was used. The detection frequency was varied such that the [NiFe] centre was in resonance from $g = 2.330$ to $g = 2.010$. The [3Fe-4S]$^{+}$ cluster has been pumped at $g = 2.0170$. The excitation bandwidth was obtained by folding the bandwidth of a 32 ns pulse with an inhomogeneous linewidth to model hyperfine coupling. This results in a 2.2 mT excitation width around the selected $g$-value for the [NiFe] centre, and 1.4 mT for the [3Fe-4S]$^{+}$ cluster. To visualize the orientation selection effect, we show the molecular orientations contributing to the ELDOR effect for the resonance condition of the detection frequency set close to the three principal values of the [NiFe] $g$-matrix. Those molecular orientations contributing are shown as dark-grey regions on a sphere (Figure 1).

The series of calculated ELDOR spectra demonstrates how the orientation selection distorts parts of the dipolar frequency spectrum. When detecting along $g_x$, mainly the perpendicular component $v_\perp$ of the Pake pattern can be observed at 5.5 MHz, but only a few molecules take part in the ELDOR experiment. This can be seen on the sphere that illustrates the fraction of molecules contributing to the ELDOR modulation at $g_x$. By shifting $g_{det}$ to higher values, the observed line splits, one part remaining at $v_\perp$, the other one shifting to lower frequencies. At $g_{det}$ values higher than $g_x$, the line at $v_\perp$ has lost intensity, but a component with frequency values

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**Figure 1 | Orientation selection**

Upper left panel: the two-pulse field-swept EPR spectrum ($\nu_{mw} = 9.7057$ GHz) of the [NiFe] centre and the [3Fe-4S]$^{+}$ cluster (multiplied by 0.02) is shown, where $g_{pump}$ corresponding to the ELDOR pump frequency $\nu_{pump}$ is indicated. Lower left panel: calculated ELDOR spectra depending on the ELDOR detection frequency $\nu_{det}$, i.e. the $g$-value $g_{det}$, assuming both spin centres as point-dipoles. Right panels: the dark areas on the spheres mark the fraction of molecular orientations participating in ELDOR experiments, when the detection frequency excites close to the principal $g$-values of the [NiFe] centre.
around $v_B$ appears. When shifting $g_{det}$ further up to $g_s$, the observed lines move strongly to lower frequencies, but also gain intensity as the fraction of participating molecules rises.

**Intra-cluster spin coupling**

Recently, we have shown that the dipolar coupling measured in an ELDOR experiment between a multinuclear metal cluster and a second spin cannot be explained by the point-dipole approximation, since the individual spins of each ion in the metal cluster and their mode of coupling have to be considered [13]. The Hamilton operator of the dipolar coupling $\hat{H}_{dip}$ between a metal cluster of $n$ ions and a spin B depends on the magnetic moments $\mu$ of each ion $i$ of the coupled system:

$$\hat{H}_{dip} = \frac{\mu_B^2}{4\pi} \sum_{i=1}^{n} \frac{1}{r_{i,B}^3}(\vec{\mu}_i \cdot \vec{\mu}_B - 3(\vec{\mu}_i \cdot \vec{n}_{i,B})(\vec{\mu}_B \cdot \vec{n}_{i,B})).$$

(2)

with

$$\vec{\mu}_i = -\beta g_i \vec{S}_i, \quad \vec{n}_{i,B} = \frac{\vec{r}_{i,B}}{r_{i,B}}.$$

In a strongly coupled metal cluster, the spins $S_i$ of the individual ions $i$ couple to an effective spin $S_{total} = \sum S_i$, where each spin can be replaced according to the Wigner–Eckart theorem as $\vec{S}_i = K_i \vec{S}_{total}$, $K_i$ being the spin projection factor of the ion $i$. Within this description, the dipolar frequency $v_d$ measured in an ELDOR experiment can be expressed in terms of the individual distances between spin B and the ions of the metal cluster and their spin projection factors as:

$$v_d = \frac{g_{det} g_{pump} B^2 \mu_B}{4\pi \hbar} \sum_{i=1}^{n} K_i \cos^2 \theta_i \frac{1}{r_{i,B}^3}.$$  

(3)

In the case of a two-centre cluster with a well-separated total spin $S$ ground state, the spin projection factors $K_i$ can be derived analytically [14]:

$$K_1 = \frac{S(S + 1) + S_i(S_i + 1) - S(S + 1)}{2S(S + 1)}$$

$$K_2 = \frac{S(S + 1) - S_i(S_i + 1) + S(S + 1)}{2S(S + 1)}$$

(4)

For a Fe(II)Fe(III) cluster with spins $S_{Fe(II)} = 2$ and $S_{Fe(III)} = 5/2$, the spin projections are $K_{Fe(II)} = -4/3$ and $K_{Fe(III)} = +7/3$ [12]. Using this model two–iron cluster, we show the influence of spin-coupling on an ELDOR experiment. In Figure 2, a system with an Fe–Fe distance of 3 Å is shown: the two spins are labelled by their spin projection factors $K_1$ and $K_2$. The centre of the metal cluster is assumed in 25 Å distance to a spin B, with $S_B = 1/2$. The orientation of the metal cluster is described by the angle $\xi$ between the Fe–Fe–connecting axis and the axis connecting the cluster with spin B. The ELDOR spectra are calculated as a function of this relative orientation.

**Figure 2** | Calculated ELDOR frequency pattern depending on the orientation of a binuclear cluster with respect to a second spin centre $S_B$

The model system is built from one spin $S_B = 1/2$, which is at a distance of 25 Å from a coupled Fe$^{3+}$Fe$^{3+}$ cluster. The Fe ions are separated by 3 Å, and have spin projection factors $K(Fe^{3+}) = -4/3$ and $K(Fe^{3+}) = +7/3$ within the $S = 1/2$ ground state of the cluster. The Fe$^{3+}$–Fe$^{3+}$ connection axis is rotated with respect to the interspin vector by the angle $\xi$.

At $\xi = 0^\circ$ and $\xi = 180^\circ$, both Fe ions have the same distance $r$ to the spin B. Owing to symmetry reasons, the spectra are equal and show an intermediate coupling with a perpendicular component of $v_\perp = 3.2$ MHz. For those orientations, the ELDOR spectrum is very similar to that in which two point-dipoles interact. The spectrum at $\xi = 90^\circ$ belongs to the orientation where the spin with projection factor $K_2$ with a small absolute value is closest to the spin B and the other one, $K_1$, is furthest away. Therefore the effective dipolar coupling is at its minimum with $v_\perp = 0.9$ MHz; considering two point-dipoles ($S = 1/2$ systems), this frequency would correspond to an interspin distance of 39 Å. When the Fe(III) ion with the large spin projection factor of $K_2 = 7/3$ is closest to the spin $S_B$ at $\xi = 270^\circ$, the dipolar coupling reaches its maximum with $v_\perp = 5.3$ MHz, which relates to a pseudo-distance between two point-dipoles of 21 Å. This shows how crucially the measured dipolar coupling depends on the spin coupling in the cluster, the location of both Fe ions and their associated spin projection factors.
Figure 3 | Spin projection factors $K_i$ for the three iron ions as a function of the mixing parameter $\alpha$ as calculated from the equations given [14] (left panel), and the calculated ELDOR spectra of hydrogenase as a function of $\alpha^2$ (right panel).

In a cluster in which more than two ions are antiferromagnetically coupled, no general solution for the ground state spin and the coupling coefficients can be given. Nevertheless, for special cases a theoretical description was found, e.g. for metal clusters comprising three metals each with high-spin $S = 5/2$ under the assumption of pure isotropic exchange coupling and an experimentally accessible total spin $S = 1/2$ [15]. Only two quantum mechanical states can fulfil these conditions, and the state of the systems can be described as a linear combination of these two, with a mixing parameter $\alpha$. The spin projection factors $K_i$ are in this case solely a function of $\alpha^2$, and are shown in Figure 3. We have applied these equations in calculating the ELDOR spectra for the $[3\text{Fe}-4\text{S}]^+$ and the $[\text{NiFe}]$ centre in hydrogenase, taking only the location of the ions and the orientation of the $g$-tensors as known. Here, we have not taken any information on the spin projection factor into account, such as using hyperfine coupling data obtained by Mössbauer on a similar $[3\text{Fe}-4\text{S}]^+$ cluster [15]. Structural data and principal $g$-values for the hydrogenase from $D. vulgaris$ Miyazaki F are described in the text.

Cluster of the hydrogenase, the Fe ion closest to the $[\text{NiFe}]$ centre has a large positive spin projection factor. A more quantitative analysis, as illustrated in Figure 3, shows that the observed 8.5 MHz dipolar coupling is only consistent with values of $\alpha^2$ in the range 0.0–0.1. Values of $\alpha^2$ in this range were obtained by Mössbauer spectroscopy on the $[3\text{Fe}-4\text{S}]^+$ cluster in ferredoxin from $A. vinelandii$ [15]. Spin projection factors of $K_i = +2.28, -0.28$ and $-1.0$ were obtained from the hyperfine interaction, which led to the conclusion that $\alpha^2 = 0.01$ [16].

The analysis presented here shows that the $[3\text{Fe}-4\text{S}]^+$ clusters in ferredoxin from $A. vinelandii$ and hydrogenase from $D. vulgaris$ Miyazaki F are very similar, and the Mössbauer-derived spin projection factors for the $[3\text{Fe}-4\text{S}]^+$ cluster in ferredoxin apply also for the $[3\text{Fe}-4\text{S}]^+$ cluster in the electron-transfer chain in the hydrogenase.

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