An Implicit TRIPLE Effect in Mims Pulsed ENDOR: A Sensitive New Technique for Determining Signs of Hyperfine Couplings

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Both the signs and magnitudes of electron−nuclear hyperfine coupling constants carry vital information about electronic and geometric structure, but only the magnitudes are available from ordinary EPR or even electron−nuclear double resonance (ENDOR) spectroscopy.[1,2] In principle, signs are available from electron−nuclear triple-resonance (TRIPLE) techniques,[3] but only rarely are these successful with the frozen-solution samples typical for metallobiomolecules.[4] We now report a new, high-sensitivity triple-resonance-type effect that is *implicit* in the Mims electron spin-echo (ESE) ENDOR technique,[5] rather than requiring the use of an additional rf field as in ordinary TRIPLE, and thus yields the signs of hyperfine couplings with significantly higher sensitivity. In the presence of strong electron spin-echo envelope modulation (ESEEM)[6,7] the Mims ESE ENDOR technique[3,5] produces complex intensity patterns that are transparently interpretable to give the signs of the hyperfine coupling constants being measured. The utility of the new "implicit-TRIPLE" effect is demonstrated by its application to revise our original assignment[8] concerning the nature of the solvent-derived OH_x ligand in the novel low-spin, non-heme iron enzyme nitrile hydratase from *Rhodococcus* sp. strain 312.[9]

A Mims ESE ENDOR signal is generated when an rf pulse at frequency ν is applied during the interval between the second and third microwave pulses of a three-pulse, stimulated-echo microwave sequence ($\pi/2-\tau-\pi/2-T-\pi/2-\tau-$Echo);[5] the ENDOR intensity of a resonant NMR transition is the difference between the magnitude of the stimulated ESE in the presence and absence of the rf field. Figure 1 presents ^{1}H Mims X-Band ENDOR spectra[10] of nitrile hydratase (NHY)[11] taken with $\tau = 0.100$ μs ($g_1 = 2.28$) for protein that is globally ^{15}N- enriched ([^{15}N]NHY) and in natural isotopic abundance ([^{14}N]NHY). The spectrum of [^{15}N]NHY (Figure 1, upper trace) is a superposition of hyperfine-split ^{1}H doublets centered at the proton Larmor frequency, ν_H, with frequencies $\nu_\pm(^1\text{H}) = |\nu_H \pm (1/2)|A(^1\text{H})||$; the + sign arises when the hyperfine and nuclear-Zeeman interactions add, the − sign when they oppose. Because the ENDOR frequencies contain no information concerning the signs of the $A(^1\text{H})$, we always denote the higher-frequency peak as ν_+. Spectra taken with a sample exchanged in D_2O show that each of the doublets labeled (X1, X2) arises from an exchangeable proton;[8] for illustration, one of the doublets from a nonexchangeable proton, Y, also is labeled.

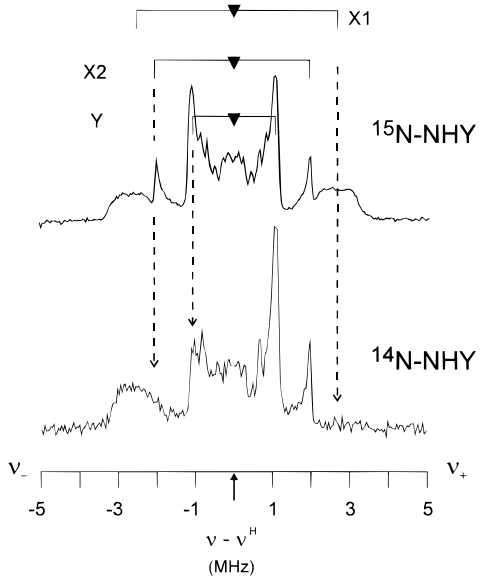

Figure 1. Mims X-Band ^{1}H ENDOR spectra of [^{15}N]NHY (upper trace) and [^{14}N]NHY (lower trace) taken with $\tau = 0.100$ μs at the low-field edge ($g_1 = 2.28$) of the EPR envelope. The labeled hyperfine-split doublets (X1, X2, Y) are discussed in the text. They are centered at the proton Larmor frequency (▼) and split by their respective $A(^1\text{H})$. The vertical (dashed) arrows show peaks present in the [^{15}N]NHY spectrum but suppressed by the implicit-TRIPLE effect in the [^{14}N]-NHY spectrum. Conditions: microwave pulse widths 16 ns; $\tau = 0.100$ μs; $T = 28.6$ μs; rf pulse width 20 μs; repetition rate 20 Hz; temperature 4 K.

For [^{15}N]NHY (Figure 1, upper trace) the $\nu_\pm$ peaks within each doublet have similar intensities, in the classical first-order ENDOR pattern for a nucleus with $I = 1/2$. In contrast, in the $\tau = 0.100$ μs ^{1}H Mims spectrum of natural-abundance enzyme ([^{14}N]NHY) (Figure 1, lower trace) for each doublet the ν_+ and ν_- peaks have significantly different intensities: for X1 the ν_- peak has the greater intensity; for X2 and Y it is the ν_+ peak. Furthermore, the relative intensities depend on τ: when $\tau = 0.240$ μs, the intensities of the ν_+ and ν_- peaks at g_1 are equal in the spectra of *both* [^{14}N]- and [^{15}N]NHY isotopomers (data not shown).

Why do the $\nu_\pm$ partners of every [^{14}N]NHY ^{1}H doublet show unequal relative intensities, with the sense of the inequality differing among the doublets, and why does this effect depend both on the N isotope present and on τ? The answer, given by a density matrix[12] analysis of the Mims ENDOR intensities, is that the anomalous intensities in Figure 1, lower trace, are an effect on ^{1}H ENDOR of the strong ^{14}N ESEEM that is present in [^{14}N]NHY but absent in [^{15}N]NHY. To see this, consider for concreteness an electron spin (ES) interacting with one or more protons along with a reference ^{14}N ($I = 1$) nucleus. The proton ENDOR frequencies are mentioned above; the ^{14}N quadrupole, hyperfine, and nuclear-Zeeman interactions further split the nuclear levels, giving rise to six ^{14}N ENDOR frequencies, three where the hyperfine and nuclear-Zeeman interactions add, and three where they oppose;[13] in analogy to the labeling

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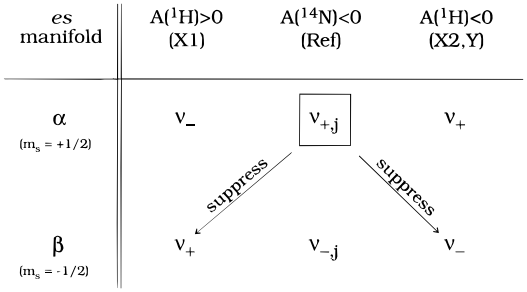

Figure 2. Suppression effects in NHY at $\tau = 0.100$ μs by a reference ^{14}N with $A(^{14}\mathrm{N}) < 0$. The lines denote the cross-manifold suppression of ^{1}H ENDOR peaks by the reference ^{14}N nucleus. More properly, the column headings are $A(g_n)/|g_n|$. As the nuclear g_n factors for ^{1}H and ^{14}N are both positive, the headings are correct as written. For ^{15}N, the g_n factor is negative, and the $\pm$ assignments would be reversed. The protons referred to in the heading are identified in Figure 1.

of the proton frequencies, we denote these, respectively, as $\nu_{\pm j}(^{14}\mathrm{N})$, where $j = 1-3$. As illustrated in Figure 2, the $\nu_\pm$ frequencies of a nucleus, ^{14}N or ^{1}H, can be associated with a particular ES manifold ($\nu_{\beta/\alpha}$: $\alpha(m_s = +1/2)$; $\beta(m_s = -1/2)$), with the correspondence being determined by the sign of the hyperfine coupling. Thus, the three ν_{+j} frequencies of the reference ^{14}N are associated with the α manifold ($\nu_{\alpha j}(^{14}\mathrm{N})$) when $A(^{14}\mathrm{N}) < 0$ (Figure 2), but with the β manifold ($\nu_{\beta j}(^{14}\mathrm{N})$) when $A(^{14}\mathrm{N}) > 0$; the reverse holds for the ν_{-j}. The association between the $\nu_\pm(^1\mathrm{H})$ ENDOR frequencies and an ES manifold similarly depends on the sign of $A(^1\mathrm{H})$ (Figure 2).

The density matrix analysis simplifies[12] because the coherences (nonsecular terms in the density matrix) decay in ~15 μs (as determined by the *T*-dependent ESEEM), which is short compared to the interval $T \approx 30$ μs in the stimulated-echo ENDOR sequence.[14] In this limit, each ENDOR transition of a proton doublet has a τ-dependent contribution (denoted ENDOR(τ, $\nu_{\alpha/\beta}(^1\mathrm{H})$)) that is proportional to the echo intensity of its associated ES manifold.[15] However, as described by eq 1 (where the χ_o term and the $\chi_{\alpha/\beta}$ have their standard form[6])

$$\begin{aligned}\mathrm{ENDOR}(\tau, \nu_\alpha(^1\mathrm{H})) &\propto {}^{\alpha}\mathrm{Echo}(\tau, \nu_\alpha(^1\mathrm{H}); \nu_{\beta j}(^{14}\mathrm{N})) \\ &\propto \chi_o/2 + \sum_j \chi_\alpha(j) \cos(2\pi\nu_{\beta j}(^{14}\mathrm{N})\tau)\end{aligned}$$

$$\begin{aligned}\mathrm{ENDOR}(\tau, \nu_\beta(^1\mathrm{H})) &\propto {}^{\beta}\mathrm{Echo}(\tau, \nu_\beta(^1\mathrm{H}); \nu_{\alpha j}(^{14}\mathrm{N})) \\ &\propto \chi_o/2 + \sum_j \chi_\beta(j) \cos(2\pi\nu_{\alpha j}(^{14}\mathrm{N})\tau) \quad (1)\end{aligned}$$

the density matrix terms that describe the intensity of the α manifold proton ENDOR transition (χ_α) are "cross- manifold" modulated by the ^{14}N ENDOR frequencies associated with the β manifold according to the functions $\cos(2\pi\nu_{\beta j}(^{14}\mathrm{N})\tau)$. Likewise, the intensity of the β manifold proton transition is modulated by $\nu_{\alpha j}(^{14}\mathrm{N})$. For [^{14}N]NHY, analysis of the ESEEM shows that, at $\tau = 0.100$ μs, the dominant effect of the modulation is a cross-manifold *suppression* of ENDOR by the $\nu_{+j}(^{14}\mathrm{N})$ frequencies. As indicated in Figure 2, the identity of the ^{1}H ENDOR signal, ν_+ or ν_-, that is thereby suppressed depends on the *relative* signs of the ^{14}N and ^{1}H hyperfine couplings. When $A(^1\mathrm{H})$ has the same sign as $A(^{14}\mathrm{N})$, the modulation by $\nu_{+j}(^{14}\mathrm{N})$ causes the intensity of the proton ν_- transition to be less than that of ν_+, whereas when the signs are opposite the ν_+ proton transition is less intense.

In the $\tau = 0.100$ μs ^{1}H spectrum of [^{14}N]NHY in Figure 1, the ν_- peaks of both X2 and Y have reduced intensity. This shows that the hyperfine couplings of these protons have the same sign, which is the same sign as $A(^{14}\mathrm{N})$ (Figure 2); the sign is negative if $A(^{14}\mathrm{N})$ is negative as expected.[16] In contrast, $\nu_+(^1\mathrm{H})$ is suppressed for X1, and thus A(X1) has the opposite (positive) sign. The $\nu_\pm(^1\mathrm{H})$ partners of the doublets in ^{1}H ENDOR spectra of [^{15}N]NHY show equal intensity, as seen in Figure 1 for $\tau = 0.100$ μs, because the ESEEM associated with the ^{15}N ($I = 1/2$) nuclei is negligible. The intensities of both ^{1}H partners are also equal in [^{14}N]NHY spectra taken at values of τ, such as $\tau = 0.240$ μs, where the net effective ^{14}N modulation is the same for $\nu_{+j}(^{14}\mathrm{N})$ and $\nu_{-j}(^{14}\mathrm{N})$ ENDOR frequencies.

The same information about the relative signs of hyperfine couplings *in principle* would emerge from a successful general-TRIPLE experiment in which one of the ^{14}N $\nu_{+j}(^{14}\mathrm{N})$ ENDOR transitions is pumped by one rf source while the ^{1}H ENDOR pattern is collected using a second source. *However*, the signal/noise ratio (S/N) of the older technique is poor for frozen solutions, and the approach usually fails. The S/N of the implicit-TRIPLE is comparable to that of the Mims ENDOR experiment itself, and thus the sign information available from implicit-TRIPLE is likely to be accessible whenever the appropriate ESEEM is provided by a reference nucleus or nuclei.

In our earlier ENDOR study of NHY, ^{17}O ENDOR disclosed the presence of a solvent-derived ligand, and the detection of two exchangeable protons (X1 and X2) led us to conclude that the ligand is bound H_2O. However, the protons of H_2O must have hyperfine couplings with the same signs when the external field is aligned along the Fe−O bond, as is true for the spectra in Figure 1, whereas the implicit-TRIPLE effect shows that X1 and X2 do not. Thus, we must revise the original assignment. One of the protons, X1 or X2, can safely be associated with a bound OH^-; the other is tentatively assigned to a N−H···S hydrogen bond to one of the two cysteinyl sulfur ligands to Fe.

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(14) This decay arises because inhomogeneous ENDOR line widths cause the loss of phase coherence.

(15) The factors of proportionality include the well-known "hyperfine suppression" factor, $\mathrm{hs} = 1 - \cos(2\pi A(^1\mathrm{H})\tau)$, which is independent of the sign of $A(^1\mathrm{H})$.

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