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Determination of the Principal g-Values of Type I or Highly-Anisotropic Low Spin (HALS) Ferriheme Centers in Frozen Solutions

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Abstract

Continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy of highly-anisotropic low spin (HALS) ferric heme centers in frozen solutions is not a very informative approach because usually only one feature is reliably observed in the spectra, that at the maximal principal g-value of, typically, 3.3 - 3.79. The other two EPR turning points are severely broadened by g-strain and are not easily observed in the first-derivative CW EPR spectra. In this work, we have explored the potential of alternative EPR techniques, the electron spin echo (ESE) field sweep and electron spin transient nutation (TN), for obtaining information about the g-tensors of such systems, using as an example a typical HALS ferric heme center, $[\text{Fe}^{\text{III}}(^{15}\text{N-coproporphyrin})(\text{CN})_2]^-$. The analysis of the experimental g-tensor of $[\text{Fe}^{\text{III}}(^{15}\text{N-coproporphyrin})(\text{CN})_2]^-$ has shown that the widths of the underlying energy distributions for this HALS center are comparable to those found for the rhombic bis-imidazole complex. The greater effect on the g-value distributions for HALS centers is determined by near degeneracy of two of the three lower-energy d-orbitals, d_{yz} and d_{xz} , which contain the unpaired electron.

Keywords

HALS heme centers; Type I heme centers; large g_{max} ; ESE; Pulsed EPR; Transient Nutation

Introduction

Highly-anisotropic low spin (HALS) [1], also known as large g_{max} [2 - 4] or Type I [5], ferric heme centers in frozen solutions present challenges for electron paramagnetic resonance (EPR) studies because the only readily observable feature in their EPR spectra is that at the maximal principal g-value (typically, $g_{\text{max}} \sim 3.3 - 3.79$). The other two EPR turning points are severely broadened by g-strain and are not easily observed in the first-derivative continuous wave (CW) EPR spectra of orientationally disordered samples, although they could be detected in single-crystal EPR measurements [6 - 8], and could be roughly estimated by Mössbauer spectroscopy [3, 9 - 12]. The difficulty in observing the

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Supporting Information The ESE field sweep spectra obtained at different mw frequencies and aligned with respect to the g-factor; the pulse sequence of the TN experiment; a formal derivation of the expression for two-pulse ESE in systems with anisotropic g-factor.

higher-field EPR turning points in frozen solutions is explained by the decreased sensitivity of the first-derivative EPR spectrum to very broad spectral features, as well as by baseline distortions due to paramagnetic ($S = 1$) dioxygen which may be dissolved in the samples, paramagnetic impurities in the sample tubes, and paramagnetic contamination of the microwave (mw) resonator. In fact, review of the original literature cited in ref. [1] shows that in some cases the estimates of the two lower principal g-values may have been inaccurate.

The (effectively) single-feature EPR spectra have been observed for the HALS heme centers in a number of important biological systems, including the three hemes of the membrane-bound cytochrome bc_1 complexes of prokaryotes and eukaryotes [13], and the cytochrome b_6f complexes of photosynthetic bacteria, algae and plants, as well as for small molecule models of these complexes. X-ray crystallographic investigations of model complexes provided the absolute correlation that HALS EPR signals arise because of the small energy splitting between the d_{xz} and d_{yz} orbitals of low-spin Fe(III) when the axial ligand planes are approximately perpendicular to each other [3 - 6, 9, 11, 13, 14] or when the axial ligands have a cylindrical symmetry with respect to the heme normal, as for bis-cyanide complexes. More recent protein X-ray crystallographic investigations of the ferriheme proteins of the bc_1 , b_6f and related complexes which exhibit the large $g_{\max}$ signals have confirmed that the axial ligand planes in *bis*-His-coordinated hemes of cytochromes b [15] and c [16], and the His-Met-coordinated cytochromes c with the same types of EPR signals [16 - 18] are also nearly perpendicular (in the case of Met-coordinated cytochromes c , the ligand plane is defined by the bisector of the methyl, sulfur, and β -C atom angle and the S-Fe bond).

While all principal g-values of the HALS centers can be obtained from single crystal EPR measurements [6 - 7], such experiments require growing single crystals (in the case of small molecules, magnetically dilute), which is not always easily accomplished. More importantly, the well-defined g-values in a crystal may be determined by the structural constraints imposed by the crystal lattice and may not reflect the whole range of g-values available for the heme in a frozen solution. To our knowledge, there are no reports in the literature where the statistical distributions of the principal g-values for the HALS centers in frozen solutions were accurately experimentally determined and analyzed. In particular, it is not clear, how the large distribution width of the g-values in the HALS centers correlates with the structural inhomogeneity. Therefore, we made it a purpose of this work to obtain the characteristics of the g-strain for HALS systems and analyze them in terms of crystal field splittings within the t_{2g} orbital set, d_{xz} , d_{yz} and d_{xy} . The results are compared with those for a bis-imidazole complex that shows moderate rhombic g-anisotropy, with the principal g-values ranging from about 1.5 to 3.

As an alternative to CW EPR detection, we have used two different pulsed EPR techniques. One of these techniques is the electron spin echo (ESE) field sweep, where the ESE signal amplitude is measured as a function of magnetic field, and the EPR spectrum is thus detected in an absorption-like mode [19]. In spite of the fact that this technique is well-known and widely used, its application to the HALS heme systems is not trivial because of the very large g-anisotropy and strong ESE envelope modulation (ESEEM) caused by the ^{14}N nuclei. Therefore, special attention is given to selecting the proper experimental parameters and to overcoming the detrimental effects of the ^{14}N ESEEM.

The other pulsed EPR technique we employed represents one of the variants of electron spin transient nutation (TN) spectroscopy, which has been occasionally used to estimate the principal g-values of transition metal complexes [20 - 22]. In this work, we apply TN spectroscopy to a HALS heme system with only one well-defined EPR turning point.

Materials and Methods

Sample preparation

^{15}N -enriched Fe(III) coproporphyrin was purchased from Frontier Scientific, and natural abundance chloroiron(III) octaethylporphyrin and chloroiron(III) tetraphenylporphyrin were purchased from Sigma-Aldrich and used as received. Imidazole was purchased from Sigma-Aldrich and used as received. Samples of $\text{K}[\text{Fe}^{\text{III}}(^{15}\text{N}\text{-coproporphyrin})(\text{CN})_2]$ (in brief, $[\text{Fe}(\text{copro})(\text{CN})_2]^-$) and $\text{K}[\text{Fe}^{\text{III}}(\text{octaethylporphyrin})(\text{CN})_2]$ (in brief, $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$) were prepared by dissolving $\text{Fe}^{\text{III}}(^{15}\text{N}\text{-coproporphyrin})$ and chloroiron(III) octaethylporphyrin each in dimethylformamide to a concentration of 10 mM and adding potassium cyanide to make the solution 20 mM in KCN. Acetonitrile was then added to this solution to create a 4:1 ratio of acetonitrile:dimethylformamide, which we have found forms good glasses. $\text{Fe}^{\text{III}}(\text{tetraphenylporphyrin})(\text{imidazole})_2\text{Cl}^-$ (in brief, $[\text{Fe}(\text{TPP})(\text{ImH})_2]^+$) was prepared by dissolving $\text{FeCl}(\text{TPP})$ in CD_2Cl_2 to a concentration of 2 mM and adding 6 equivalents of imidazole. All samples were transferred to quartz EPR sample tubes (Wilmad) and immediately frozen in liquid nitrogen, and stored in that medium.

EPR experiments and simulations

The experiments were performed on a homebuilt broadband pulsed EPR spectrometer operating over the range of mw frequencies from 2 to 40 GHz (S to K_a bands) [23]. The spectrometer is equipped with a flow cryostat CF935 (Oxford Instruments). The measurement temperature was 4.2 K. The detailed experimental conditions are indicated in the figure legends. The numerical simulations of the data obtained in EPR experiments (ESE field sweeps, TN spectra) were performed using the specially written software utilizing the rigorous theoretical expressions appropriate for the systems with strong g-anisotropy (see Supporting Information).

Results and Discussion

1. Two-pulse ESE field sweeps

While ESE detection, as opposed to first-derivative CW EPR detection, makes the sensitivity less dependent on the sharpness of the spectrum features (the ESE amplitude is proportional to the inverse linewidth, while the CW EPR signal is proportional to the square of the inverse linewidth), another serious problem arises. The heme complexes are notorious for their very deep ESEEM caused by the heme pyrrole nitrogens and the nitrogens of the axial ligands. Protons also heavily contribute to the ESEEM at lower magnetic fields. Since the ESEEM depends on the EPR position (which determines both the nuclear Zeeman frequency and the range of orientations of the paramagnetic center contributing to the ESE signal), measuring a field-sweep spectrum at any given separation between the mw pulses results in significantly distorted EPR spectrum shape. For the heme centers with small to moderate g-anisotropy (*e.g.*, a bis-imidazole complex) this usually does not represent a problem because the EPR turning points are relatively sharp and readily discernible (at least, in X-band experiments). For the HALS centers, however, the g-strain is much stronger, and the spectrum amplitude undulations caused by ESEEM can efficiently hide the turning points corresponding to the two smaller principal g-values.

Three measures were taken in this work to reduce the ESEEM effects and enable the detection of the high-field EPR turning points. First, most of the measurements were performed for the complex with ^{15}N -enriched coproporphyrin. Unlike ^{14}N , the ^{15}N isotope has spin $I = 1/2$ and no nuclear quadrupole interaction (nqi), which eliminates one of the main causes of the strong nitrogen ESEEM in the heme systems. Second, axial cyanide ligands were used to reduce the overall number of directly coordinated nitrogens. Finally,

the ESEEM effects were further suppressed by recording a two-dimensional (2D) field sweep spectrum, B_0 vs. τ (where τ is the time interval between the mw pulses) and obtaining a 1D spectrum out of it by integrating over τ . For brevity, we will call such a 1D field sweep an integrated field sweep spectrum.

Before considering the experimental results, we have to briefly discuss the effect of the amplitude of the mw field, B_1 , on the shape of the field-sweep spectra. This effect can be qualitatively understood from the expression describing the two-pulse ESE signal amplitude as a function of the turning angles of the mw pulses. For the common case of two pulses of the same amplitude and of durations t_p for the first pulse and $2t_p$ for the second one, the well-known expression is [24]:

$$V \propto \sin \theta \cdot \sin^2 \frac{2\theta}{2} = \sin^3 \theta \quad (1)$$

where the first pulse turning angle $\theta = 2\pi g_{B1} \beta B_1 t_p$ (β is the Bohr magneton) is proportional to the mw field amplitude, B_1 , and to the g-factor, g_{B1} , corresponding to the direction along $\mathbf{B}_1$ (the explicit expression for g_{B1} is given by Eq. (S18) of the Supporting Information). We should note that while Eq. (1) is rather approximate for systems with strong g-anisotropy (a rigorous expression is given by Eq. (S16) of the Supporting Information; also see ref. [25]), it is quite sufficient for a qualitative consideration.

It follows from Eq. (1) that the largest ESE signal is obtained for $\theta = 90^\circ$. This condition, however, can be satisfied for all resonant spins at once only in two cases, either for an isotropic g-factor (in which case $g_{B1} = g_{iso}$), or for a system with an axial g-tensor, when the static magnetic field $\mathbf{B}_0$ is oriented along the axis of $g_{//}$, while $\mathbf{B}_1$ is in the $g_{\perp}$ plane, so that $g_{B1} = g_{\perp}$. In all other cases, different orientations of $\mathbf{B}_1$ (obtained by rotation around $\mathbf{B}_0$) will correspond to different values of g_{B1} , and thus, to a range of turning angles θ contributing to the ESE signal. For the HALS systems the practical implications of these considerations are especially important because the g-anisotropy is so large. The mw amplitude required to optimize the ESE signal at the low-field EPR turning point ($g_3 = g_{max}$, $g_2 = g_1$) may be several fold as large as that needed to optimize the ESE signal at the high-field regions of the spectrum, which can lead to strong distortions of the recorded lineshapes. The model simulations presented in Fig. 1 show that, in order to detect a relatively undistorted lineshape (*i.e.*, having the ESE signal amplitude of the same sign throughout the whole spectrum and showing all of the turning points), one has to select B_1 in such a way as to optimize the ESE amplitude at the EPR positions corresponding to $g_{\perp}$.

Now let us consider the experimental ESE field sweeps. Traces 1 and 2 in Fig. 2 show integrated field sweep spectra of the ^{15}N -enriched $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ obtained at X-band. It is expected from the symmetry of this complex that the g-tensor should, on average, be axial (with the main axis perpendicular to the heme plane), while the theoretical analysis of the electronic configuration for a system with the largest g-factor of ~ 3.5 predicts $g_{//} > g_{\perp}$ [6]. Because of the slight structural variations in the ligand and heme geometry (leading to the g-strain), however, each statistical realization of the g-tensor is not necessarily axial, and we will therefore also denote the principal g-values in a more general way as g_3, g_2, g_1 . Specifically, for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ both notations are related by $g_{//} = \langle g_3 \rangle$, $g_{\perp} = \langle g_1 \rangle = \langle g_2 \rangle$.

Spectrum 1 of Fig. 2 was recorded with B_1 maximizing the ESE signal amplitude at the low-field EPR turning point ($g_{//} = 3.5$). At this EPR position $\mathbf{B}_0$ is parallel to the axis of $g_{//}$, while the $\mathbf{B}_1$ vector is in the $g_{\perp}$ plane. Based on the qualitative considerations above, one can expect that the high-field parts of the spectrum will be significantly attenuated at this B_1 setting, and this is confirmed by the field sweep. In order to be able to detect the high-field

turning point(s), it is necessary to set B_1 to maximize the ESE signal at $g \sim g_{\perp}$. Without knowing $g_{\perp}$ in advance, one can only maximize the ESE signal at a possibly high B_0 value (in these experiments, ~ 1.2 T). Trace 2 in Fig. 2 shows the spectrum obtained at such B_1 setting. This spectrum reveals a broad peak at $B_0 \sim 600$ mT potentially attributable to $g_{\perp} \sim 1$. However, since this peak could also be related to residual effects of the ESEEM (remaining after integration over τ), we have repeated the measurements at other mw frequencies. Because the measurements at higher mw frequencies would effectively reduce the portion of the field sweep spectrum available for observation, we performed measurements at lower frequencies of 6.4 and 4.7 GHz (C-band). The spectra obtained are shown in Fig. 2 by traces 3 and 4, respectively. In both cases the mw field was set to maximize the ESE signal at a possibly high B_0 . One can see that both spectra show a singularity near $g = 1$. We therefore can confidently assign this singularity to a canonical orientation of the g-tensor ($g_{\perp}$).

Rescaling the C-band spectra to the X-band mw frequency, $\nu_{mw} = 9.45$ GHz, shows that the shapes of these spectra at $g < 1$ (in the region where they overlap) are identical (see Fig. S1 of the Supporting Information). This shows that the ESEEM oscillations are efficiently averaged out, and we can therefore append the high-field part of the rescaled 4.7 GHz spectrum to the X-band spectrum to obtain the X-band spectrum as if it were recorded up to $B_0 = 2.2$ T. Such a composite spectrum is shown by the solid trace in Fig. 3. This spectrum was used for comparison with the results of numerical simulations aimed at determining the principal g-values and their distribution widths.

Figure 4 compares the X-band integrated field-sweep spectrum of the ^{15}N -enriched $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ (trace 1) with that of $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$ (trace 2). The latter compound has the natural abundance of nitrogen isotopes, which results in greater distortions of the spectrum compared with an ideal lineshape. In particular, the turning points are better defined at the expense of the reduced signal amplitude at the intermediate field positions. These distortions, however, are not very large, and one can readily estimate for $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$ $g_{\perp} \sim 1$, similar to that found for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$.

2. Transient nutation spectra

An alternative approach for determining the principal g-values is based on a TN experiment. The idea of this experiment consists in the fact that the longitudinal magnetization, M_z , produced by the resonant spins, changes periodically during the mw pulse:

$$M_z \propto \cos \theta \quad (2)$$

where $\theta = 2\pi g_{B1} \beta B_1 t_p$ (see Eq. (S18) in the Supporting Information for explicit expression). The magnetization at the end of the mw pulse of variable length t_p can be measured as a function of t_p , and then Fourier transformed. The resulting spectrum will give the nutation frequencies of the resonant spins, $\nu_N = g_{B1} \beta B_1$.

As discussed above, the range of g_{B1} values contributing to M_z depends on EPR position, and so does the range of nutation frequencies. Figure 5 shows examples of 2D, ν_N vs. B_0 , TN spectra simulated numerically for the situations of axial g-tensors with $g_{\perp} > g_{\parallel}$ and $g_{\perp} < g_{\parallel}$ (panels a and c, respectively) and a rhombic g-tensor (panel b). One can see that the shape of the spectrum can be described as a triangle (for an axial g-tensor) or a hexagon, whose corners are located at positions (ν_N, B_0) corresponding to various combinations of principal g-values (g_1, g_k). Thus, in principle, the measurement of ν_N at one of the turning points of a g-anisotropic EPR spectrum allows one to measure the g-factors corresponding to two other turning points. Specifically for the case of a rhombic g-tensor (panel b) the B_0 position of the middle corner of the high-frequency ridge of the TN spectrum gives an estimate for g_2 .

While various modifications of the TN experiment are possible [26], we used in this work the ESE-detected TN technique [27] employing the pulse sequence shown in the Supporting Information (Fig. S2). The longitudinal magnetization was altered by the preparation pulse of variable length, and then monitored using the amplitude of the ESE signal produced by a two-pulse observation sequence. In a practical experiment, it is essential to create conditions when the oscillations given by Eq. (2) are observed as cosine functions of the same sign. For this, the observation B_1 should be set in the same way as for the field-sweep experiment described above. Under such conditions, however, the low nutation frequencies also correspond to small ESE amplitudes obtained by the detection two-pulse sequence (as they correspond to small turning angles θ in Eq. (1)). Therefore, it would probably be overoptimistic to hope to detect the nutation frequency corresponding to the very low principal g -values in HALS ferriheme systems, and indeed, the experimental 2D TN spectra (see below) were significantly weighted toward the higher nutation frequencies and have mostly shown a prominent ridge corresponding to the larger g -values.

The 2D TN spectrum obtained for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ is shown in Fig. 6. The nutation frequency axis in this spectrum was scaled in g -factor units, using for calibration the largest principal g -value that is known from CW EPR with good accuracy. In spite of the effective axial symmetry of the g -tensor, the TN spectrum does not look like that simulated for an axial g -tensor (Fig. 5c), but rather like that simulated for a general rhombic g -tensor (Fig. 5b). This is explained by the broad statistical distribution of the two lower g -values, which results in the overwhelming majority of individual centers having a rhombic g -tensor. This leads to the appearance of the horizontal ridge in the spectrum, the extent of which can serve as a characteristic of the distribution width. The position of the corner in the high-frequency ridge gives an estimate of $g_\perp$ from above: $g_\perp \sim 1.5$, which is significantly larger than the qualitative estimate obtained from the field sweep.

Similar field sweep and TN data were obtained for $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$. As shown below, both kinds of spectra, ESE field sweep and 2D TN, can be simulated with the same set of parameters.

3. Simulations of ESE field-sweep and 2D TN spectra

While certain qualitative conclusions have been made from the field sweep and TN experiments described above, accurate estimates of the average high-field g -values and their distribution widths can only be made using numerical simulations. Such simulations were performed using the rigorous expressions given in the Supporting Information and employing a model of uncorrelated Gaussian distributions of principal g -values. The mean g -tensor of $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ was assumed to be axial ($\langle g_{\parallel} \rangle = \langle g_3 \rangle$; $\langle g_{\perp} \rangle = \langle g_1 \rangle = \langle g_2 \rangle$), which corresponds to the symmetry of this complex. Since the field-sweep spectrum shape depends on B_1 , the simulations had to be performed in a 2D fashion: B_0 vs. B_1 . The target 1D spectrum was then represented by the slice of the 2D spectrum along B_0 corresponding to the B_1 value maximizing the ESE amplitude at $B_0 \sim 1.2$ T, thus emulating the experimental choice of B_1 .

The simulations have resulted in $(\langle g_1 \rangle, \langle g_2 \rangle, \langle g_3 \rangle) = (0.6, 0.6, 3.5)$ and $(\Delta g_1, \Delta g_2, \Delta g_3) = (0.55, 0.55, 0.2)$, where Δg_k are the Gaussian widths between the maximum slope points. The simulated field-sweep spectrum is shown by dashed trace in Fig. 3. The simulated 2D TN spectrum is shown in Fig. 7. It reasonably reproduces the experimental spectrum shown in Fig. 6, in particular, the position of the corner in the high-frequency ridge of the TN spectrum at B_0 corresponding to $g \sim 1.5$.

One has to note that the very broad statistical distribution of the two smaller g -tensor components actually makes it impossible to accurately estimate their mean values without

explicitly specifying the symmetry of the g-tensor. For example, for a general rhombic g-tensor one can reasonably fit the shape of the field sweep spectrum even for $\langle g_1 \rangle$ approaching 0, in which case $\langle g_2 \rangle \approx 0.9$ is estimated, significantly larger than $\langle g_{\perp} \rangle = 0.6$ obtained for the axial tensor.

One also has to comment on the g-strain model based on uncorrelated Gaussian distributions of the principal g-values. While this model is convenient and allows one to fit the experimental spectra, it may not be entirely appropriate for the situation when the distribution width is comparable (or even exceeds) the mean g-value. Although we are not aware of any obvious physical arguments as to why this model should be wrong, it is possible that some sort of a skewed truncated distribution would be more appropriate for the lower principal g-values. However, given the accuracy with which the two lower principal g-values can be formally estimated from the numerical simulations (see above), the simulations are unlikely to provide much guidance in establishing the detailed properties of the actual distribution. In spite of this uncertainty, we believe that the simple g-strain model used can provide at least the characteristic mean values and distribution widths of the relevant energies in the d-orbital system (see the next section), if not the accurate shapes of these distributions.

4. Analysis

The simulation of the experimental ESE field sweeps has resulted in the principal g-values and the widths of their statistical distributions. In this section we will analyze these parameters in terms of the energies and wave functions of the lower d-orbital triplet (t_{2g}) populated by the unpaired electron.

While the signs of the principal g-values were not important for the EPR simulations, they become important for the theoretical analysis. The relative signs of the principal g-values are chosen in such a way as to ensure the correct relative magnitudes of the tetragonal splitting, Δ , and the rhombic splitting, V , in the t_{2g} triplet of the lower d-orbitals: $V/\Delta = 2/3$, which corresponds to the so-called proper axis system [28]. The relative signs of the principal g-values depend on the choice of phases in the wavefunctions [29, 30]. In this work we used the expressions derived by Taylor [28], and with his parametrization a system with an axial g-tensor corresponding to the proper coordinate system is actually described by a g-tensor with opposite signs of the “perpendicular” components.

Figure 8 shows the results of the calculations of the rhombic and tetragonal splittings obtained from the experimental distributions of the g-values. For comparison, the distributions of these quantities were also calculated for the rhombic EPR signal of the bis-imidazole complex of Fe(III)(tetraphenylporphyrin), $[\text{Fe}(\text{TPP})\text{ImH}_2]^+$, which has $(\langle g_3 \rangle, \langle g_2 \rangle, \langle g_1 \rangle) \approx (3, 2.22, 1.42)$ and shows much smaller distribution widths ($\Delta g_3, \Delta g_2, \Delta g_1 \approx (0.1, 0.15, 0.15)$). One can see that the widths of the underlying energy distributions are actually quite comparable in both cases. Given the difference between the ligands in these complexes (planar imidazoles vs. linear cyanides), one can conclude that these energy distributions should be mostly unrelated to the statistical variations of the angle between the ligand planes, and are mostly caused by other structural degrees of freedom, the ranges of the latter structural variations being similar (while very small [6]) in both types of complexes. The greater effect on the g-value distributions for HALS centers is determined by near-degeneracy of two of the lower d-orbitals.

Conclusion

In this work we have addressed the problem of measuring the g-tensors of HALS Fe(III) heme centers with large statistical distribution of the two lower principal g-values. It was

shown that the use of ESE field sweeps with integration over the time interval between the mw pulses allows one to dramatically reduce the ESEEM-related distortions and unambiguously observe the high-field EPR turning points. Without an isotopic substitution of the heme pyrrole nitrogens by ^{15}N , however, this integration may not be sufficient for obtaining a relatively undistorted overall lineshape that could be used as a reference in numerical simulations. Since the shape of the field-sweep spectrum depends on B_1 , the simulations have to be performed in a 2D fashion, B_0 vs. B_1 , with the target 1D spectrum representing a slice along B_0 at a proper relative B_1 .

In addition to the ESE field sweep, an alternative technique based on measuring the electron spin transient nutation frequencies was used. In principle, the TN technique was perceived as a tool that gives the possibility to estimate high-field principal g-values from a single measurement at the low-field principal g-value. In this way, one can disentangle the overlapped spectra of ferriheme centers that are sometimes observed in proteins. This approach can undoubtedly work for the systems with well-defined EPR turning points. However, as shown in this work, for the systems with significant g-strain the information obtained from the TNs is mostly limited to qualitative observations regarding the overall spread of the lower g-values. More detailed quantitative information about the mean principal g-values and their statistical distributions could potentially be obtained from the TN spectra, but such numerical simulations would be significantly more involved than those performed in this work for the two-pulse field-sweep spectra.

The analysis of the experimental g-tensors of a representative HALS center, $[\text{Fe}(\text{copro})(\text{CN})_2]^-$, has shown that the widths of the underlying energy distributions for HALS centers are similar to those found for the rhombic bis-imidazole complex. The greater effect on the g-value distributions for HALS centers is determined by near degeneracy of two of the lower d-orbitals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- ▶ Highly-anisotropic low-spin (HALS) heme centers have a very large g-strain.
- ▶ Pulsed EPR was used to determine the g-tensor of a HALS heme center.
- ▶ Analysis shows that the large g-strain is caused by the degeneracy of d_{π} orbitals.

Figure 1.

Two-pulse ESE field sweeps simulated for $(g_1, g_2, g_3) = (0.4, 1.5, 3.5)$ and $\nu_{\text{mw}} = 9.45$ GHz. Solid, long-dashed, and short-dashed traces correspond to the B_1 values maximizing the ESE signal at g_3 , g_2 , and g_1 , respectively.

Figure 2.

Integrated (over τ) two-pulse ESE field sweep spectra of ^{15}N -enriched $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ at different mw frequencies. Trace 1, B_1 is set to maximize the ESE amplitude at g_3 . Traces 2 through 4, B_1 is $1/2.8$ of that maximizing ESE amplitude at g_3 ($B_1 = B_{1\text{max}}/2.8$; 9 db relative power attenuation). The mw frequencies for traces 1 through 4 are 9.450 GHz, 9.450 GHz, 6.279 GHz and 4.679 GHz, respectively. The mw pulse durations were 2×15 ns for traces 1 and 2, and 2×30 ns for traces 3 and 4. The range of intervals τ included in the integration was 150 – 630 ns for traces 1 and 2, and 204 – 1220 ns for traces 3 and 4. The data acquisition time was ~ 30 min per spectrum. The dashed arrows trace the $g = 1$ position as a function of the mw frequency.

Figure 3.

Solid trace, integrated (over τ) two-pulse ESE field sweep spectrum of ^{15}N -enriched $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$ at $B_1 = B_{1\text{max}}/2.8$ obtained by concatenation of the X-band spectrum (trace 2 of Fig. 2) and the high-field part of the C-band spectrum detected at $\nu_{\text{mw}} = 4.679$ GHz (trace 4 of Fig. 2). The effective mw frequency for this composite spectrum is $\nu_{\text{mw}} = 9.450$ GHz. Dashed trace, simulated (using Eq. (S16)) for $(\langle g_3 \rangle, \langle g_2 \rangle, \langle g_1 \rangle) = (3.5, 0.6, 0.6)$ and $(\Delta g_3, \Delta g_2, \Delta g_1) = (0.2, 0.55, 0.55)$. The B_1 used in the simulations was similar to that used in the experiments ($B_1 = B_{1\text{max}}/2.8$).

Figure 4.

Integrated (over τ) two-pulse ESE field sweep spectra of ^{15}N -enriched $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ (trace 1, reproduces trace 2 in Fig. 1) and $[\text{Fe}(\text{OEP})(\text{CN})_2]^-$ (trace 2). The latter sample had the natural abundance of the nitrogen isotopes. Experimental conditions for trace 1 are shown in the legend for Fig. 1. Experimental conditions for trace 2: $\nu_{\text{mw}} = 9.448 \text{ GHz}$; mw pulses, $2 \times 15 \text{ ns}$; $B_1 = B_{1\text{max}}/2.8$; range of time intervals τ included in the integration, 150 – 850 ns. The data acquisition time was $\sim 30 \text{ min}$ per spectrum.

Figure 5. Simulated 2D TN spectra for (a) $\mathbf{g} = (3.5, 3.5, 0.8)$; (b) $\mathbf{g} = (3.5, 1.5, 0.8)$ and (c) $\mathbf{g} = (3.5, 0.8, 0.8)$. $\nu_{\text{mw}} = 9.450$ GHz.

Figure 6.

2D TN spectrum of ^{15}N -enriched $[\text{Fe}(\text{copro})(\text{CN})_2]^-$. Experimental conditions: $\nu_{\text{mw}} = 9.450$ GHz; observation mw pulses, 2×80 ns; $B_1 = B_{1\text{max}}/4$ (12 db relative power attenuation). The data acquisition time was ~ 90 min.

Figure 7.

Simulated 2D TN spectrum for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$. The simulation parameters are the same as for the dashed trace in Fig. 3: $\nu_{\text{mw}} = 9.450$ GHz, $(\langle g_3 \rangle, \langle g_2 \rangle, \langle g_1 \rangle) = (3.5, 0.6, 0.6)$ and $(\Delta g_3, \Delta g_2, \Delta g_1) = (0.2, 0.55, 0.55)$.

Figure 8.

The distributions of (a) rhombic energy splittings $V = E(d_{XZ}) - E(d_{YZ})$ and (b) tetragonal energy splitting $\Delta = (E(d_{XZ}) + E(d_{YZ}))/2 - E(d_{XY})$ for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ (solid traces) and $[\text{Fe}(\text{TPP})(\text{ImH})_2]^+$. The g-tensors used in the simulations were $(\langle g_1 \rangle, \langle g_2 \rangle, \langle g_3 \rangle) = (-0.6, 0.6, 3.5)$ and $(\Delta g_1, \Delta g_2, \Delta g_3) = (0.55, 0.55, 0.2)$ for $[\text{Fe}(\text{copro})(\text{CN})_2]^-$ and $(\langle g_1 \rangle, \langle g_2 \rangle, \langle g_3 \rangle) = (1.42, 2.22, 3.0)$ and $(\Delta g_1, \Delta g_2, \Delta g_3) = (0.15, 0.15, 0.1)$ for the bis-imidazole complex. λ is the spin-orbit coupling constant.