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Magnetic Circular Dichroism Evidence for a Weakly Coupled Heme-Radical Pair at the Active Site of Cytochrome *cd*$_1$, a Nitrite Reductase

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In nitrite-treated cytochrome *cd*$_1$ nitrite reductase, heme *d*$_1$ is electron paramagnetic resonance silent but paramagnetic. Analysis of the unusual temperature dependence of the magnetic circular dichroism spectra unambiguously demonstrates that the heme *d*$_1$ is not in the oxoferryl ($Fe^{IV}{=}O$) state but is low-spin Fe^{III} weakly coupled to a radical species. This species could be either a protein-bound radical generated by a nitrite ion reacting with a heme group resulting in a one-electron oxidation of an amino acid residue, possibly tyrosine or tryptophan, adjacent to heme *d*$_1$, or a heme *d*$_1$ $Fe^{III}NO$ complex.

Cytochrome *cd*$_1$ (*cd*$_1$) is a soluble homodimeric enzyme involved in bacterial denitrification,[1] where it catalyzes the one-electron reduction of nitrite ion (NO_2^-) to nitric oxide (NO):

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \qquad (1)$$

The enzyme possesses two heme groups: heme *c* transfers electrons from donor proteins to heme *d*$_1$, the novel iron dioxoisobacteriochlorin at the active site. In oxidized *cd*$_1$, *Paracoccus pantotrophus* (*Pp*), heme *c* has *bis*-histidine ligation and Fe^{III} heme *d*$_1$ [*d*$_1$(Fe^{III})] is bound by histidine and tyrosine.[2] Both give rise to a low-spin Fe^{III} electron paramagnetic resonance (EPR) spectrum.[2] Reduction of both hemes to the Fe^{II} state results in the replacement of one heme *c* histidine ligand with methionine and the concomitant dissociation of the distal tyrosine ligand from heme *d*$_1$, creating a vacant coordination site where substrate NO_2^- can bind.[3] Because enzyme activity is associated with the histidine−methionine coordination of heme *c*, it has been assumed that the activation process is driven by the reduction of both heme groups.[4] However, recently it has been shown[5] that oxidized *cd*$_1$ will react directly with NO_2^- to drive the ligand switch at heme *c* without the necessity of reduction of either heme.

The resulting heme *d*$_1$ species, termed *cd*$_1$*-X, no longer gives rise to an EPR spectrum, but ratio data magnetic circular dichroism (RD-MCD) spectroscopy shows that it remains paramagnetic with a spin $S \neq {}^1/_2$.[5] There are two plausible models for the nature of heme *d*$_1$ in *cd*$_1$*-X, which may explain these observations. Oxidation of *d*$_1$(Fe^{III}) by NO_2^- could yield an $S = 1$ oxoferryl form of heme *d*$_1$, as has been postulated to occur during the reoxidation of reduced *cd*$_1$ by dioxygen.[6] Alternatively, a single turnover of NO_2^- (eq 1) using an electron abstracted from a nearby residue would produce two radical species: the protein-based radical itself and the product molecule NO. Dissipation of the protein-radical OR dissociation of NO would leave a single spin to exchange-couple with the low-spin *d*$_1$(Fe^{III}) and so preclude observation of an EPR spectrum. The absorption spectrum of *cd*$_1$*-X is consistent with a model in which heme *d*$_1$ is in the low-spin Fe^{III} state.[5] However, there is only one report of a $Fe^{IV}{=}O$ species in this enzyme[6] and no absorption data that could rule out this state in *cd*$_1$*-X. When reductant is added to the *cd*$_1$*-X species, the enzyme turns over because substrate is present. The heme *d*$_1$ therefore becomes immediately reduced but EPR-silent. This paper describes an analysis of the variable-temperature (VT)-MCD

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spectra, in the spectral region of the heme d_1, of cd_1*-X that allows a discrimination between these two models.

MCD is a powerful tool for the analysis of the non-Kramers paramagnetic ground states of high-spin single metal ions[7−9] and of exchange-coupled spins.[9,10] For low-spin Fe^{III} hemes c and d_1, all transitions of appreciable intensity in the UV−visible spectra are xy-polarized, and according to the general theory,[7,8] their MCD intensities are proportional to

$$\langle S_z^{\mathrm{Fe}}(T,H)\rangle = \frac{g_{zz}}{4\pi}\int_0^{\pi}\int_0^{2\pi}\frac{\cos^2\theta\sin\theta}{g(\theta,\phi)}\tanh\left[-\frac{\beta H}{2kT}g(\theta,\phi)\right]\mathrm{d}\theta\,\mathrm{d}\phi \quad (2)$$

where $g(\theta,\phi) = [g_{zz}^2\cos^2\theta + (g_{xx}^2\cos^2\phi + g_{yy}^2\sin^2\phi)\sin^2\theta]^{1/2}$ and contains the principal components of the **g** tensor.

The integral contains the ratio of field to temperature, H/T, as a parameter and, hence, by recording of the MCD intensity at fixed ratios of H/T (called RD-MCD[10]), no changes in the MCD intensity of $S = {}^1/_2$ species will be observed, i.e., when both hemes are in the low-spin Fe^{III} state. Both RD-MCD[5] and VT-MCD data (Figure 1) show that heme d_1 is not a simple isolated $S = {}^1/_2$ species.

To rationalize these observations, we have considered the two models of a non-Kramers system, namely, a ferryl form of heme d_1, $S = 1$, d_1(Fe^{IV}═O) and an exchange-coupled low-spin d_1(Fe^{III})-radical pair.

Both the temperature-independent component and the contribution from low-spin Fe^{III} heme c were eliminated from the MCD spectra.[10] The normalized temperature dependence of the remaining VT-MCD intensity, measured at the 654−620 nm peak-to-trough distance, together with simulations based on the two different models, are shown in Figure 1.

Only the spin-coupled model fits the temperature dependence curve. We have applied the spin Hamiltonian

$$\hat{H} = \lfloor\beta H\mathbf{g}\hat{S}\rfloor\mathrm{Fe} + \lfloor\beta H\mathbf{g}\hat{S}\rfloor^{\mathrm{rad}} + \hat{S}^{\mathrm{Fe}}\cdot\mathbf{J}\cdot\hat{S}^{\mathrm{rad}} \quad (3)$$

to a basis set comprising products of single-ion spin-state wavefunctions with typical values of low-spin d_1(Fe^{III}) **g**-tensor components $g_{zz} = 2.52$, $g_{yy} = 2.19$, and $g_{xx} = 1.84$,[2,4,6] together with a spin radical with the g factor of a free electron. In the case of coupled spins, only the contribution of $\langle S_z^{\mathrm{Fe}}\rangle$ from heme d_1 to $\langle S_z\rangle = \langle S_z^{\mathrm{Fe}}\rangle + \langle S_z^{\mathrm{rad}}\rangle$ of the overall spin $\langle\mathbf{S}\rangle$ must be calculated and used in the expressions for MCD.[7,8,10] MCD provides directional selectivity even for randomly oriented molecules in frozen solution.[7,8] The principal components of the **J** tensor, in the heme frame, obtained from fitting of the MCD temperature dependence, appear to be antiferromagnetic and anisotropic, with the smallest component directed along the heme normal. No EPR signal has been observed at X-band frequencies up to 0.7 T. Therefore, the zero-field-splitting (ZFS) spin levels should be well separated. This is achieved by the following conditions: $|\mathbf{J}_{ii} - \mathbf{J}_{jj}| > 2h\upsilon$, where υ is the X-band microwave frequency, ~9.5 GHz. An important feature of VT-MCD is the lack of saturation of the MCD intensity even at a very low temperature, 1.5 K. This is unusual behavior for the MCD temperature dependence observed for single-ion paramagnetic sites with $S \geq {}^1/_2$.[7] The energy-level diagram (Figure 2a) shows the magnetic field dependence of the ground-state energy levels arising from exchange coupling between two $S = {}^1/_2$ spins, d_1(Fe^{III}), and a radical, whereas Figure 2b shows the case of a ferryl form of d_1(Fe^{IV}═O), $S = 1$.

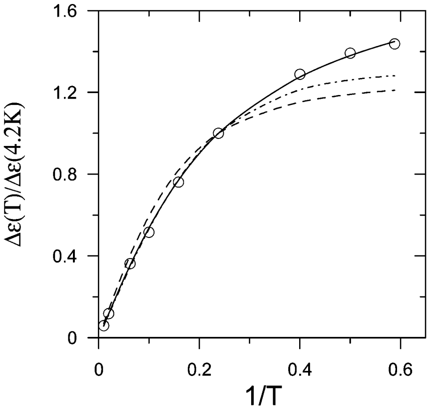

Figure 1. Comparison between the experimental (○) and simulated MCD temperature dependence of heme d_1. Simulation using eq 2 indicating that heme d_1 is not a simple isolated $S = {}^1/_2$ species (− • −). Simulation model of coupled spins with $J_{xx/yy/zz}$ = [4.8, 3.6, 1.5] cm^{-1} (—). Simulation for an $S = 1$ single-ion model using the following parameters: $D = 2$ cm^{-1}; $E = 0.5$ cm^{-1} (− −). The values of D and E were chosen to ensure that no EPR resonances can be observed between these states up to 0.7 T.

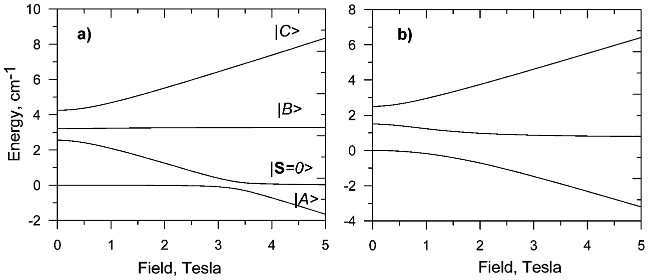

Figure 2. Energy levels as a function of the field of (a) the exchange-coupled system and (b) a single-ion spin $S = 1$.

The unique feature of exchange-coupled systems with different spin states lying in close proximity (Figure 2a) is that the slope of the MCD curve does not saturate at low temperature. It is sensitive to the energy difference between the $S = 0$ and $|A\rangle$ states at 5 T and, therefore, provides a good estimate of the isotropic part $J_{\mathrm{iso}} = (J_{xx} + J_{yy} + J_{zz})/3$ of the **J** tensor, which is calculated from the data to be 3.3

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cm^{-1}. Because of orientational averaging, the main contribution to the MCD signal intensity will arise from field orientations at about $\theta = 45°$ to the heme normal.[8] Figure 2 shows energy levels as a function of the field, with the direction of the magnetic field relative to the heme frame described by the following angles: $\theta = 45°$ and $\phi = 0°$.

At this orientation for an arbitrary spin, manifold neighboring states, differing by $\Delta M = \pm 1$, will undergo mixing and repulsion under the Zeeman interaction.

In particular, all three wavefunctions $|A\rangle$, $|B\rangle$, and $|C\rangle$ in Figure 2a are superpositions of the states $|{}^{1}/_{2}, {}^{1}/_{2}\rangle$, $|-{}^{1}/_{2}, -{}^{1}/_{2}\rangle$, and $1/\sqrt{2}(|{}^{1}/_{2}, -{}^{1}/_{2}\rangle + |-{}^{1}/_{2}, {}^{1}/_{2}\rangle)$ of the triplet. Consequently, at high fields the lowest state is substantially separated from the next. This is illustrated by the energy-level diagram for the $S = 1$ system when the axial ZFS parameter $D = 2$ cm^{-1} (Figure 2b). Under the condition $g\beta H \geq D$, the separation between the two lowest levels is not very sensitive to variations in D. As a result, at high field and low temperatures ($T \leq 3$ K), the Boltzmann population of the lowest state will approach 100%. In contrast, for the case of the exchange-coupled pair (Figure 2a), the energy of an additional singlet state $S = 0$ arising from $\hat{S}^{\mathrm{Fe}} + \hat{S}^{\mathrm{rad}} = 0, 1$ will remain unchanged because the Zeeman interaction does not mix states of different spins. The avoided level crossing, observed in Figure 2a, is due to the anisotropy of the **g** tensor of Fe$^{\mathrm{III}}$. Because optical transitions from the $S = 0$ level have vanishingly weak MCD intensities, such a level positioned close to $|A\rangle$, the lowest state, will share the population with it (~20% for $S = 0$ vs ~80% for $|A\rangle$ at ~1.6 K) and lower the overall MCD intensity. However, with a further decrease in the temperature, $|A\rangle$ gains more population and the MCD signal increases. Thus, the MCD intensity does not saturate even at extremely low temperatures.

The anisotropic part of the **J** tensor, however, is responsible for the ZFS of the triplet state in the coupled system, which involves substantial spin−orbit coupling between the ground- and excited-state magnetic d orbitals of the Fe$^{\mathrm{III}}$ heme.[11] The ZFS between the levels also ensures that no EPR intensity will be detected at the X band at low magnetic fields, which is supported by our continuous-wave EPR simulation.

Thus, this analysis clearly shows that the unusual magnetic properties arise from low-spin d_1(Fe$^{\mathrm{III}}$) weakly coupled to an $S = {}^{1}/_{2}$, $g \sim 2.0$, radical species. One clear possibility for this species is an amino acid that has been oxidized to generate an organic radical. The X-ray structure of the oxidized nitrite-bound form of *cd*$_1$, *Pp*, shows both tyrosine and tryptophan residues in close proximity to two O atoms of the d_1 porphyrin macrocycle (Figure 3). Both residues could generate a radical and form similar exchange pathways to Fe$^{\mathrm{III}}$ consisting of the carbonyl O−C−C−N atoms of the heme plane.

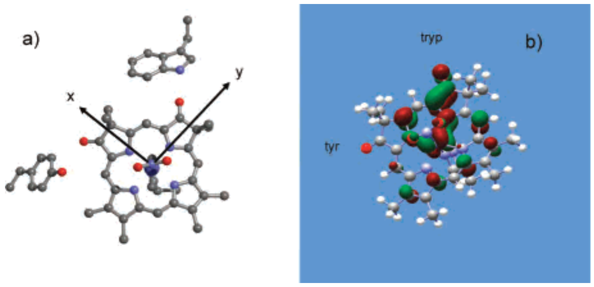

Figure 3. (a) Structure of heme d_1 showing distances between the heme d_1 carbonyl O atoms and the heteroatoms of the close-lying tyrosine and tryptophan residues (2.7 and 3.18 Å, respectively; structure file 1AOQ·pdb).[3] (b) Illustrative example of LUMO from the DFT calculation for nitrite-bound low-spin d_1(Fe$^{\mathrm{III}}$) heme. Note that the nature and orientation of an axial ligand to heme d_1 could direct the spin density toward either tryptophan or tyrosine.

We have performed spin-unrestricted density functional theory (DFT) calculations with a B3LYP hybrid functional with the 6-311G basis set for all atoms.[12] The calculated lowest unoccupied molecular orbital (LUMO), equivalent to the singly occupied molecular orbital in a spin-restricted method, reflects the ground-state wavefunction and its spin distribution. This wave function is mainly a mixture of the Fe$^{\mathrm{III}}$ d$_{xz}$ + d$_{yz}$ orbitals (plus, a small percentage of d$_{xy}$) partially delocalized toward the heme ring carbonyl O atom close to either tryptophan or tyrosine depending on the orientation of the O−N−O plane of the nitrate molecule. Figure 3b shows the LUMO from the DFT calculation on the structure with dihedral angles of ~40° and ~60° between this plane and *yz* and *xz* planes, respectively. In this case, the spin density is directed toward the tryptophan residue. The other possibility is that the species consists of NO bound to low-spin d_1(Fe$^{\mathrm{III}}$) but weakly coupled, such as, for example, in a complex of linear Fe−N−O geometry with one unpaired electron in the π* NO orbital and the other in the d$_{xy}$ orbital of heme d_1. This work does not distinguish between these possibilities.

In conclusion, an unusual temperature dependence of the MCD intensity has been observed for the nitrite-treated form of cytochrome *cd*$_1$ nitrate reductase. Theoretical analysis and simulation of MCD data show that the lack of saturation at high field of the MCD signals at ultralow temperatures arising from heme d_1 of its oxidized form after reaction with a nitrite ion is characteristic of a weakly coupled spin system ($S_1 = {}^{1}/_{2}$ plus $S_2 = {}^{1}/_{2}$) rather than a ferryl, Fe$^{\mathrm{IV}}$=O, form of heme d_1.

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Supporting Information Available: Experimental procedure. This material available free of charge via the Internet at http://pubs.acs.org.

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