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Rapid Freeze-Quench ENDOR Study of Chloroperoxidase Compound I: The Site of the Radical

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Chloroperoxidase (CPO) is a versatile hemoprotein that exhibits peroxidase, catalase, and cytochrome P450-like activities in addition to catalyzing halogenation reactions in the biosynthesis of halogen-containing compounds.[1] During catalysis, CPO forms the classical heme-monooxygenase intermediate, compound I (Cpd-I), which is doubly oxidized above the resting ferric state, one equivalent being associated with a ferryl center, $[Fe{=}O]^{2+}$ ($^{Fe}S = 1$), and the other with an active-site radical ($^{R}S = 1/2$). Cpd-I of CPO (CPO−I) is the only thiolate-ligated enzyme (Figure 1) whose Cpd-I has been characterized through absorption,[2] resonance Raman,[3] EPR, and Mossbauer spectroscopic measurements.[4] The EPR spectrum of CPO−I freeze-quenched during reaction with peracetic acid displays a novel signal with $g_{\|} \approx 1.99$ (along Fe−O) and $g_{\perp} \approx 1.73$ (in heme plane), which was shown to result from exchange coupling between the radical and the ferryl ion. It was modeled with the spin Hamiltonian[5]

$$H = J^{Fe}\boldsymbol{S}\cdot{}^{R}\boldsymbol{S} + D({}^{Fe}S_z^2 - 2/3) \qquad (1)$$

where $D > 0$ is the zero-field splitting (zfs) of the ferryl ion, $J > 0$ (antiferromagnetic (AF) coupling) is the exchange parameter;[4] the observed g values, in combination with Mossbauer data, indicate that $J/D = +1.6$ ($J \approx 58$ cm^{-1}).[4,6]

The $^{R}S = {}^{1}/_{2}$ site of CPO−I is generally presumed to be a porphyrin π-cation radical, although the optical spectrum of CPO−I differs markedly from that of the prototypical porphyrin π-cation radical exchange-coupled to a ferryl ion in horseradish peroxidase Cpd-I (HRP−I).[4a] Theoretical calculations on models of a Cpd-I with a thiolato axial ligand have presented divergent views about its electronic structure. A recent DFT calculation[8] suggested that the radical species is sulfur based, with 80% of spin density on the sulfur of the cysteine ligand of heme. By contrast, other DFT QM/MM studies that include hydrogen bonding to sulfur in the calculations predicted that the radical spin is distributed between porphyrin and sulfur.[9−12] Indeed, some calculations have suggested that the nature of the Cpd-I electronic state can be controlled by its environment, with computations yielding the sulfur-based radical for the active site in vacuo but the porphyrin radical when the active site is embedded in the P450cam protein (for review[13]).

ENDOR spectroscopy of a trapped intermediate provides an ideal means of determining the distribution of spin density in a trapped intermediate and, hence, of characterizing the radical in a Cpd-I.[14,15] As CPO−I is to date the only Cpd-I of a heme enzyme with a thiolate axial ligand that has been trapped in quantities amenable to ENDOR investigation, its characterization is of particular importance. Here we present the first results of rapid freeze-quench ENDOR studies of CPO−I.

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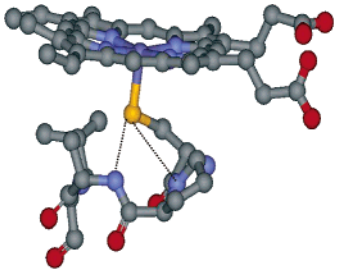

Figure 1. The crystal structure of CPO.[7] The dotted lines represent hydrogen bonds from Ala31 and Pro30 to the cysteinyl sulfur.

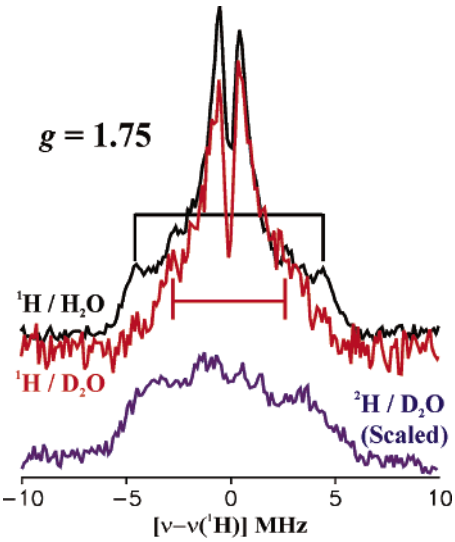

Figure 2. Top: Stochastic Davies[20] ^{1}H ENDOR of CPO−I in H_2O (black line: frequency = 35.086 GHz; $t(\pi/2) = 40$ ns; 60 μs rf; $\tau = 460$ ns; repetition rate = 25 Hz) and D_2O (red line; same conditions). Black brace, largest exchangeable doublet, $A \sim 10$ MHz; red brace, largest nonexchangeable doublet, $A \sim 6$ MHz. Bottom: Mims ^{2}H ENDOR of CPO−I in D_2O (frequency = 35.144 GHz; $\tau = 500$ ns; 20 μs rf; repetition rate = 50 Hz; $t(\pi/2) = 52$ ns); $T = 2$ K.

Figure 2 shows ^{1}H Q-band pulsed ENDOR spectra taken at a field near $g_{\perp}$ for CPO−I freeze-quenched in H_2O and D_2O buffer.[16−19]

Such spectra are a superposition of $\nu_{\pm}$ doublets centered at ν_H, each split by its hyperfine coupling, A: $\nu_{\pm} = \nu_H \pm A/2$. The $g_{\perp}$ spectrum of CPO−I in H_2O buffer contains features associated with a number of doublets with couplings ranging in magnitude to a maximum of $A(g_{\perp}) \sim 10$ MHz. The corresponding spectrum from CPO−I in D_2O buffer shows a loss of the most strongly coupled doublet. The attribution of this signal to an exchangeable proton(s) is confirmed by the appearance of a ^{2}H ENDOR signal with the same coupling (when scaled by the nuclear g factors); the ^{2}H

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lines are sufficiently broad that a ^{2}H quadrupole splitting is not resolved. The remaining ^{1}H doublets thus are from constitutive protons of the heme and/or proximal cysteinyl ligand; as indicated, the largest coupling constant for those protons is $A(g_\perp) \approx 6(1)$ MHz. Similar results are obtained with CW ENDOR (Figure S1).

The ^{1}H ENDOR measurement immediately allows us to estimate an upper bound for the spin density, ρ_S, on the cysteinyl sulfur. As first discussed for the blue Cu proteins,[18,21] when spin density is delocalized into the p$-\pi$ orbital of a metal-bound thiolate, the intrinsic hyperfine couplings to the β protons, a_{int}, are essentially isotropic and are proportional to ρ_S:

$$a_{int} = B \cos^2(\theta)\, \rho_S \qquad (2)$$

where $B \approx 100$ MHz and θ is the dihedral angle between the spin-bearing p orbital of sulfur and the C$-$H bond. On the basis of a crystal structure of CPO,[7] proton H$^{\beta 1}$, with $\theta(H^{\beta 1}) = -43°$ ($[\cos\theta]^2 = 0.53$), has the larger β proton coupling. However, a measured hyperfine coupling cannot be directly entered into eq 2 to determine ρ_S, for it is altered from the intrinsic values by the spin coupling between the radical and the [Fe$=$O]$^{2+}$ ion.

The spin-coupling model of eq 1[4b,5] incorporates a "competition" between exchange and zfs interactions, and the g values and observed hyperfine couplings of the ground-state Kramers doublet are functions of $q = J/D$. As a result, even if a nucleus is hyperfine-coupled to one of the interacting spin centers (i = Fe or R) by an isotropic intrinsic interaction, a_{int}, the observed coupling can become anisotropic.[4b] The couplings can be written as

$$^iA_j = {}^iK(q)_j \cdot ({}^ia_{site})$$
$$({}^ia_{site}) = (a_{int})/(2^iS) \qquad (3)$$

where $j = \perp$ or $||$ for hyperfine components (^iA_j) perpendicular or parallel to the Fe$-$O (zero-field splitting) direction, which are modified from the intrinsic values by spin-coupling coefficients ($^iK(q)_j$)[22,23] that are functions of q. For weak AF exchange, $0 \le q \lesssim 0.3$, the hyperfine couplings of the radical are negligibly altered by exchange: $^RK_j \sim 1$. In the limit of strong AF coupling, $q \gg 0$, the exchange leads to an $S = {}^1/_2$ ground state which has the well-known coefficients, $^RK_\perp = {}^RK_{||} = -{}^1/_3$.[22] In the intermediate coupling range, $0.3 \lesssim q \lesssim 3$, the g_j and $^iK(q)_j$ of the active site are complicated functions of q. For $q = 1.6$ (see above), we find the perpendicular coupling to a radical nucleus to be $^RA_\perp = (0.544) \times (^Ra_{int})$.[24]

Assignment of the maximum observed proton hyperfine coupling to cysteinyl proton H$^{\beta 1}$ interacting with the radical ($S = {}^1/_2$) spin sets an upper bound to the intrinsic isotropic hyperfine coupling based on the canonical assumption that the spin density on sulfur is associated with the radical center: $^Ra_{int}{}^{max} = (\sim 6)/(0.544) \approx 12$ MHz. From this value for $^Ra_{int}{}^{max}$, we can derive a maximum value for the sulfur spin density, $\rho_S{}^{max}$, from the semiempirical eq 2: $^R\rho_{Smax} \approx 0.23$. For ρ_S with such a value, we can assign the exchangeable proton to the peptide hydrogen bonds from Ala31 and Pro30 to the cysteinyl sulfur (Figure 1).

This value is consistent with those derived by QM/MM DFT computations on the corresponding Cpd-I of P450cam, where the heme site is embedded in the protein (p). These find the radical site to be mainly on the porphyrin, with delocalization to sulfur only to the extent that $^p\rho_S \sim {}^1/_4 - {}^1/_3$.[9-12] In contrast, for CPO$-$I and P450$-$I heme sites in the gas phase (g), the radical is found to be associated with the axial cysteinyl ligand, $^g\rho_S \sim {}^2/_3$ or higher.[8-12]

We suggest that the results presented here settle the question, does CPO$-$I contain a porphyrin π-cation radical or an iron-bound cysteinyl radical: the radical is predominantly on the porphyrin, with $\rho_S \le \rho_{Smax} \approx 0.23$,[25] consistent with the bleaching of the Soret band in CPO$-$I.[4a] As the active site of CPO is essentially identical to that of cytochromes P450, we further suggest that the same answer applies to P450$-$I.[26] A fuller picture of the CPO$-$I active site will be provided by additional ^{1,2}H and ^{14}N ENDOR studies.

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Supporting Information Available: One figure with CW ^{1}H ENDOR spectra of CPO$-$I in H_2O and D_2O buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

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