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ENDOR Determination of Heme Ligation in Chloroperoxidase and Comparison with Cytochrome P-450$_{Cam}$

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Chloroperoxidase (CPO) isolated from *Caldariomyces fumago* is a hemoprotein that catalyzes halogenation reactions in the biosynthesis of halogen-containing compounds[1] and recently has been shown to catalyze olefin epoxidations.[2] As a result, the active-site environment of the enzyme has been the subject of intensive studies.[3-6] Electron paramagnetic resonance (EPR),[3,4] magnetic circular dichroism,[4] and EXAFS[5] studies indicate that the protein donates a cysteinyl thiolate ligand to the heme.[6] However, the identity of the exogenous sixth ligand of low-spin chloroperoxidase still is not known, with the most recent proposal being a carboxylate ligand.[4] Through the use of 35 GHz continuous-wave (CW) and 9 GHz Davies pulses ^{1}H electron-nuclear double resonance (ENDOR) spectroscopy[7,8] we identify the axial ligand of native low-spin chloroperoxidase as an aqua ligand with two major conformations and add support to the proposal of an endogenous cysteinyl axial ligand. These results, along with exceptionally well-resolved ^{14}N ENDOR spectra of pyrrole nitrogens, show that the heme iron coordination spheres of CPO and cytochrome P-450$_{Cam}$ are identical, but that the structures differ significantly in detail.

The low-temperature EPR spectrum of CPO at pH = 5.2 arises mostly from a low-spin form with $g_{1,2,3} = 2.61, 2.26, 1.84$. Figure 1B shows a single-crystal-like[7] ^{1}H ENDOR spectrum of low-spin CPO in pH = 5.2 potassium acetate buffer taken in a 35 GHz CW spectrometer[9] at the low-field edge of the EPR envelope ($g_1 = 2.61$). Proton ENDOR spectra in principle exhibit a hyperfine-split doublet centered at ν_H, with frequencies $\nu_\pm(H) = \nu_H \pm A(H)/2$, where $A(H)$ is the hyperfine coupling constant.[7] The two broad ENDOR peaks seen in Figure 1B at $\delta\nu \equiv \nu - \nu_H = -5.0$ and -7.6 MHz correspond to the $\nu_-(H)$ partners of doublets with coupling constants $A(H) = 10$ and 15.2 MHz. Although the $\nu_+(H)$ branch of the proton pattern is not clearly observable in the CW ^{1}H spectrum of Figure 1B,[9] it is clearly seen in a 9-GHz Davies pulsed ENDOR[7,8] spectrum taken at the same g value (Figure 1A). The strongly-coupled protons are exchangeable as shown by their loss in D_2O buffer, Figure 1C. For comparison,

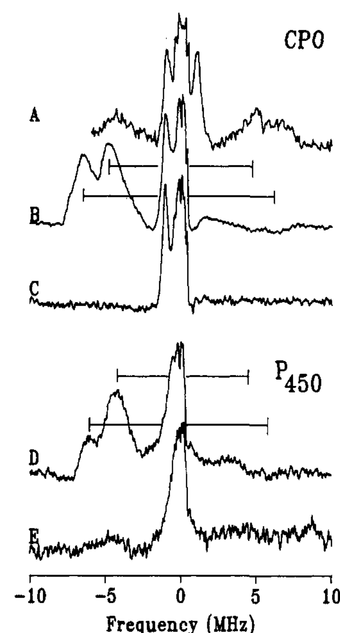

Figure 1. ^{1}H ENDOR spectra at $g_1 = 2.61$ of CPO (A–C) and $g_1 = 2.45$ of P-450$_{Cam}$ (D, E) as plotted relative to the proton Larmor frequency (~11 MHz at X band, ~41 MHz at 35 GHz). The braces represent the hyperfine splittings, $A(H_a)$ and $A(H_b)$. (A) ^{1}H X-band Davies pulsed ENDOR spectrum of CPO (pH = 5.2 KAc buffer) showing ν_+ branch and part of ν_- branch; the remainder of ν_- is overlapped with ^{14}N signals (not shown). Conditions: 9.415 GHz; 0.2577 T; π microwave pulse, 120 ns; $\tau_{12} = 23.7$ μs; $\tau_{23} = 280$ ns; repetition rate, 25 Hz; rf pulse, 20 μs; 256 data points per spectrum; 66 scans; T, 2 K. (B) 35.185 GHz CW ^{1}H ENDOR spectrum of CPO in ^1H_2O buffer. Conditions: 0.9641 T; microwave power, 0.5 mW; modulation amplitude, 0.2 mT; scan speed, 1.0 MHz/s; time constant, 32 ms; rf power, 30 W; 50 scans; T, 2 K. (C) 35.25-GHz CW ^{1}H ENDOR spectrum of CPO in the D_2O buffer. Conditions: as in spectrum B. (D) 35.21 GHz CW ^{1}H ENDOR spectrum of P-450$_{Cam}$ in ^1H_2O buffer. Conditions: as in spectrum B except 1.029 T; modulation amplitude, 0.32 mT; 100 scans. (E) 35.15-GHz CW ^{1}H ENDOR spectrum of P-450$_{Cam}$ in the D_2O buffer taken at $g_1 = 2.45$. Conditions: as in spectrum D.

we present 35 GHz CW ^{1}H ENDOR taken at $g_1 = 2.45$ for substrate-free (native) cytochrome P-450$_{Cam}$. LoBrutto *et al.*[10] reported that this enzyme exhibits an exchangeable-proton signal with large hyperfine couplings, ~10 MHz. The enhanced sensitivity of the 35-GHz spectrometer to large ^{1}H couplings[9] in fact discloses that the ^{1}H patterns of *both* CPO and P-450$_{Cam}$ show resolved ^{1}H doublets from two exchangeable protons, H_a and H_b (Figure 1), with slightly smaller couplings for P-450$_{Cam}$, $A(H) \sim 19$ and 12 MHz, than for CPO, $A(H) \sim 10$ and 15 MHz. X-ray diffraction studies of P-450$_{Cam}$[11] confirm that the exchangeable ^{1}H resonances arise from H_2O as the sixth heme ligand.[12] The similarity of the ^{1}H ENDOR data for the two enzymes requires the same assignment for CPO.

To further characterize this aqua ligand, for each protein a set of ^{1}H spectra was taken across the EPR envelope and analyzed[7] to give complete hyperfine tensors for the two classes of protons.[13] The largest tensor component points roughly along g_1 as expected for a contribution from a through-space dipolar interaction where g_1 lies closely along the Fe–O bond. In fact, this has been shown by single-crystal EPR to be the case for P-450$_{Cam}$,[14] and thus the same is inferred for CPO. It is possible that a "tilt" of the H_2O ligand causes the two protons to appear as two ^{1}H classes, H_a and

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(13) To be published.

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H_b. However, we tentatively prefer the view that each protein shows two major (tier 0) conformational substates[15] of the H_2O cluster in the distal heme pocket, each with different properties of the aqua ligand and thus a distinct ENDOR signal. The large breadth of the individual proton resonances for both CPO and P-450$_{Cam}$ (e.g., for g_1 see Figure 1) suggests that the major conformations of the aqua ligands (or solvent network) exhibit a further (tier 1) distribution of orientations.[15] The ENDOR peaks of the exchangeable protons at other fields are narrower for CPO than P-450$_{Cam}$, an indication that this distribution is tighter in CPO.

We have compared the remainder of the ferriheme coordination spheres of CPO and P-450$_{Cam}$ by collecting CW 35 GHz ^{14}N spectra. Both P-450$_{Cam}$ and CPO show resonances in the 4-8 MHz region from heme-pyrrole ^{14}N, with the resolution of the CPO ^{14}N spectrum being quite extraordinary (Figure 2). LoBrutto *et al.*[10] showed with 9 GHz CW ENDOR that P-450$_{Cam}$ does not exhibit the ^{14}N resonances expected for a histidyl imidazole fifth ligand, and the X-ray structure determination established that the fifth ligand is a cysteinyl mercaptide, not histidine. Likewise, CPO shows no such histidyl resonances (data not shown), supporting the proposal[6] of a cysteine endogenous ligand.

Figure 2 indicates the assignment of the heme ^{14}N resonances for the two proteins as resolved, overlapping ^{14}N spectra from distinct heme nitrogens.[16] It is common for a low-spin heme to show two[17] or even three pyrrole resonances, but the eight sharp ^{14}N peaks exhibited by CPO at g_1 correspond to signals from *four* distinguishable ^{14}N heme nitrogens with well-resolved quadrupole splitting. The average hyperfine and quadrupole coupling constants along g_1 for these nitrogens is typical of those found in low-spin ferric heme complexes ($\bar{A}_1(^{14}N) \approx 6.1$ MHz; $3\bar{P}_1(^{14}N) \approx 1.0$ MHz).[17] The ^{14}N resonances of P-450$_{Cam}$ are less resolved than those of CPO (Figure 2B), and the best assignment of the pattern is to only two resolved types of nitrogen as indicated, suggesting that the local environment of the heme iron is more symmetric than in CPO. The average heme pyrrole ^{14}N hyperfine coupling for P-450$_{Cam}$, $\bar{A}_1(^{14}N) \approx 5.7$ MHz, is significantly less than that for CPO, and the same is true for the quadrupole coupling, $3\bar{P}_1(^{14}N) \sim 0.9$ MHz. We take these differences to arise from differences between the metal out-of-planarity and/or heme ruffling in the two enzymes.

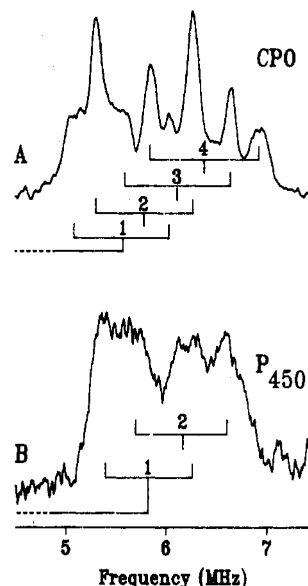

Figure 2. The $\nu_+(m)$ branch (ref 15) of 35-GHz CW ^{14}N ENDOR spectra. (A) CPO taken at $g_1 = 2.61$. Conditions: as in Figure 1B except modulation amplitude, 0.32 mT; 100 scans. (B) P-450$_{Cam}$ taken at $g_1 = 2.45$. Conditions: as in Figure 1D. The quadrupole splittings ($3P(^{14}N)$) are indicated by "goal post" symbols where the central vertical mark corresponds to $\nu_+ = A(^{14}N)/2 + \nu(^{14}N)$, and $\nu(^{14}N) = 2.95$ MHz at 0.96 T. Numbers in figures label the resolved types of heme nitrogen.

It is plausible that each of the four pyrrole ^{14}N seen in the highly-resolved CPO spectra might represent an individual pyrrole ^{14}N. Alternatively, the two major substates inferred from the ^1H ENDOR could each be exhibiting resonances from two pairs of ^{14}N, thereby doubling the number of ^{14}N signals. Analogous possibilities arise for P-450$_{Cam}$: two major classes of ^{14}N signals from one conformation or one each from two conformations. Experiments are under way to address these questions. In any event the ^{14}N data show distinct differences between the two proteins, with the CPO heme apparently having a lower symmetry (fewer classes of ^{14}N) and a more precisely defined structure (sharper ^1H and ^{14}N resonances). Thus, the present study not only has established the conjecture that CPO and P-450$_{Cam}$ share a common metal coordination environment but also has provided insights into the means whereby the protein environment acts to modify the heme of CPO, giving it ligand-binding properties like those of a hydroperoxidase[6] and the remarkable ability to chlorinate organic substrates.

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