Q-Band ENDOR (Electron Nuclear Double Resonance) of the Heme *o3* Liganding Environment at the Binuclear Center in Cytochrome *bo3* from *Escherichia coli*

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Abstract: The liganding environment and electronic structure of high-spin ferric heme *o3* in the binuclear center of *bo3* ubiquinol oxidase were probed with Q-band (34.1 GHz) ENDOR. We studied forms of the enzyme where reduction eliminated antiferromagnetic coupling to the nearby Cu_B center. ENDOR comparisons were made to ^{14}N heme and histidine nitrogen features, to exchangeable proton features, and to the ^{17}O-water feature of aquometmyoglobin, a high-spin ferric heme protein with a known axial water ligand. Nitrogen features observed from heme and proximal histidine of cytochrome *o3* occurred in the range of frequencies where they had previously been observed for aquometmyoglobin. However, the proximal histidine of cytochrome *o3* was notable in revealing more disorder and a wider range in its hyperfine couplings than in aquometmyoglobin. Di-oxygen-induced turnover of the *bo3* enzyme altered both the heme and histidine electronic structure so as to show after turnover a simpler, better resolved heme and histidine pattern with greater similarity to the pattern found in aquometmyoglobin. We saw no evidence from cytochrome *o3* for the 6 MHz exchangeable water proton coupling and the 17.5 MHz ^{17}O-water coupling exhibited by aquometmyoglobin. A plausible conclusion from such a negative result is that the high-spin ferric *o3* heme which we studied has no covalently attached axial sixth OH_X ligand when magnetically decoupled from Cu_B. Comparison of cytochrome *o3* in protonated and deuterated solvents definitively indicated no exchangeable proton couplings greater than 3.5 MHz. An implication of our study is that in the magnetically decoupled high-spin ferric cytochrome *o3* there is either no sixth OH_X ligand or, if there is any "sixth" OH_X ligand to cytochrome *o3* that can exchange with ^{17}O-water, it would have to be off-axis, disordered, and weakly liganded to the heme.

Introduction

Cytochrome *bo3* ubiquinol oxidase from *Escherichia coli* belongs to the heme-copper superfamily of respiratory oxidases that have a conserved binuclear (heme *o3*-Cu_B) center where dioxygen reduction occurs.[1] The binuclear center is the site of the oxygen reduction which is a source of free energy for proton translocation. Crystal structures of related cytochrome *c* oxidases from *Paracoccus denitrificans*[2] and beef heart[3] are available. For the oxidized enzymes different interpretations remain within the 2.3−2.8 Å crystallographic resolution over the structure and existence of bridging oxygen atoms between the two metals of the binuclear site. Water and hydroxide have been suggested for the *Paracoccus* enzyme[4] and peroxide for the beef heart enzyme.[5] In the completely reduced form of the enzyme no ligand is evident between the heme iron and Cu_B of the binuclear center,[5] but there is evidence for change in Cu_B ligation upon reduction.[6] Given the necessity for binding various products of oxygen reduction, the binuclear site would appear to be structurally labile. The lability is reflected in the uncertainty over the existence and location of oxygen reduction products at the binuclear center, and it may be reflected in the various models for transient changes that occur as reduction of the binuclear metals, oxygen binding, and oxygen reduction proceed.[7−9]

Strong antiferromagnetic coupling between ferric heme and cupric copper of the binuclear site largely eliminates EPR (electron paramagnetic resonance) signals under the oxidizing "resting" conditions used to prepare *bo3* and cytochrome *c* oxidases. Partial reduction of the completely oxidized binuclear center is known to do away with these antiferromagnetic couplings[10,11] so that a decoupled high-spin ferric *o3* heme or

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a cupric Cu_B (the subject of a separate study) can then be observed by EPR-ENDOR. The ENDOR (electron nuclear double resonance) technique which requires a paramagnetic center has the advantage as a complement to protein X-ray crystallography of resolving small variations in geometrical and electronic structure and of detecting protons. We chose *bo3* quinol oxidase because it lacks the additional Cu_A of cytochrome *c* oxidase whose signal would otherwise overlap and interfere with the desired high-spin ferric heme signal in the informative $g_{||} = 2.00$ region. Primarily focusing on this $g_{||} = 2.00$ region, we probed by ENDOR the environment of the high-spin ferric heme *o3* in partially reduced *bo3* oxidase. We have compared our ENDOR spectra of cytochrome *o3* to spectra of ^{14}N, ^{1}H, and ^{17}O-water from aquometmyoglobin, which we take as a model for a high-spin ferric heme protein known to have an oriented sixth water ligand.[12] For aquometmyoglobin, the hyperfine couplings to the heme and histidine ^{14}N nitrogens were already studied by X-band ENDOR in frozen solution[13,14] and with single-crystal ENDOR.[15] For comparison to ENDOR of cytochrome *o3*, there is also ENDOR evidence of the axial water from the exchangeable proton hyperfine couplings of aquometmyoglobin,[14] and as presented here, from ^{17}O-enriched water.

Experimental Section

Materials. Cytochrome *bo3* was made by the method of ref 16 with modifications detailed in ref 6. The total stock of 0.8 mM enzyme was ~0.8 mL, limiting us to small (~50 μL) Q-band samples. Samples were prepared in pH 7.4, 100 mM HEPES buffer containing 0.1% *n*-dodecyl-β-D-maltoside detergent (Anatrace, Inc., Maumee, OH). Deuteration to approximately 90% was carried out by ultrafiltration (Microcon 3 Concentrator, Millipore, Burlington, MA) vs pD 7.6, 100 mM HEPES buffer (made with 99.9% D_2O, Cambridge Isotopes, Cambridge, MA). Enrichment to approximately 33% in $H_2{}^{17}O$ was also carried out by ultrafiltration versus 50% enriched $H_2{}^{17}O$ (Monsanto, Miamisburg, OH). Following repeated pump-flushing with pure argon, samples were anaerobically reduced by stoichiometric additions of NADH (Sigma) in the presence of 3 μM PMS (phenazinemethosulfate, Sigma). Samples were transferred by gastight Hamilton syringe to argon-flushed Q-band sample tubes (2.0 mm inner diameter, 2.4 mm outer diameter, VitroCom, Inc., Mountain Lakes, NJ) and anaerobically frozen. Sample preparations that revealed the decoupled high-spin *o3* ferric heme used one reducing electron per *bo3* molecule to eliminate antiferromagnetic coupling within the *o3*-Cu_B center. Preparations that also showed the decoupled *o3* ferric heme signal, albeit with an altered nitrogen ENDOR signal, used four reducing electrons per *bo3* molecule followed by dioxygen turnover and subsequent freezing. Although there was a small residual high-spin ferric heme signal in the fully oxidized *bo3* oxidase, our methods of preparation provided a high-spin ferric heme signal that was 3−5 times larger than the residual signal. The implication is that our methods did indeed eliminate antiferromagnetic coupling within the binuclear center and gave rise to a decoupled high-spin ferric *o3* heme signal, whose concentration was estimated at ~100 μM by integration in the $g_\perp = 6$ region and comparison to an aquometmyoglobin standard. Q-band EPR showed that aquometmyoglobin had $g_\perp = 5.88$ (at its derivative zero crossing) and a line width (ΔH between derivative extrema) of 0.09 T. The corresponding $g_\perp$ and ΔH of high-spin ferric cytochrome *o3* were 5.83 and 0.22 T for the 1-electron-reduced cytochrome *o3* and 5.83 and 0.19 T for the 4-electron-reduced cytochrome *o3* that had been turned over with dioxygen. The latter sample showed a small (~10%) rhombic component to its high-spin ferric heme signal having $g_x = 6.5$ and $g_y = 5.3$. The sperm whale aquometmyoglobin samples of approximately 3 mM concentration were prepared as previously,[14] and an aquometmyoglobin sample enriched to approximately 25% in $H_2{}^{17}O$ was also made.

Methods. Q-band (34.1 GHz) EPR-ENDOR measurements were performed at 2.1 K with a spectrometer having a cryogenically tunable TE_{011} Q-band resonator.[17] The cavity was located in an immersion double dewar (Janis, Inc., Wilmington, MA) filled with pumped liquid helium at 2.1 K. ENDOR signals were obtained under rapid passage (χ') conditions with 100 kHz field modulation[18] in the 0.1−0.2 mT ptp (peak-to-peak) range. The ENDOR radio frequency field typically had a ptp amplitude of 0.1 mT. The microwave power was in the 0.3 μW range. Approximately 20 min of signal averaging was used per spectrum. EPR frequencies were measured with an EIP Model 548 microwave frequency counter (San Jose, CA); *g* values were calibrated versus a DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma) sample having a known *g* value of 2.0036. The ENDOR measurements were done on the high-spin ferric heme signals at a *g*-value of 2.000 with a magnetic field of 1.218 T and EPR frequency of 34.10 GHz.

Spin Hamiltonian Theory for ENDOR

Commonly used first-order expressions for ^{14}N ENDOR frequencies are as follows: $^{14}\nu^{+}_{ENDOR} = |^{14}A/2 \pm 3Q/2 + {}^{14}\nu_{NMR}|$ and $^{14}\nu^{-}_{ENDOR} = |^{14}A/2 \pm 3Q/2 - {}^{14}\nu_{NMR}|$. ^{14}A is the electron−nuclear hyperfine coupling, which in aquometmyoglobin (along the $g_{||}$ heme normal direction) is of order 7.0 MHz for the heme nitrogen and 11.5 MHz for the proximal histidine nitrogen.[15] Q is the quadrupolar coupling which in aquometmyoglobin (along the $g_{||}$ heme normal direction) is of order 0.3 MHz for heme nitrogens and 1.1 MHz for the proximal histidine nitrogen.[15] $^{14}\nu_{NMR}$ is the ^{14}N nuclear Zeeman frequency (=3.75 MHz at 1.218 T). At Q-band (34.1 GHz), the $^{14}\nu^{+}_{ENDOR}$ branch, having the positive nuclear Zeeman contribution, has often been the only branch observed for ^{14}N by us and others.[19] For the heme and histidine nitrogens of aquometmyoglobin, the branch we have observed here is also the $^{14}\nu^{+}_{ENDOR}$ branch whose features are predicted, on the basis of Spin Hamiltonian parameters in ref 15, to occur at Q-band above 6 MHz. The features of the $^{14}\nu^{-}_{ENDOR}$ branch, having the negative nuclear Zeeman contribution, are all predicted to lie well below 5 MHz and were not observed here. The heme and histidine hyperfine and quadrupolar tensors from single-crystal ENDOR of aquometmyoglobin are given in Table 3 of ref 15. It will be noted in ref 15 that the four heme nitrogens of aquometmyoglobin showed a resolved hyperfine inequivalence of approximately 5% among themselves. Equation 4b of ref 15 (given and discussed in footnote 20 here), which includes a second-order correction to the first-order expressions above, was used to estimate aquometmyoglobin heme and histidine $^{14}\nu^{+}_{ENDOR}$ frequencies. The predicted $^{14}\nu^{+}_{ENDOR}$ frequencies for aquometmyoglobin heme nitrogens were as follows: 6.87, 6.94, 6.98, 6.99, 7.62, 7.71, 7.77, and 7.94 MHz. (The uncertainty in these individual predicted frequencies, which reflected experimental uncertainty in the spin Hamiltonian parameters of ref 15, was ±0.1 MHz.) The quadrupolar term in the expression for $^{14}\nu^{+}_{ENDOR}$ accounts

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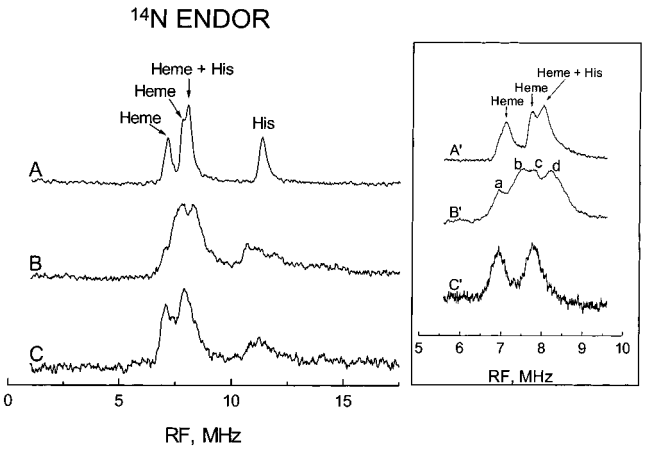

Figure 1. This figure presents a comparison of the heme and histidine nitrogen ENDOR spectra from (A, A′) aquometmyoglobin with the ENDOR spectra (B, B′ and C, C′) of heme and histidine from cytochrome *bo3*. Spectra B, B′ and C, C′ are respectively from enzyme that had been simply reduced by 1 e^-/*bo3* molecule and from enzyme that had been reduced by 4 e^-/*bo3* molecule and then allowed to react with O_2. The labeling and assignment of the heme and histidine features for aquometmyoglobin is explained in the Spin Hamiltonian Theory section; the features in spectra A and A′ labeled Heme, Heme, Heme+His, and His, respectively, occurred at 7.00, 7.69, 7.94, and 11.13 MHz. The inset containing spectra A′, B′, and C′ more intimately compares the heme nitrogen features in the 5.5−9.5 MHz range for the three samples; in spectrum B′ the features a, b, c, d occurred respectively at 6.81, 7.38, 7.75, and 8.15 MHz. Experiments were carried out under conditions of field, EPR frequency, and *g*-value given in the Methods section.

for the ∼0.9 MHz separation between the collection of heme features near 6.9 and the collection of heme features near 7.8 MHz. The predicted $^{14}\nu^{+}_{\mathrm{ENDOR}}$ frequencies for the histidine nitrogen were 7.84 and 11.20 MHz, and the 3.4 MHz difference between these two frequencies was quadupolar. These predicted ENDOR frequencies appear to fall in groups, notably: The first four predicted heme nitrogen ENDOR frequencies would indicate a ^{14}N heme feature at 6.95 ± 0.05 MHz; the next three heme nitrogen ENDOR frequencies would indicate a ^{14}N heme feature at 7.70 ± 0.08 MHz; the highest frequency heme and the lower histidine frequency would combine to give a ^{14}N feature at 7.89 ± 0.08 MHz. Finally there is the "stand-alone" histidine feature predicted at 11.20 ± 0.10 MHz.

First-order proton ENDOR frequencies, $^{\mathrm{P}}\nu_{\mathrm{ENDOR}}$, center at the proton Larmor frequency, $^{\mathrm{P}}\nu_{\mathrm{NMR}}$ (= 51.84 MHz for fields used at Q-band), and split away from the proton Larmor frequency by ±*A*/2, the electron−proton hyperfine coupling. Thus to first-order, $^{\mathrm{P}}\nu_{\mathrm{ENDOR}} = |^{\mathrm{P}}\nu_{\mathrm{NMR}} \pm A/2|$.

First-order ^{17}O ENDOR frequencies for a ^{17}O $I = {}^5/_2$ nucleus are as follows: The $^{17}\nu^{+}_{\mathrm{ENDOR}}$ branch occurs at $|^{17}A/2 + {}^{17}\nu_{\mathrm{NMR}}|$, $|^{17}A/2 + {}^{17}\nu_{\mathrm{NMR}} \pm 3\mathrm{Q}|$, $|^{17}A/2 + {}^{17}\nu_{\mathrm{NMR}} \pm 6\mathrm{Q}|$; the $^{17}\nu^{-}_{\mathrm{ENDOR}}$ branch occurs at $|^{17}A/2 - {}^{17}\nu_{\mathrm{NMR}}|$, $|^{17}A/2 - {}^{17}\nu_{\mathrm{NMR}} \pm 3\mathrm{Q}|$, $|^{17}A/2 - {}^{17}\nu_{\mathrm{NMR}} \pm 6\mathrm{Q}|$. ^{17}A is the hyperfine coupling, $^{17}\nu_{\mathrm{NMR}}$ (=7.02 MHz at 1.218 T) is the ^{17}O nuclear Zeeman interaction, and *Q* is the quadrupolar coupling. At Q-band the $^{17}\nu^{+}_{\mathrm{ENDOR}}$ branch is again the commonly observed branch.

Results

We compared the heme and histidine ^{14}N features of aquometmyoglobin (Figure 1A,A′) as measured at $g_{\|} = 2.000$ with those of the high-spin ferric cytochrome *o3* (Figure 1B,B′,C,C′). The arrows in Figure 1 A,A′ indicate features assigned to the $^{14}\nu^{+}_{\mathrm{ENDOR}}$ branches of heme and histidine nitrogens from aquometmyoglobin [at 7.00, 7.69, 7.94, and 11.13 (±0.05) MHz]. The assignment of these features was made according to predictions of the previous ENDOR Theory section that were based on the Spin Hamiltonian parameters in ref 15. The general features in the heme and histidine frequency regions are similar for aquometmyoglobin and for high-spin ferric cytochrome *o3*, but especially for the 1-electron-reduced cytochrome *o3*, were split and broadened. The separately resolved high-frequency proximal histidine feature of the 1-electron-reduced cytochrome *o3* had its major peak at 10.5 ± 0.1 MHz, about 0.6 MHz lower than the stand-alone high-frequency aquometmyoglobin histidine feature. The histidine feature of 1-electron-reduced cytochrome *o3* showed a tail stretching to higher frequency with reproducible smaller peaks at 10.9 and 11.7 MHz. In the 6.5−8.5 MHz region, peaks from the 1-electron-reduced cytochrome *o3* occurred at 6.81, 7.38, 7.75, and 8.15 ± 0.05 MHz as respectively indicated in Figure 1, spectrum B′, by letters a, b, c, d. Even in aquometmyoglobin there is an approximate 5% difference in hyperfine couplings between heme nitrogens.[15] Thus, it is not immediately obvious which of these four features from 1-electron-reduced cytochrome *o3* in the 6.5−8.5 MHz region might be from heme and which might be the histidine lower frequency quadrupole partner, occurring at 3Q below the 10.5 MHz histidine feature. If the histidine quadrupole coupling stays the same as in aquometmyoglobin, then the feature closest to being 3*Q* (=3.3 MHz) below the 10.5 MHz histidine feature would be the feature (b in Figure 1B′) at 7.38 MHz. As indicated by comparison of the ENDOR features in Figure 1B,B′ with those in Figure 1C,C′, there was a notable change between cytochrome o_3 which had been reduced by 4 electrons and then turned over by dioxygen and the 1-electron-reduced cytochrome *o3*. For the cytochrome o_3 which had been reduced by 4 electrons and then turned over by dioxygen the histidine peak above 10 MHz moved to a frequency of 10.8 MHz, closer to where it occurred in aquometmyoglobin, and it narrowed, albeit not becoming as narrow as the stand-alone histidine signal from aquometmyoglobin. The features (b and d) at 7.38 ± 0.05 and 8.15 ± 0.05 MHz in Figure 1B,B′ disappeared. The peaks at 6.81 ± 0.05 and 7.75 ± 0.05 MHz (Figure 1C,C′) which remained are consistent with the average quadupole-split heme frequencies of aquometmyoglobin.

As shown here in Figure 2A, and previously at X-band,[14] aquometmyoglobin had a readily identifiable water proton coupling of 6 MHz (i.e., features ±3 MHz away from $^{\mathrm{P}}\nu_{\mathrm{NMR}}$). Such an exchangeable proton feature was not observed from our high-spin ferric cytochrome *o3* sample in protonated solvent (as indicated in Figure 2B).[21] Comparison of Figure 2B with a deuterated cytochrome o_3 sample of Figure 2C gave additional negative evidence for any exchangeable proton coupling greater than 3.5 MHz, and little evidence, if any, of any exchangeable protons with coupling less than 3.5 MHz. (The method of preparation of the decoupled heme *o3* signal, either by 1-electron

(20) There are two quadrupole-split $^{14}\nu^{+}_{\mathrm{ENDOR}}$ partners. The first partner is $^{14}\nu^{+}_{\mathrm{ENDOR}} = {}^{14}A_{33}/2 - \mathbf{3Q_{33}/2} + {}^{14}\nu_{\mathrm{NMR}} + (Q_{11} - Q_{22})^2/[4(^{14}A_{33} + 2{}^{14}\nu_{\mathrm{NMR}})]$. The second partner is $^{14}\nu^{+}_{\mathrm{ENDOR}} = {}^{14}A_{33}/2 + \mathbf{3Q_{33}/2} + {}^{14}\nu_{\mathrm{NMR}} + (Q_{11} - Q_{22})^2/[4(^{14}A_{33} + 2{}^{14}\nu_{\mathrm{NMR}})]$. $^{14}A_{33}$ and Q_{33} are ^{14}N hyperfine and quadrupolar couplings along the heme normal, and $^{14}A_{11}$, $^{14}A_{22}$, Q_{11}, and Q_{22} are the respective hyperfine and quadrupolar elements that are along directions described in ref 15 perpendicular to the heme normal. A second-order quadrupolar correction in $(Q_{11}-Q_{22})^2$ is included here from ref 15, but the second-order hyperfine correction from ref 15 is not needed here since its magnitude (∼0.003 MHz) is much less than the experimental accuracy in ENDOR frequencies. Values for all of the hyperfine and quadrupolar tensor elements for all the heme nitrogens and for the proximal imidazole nitrogen are explicitly given in Table 3 of ref 15. $^{14}\nu_{\mathrm{NMR}}$ is the nuclear Zeeman frequency (=3.75 MHz here).

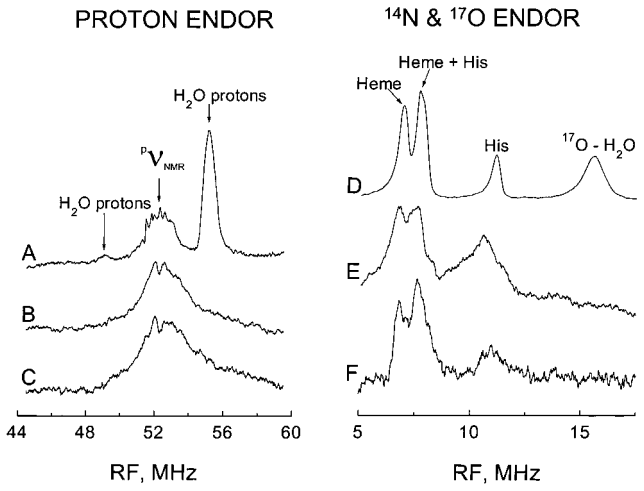

Figure 2. Spectra A and B respectively compare proton features including the exchangeable water of aquometmyoglobin with the proton features of the ferric heme *o3* of cytochrome *bo3*; spectrum B was obtained from a *bo3* sample prepared in protonated buffer by 1-electron reduction. Spectrum C was obtained for comparison with spectrum B from a sample of *bo3* oxidase prepared in deuterated buffer by 4-electron reduction followed by dioxygen. Spectra D and E respectively compare the ENDOR of aquometmyoglobin prepared in ^{17}O-enriched $H_2{}^{17}O$ with cytochrome *bo3* which had been prepared in ^{17}O-enriched $H_2{}^{17}O$ solvent and then reduced by 4 electrons and turned over by dioxygen. Spectrum F, for comparison with spectrum E, was from a sample of cytochrome *bo3* which had been prepared similarly to that in spectrum E but in standard $H_2{}^{16}O$ solvent. Experiments were carried out under conditions of field, EPR frequency, and *g*-value given in the Methods section.

reduction or by 4-electron reduction followed by dioxygen, gave no differences in exchangeable proton features.) At $g_{||} = 2.000$ the ^{17}O ENDOR feature of the aquo ligand is obvious near 15.8 MHz from aquometmyoglobin as shown in Figure 2D; no additional splittings, potentially of a quadrupolar nature, could be discerned from this ^{17}O aquometmyoglobin feature. This ^{17}O ENDOR feature of aquometmyoglobin provides a hyperfine coupling ($^{17}A_{||}$) of about 17.5 MHz (see ENDOR Theory section), where the parallel direction is along the metal (d_{z^2})-oxygen bond and where the quadrupole coupling $Q_{||}$ is approximately zero. The value of $^{17}A_{||}$ is ~70% higher than the corresponding $^{17}A_{||}$ hyperfine coupling to ^{17}O of water liganded to high-spin d^5 hexaquo Mn(II), where the hyperfine coupling was predominantly due to covalent transfer of spin in a σ orbital to the oxygen 2s orbital.[22] The sample of *bo3* oxidase for Figure 2E was prepared in buffer having similar enrichment in $H_2{}^{17}O$ to the enrichment in $H_2{}^{17}O$ used for aquometmyoglobin of Figure 2D. This *bo3* sample in $H_2{}^{17}O$-enriched buffer was one which had been reduced by 4 electrons and turned over by dioxygen. (The isotopic nature of the dioxygen gas used for turnover, whether 80% enriched in $^{17}O_2$ or standard $^{16}O_2$, made no difference to the ENDOR spectrum.) For comparison to Figure 2E, a spectrum from *bo3* oxidase similarly prepared in standard $H_2{}^{16}O$ is given in Figure 2F. If there is a ^{17}O feature in Figure 2E from cytochrome o_3 in the general region where ^{17}O coupling is seen for aquometmyoglobin, it is barely discernible above the baseline. Plausibly, on comparison of Figures 2E and 2F, the stand-alone feature of the histidine near 11 MHz in Figure 2E could be underlain by a broad ^{17}O feature. If that were the case, then there would be weaker coupling to the ^{17}O in cytochrome *o3* than to the ^{17}O-water of aquometmyoglobin, and the weaker coupling would be about 8 MHz vs 17.5 MHz for ^{17}O-water of aquometmyoglobin. Experimentally, any ^{17}O feature from cytochrome *o3* is definitely not the well-resolved ^{17}O-water feature of aquometmyoglobin.

Discussion

In cytochrome *o3* the greater breadth of the histidine feature of all cytochrome *o3* samples studied indicated less order in histidine ligation than found in aquometmyoglobin and indicated a range of covalent interactions with the proximal histidine. Especially in the 1-electron-reduced cytochrome *o3*, the breadth and existence of additional ENDOR features in the 6.5−8.5 MHz region where heme nitrogen ENDOR occurs and in the 10−12 MHz region where histidine nitrogen ENDOR occurs was an additional sign for a distribution of hyperfine couplings. A major part of the histidine ENDOR signal from the 1-electron reduced cytochrome *o3* occurred with a frequency of about 10.5 MHz (compared to 11.13 MHz for aquometmyoglobin). Assuming that the histidine nitrogen quadrupole coupling is constant, this histidine would have an approximate 10% decrease in covalent spin transfer from heme iron to histidine as compared to aquometmyoglobin.[15] The change and simplification of the overall heme and histidine spectrum after turnover with oxygen (Figures 1C and 1C′ vs Figures 1B and 1B′) may be an electronic structural aspect, as reflected by altered spin density at heme and histidine nitrogens, of the "fast" or "pulsed" phenomenon. The "fast" or "pulsed" phenomenon is noted where terminal oxidase that has been recently enzymatically turned over shows greater activity than initially prepared "resting" enzyme.[23]

The simplest explanation for the lack of any resolved ^{17}O or exchangeable proton feature from cytochrome *o3* is that there is no OH_X ligand to the heme *o3* when the heme *o3* and Cu_B are magnetically decoupled. If there were any "sixth" OH_X ligand to heme *o3* in our partially reduced samples, it is certainly not such an ordered axial OH_X as yields the well-resolved proton and ^{17}O hyperfine couplings from aquometmyoglobin. Since the cytochrome *o3* which we study is in high-spin ferric form, it would be unlikely that its sixth ligand is a hydroxide (OH^-) ligand because the hydroxide ligand favors low-spin ferric heme, as will happen with metmyoglobin at high pH. The exchangeable proton hyperfine couplings to water of aquometmyoglobin are due to protons that lie off the heme normal (estimated to lie 16° away from the $g_{||} = 2.000$ direction in ref 14), yet their ENDOR features (Figure 2A) are exceptionally well resolved. The reasons for good proton ENDOR resolution are good angular orientation brought on by the *g*-anisotropy of high-spin ferric heme and minimal angular dispersion in the precise localization of the water protons within the heme pocket of aquometmyoglobin. The precise alignment of the sixth ligand in myoglobin is thought to result from specific interactions within the proximal pocket, notably from the distal histidine.[24−26]

(21) The oxidase study shown here was done on bo_3 oxidase at $g = 2.000$, but ENDOR spectra were also taken in the $g_\perp = 6$ region of high-spin ferric heme from partially reduced bo_3 oxidase and from partially reduced beef heart cytochrome *c* oxidase (prepared as in the following: Fan, C.; Bank, J.; Dorr, R.; Scholes, C. P. *J. Biol. Chem.* **1988**, *263*, 3588−3591) and compared to $g_\perp = 6$ ENDOR spectra of aquometmyoglobin. Although at $g_\perp$ nonexchangeable heme meso proton features were identified in all ferric heme samples, there was *no evidence* from either cytochrome *c* or *bo3* oxidase for exchangeable proton features, while exchangeable proton features were obvious from aquometmyoglobin.

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In the cytochrome *o3* that is antiferromagnetically decoupled from the Cu_B there is probably no distal, strongly orienting moiety for a sixth ligand. A reason the ^{17}O-water of aquometmyoglobin has well-resolved ENDOR is that the $g_{||}$ direction where we observe ENDOR is coincident with the heme-to-water axis and is also along a minimal quadrupole coupling direction. If the water ligand were tilted off axis, this would lead to noncollinearity of the electronic *g* and ^{17}O hyperfine axes and to a concomitant larger quadrupolar broadening. One would also expect there to be less covalent σ-bonding overlap between oxygen and iron orbitals.

Conclusion

Comparison of ^{14}N heme and histidine ENDOR features from aquometmyoglobin with those of cytochrome *o3* indicated a spreading of hyperfine couplings for cytochrome *o3*, especially for the proximal histidine of 1-electron-reduced cytochrome *o3*. A simplification and narrowing in these nitrogen ENDOR features was noted after turnover of 4-electron-reduced cytochrome *o3* with dioxygen. We suggest that such a change may reflect spin density changes at *o3* heme and histidine nitrogens associated with the "fast" or "pulsed" phenomenon where oxidase that has recently been turned over shows greater activity than "resting" oxidase.[23] For high-spin ferric cytochrome o3 that had been magnetically decoupled from Cu_B, there was a lack of any identifiable exchangeable proton or ^{17}O features such as found from axial water of aquometmyoglobin. The conclusion is that if there is any "sixth" OH_X ligand at all to heme *o3* in our partially reduced samples, it is not an ordered axial OH_X such as is seen with the exchangeable water protons and ^{17}O of aquometmyoglobin, and it would have to be off-axis, weakly liganded to the heme with less covalent spin transfer, and probably disordered.[27] The implication of our findings is that the process that magnetically decouples ferric heme *o3* from cupric Cu_B prepares the way for dioxygen binding to heme, either by eliminating a preexisting sixth OH_X ligand or by favoring a weakly bound, disordered sixth OH_X ligand.

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