Hydroperoxy-Heme Oxygenase Generated by Cryoreduction Catalyzes the Formation of α-*meso*-Hydroxyheme as Detected by EPR and ENDOR

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The degradation of heme by heme oxygenase (HO) involves the formation of a heme−protein complex in which the heme-iron coordinates to a neutral imidazole of histidine as in myoglobin and hemoglobin,[1,2] followed by three cycles of oxygenation in which the heme binds and activates O_2.[3−5] The first monooxygenation step of HO catalysis is believed to convert the heme to α-*meso*-hydroxyheme, and it has been proposed to proceed by reduction of the O_2-bound complex to a hydroperoxy-ferric active intermediate, rather than the oxo-ferryl form believed to occur in P450.[3,4] However, to date α-*meso*-hydroxyheme has *not* been detected during physiological HO catalysis. We here use EPR and ENDOR of oxy-ferrous HO reduced at 77 K to establish that hydroperoxy-HO indeed catalyzes the formation of α-*meso*-hydroxyheme, and show that it can do so in situ at temperatures above 200 K.

One-electron radiolytic cryoreduction[6,7] of diamagnetic oxy-hemoproteins in frozen solution at 77 K [8−13] creates a paramagnetic species in the environment of the precursor oxy-heme, before relaxation of the heme pocket to equilibrium conformational state.[8−11] Figure 1 presents 2 K Q-band EPR spectra[14] of the species generated by 77 K cryoreduction[7] of the dioxygen complexes[5,15] of hemoglobin β chains (oxy-β-Hb; **1A**) and of HO (**1C**), along with the spectra of these samples after annealing to

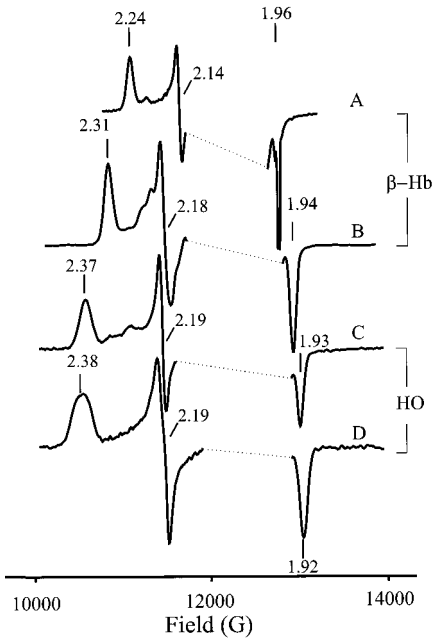

Figure 1. Q-band EPR spectra[14] of oxy-β-Hb (A) and oxy-HO (C) radiolytically reduced at 77 K. (B) and (D) are EPR spectra of these same samples after annealing at 200 K and 180 K, respectively. Free radical signals around $g = 2$ are omitted for clarity. *Conditions:* $T = 2$ K, 35.12 GHz, 100 kHz detection. Modulation amplitude 2 G. The derivative presentation was obtained digitally from absorption mode EPR envelopes detected under these rapid passage conditions.

200 K (Figure 1, parts B and D, respectively). Each of the spectra has the unmistakable signature of a low-spin ferriheme species, $g_1 > g_2 > g_e > g_3$ in a strong ligand field (small g dispersion),[16] rather than that of a center where the major unpaired-spin density resides on the dioxygen moiety, $g_1 = g_{par} > g_2$, $g_3 \approx g_e$, as in dioxygen adducts of Co(II) complexes.[17]

Following the arguments in a companion study of P450cam,[13] the ferri-heme primary product of cryoreduction of oxy-β-Hb at 77 K, whose EPR spectrum is characterized by $\mathbf{g} = [2.24, 2.14, 1.96]$ (Figure 1A), is assigned as the end-on, (formally) "ferric-peroxo", species (denoted $\mathbf{[FePO_2]_{red}}$). As shown earlier,[8,10] at 180−200 K this converts to the hydroperoxy-ferriheme complex whose EPR spectrum has a larger g-spread, $\mathbf{g} = [2.31, 2.18, 1.94]$ (Figure 1B). Proton ENDOR spectra[14,18,19] of the β-Hb $\mathbf{[FePO_2]_{red}}$ in H_2O (Figure 2A) and D_2O (not shown, but see Figure 2, $\mathbf{D_2O}$) show the presence of a doublet for an exchangeable proton with strong hyperfine coupling ($A(g_1) \approx 14$ MHz), assigned to a hydrogen bond from the distal histidine to the "peroxo" moiety.[10] The Q-band ENDOR measurements further show a signal from the exchangeable proton of the β-Hb hydroperoxo-ferric moiety,

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(14) Q-band (35 GHz) CW EPR and ENDOR spectra were recorded with a spectrometer of local design that employes 100 kHz field modulation and, for ENDOR, no frequency modulation (Werst, M. W.; Davoust, C. E.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 1533−1538). To first order, a proton ENDOR spectrum consists of a doublet split by the hyperfine coupling and centered at the proton Larmor frequency. At Q-band the two branches often have unequal intensities, as seen here.
(15) Typically the samples contained 2−3 mM oxyhemoproteins in 20% glycerol/buffer (0.05 M KPO_4, pH 7.6) (Migita, C. T.; Mansfield Matera, K.; Ikeda-Saito, M.; Olson, J. S.; Fujii, H.; Yoshimura, T.; Zhou, H.; Yoshida, T. *J. Biol. Chem.* **1998,** *273*, 945−949).
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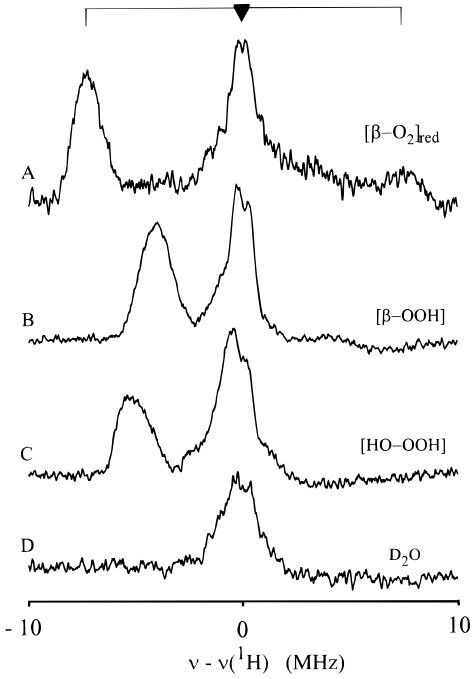

Figure 2. Q-band CW proton ENDOR spectra[14] taken at g_1 for reduced oxy-β-Hb (A) and for hydroperoxy-HO (C), each generated by cryoreduction at 77 K. The (▼) represents the proton Larmor frequency (~50 MHz); ($\lceil\ \rceil$), the hyperfine splitting, A. (B) is the ENDOR spectrum of hydroperoxy-β-Hb produced by annealing cryoreduced oxy-β-Hb at 200 K. The ENDOR spectrum (D), labeled **D_2O**,was taken with hydroperoxy-HO in D_2O buffer; a similar result is obtained with reduced oxy-β-Hb in such buffer. Conditions: $T = 2$ K; field modulation amplitude, 2G.

with a significant, although lessened, hyperfine coupling (Figure 2B; $A(g_1) \approx 8.2$ MHz). Preliminary 2-D sets of spectra collected across the EPR envelope[18] indicate that the decrease reflects a reduced isotropic interaction. The decrease in coupling, despite the change from H-bond to covalent bond, likely reflects in part a diminished spin density on the hydroperoxy moiety compared to that on the peroxo one.

The EPR spectrum of oxy-HO reduced at 77 K (Figure 1C) has a spread in g-values that is greater than that of even the β-Hb hydroperoxo-ferriheme complex, and indeed is substantially greater than that of all previously studied cryo-reduced oxyhemoproteins and hydroperoxo-heme complexes.[20] ENDOR spectroscopy reveals an interaction of an exchangeable proton, Figure 2C, whose hyperfine coupling is comparable to that of the proton of the ferric-hydroperoxo-β-Hb, Figure 2B. Unlike β-Hb, annealing the cryoreduced HO sample to 180 K causes only a minimal broadening in the EPR spectrum of cryoreduced HO, Figure 1D, attributed to subtle structural relaxation of the heme pocket; there is no change in the proton coupling. From these observations we conclude that the O_2 moiety of the precursor oxy-HO is stabilized by an H-bond, as found previously,[21] and that reduction of oxy-HO at 77 K initially produces an H-bonded ferric-peroxo species, **$[FePO_2]_{red}$**, that is not detected. Rather, it promptly converts *at 77 K*, to the hydroperoxo-ferric-HO, which is the species actually observed. A similar observation has been made for P450cam and its T252A mutant.[13] Given the absence of a distal histidine in HO,[22] the source of this proton is likely a sequestered water molecule. The difference in **g** tensors for the hydroperoxo-ferric-β-Hb and HO forms is attributed to a difference in the Fe−O−O angle of the hydroperoxo ligand induced by differing interactions with the distal pocket.[23]

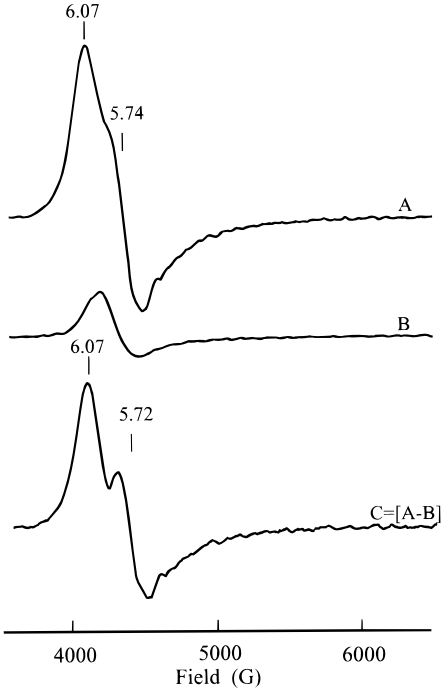

Figure 3. Low-field Q-band EPR spectra of cryoreduced HO−O_2 after annealing at 180 K (B) and 238 K (A); (C**)** = (A) − (B). *Conditions:* as in Figure 1.

Upon annealing the hydroperoxo-ferric-HO to temperatures above 200 K its EPR spectrum disappears; it is gone after warming from 77 K to 238 K for 1 min and then re-cooling to 77 K. To determine the fate of this species we collected EPR spectra in the $g \approx 6$ region, where high-spin ferrihemes show their characteristic $g_\perp$ signals. Oxy-HO cryoreduced at 77 K gives a low-intensity, axial high-spin spectrum from residual ferric-HO which is unchanged by annealing to ~200 K, Figure 3B. The loss of the signal from the hydroperoxo-ferric-HO intermediate caused by annealing to 238 K is paralleled by a corresponding increase in that from high-spin ferriheme, with a change in the high-spin signal to that characteristic of a species with a rhombic splitting of its **g** tensor, Figure 3A. Subtraction of the spectrum of the residual ferric-HO (Figure 3B) from that of the 238 K-annealed sample gave Figure 3C, which corresponds to a high-spin EPR species with $g_1 = 6.07$ and $g_2 = 5.72$. *This is the spectrum of high-spin ferric* α-meso-*hydroxyheme-HO, which is the only species in the HO catalytic cycle that gives such a rhombic spectrum.*[24] *We conclude that the heme of hydroperoxo-ferric-HO self-hydroxylates to form the* α-meso-*hydroxoheme-HO, in situ at temperatures above 200 K.*

This work for the first time demonstrates that one-electron reduction of oxyferrous-HO yields α-*meso*-hydroxyheme, thereby establishing that heme is catabolized through the α-*meso*-hydroxyheme intermediate in HO catalysis. Our results further corroborate the early proposal[3] that HO falls into a new class of heme-containing oxygenases with the hydroperoxo-ferric active species.

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