EPR and ENDOR of Catalytic Intermediates in Cryoreduced Native and Mutant Oxy-Cytochromes P450cam: Mutation-Induced Changes in the Proton Delivery System

Roman Davydov,[†] Iain D. G. Macdonald,[‡] Thomas M. Makris,[‡] Stephen G. Sligar,*[,‡] and Brian M. Hoffman*[,†]

The Department of Chemistry, Northwestern University Evanston, Illinois 60201
The Beckman Institute
Departments of Chemistry and Biochemistry and the Center for Biophysics, University of Illinois Urbana Illinois 61801

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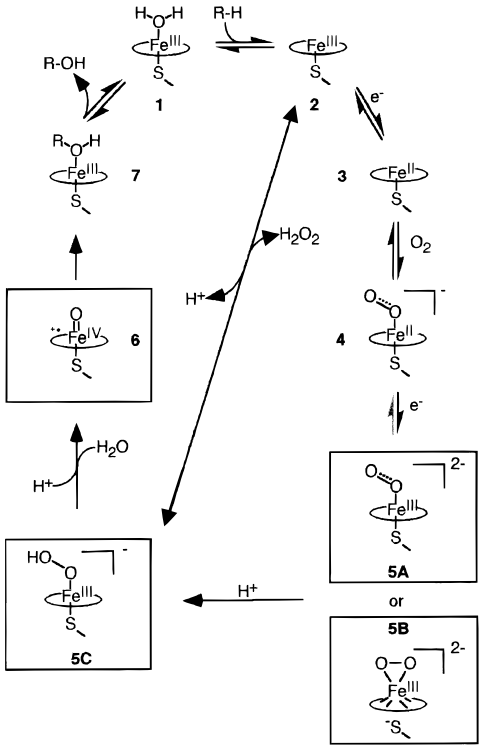
Members of the cytochrome P450 monooxygenase family play vital roles in the synthesis and degradation of many physiologically important compounds and xenobiotics.[1] The P450 hydroxylation reaction is believed to follow the mechanism of Scheme 1,[2] in which the overall rate-limiting step for hydroxylation by the native enzyme is the reduction of the dioxygen-bound ferrous state (**4**).[2] Catalytic efficacy is critically dependent on proton delivery to the catalytic site. For instance, the site-specific mutant T252A of P450cam shows an uncoupling of O_2 consumption from product formation,[3,4] which is thought to arise from disruption of a hydrogen-bond network that involves T252 and sequestered water.[5] In a second P450cam mutant that perturbs this network, D251N, the overall rate of catalysis is drastically slowed, while the observed solvent isotope effect is dramatically increased. These results were rationalized by a proposal of an alternate mechanism for proton delivery in this mutant.[6]

We here describe the first stage of an investigation of the proton delivery system in P450 by EPR and ENDOR spectroscopy of cytochrome P450cam intermediate states prepared by radiolytic cryoreduction[7−13] of **4** in frozen solution at 77 K. For P450 the product of this reduction is the reduced-oxy intermediate, **5**, as it occurs at 77 K, unaltered by higher-temperature thermal relaxation. The results for cryoreduced and annealed oxy-P450cam-WT enzyme and the site-specific T252A, and D251N mutants, as compared to those for oxyMb, allow us to to characterize both the P450cam state **5**, and the mutation-induced changes in the P450cam catalytic proton delivery system.

Scheme 1



The primary product of cryoreduction of oxy-Mb, oxy-Hb, and Hb oxy-beta-chains[7,9,14] at 77 K is an end-on "ferric-peroxo (formally)" species, corresponding to **5A** in Scheme 1, whose EPR spectrum is characterized by $\mathbf{g} \approx [2.24, 2.14, 1.96]$. The assignment for this intermediate[7] is based on the observations that the EPR spectra have the unmistakable signature of a low-spin ferriheme species,[15] $g_1 > g_2 > g_e > g_3$ in a strong ligand field (small g dispersion), rather than that of a "ferrous-superoxy" center where the major unpaired-spin density resides on the dioxygen moiety, $g_1 > (g_2, g_3) \sim g_e$, as in dioxygen adducts of Co(II)complexes,[16] or of a side-on "ferric-peroxo" form, **5B**, which would have a rhombic high-spin, "g-4.3", signal.[17] Upon annealing such a sample to a temperature of ∼200 K, **5A** abstracts a proton from within the heme pocket to yield the hydroperoxy-ferri−protein complex whose EPR spectrum has a larger g-spread, $\mathbf{g} \approx [2.30, 2.18, 1.94]$.[7,9,14]

Figure 1 presents the 2 K Q-band EPR spectra[18] obtained after cryoreduction of the dioxygen complexes of P450cam-WT[12,19] and the two P450cam mutants, T252A and D251N.[20,21] The spectrum of reduced-oxyP450-D251N, with $\mathbf{g} = [2.25, 2.16, 1.96]$, is very similar to that of reduced-oxyMb, whereas the spectra of reduced-oxyP450-WT and T252A are themselves very similar, with $\mathbf{g} \approx [2.3, 2.16, 1.96]$, but are quite distinct from that of D251N. The catalytic competence of the species **5** formed by cryoreduction is confirmed by GC analysis which shows that warming of the WT and D251N cryoreduced samples leads to

[†] Northwestern University
[‡] University of Illinois

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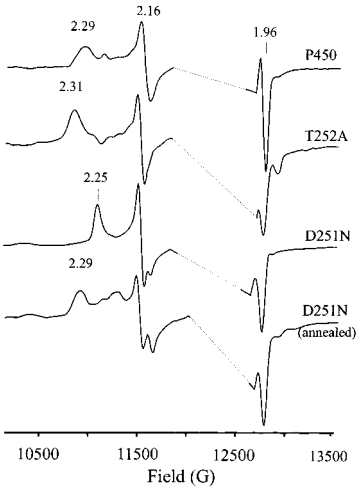

Figure 1. Q-band CW-EPR spectra of oxy-P450cam and mutants cryoreduced and stored at 77 K, plus one for the D251N mutant which had been annealed at 180 K for 1 min. Free-radical signals around $g = 2$ were omitted for clarity. Conditions: $T = 2$ K, 35.0 GHz; 100 kHz detection, modulation amplitude, 2 G. The derivative presentation was obtained digitally from the absorption-mode EPR envelopes detected under these rapid-passage conditions.

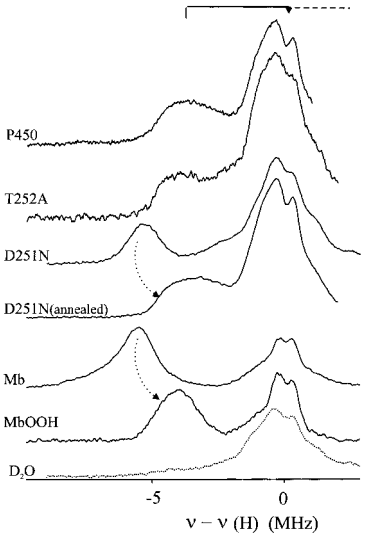

Figure 2. Q-band CW proton ENDOR spectra taken at the g_1 field for each of the cryoreduced oxyhemoproteins; dotted arrow indicates annealing as in caption for Figure 1 (see text). The spectrum labeled "D_2O" was taken with reduced oxyMb in D_2O buffer; a similar result is obtained with the reduced oxyP450cam samples in such buffer. Asymmetry in Q-band ENDOR spectra is common and occurred here. As a result, only the ν_- regions are shown, with frequencies referenced to the proton Larmor frequency. Conditions: $T = 2$ K; field modulation amplitude, 2 G.

the formation of the stereoselective enzymatic product, whereas the T252A sample shows no product, consistent with room-temperature observations of catalysis.[3,4,6]

The differences in **g** tensor values among Mb, P450, and the mutants, considered by themselves, might be interpreted as reflecting solely a competition between the proximal influence on **5A** of the axial ligands, histidyl-nitrogen in Mb versus cysteinyl-thiolate in P450, and the distal influence of differing H-bonding networks. However, the spectra for the reduced-oxyP450-WT and T252A are essentially the same as for the hydroperoxo-ferriMb and Hb (see above), and have similar **g** tensors, most particularly g_1, to those for *n*- and *tert*-butylperoxo-ferriheme-thiolate model compounds reported by Tajima: $\mathbf{g} \approx [2.29, 2.21, 1.96]$.[22] Moreover, the spectra for both the WT and T252A enzymes do not change upon annealing to 180 K, but that for the reduced-oxyD251N mutant largely converts (Figure 1) to such a hydroperoxo-ferric-like spectrum. This suggests that the species **5A** produced by cryoreduction of oxyP450-WT and T252A accept a proton from the P450 proton delivery system *at 77 K* to form the hyroperoxo-ferri P450, **5C** (a similar finding has been made for heme oxygenase[14]), whereas the altered proton delivery system of the D251N mutant forestalls that reaction and permits observation of the end-on ferric-peroxo species, **5A**.

To test this hypothesis we employed Q-band CW proton ENDOR spectroscopy.[18,23] The ENDOR spectrum taken at g_1 for each cryoreduced oxy-hemoprotein in H_2O buffer shows an intense ^{1}H signal centered at the proton Larmor frequency and of ~4−5 MHz breadth, along with a doublet signal from more strongly coupled exchangeable proton(s) (see Figure 2). The exchangeable proton seen in the spectrum of ferric-peroxo-Mb in H_2O (Figure 2, $A(g_1) \approx 14$ MHz) is assigned to a hydrogen bond between the distal histidine and the peroxo moiety.[9] Contrary to earlier report,[9] the hydroperoxo-ferric-Mb gives a signal from the exchangeable proton with a significant, although lessened, hyperfine coupling (Figure 2, $A(g_1) \approx 8.2$ MHz). (The decrease in coupling, despite the change from H-bond to covalent bond, likely reflects a diminished spin density on the hydroperoxy moiety compared to that on the peroxo one.) The exchangeable protons in P450cam-WT, the T252A mutant, and the annealed D251N mutant give the same coupling as that of the ferric-hydroperoxo-Mb, $A(g_1) \approx 8$ MHz, rather than the larger coupling for ferric-peroxo-Mb and for the D251N mutant as prepared at 77 K, $A(g_1) \approx 11$ MHz. In short, the ENDOR results corroborate the above hypothesis.

We have interpreted the EPR spectra *and* ^{1}H couplings for cryoreduced-oxy-P450cam-WT and the T252A mutant as indicating that the species trapped at 77 K is the hydroperoxo-ferric-intermediate, **5C**; thus, the first proton of catalysis can be delivered to **5A** even at this temperature. However, cryoreduced T252A mutant does not form product upon warming, whrereas the WT enzyme does. From this we infer that the T252A mutation does not interfere with the formation of **5C** and that the uncoupling of O_2 consumption from product formation, the dissociation of H_2O_2 rather than O−O bond cleavage, instead reflects a perturbation in a subsequent step of catalysis. In contrast, after 77 K cryoreduction of **4** in the D251N mutant, one observes the peroxo-ferric species, **5A**; delivery of the first catalytic proton to form **5C** is impeded at 77 K, and requires thermal activation. This suggests to us that the drastic reduction in the overall rate of catalysis and increase in the observed solvent isotope effect for this mutant[6] may reflect an inhibition in the delivery of the *first* proton of catalysis.

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