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The Use of Deuterated Camphor as a Substrate in ^{1}H ENDOR Studies of Hydroxylation by Cryoreduced Oxy P450cam Provides New Evidence of the Involvement of Compound I

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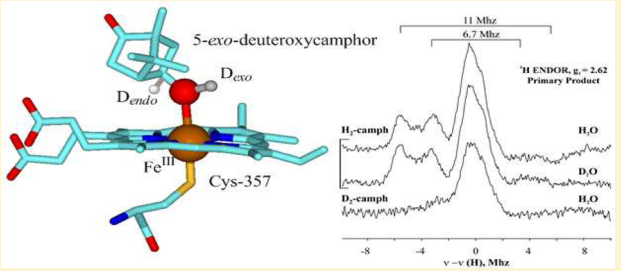
Supporting Information

ABSTRACT: Electron paramagnetic resonance and ^{1}H electron nuclear double resonance (ENDOR) spectroscopies have been used to analyze intermediate states formed during the hydroxylation of (1*R*)-camphor (H_2-camphor) and (1*R*)-5,5-dideuterocamphor (D_2-camphor) as induced by cryoreduction (77 K) and annealing of the ternary ferrous cytochrome P450cam−O_2−substrate complex. Hydroxylation of H_2-camphor produced a primary product state in which 5-*exo*-hydroxycamphor is coordinated with Fe(III). ENDOR spectra contained signals derived from two protons [Fe(III)-bound C5-OH_{exo} and C5-H_{endo}] from camphor. When D_2-camphor was hydroxylated under the same condition in H_2O or D_2O buffer, both ENDOR H_{exo} and H_{endo} signals are absent. For D_2-camphor in H_2O buffer, H/D exchange causes the C5-OH_{exo} signal to reappear during relaxation upon annealing to 230 K; for H_2-camphor in D_2O, the magnitude of the C5-OH_{exo} signal decreases via H/D exchange. These observations clearly show that Compound I is the reactive species in the hydroxylation of camphor in P450cam.



The P450 cytochromes form a superfamily of cysteine thiolate-ligated heme enzymes that catalyze reductive activation of dioxygen for the insertion or addition of a single oxygen atom into a wide variety of substrates. These reactions include hydroxylations, epoxidations, sulfoxidations, and many more.[1] P450 enzymes are critical to many biological processes, including steroid hormone biosynthesis, drug metabolism, and the detoxification of xenobiotics, and are found in most classes of organisms.[1−5]

The reaction cycle of cytochrome P450s and the nature of the catalytically active state have been the object of intense study for more than three decades.[6−13] The classic cytochrome P450 catalytic cycle is initiated by binding of substrate to the ferric state followed by one-electron reduction to the ferrous state by NAD(P)H-dependent reductases. Binding of molecular oxygen gives the ferrous−dioxygen (**1**) complex, which has been observed and characterized (Scheme 1). The addition of a second electron to the oxyferrous complex leads to a peroxoferric (**2**), which is protonated to generate a hydroperoxoferric (**3**) intermediate. Protonation of the distal oxygen in the hydroperoxo intermediate then leads to heterolytic O−O bond cleavage with loss of water and generation of iron(IV)oxo porphyrin π-cation radical [Fe(IV)═O $P^{+\bullet}$] center, known as Compound I (Cpd I) (**4**). In this classical scheme, Cpd I is the catalytically active species in substrate hydroxylation,[2−5] but it has also been proposed that the peroxo or hydroperoxo intermediates can function as the hydroxylating species in some enzymes and/or with specific classes of substrates.[2−5,14]

The short lifetime of intermediates **2**−**4** makes it difficult to characterize them and their reactions with substrate by conventional kinetic and spectroscopic techniques under physiological conditions. Thus, until recently, the oxyferrous complex was the last observable intermediate in the reaction cycle of heme-containing monooxygenases; Cpd I now has been detected by direct oxidation of substrate-free ferric P450cam and Cyp 119 with *m*-chloroperoxobenzoic acid.[15−17] These limitations have been largely overcome by application of radiolytic cryoreduction at 77 K, which both generates and traps one-electron-reduced ternary ferrous heme monooxygenase−O_2−substrate complexes that are catalytically competent while being annealed to higher temperatures.[14,18−25] Cryogenic stabilization of intermediates generated in this way has made possible the direct spectroscopic and structural characterization of peroxoferric and hydroperoxoferric intermediates, as well as measurement of the kinetics of their interconversion and the formation of product.[14,26] In contrast, no spectroscopically detectable amounts of Cpd I are observed during these conversions in the presence of substrate because of the rapid

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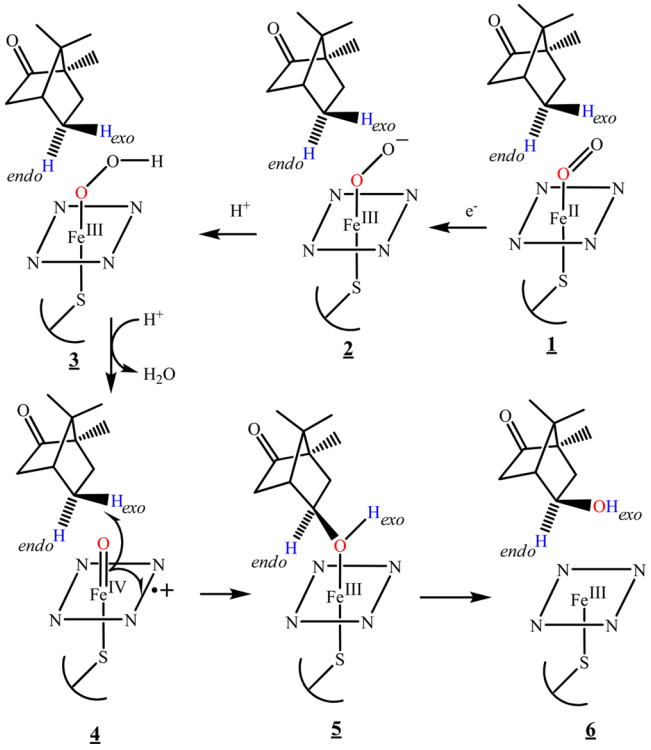
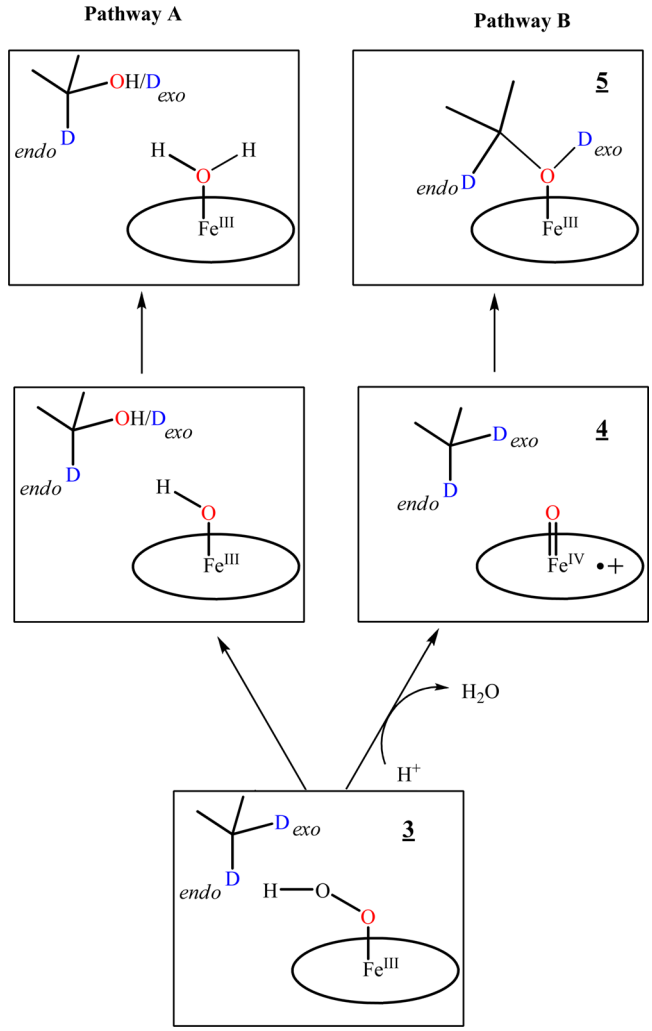
Scheme 1. Cytochrome P450 Intermediates in the Presence of D_2-Camphor[a]



[a] **1**, oxyferrous; **2**, peroxoferric; **3**, hydroperoxoferric, **4**, Compound I.

Scheme 2. Alternative Reaction Pathways during Hydroxylation of D_2-Camphor by the Hydroperoxoferric P450 (pathway A) Intermediate and Cpd I (pathway B)



reaction with bound substrate, although Cpd I again has been observed during annealing of cryoreduced oxyferrous dehaloperoxidase in the absence of bound substrate.[23] Nonetheless, the cryoreduction approach in combination with electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopy permits identification of the reactive species involved in conversion of bound substrate to product.[14]

Identification of the active oxidizing species in cytochrome P450 reactions through cryoreduction techniques has been based on EPR and ENDOR on the solvent isotope of the isotopic composition of the primary product state trapped during annealing of the cryoreduced ternary complex of oxyferrous heme monooxygenase with bound substrate.[14] If the product is formed by insertion of the ferryl oxygen of Cpd I into a C−H bond of substrate, the primary product state trapped by cryoreduction and/or annealing at low temperatures contains the product alcohol bound to the heme iron(III) (Scheme 2, pathway B). When reaction occurs with deuterated substrate in H_2O, the product hydroxyl group contains the D abstracted from the reaction mixture; when reaction occurs in D_2O with protonated substrate, the product hydroxyl contains the H abstracted from the substrate. In the relaxed product state that forms during annealing to higher temperatures, as visualized in the previously reported crystal structure of cytochrome P450cam with its product, 5-*exo*-hydroxycamphor bound by its OH group to the low-spin Fe(III) heme,[27] the hydroxyl H or D of the primary product can exchange with solvent. The source of the exchangeable hydroxyl H or D in the bound product can be determined by ^{1}H ENDOR spectroscopy.

If, instead, a peroxo−hydroperoxoferric heme intermediate is the reactive species (Scheme 2, pathway A), the primary product trapped at a low temperature contains a hexacoordinated aqua ferriheme, while the hydroxylated substrate is sequestered remotely from the heme in the distal pocket of the active site.[14] The same is true for the "somersault" mechanism suggested by Bach.[13] In this case, the bound water incorporates H or D from solvent, not substrate, and again this can be revealed by ^{1}H ENDOR, while the remote hydroxylated substrate in this state would at most show a ^{1,2}H ENDOR signal with weak hyperfine coupling.

Previously, a cryoreduction−ENDOR study that employed such a variation in solvent isotope provided evidence that Cpd I is the active state in the hydroxylation of bound camphor by the cryoreduced oxyferrous mutant D251N P450cam.[19] We herein cleanly demonstrate the accuracy of this conclusion through a complementary study that uses cryoreduction−ENDOR techniques to determine the variation of the isotopic composition of the primary product of hydroxylation with the isotopic composition of the substrate. Comparison of the primary product formed with (1*R*)-camphor (H_2-camphor) and (1*R*)-5,5-dideuterocamphor (D_2-camphor) by ENDOR spec-

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troscopy directly reveals the agency of Cpd I as the reactive intermediate.

EXPERIMENTAL PROCEDURES

Chemicals. Solid $Na_2S_2O_4$, chromium trioxide, d_4-hydrazine, (*R*)-camphor, and all other chemicals and reagents were purchased from standard commercial sources (Aldrich) and used as received. O_2 gas was obtained from Matheson Co.

Materials. The synthesis of (1*R*)-5,5-dideuterocamphor (D_2-camphor) was conducted in two steps. In the first step, to (1*R*)-(+)-camphor (10 g) in refluxing glacial acetic acid (150 mL) was cautiously added chromium trioxide (20.7 g) in three portions over 30 min.[28] Refluxing was continued for 1 h. The solution was then cooled and diluted with water (150 mL). The organic layer was extracted with ether (3 × 100 mL). The combined extracts were washed with a saturated sodium bicarbonate solution (6 × 50 mL) and brine (100 mL), dried over anhydrous $MgSO_4$, and filtered, and the solvent was removed under vacuum. Flash chromatography using silica and a hexane/ethyl acetate mixture (80:20) gave the product, 5-oxo-(1*R*)-(+)-camphor (5-ketocamphor), as a white solid: GC–MS (6.1 min) *m*/*z* 166 (M), 151, 123, 109, 95, 83, 69, 55; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93 (s, 3H), 1.03 (d, *J* = 3 Hz, 6H), 2.01 (d, 1H, *J* = 19 Hz, 6-*exo*), 2.13 (d, *J* = 17.5 Hz, 1H, 3-*endo*), 2.31 (d, *J* = 18.5 Hz, 1H, 6-*endo*), 2.55 (m, 2H, 3-*exo*, 4-bridge); ^{13}C NMR δ 214.3, 212.5, 58.1, 57.6, 46.1, 42.7, 36.6, 19.5, 18.3, 9.0[29] (Figure S1 of the Supporting Information)

In the second step, 5-oxo-(1*R*)-(+)-camphor (0.05 g), d_4-hydrazine (0.0432 g), and potassium *tert*-butoxide (0.134 g) were taken in DMSO-d_6 (10 mL). The mixture was refluxed for 18 h under anhydrous conditions (bath temperature maintained at 175 °C). D_2O (10 mL) and DCl (30%, 1 mL) were added to the mixture, and the mixture was stirred for 30 min at room temperature. The product was examined by TLC and purified by flash chromatography using silica and a hexane/ethyl acetate mixture (90:10) as the solvent: GC–MS (9.37 min) *m*/*z* 154 (M), 109, 97, 83, 69, 55 (Figure S2 of the Supporting Information).

The *Escherichia coli* expression system was used to express and purify P450cam protein as previously described.[24]

Sample Preparation. EPR–ENDOR samples of oxyferrous P450cam complexes with H_2-camphor and D_2-camphor were prepared as described previously.[24] The oxyferrous P450 samples were reduced at 77 K by γ-irradiation with a ^{60}Co source to a dose of 3 Mrad, and the annealing protocol for the irradiated samples also has been described previously.[24]

Spectroscopic Techniques. EPR–ENDOR measurements of cryoreduced samples were conducted as previously described.[25,30]

RESULTS AND DISCUSSION

Studies of the catalytic intermediates in the P450cam catalytic cycle begin with the substrate-bound oxyferrous state of the protein. Figure 1 shows EPR spectra of the cryoreduced ternary oxyferrous P450–camphor complex taken during its progressive annealing at 190 K. The 77 K cryoreduced ternary complex shows a rhombic EPR signal with **g** = [2.30, 2.18, 1.9], characteristic of the $S = {}^1/_2$ hydroperoxoferric heme species.[18,19,24] As seen previously, during annealing at 190 K, the hydroperoxoferric intermediate converts into the primary product, with **g** = [2.62, 2.25, 1.89], which then relaxes to the equilibrium state (Figure 1).[19] This relaxed complex shows two

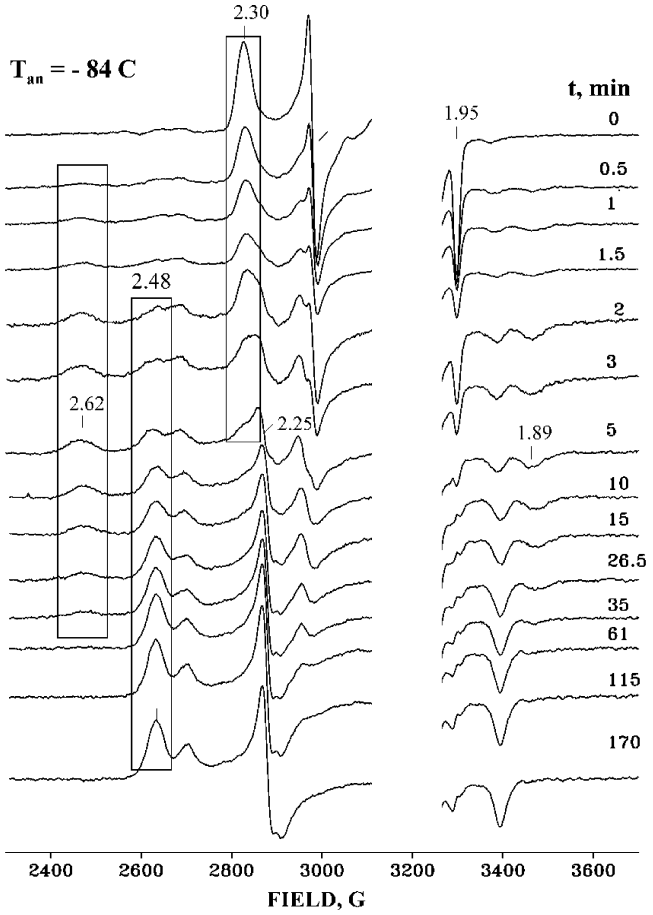

Figure 1. X-Band EPR spectra of the cryoreduced oxyferrous P450cam–camphor complex taken during progressive annealing at 190 K at indicated times. Boxes isolate the loss of the g = 2.30 signal from the hyrdoxoferric state, the appearance and loss of the g = 2.62 signal from the primary product state, and the appearance of the g = 2.48 signal from the relaxed product state. Instrument conditions: T = 29 K, microwave frequency of 9.369, microwave power of 13 db, and modulation amplitude (A_m) of 10 G.

low-spin EPR signals with **g** = [2.48, 2.25, 1.90] and [2.41, 2.23 1.96], assigned to the ferric P450–5-hydroxycamphor complex and aquo ferric P450cam, respectively. Decay of the hydroperoxoferric heme intermediate during annealing at 190 K was earlier shown to be 2-fold slower in D_2O solvent,[18] establishing that the rate-limiting step of the decay of the hydroperoxo intermediate involves activation of the hydroperoxo moiety by transfer of a proton to it, as presumed for Cpd I formation.

According to Scheme 2 (pathway B), hydroxylation of H_2-camphor by Cpd I should produce a primary product with ENDOR signals from two protons derived from camphor, one from the C5-OH_{exo} group coordinated to Fe(III) and the other from the remaining C5-H_{endo} group, whereas if hydroxylation occurs by the hydroperoxyferric heme, in the primary product the heme iron would be coordinated by water and the C5-H_{endo} proton of hydroxycamphor will be too distant to give an appreciable hyperfine interaction.

Our previous studies of the primary product complex formed in H_2O buffer with C5-H_2-camphor showed two well-resolved, strongly coupled ^{1}H ENDOR signals. This observation is repeated in this work. Figure 2 presents ^{1}H ENDOR spectra taken at the canonical g_1 = 2.62 field position for the primary product intermediate. It shows signals from two protons, with

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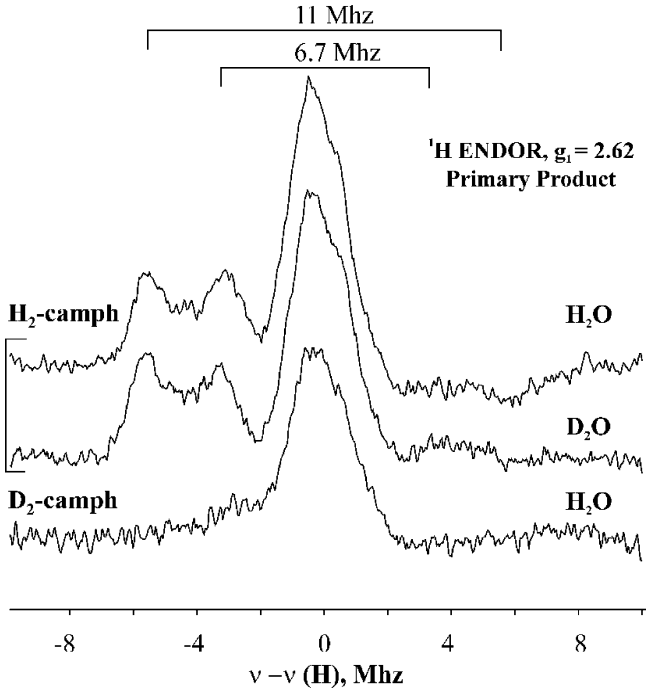

Figure 2. ^{1}H continuous wave 35 GHz ENDOR spectra taken at g_1 = 2.62 field for the primary product trapped upon annealing at 190 K of the cryoreduced oxyferrous P450cam−camphor complex in H_2O and D_2O and the cryoreduced oxyferrous P450cam−D_2-camphor complex in H_2O. Instrument settings: T = 2 K, 35.1 GHz, field modulation of 100 kHz, modulation amplitude of 2 G, scan speed of 1 MHz/s, and 100 kHz broadening of radiofrequency excitation.

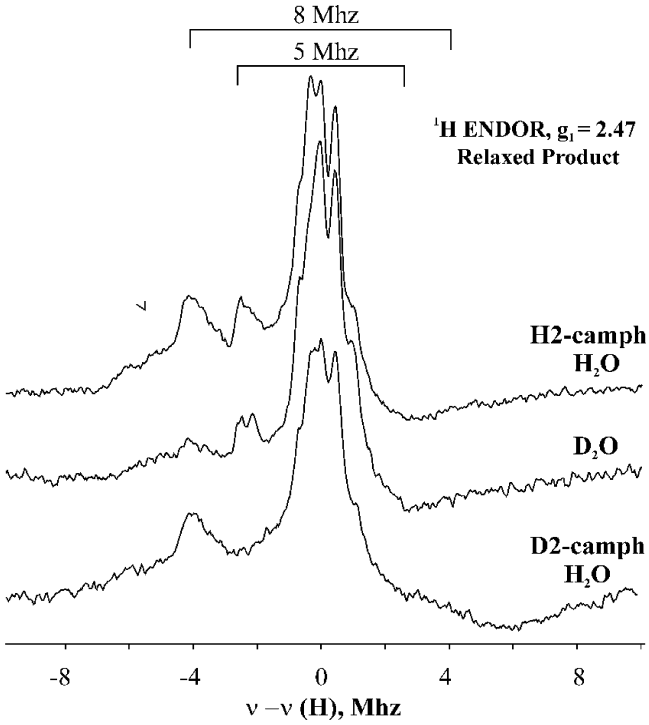

Figure 3. ^{1}H continuous wave 35 GHz ENDOR spectra taken at g_1 = 2.47 field for the relaxed product complex formed upon annealing at 230 K of the cryoreduced oxyferrous P450 cam−camphor complex in H_2O and D_2O and the cryoreduced oxyferrous P450cam−D_2-camphor complex in H_2O. Instrument settings were the same as those described in the legend of Figure 2.

hyperfine coupling [A(H1) = 11 MHz, and A(H2) = 6.7 MHz], as previously reported. Figure 2 also shows the complementary ENDOR spectrum obtained when the primary product was instead formed with D_2-camphor in H_2O buffer: both the H1 and H2 signals disappear, unambiguously confirming that the two ^{1}H signals seen in the H_2-camphor substrate arise from the C5 protons of the substrate, as they both are absent when product is formed by hydroxylation of D_2-camphor.

Relaxation of the primary product formed in H_2O buffer with C5-H_2-camphor gives the equilibrium product complex, which also shows two well-resolved, strongly coupled ^{1}H ENDOR signals, but with slightly different couplings [A_{max}(H1) = 9.2 MHz, and A_{max}(H1) = 5.7 MHz at g_1 = 2.48], as seen in Figure 3. Relaxation experiments previously conducted with D_2O buffer and repeated in Figure 3 show that the H1 signal is lost in the relaxed product complex but H2 is not. This indicates that H2 = H_{endo}(C5) of hydroxycamphor, and upon relaxation, H1 exchanges with solvent; therefore, H_{exo} is the proton of the hydroxycamphor hydroxyl coordinated to heme iron(III).[19] The differences in hyperfine couplings of the two protons and **g**-tensor components of the EPR signals of the primary and relaxed (g_1 = 2.48) product complexes reflect different geometries of hydroxycamphor coordinated to Fe(III).[19]

Correspondingly, when D_2-camphor is the substrate, the primary product state formed in H_2O buffer during hydroxylation shows neither H_{exo} or H_{endo} signals, but when it is annealed at 230 K, the H_{exo} ENDOR signal reappears as the Fe-bound hydroxyl of 5,5′-OD,D-camphor in the primary product exchanges with H of solvent to form 5,5′-OH,D-camphor (Figure 3). As required, the H_{endo} signal from the nonexchangeable H_{exo}(C5) does not reappear (Figure 3).

In summary, complementary cryoreduction−ENDOR approaches in which the isotopic composition of the primary product of camphor hydroxylation by P450cam is correlated with either the solvent or substrate isotopic composition demonstrate that Cpd I is the reactive hydroxylating species according to Scheme 2 (pathway B). Insertion of the ferryl oxygen of Cpd I into the camphor C(5)-H_{exo} bond of bound camphor leads to formation of 5-OH-camphor with its hydroxyl coordinated to Fe(III), and with the hydroxyl proton derived from substrate. At the reaction temperature of 190 K, the hydroxyl proton does not exchange with solvent, but during annealing of the primary complex at 230 K, the conformation of the product complex relaxes and the OH/D group of the 5-hydroxycamphor product is exchanged with solvent. The ENDOR results are those predicted for Cpd I as the active hydroxylating intermediate (Scheme 2, pathway B) and rule out the peroxo/hydroperoxo state (pathway A) for that role.

■ ASSOCIATED CONTENT

Supporting Information

Additional supporting spectral data pertaining to the synthesis of (1*R*)-5,5-dideuterocamphor. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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