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Quantitative Production of Compound I from a Cytochrome P450 Enzyme at Low Temperatures. Kinetics, Activation Parameters, and Kinetic Isotope Effects for Oxidation of Benzyl Alcohol

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Abstract: Cytochrome P450 enzymes are commonly thought to oxidize substrates via an iron(IV)-oxo porphyrin radical cation transient termed Compound I, but kinetic studies of P450 Compounds I are essentially nonexistent. We report production of Compound I from cytochrome P450 119 (CYP119) in high conversion from the corresponding Compound II species at low temperatures in buffer mixtures containing 50% glycerol by photolysis with 365 nm light from a pulsed lamp. Compound I was studied as a reagent in oxidations of benzyl alcohol and its benzylic mono- and dideuterio isotopomers. Pseudo-first-order rate constants obtained at −50 °C with concentrations of substrates between 1.0 and 6.0 mM displayed saturation kinetics that gave binding constants for the substrate in the Compound I species (K_{bind}) and first-order rate constants for the oxidation reactions (k_{ox}). Representative results are $K_{bind} = 214\ M^{-1}$ and $k_{ox} = 0.48\ s^{-1}$ for oxidation of benzyl alcohol. For the dideuterated substrate $C_6H_5CD_2OH$, kinetics were studied between −50 and −25 °C, and a van't Hoff plot for complexation and an Arrhenius plot for the oxidation reaction were constructed. The H/D kinetic isotope effects (KIEs) at −50 °C were resolved into a large primary KIE ($P = 11.9$) and a small, inverse secondary KIE ($S = 0.96$). Comparison of values extrapolated to 22 °C of both the rate constant for oxidation of $C_6H_5CD_2OH$ and the KIE for the nondeuterated and dideuterated substrates to values obtained previously in laser flash photolysis experiments suggested that tunneling could be a significant component of the total rate constant at −50 °C.

Introduction

Cytochrome P450 enzymes (P450s or CYPs) are heme-containing enzymes that are widely distributed in nature and catalyze a wide range of reactions including numerous oxidations.[1,2] In humans, P450s are the major oxidants of drugs, pro-drugs, and xenobiotics,[3−5] generating great interest from the pharmaceutical perspective in regard to toxicology and drug dosing. P450s also are of medicinal interest due to the relationships of P450 overexpression with cancer and other disease states.[6−8]

The active oxidizing forms of P450 enzymes have not been detected under turnover conditions, but they are widely believed to be iron(IV)-oxo species with the porphyrin oxidized to a radical cation.[1,9] Such species, termed Compounds I, are analogous to known intermediates formed in other heme-containing enzymes, such as peroxidases and catalases, upon reaction of the enzymes with hydrogen peroxide.[10] Unlike peroxidases and catalases, P450s typically are activated by a sequence of reactions composed of reduction of the enzyme to the ferrous state, oxygen binding, a second reduction step, and protonation steps. Nonetheless, activations of P450s with peroxy compounds have been known for many years to support substrate oxidations,[11,12] and attempts to detect Compounds I in P450s by chemical oxidations with peroxy compounds were conducted as early as three decades ago.[13] Despite indications that a transient was formed in reactions of $P450_{cam}$ (CYP101) with hydroperoxy compounds,[14,15] detailed spectroscopic analyses revealed that Compounds I did not accumulate to detectable

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(1) *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 3rd ed.; Ortiz de Montellano, P. R., Ed.; Kluwer: New York, 2005.
(2) Guengerich, F. P. *Chem. Res. Toxicol.* **2008**, *21*, 70–83.
(3) Wrighton, S. A.; Stevens, J. C. *Crit. Rev. Toxicol.* **1992**, *22*, 1–21.
(4) Gonzalez, F. J.; Gelboin, H. V. *Drug Metab. Rev.* **1994**, *26*, 165–183.
(5) Guengerich, F. P. In *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 3rd ed.; Ortiz de Montellano, P. R., Ed.; Kluwer: New York, 2005; pp 377−530.
(6) Guengerich, F. P. *Cancer Res.* **1988**, *48*, 2946–2954.
(7) Dumitrescu, R. G.; Cotarla, I. *J. Cell. Mol. Med.* **2005**, *9*, 208–221.
(8) Rodriguez-Antona, C.; Ingelman-Sundberg, M. *Oncogene* **2006**, *25*, 1679–1691.
(9) Makris, T. M.; von Koenig, K.; Schlichting, I.; Sligar, S. G. *J. Inorg. Biochem.* **2006**, *100*, 507–518.
(10) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841–2887.
(11) Coon, M. J.; Ballou, D. P.; Guengerich, F. P.; Nordblom, G. D.; White, R. E. *Adv. Exp. Med. Biol.* **1976**, *74*, 270–280.
(12) Nordblom, G. D.; White, R. E.; Coon, M. J. *Arch. Biochem. Biophys.* **1976**, *175*, 524–533.
(13) Coon, M. J.; Blake, R. C.; Oprian, D. D.; Ballou, D. P. *Acta Biol. Med. Ger.* **1979**, *38*, 449–458.
(14) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1464–1469.
(15) Spolitak, T.; Dawson, J. H.; Ballou, D. P. *J. Biol. Chem.* **2005**, *280*, 20300–20309.

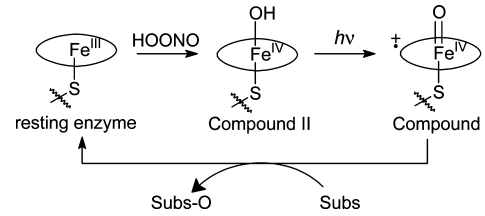
levels in chemical oxidations of either $P450_{cam}$ or $P450_{BM3}$ (CYP102).[16−19] Reaction of another P450, CYP119, with *m*-chloroperoxybenzoic acid also provided evidence for formation of a short-lived Compound I species.[20]

Cytochrome P450 enzymes and their models have been extensively studied from chemical and biochemical perspectives, generating thousands of reports per year. P450s have been emulated in catalytic oxidation chemistry, are potentially viable reagents for the synthesis of high-value-added intermediates in the drug industry, and are the operative oxidizing enzymes in many organisms employed in bioremediation. Nonetheless, almost all kinetic studies of P450s have been performed under catalytic turnover conditions, where the rate-limiting step in the overall conversion is not necessarily identified. Kinetic studies of P450s under single turnover, or stoichiometric, conditions are limited, and stoichiometric studies of the P450 Compounds I are essentially nonexistent.

Given difficulties in preparation of P450 Compounds I, our group explored an alternative to chemical oxidation of resting enzymes with hydroperoxy compounds. Reactions of some P450 enzymes with peroxynitrite give iron(IV)-oxo neutral porphyrin intermediates known as Compounds II,[21,22] which are one electron reduced in the oxidation state from Compounds I. Photolysis of Compounds II resulted in photoejection of an electron to give Compound I in model systems[23] and in P450 enzymes.[24−27] Due to the reactivity of Compounds I and their analogues, initial photo-oxidation studies were conducted at ambient temperature employing laser flash photolysis (LFP) methods.

In the present work, we report an extension of the photoejection preparative method to low temperatures that employs a high-powered pulsed lamp for photolyses of Compound II. Using glycerol−buffer mixtures at temperatures between −25 and −50 °C, we prepared CYP119 Compound I in high conversion and studied Compound I as a reagent. Substrate binding constants, rate constants, and kinetic isotope effects for oxidations of benzyl alcohol and its benzyl-deuterated isotopomers were measured at −50 °C. The α,α-dideuteriobenzyl isotopomer was studied between −50 and −25 °C, yielding a temperature-dependent function for binding and the first activation parameters measured for any P450 Compound I reaction.

Scheme 1

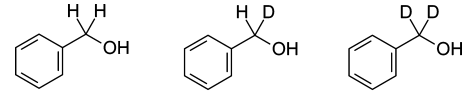


The results suggest that the benzyl alcohol oxidation at −50 °C involves a considerable amount of tunneling, similar in magnitude to the tunneling found for oxidations of benzyl alcohol by an iron(IV)-oxo porphyrin radical cation model of a heme enzyme Compound I species.[28]

Results and Discussion

The reaction sequence for production of CYP119 Compound I is shown in Scheme 1. Reaction of the resting enzyme with peroxynitrite (PN) gives the Compound II derivative, which is best formulated as an hydroxyiron(IV) species based on its XAFS spectrum that indicated a long (1.82 Å) Fe−OH bond.[22] Irradiation of Compound II gave Compound I in a photochemical oxidation reaction that involves loss of an electron from the porphyrin and loss of a proton from the hydroxyiron group. Whether the process involves a proton-coupled electron transfer (PCET) or two distinct steps is not known. Compound I reacted with substrate to return the ferric enzyme. Under the conditions of this study, substrate binding was reversible, resulting in saturation kinetics that permitted solution of binding constants and first-order oxidation rate constants.

Materials. The substrates studied in this work were isotopomers of benzyl alcohol. Nonlabeled benzyl alcohol (BA-d_0) was a commercial sample. The deuterated isotopomers BA-d_1 and BA-d_2 are known compounds that were prepared by $LiAlD_4$ reduction of benzaldehyde and methyl benzoate, respectively. NMR spectra of BA-d_1 and BA-d_2 and mass spectral fragmentation patterns for the three substrates are in the Supporting Information. Previous studies established that benzyl alcohol is oxidized to benzaldehyde under shunt conditions by the P450 enzyme used in this study.[25]



The P450 enzyme employed was CYP119, which was expressed in *Escherichia coli* and purified as previously reported.[24,29,30] CYP119 was originally thought to be from the thermophile *Sulfolobus solfataricus*, but more recently, it was reported to be from the related thermophile *S. acidocaldarius*.[31] The CYP119 used here was judged to be high purity by its *RZ* value, the ratio of absorbance at λ_{max} of the Soret band to that at 280 nm, which was ≥1.5.

Methods. In previous work at ambient temperature, laser flash photolysis (LFP) methods were necessary due to the high

(16) Schünemann, V.; Jung, C.; Trautwein, A. X.; Mandon, D.; Weiss, R. *FEBS Lett.* **2000**, *479*, 149–154.
(17) Schünemann, V.; Trautwein, A. X.; Jung, C.; Terner, J. *Hyperfine Interact.* **2002**, *141*, 279–284.
(18) Jung, C.; Schünemann, V.; Lendzian, F.; Trautwein, A. X.; Contzen, J.; Galander, M.; Bottger, L. H.; Richter, M.; Barra, A. L. *Biol. Chem.* **2005**, *386*, 1043–1053.
(19) Jung, C.; Schünemann, V.; Lendzian, F. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 355–364.
(20) Kellner, D. G.; Hung, S. C.; Weiss, K. E.; Sligar, S. G. *J. Biol. Chem.* **2002**, *277*, 9641–9644.
(21) Mehl, M.; Daiber, A.; Herold, S.; Shoun, H.; Ullrich, V. *Nitric Oxide* **1999**, *3*, 142–152.
(22) Newcomb, M.; Halgrimson, J. A.; Horner, J. H.; Wasinger, E. C.; Chen, L. X.; Sligar, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 8179–8184.
(23) Zhang, R.; Chandrasena, R. E. P.; Martinez, E.; Horner, J. H.; Newcomb, M. *Org. Lett.* **2005**, *7*, 1193–1195.
(24) Newcomb, M.; Zhang, R.; Chandrasena, R. E. P.; Halgrimson, J. A.; Horner, J. H.; Makris, T. M.; Sligar, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 4580–4581.
(25) Sheng, X.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **2008**, *130*, 13310–13320.
(26) Sheng, X.; Zhang, H. M.; Hollenberg, P. F.; Newcomb, M. *Biochemistry* **2009**, *48*, 1620–1627.
(27) Sheng, X.; Zhang, H.; Im, S.-C.; Horner, J. H.; Waskell, L.; Hollenberg, P. F.; Newcomb, M. *J. Am. Chem. Soc.* **2009**, *131*, 2971–2976.
(28) Pan, Z.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **2008**, *130*, 7776–7777.
(29) McLean, M. A.; Maves, S. A.; Weiss, K. E.; Krepich, S.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166–172.
(30) Maves, S. A.; Sligar, S. G. *Protein Sci.* **2001**, *10*, 161–168.
(31) Rabe, K. S.; Kiko, K.; Niemeyer, C. M. *ChemBioChem* **2008**, *9*, 420–425.

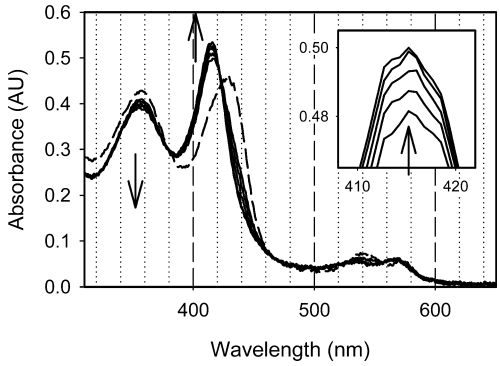


Figure 1. UV−visible spectra of CYP119 species in the reaction of BA-d_1 at −50 °C. The dashed line is Compound II, and the solid lines show the evolution of signals as Compound I reacts with the substrate to give resting enzyme. The inset shows an expansion of the large Soret band for reaction of 3 mM substrate where the time slices are 2.4, 6, 12, 20, and 30 s after irradiation.

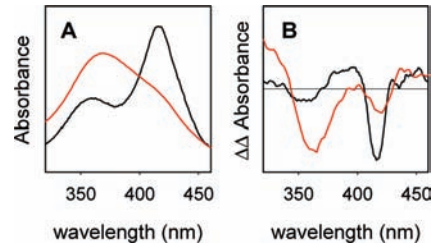


Figure 2. (A) Expansion of Soret band region and (B) second derivative spectra of CYP119 Compound I (black line) and CPO Compound I (red line). Absorbances were adjusted arbitrarily to place the spectra on the same scales. The ΔΔAbs equal zero line is shown in B.

reactivity of CYP119 Compound I.[24−27] In the present studies at low temperature, we employed a high-powered, pulsed UV lamp designed for commercial photoset bonding applications. The unit controlled the pulse duration and power of a mercury lamp, which was filtered through a 365 ± 5 nm cutoff filter. For example, the unit produced 2 J in a 0.5 s pulse in the studies in this work. The light was delivered through a flexible waveguide that was mounted in a temperature-controlled reaction box that held a microcuvette. Reactions were monitored with a diode array UV−visible spectrometer using fiber optics cables that were mounted in the reaction box orthogonal to the waveguide.

In order to study reactions at reduced temperatures, the samples were prepared in mixtures of glycerol and phosphate buffer. Thus, samples of CYP119 and substrate (if desired) in 1:1 mixtures of glycerol and 100 mM phosphate buffer (pH 7.0) were cooled to ca. −8 °C, and basic peroxynitrite (PN) solutions[32] were added via pipet. The final concentration of enzyme was 5 μM, and the final concentration of PN was 125 μM. The basic PN solution increased the pH of the mixture to 7.4.

Formation of Compound II was monitored by the increasing absorbance at 430 nm, which is λ_{max} for this species.[24,25] This reaction was complete within a few seconds after mixing, and Compound II was stable at −8 °C for minutes. Excess PN, which decomposes rapidly in neutral media, was depleted in less than 1 min. After the PN had decayed, the mixtures were cooled to the desired reaction temperature between −50 to −25 °C, and the samples were irradiated with 2.0 J of 365 ± 5 nm light delivered in 0.5 s. Conversion of Compound II to Compound I was monitored by UV−visible spectroscopy.

Spectra. CYP119 Compound II has λ_{max} for the major Soret band at 433 nm, and this species is readily distinguished from CYP119 resting enzyme and Compound I, both of which have a split Soret band with absorbance maxima at about 360 and 416 nm.[25] Figure 1 shows one set of results containing the spectra for all three species. In this example, CYP119 Compound I was produced in the presence of 3.0 mM BA-d_1 and converted completely to resting enzyme in about 30 s.

CYP119 Compound I was previously prepared in LFP studies where the laser pulse partially converted Compound II to Compound I.[24,25] It also was detected as a transient from reaction of *m*-chloroperoxybenzoic acid with the resting enzyme in stopped-flow mixing studies.[20,25] In the rapid mixing studies, the UV−visible spectrum of Compound I was obtained by deconvolution of the spectral files, which gave highly variable results that were dependent on the initial guess for the reaction rate constants.[25] In the LFP studies, a more reliable spectrum of Compound I was obtained from the difference spectra observed before and after laser irradiation.[24,25] In the present work, CYP119 Compound I was formed quantitatively, and the UV−visible spectrum of a P450 Compound I was measured directly for the first time. We note that the Soret absorbances of CYP119 resting enzyme and Compound I are centered at the same wavelengths.[25] At ca. 355 nm, the absorbance of Compound I is greater than that of the resting enzyme, whereas at ca. 416 nm, the absorbance of Compound I is less than that of the resting enzyme. The change in absorbance upon reaction of Compound I is shown in the inset in Figure 1.

Before a UV−visible spectrum of a P450 Compound I was available, it was assumed that the spectrum would display a broad absorbance with λ_{max} = 367 nm. This assumption was based on the UV spectrum of Compound I from the heme-thiolate enzyme chloroperoxidase (CPO) reported by Hager in 1981.[33] Later, however, Egawa and co-workers showed that the Soret band in the CPO Compound I spectrum actually was composed of two overlapping absorbances.[34] The overlapping absorbances of CPO Compound I Soret band were shown to be readily detected in the second derivative spectrum,[25] as illustrated in Figure 2. The Soret absorbances from CYP119 Compound I and its second derivative also are shown in Figure 2, and the great similarity of the two spectra are apparent. In fact, the UV−visible spectrum of CYP119 Compound I is similar to spectra of Compound I species from other heme enzymes such as lignan peroxidase, which has λ_{max} at ca. 409 nm for Compound I.[35]

Kinetics. Kinetic studies were conducted by monitoring the signal growth at 416 nm. Figure 3 shows a typical set of results. As the concentration of benzyl alcohol increased, the rate of reaction of the Compound I species increased. The kinetic traces were well-solved for double exponential signal growth, where the predominant reaction (60−80%) was dependent on the concentration of benzyl alcohol, and the minor reaction was independent of substrate concentration. The origin of the minor kinetic component is not clear, but its lack of dependence on substrate concentration suggests that it is an enzyme relaxation phenomenon of some type.

(32) Uppu, R. M.; Pryor, W. A. *Anal. Biochem.* **1996**, *236*, 242–249.
(33) Palcic, M. M.; Rutter, R.; Araiso, T.; Hager, L. P.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1123–1127.
(34) Egawa, T.; Proshlyakov, D. A.; Miki, H.; Makino, R.; Ogura, T.; Kitagawa, T.; Ishimura, Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 46–54.
(35) Khindaria, A.; Aust, S. D. *Biochemistry* **1996**, *35*, 13107–13111.

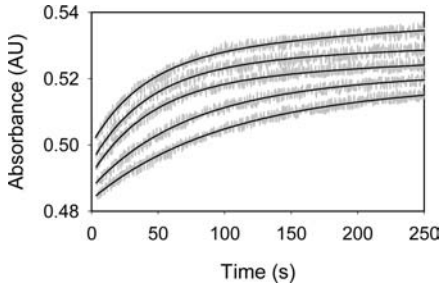


Figure 3. Kinetic traces at 416 nm for reactions of CYP119 Compound I at −50 °C with (from the top) 6.0, 5.0, 4.0, 2.0, and 1.0 mM benzyl alcohol-d_2. The gray lines are the kinetic traces, and the black lines are double exponential fits.

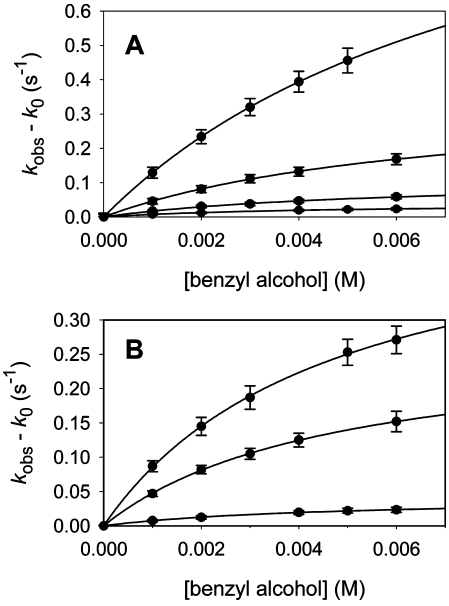


Figure 4. Observed rate constants for reactions of CYP119 Compound I with benzyl alcohol. (A) Rate constants for reactions of BA-d_2 at (from the top) −25, −35, −43, and −50 °C. (B) Rate constants at −50 °C for reactions of (from the top) BA-d_0, BA-d_1, and BA-d_2.

Detailed kinetic results for oxidations of benzyl alcohol and its deuterated isotopomers are listed in Table S1 in Supporting Information. The reactions of the nondeuterated substrate (BA-d_0) and monodeuterated substrate (BA-d_1) were studied only at −50 °C because reactions of high concentrations of these substrates at higher temperatures would be largely completed during the 0.5 s duration of the light pulse. Reactions of the dideuterated substrate BA-d_2 were relatively slow due to a sizable kinetic isotope effect, and this substrate was studied over the temperature range of −50 to −25 °C.

Figure 4 shows graphic representations of the kinetic results. For all cases, saturation kinetics were observed, which are described by eq 1, where k_{obs} is the observed pseudo-first-order rate constant, k_0 is the rate constant in the absence of substrate, K_{bind} is the equilibrium constant for formation of the reactive complex, k_{ox} is the first-order rate constant for reaction of the complex, and [Subs] is the concentration of substrate. The kinetic data were solved by nonlinear least-squares regression analysis to give the results in Table 1, and these values for K_{bind} and k_{ox} were used to generate the line plots in Figure 4. For the three isotopomers, the binding constants at −50 °C differed by less than the standard deviations of their fits, which indicates a high precision in the method. The consistency of the binding

Table 1. Equilibrium Binding Constants and First-Order Rate Constants for Reactions of CYP119 Compound I with Isotopomers of Benzyl Alcohol

substrate	temp (°C)[a]	K_{bind} (M^{-1})[b]	k_{ox} (s^{-1})[b]
BA-d_0	−50.0	210 ± 20	0.48 ± 0.02
BA-d_1	−50.0	210 ± 10	0.27 ± 0.01
BA-d_2	−50.0	220 ± 20	0.042 ± 0.002
BA-d_2	−43.0	180 ± 30	0.11 ± 0.01
BA-d_2	−35.0	150 ± 10	0.36 ± 0.02
BA-d_2	−25.0	115 ± 2	1.25 ± 0.01

[a] ±0.2 °C. [b] Errors are 2σ.

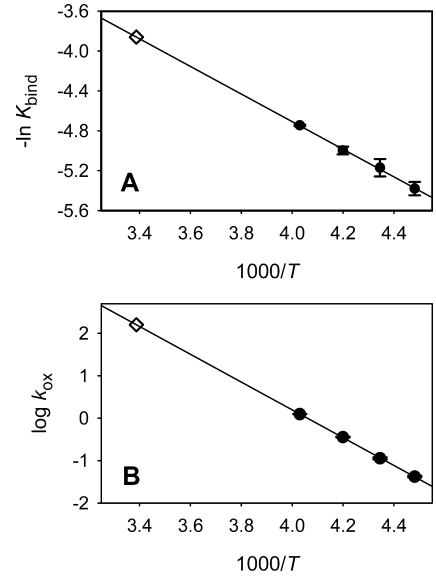


Figure 5. Binding constants (A) and rate constants (B) for reactions of BA-d_2 with CYP119 Compound I. Error bars at 1σ are obscured by the symbols in some cases. The open diamonds show extrapolations to 22 °C.

constants requires that substrate equilibration in the active site of Compound I was rapid relative to the oxidation step; if equilibration was not rapid, then the analysis would give apparent binding constants, with $K_{bind(app)} = k_{on}/(k_{off} + k_{ox})$, which varied due to differences in the values of k_{ox} for each isotopomer.

$$k_{obs} - k_0 = (K_{bind}k_{ox}[Subs])/(K_{bind}[Subs] + 1) \quad (1)$$

The variable temperature studies with BA-d_2 permit analysis of the binding constants and rate constants to determine the energy of complexation (van't Hoff plot, Figure 5A) and the activation parameters for the oxidation reaction (Arrhenius plot, Figure 5B). The temperature-dependent function for the equilibrium binding constant K_{bind} is given in eq 2, and the temperature-dependent function for the rate constant k_{ox} is given by eq 3; in these equations, the error limits are at 1σ. The enthalpy of binding is −0.7 kcal/mol, and the enthalpy of the activated complex ($\Delta H^{\ddagger} = E_a - RT$) is 14.5 kcal/mol at ambient temperature.

$$-\ln K_{bind}/M^{-1} = (0.83 \pm 0.19) - (1390 \pm 50)/T \quad (2)$$

$$\log k_{ox}/s^{-1} = (13.31 \pm 0.15) - (15.06 \pm 0.16)/2.313RT \text{ (kcal/mol)} \quad (3)$$

The CYP119 Compound I binding constants can be assumed to be equal for all BA isotopomers given the excellent agreement

in K_{bind} results at −50 °C. It is noteworthy that, for the aromatic substrate BA, extrapolation of K_{bind} to ambient temperature gives a value that is about an order of magnitude smaller than that for lauric acid.[25] The CYP119 Compound I binding constants are smaller than the binding constants for the same substrates in the resting enzyme,[36] but the order of substrate binding appears to be the same.

The type of kinetics and energetics information available from the variable temperature studies of reactions of CYP119 Compound I was not available previously for any P450 enzyme, and it represents important benchmark results for computational studies. In that regard, it must be noted that the binding constants for substrates in the activated CYP119 Compound I are not the same as those for substrates binding in the resting CYP119 enzyme. In the case of lauric acid, for example, the binding constant for resting CYP119[36] is much larger than the binding constant for CYP119 Compound I.[25] Because experimentally derived apparent second-order rate constants are the products of the binding constant and the first-order rate constant (see below for examples), one cannot predict rate constants for P450 Compound I oxidations without estimating both the binding constant and activation energy values. Computations of the first-order rate constants might give general results that apply for various P450s, but binding constants for different P450s will inevitably be highly variable and difficult to predict.

When the product of the binding constant and substrate concentration is small, the denominator in eq 1 reduces to unity, and a series of studies at varying concentration of substrate under pseudo-first-order conditions give k_{obs} values that are described by a straight line. In such a case, eq 1 reduces to eq 4, where the linear result is the tangent to the curve for saturation kinetic results. The straight line in a plot described by eq 4 gives an apparent second-order rate constant (k_{App}), which is the product of the binding constant and the first-order oxidation rate constant. In LFP studies of CYP119 Compound I at 22 °C, a linear dependence on substrate concentration was found for BA oxidation reactions,[25] and one can compare those results to results from this study extrapolated to 22 °C.

$$k_{obs} - k_0 = K_{bind}k_{ox}[\text{Subs}] = k_{App}[\text{Subs}] \qquad (4)$$

Extrapolation of the BA-d_2 results to ambient temperature is shown in the plots in Figure 5. At 22 °C, the binding constant is $K_{bind} = 48\ M^{-1}$, and the rate constant for oxidation of BA-d_2 is $k_{ox} = 160\ s^{-1}$. These results predict that the apparent second-order rate constant for BA-d_2 oxidation at 22 °C will be $7.7 \times 10^3\ M^{-1}\ s^{-1}$. In fact, the experimental value for oxidation of perdeuterated BA was $k_{App} = 1.17 \times 10^4\ M^{-1}\ s^{-1}$,[25] and the predicted result from the present work represents a 34% reduction in the rate constant from the measured apparent second-order rate constant found in the LFP study at ambient temperature. The origin of the difference in the kinetic values is not known with certainty, and there are at least two possible explanations.

It is possible, in principle, that the different buffer solutions in the two studies resulted in slightly different binding constants. In the present work, the buffer contained 50% glycerol, but no glycerol was added to the buffer in the LFP study. Although this rationalization cannot be excluded, we favor a second possibility involving a considerable amount of tunneling at −50 °C.

If tunneling is significant, then the Arrhenius plot in Figure 5B would be the tangent to a curve with the calculated activation energy smaller than the true value for the chemical reaction,[37] and accordingly, the extrapolated apparent second-order rate constant at ambient temperature would be smaller than the actual value. Our view is that this rationalization is reasonable because tunneling in BA oxidations by a model for Compound I, an iron(IV)-oxo porphyrin radical cation, was considerable.[28] In addition, as discussed later, comparison of the kinetic isotope effects at −50 and 22 °C also suggests a significant tunneling component in the hydroxylation reaction at low temperature.

The first-order rate constants for oxidations of the benzyl alcohol isotopomers are perhaps the most important findings in this work. The bond dissociation energy (BDE) of the benzylic C−H bonds in benzyl alcohol is relatively small, estimated to be only 79 kcal/mol,[38] but oxidation of this substrate by CYP119 Compound I is not phenomenally fast. In fact, CYP119 Compound I reactions with BA, lauric acid,[25] benzphetamine,[27] and a methylcyclopropyl group[26] are about 2 orders of magnitude faster than reactions of Compound I models lacking the thiolate ligand.[39]

It is noteworthy for analysis of P450 Compound I reactions, in general, that the rate constants obtained for oxidations by CYP119 Compound I are not a special feature of the particular P450 enzyme. In two kinetic studies,[26,27] rate constants for oxidations by Compound I of the mammalian hepatic P450 enzyme CYP2B4 and those for CYP119 Compound I were directly compared. For oxidations of benzphetamine and a methylcyclopropane, the rate constants for oxidation by Compound I from CYP2B4 were greater than those for oxidations by Compound I of CYP119 by factors of 1.5 and 1.2, respectively.[26,27] The differences in activation free energies for the two P450 Compounds I were small in these examples, $\Delta\Delta G^{\ddagger} < 0.3$ kcal/mol, and, in fact, smaller than the difference for the rate constants for oxidation of benzphetamine by Compounds I from CYP2B4 and its F429H mutant.[27] In another kinetic study that can be used for comparisons of Compounds I,[40] rate constants for oxidations by Compound I of the heme-thiolate enzyme chloroperoxidase (CPO) from *Caldariomyces fumago* were similar to those found for CYP119 Compound I. From all kinetic data now available, Compound I from CYP119 appears to be normal in its C−H oxidizing reactivity.

The difference between the measured reactivities of Compounds I of CYP119, CYP2B4, and CPO and the inferred reactivities of the oxidizing transients in other P450 enzymes is a contradiction, the resolution of which will require further study. The inability to detect P450 Compound I from reactions of resting enzyme with hydroperoxy compounds might be rationalized as due to a fast oxidase-type reaction where Compound I reacts with a second molecule of hydroperoxy compound to give molecular oxygen and a second molecule of reduced hydroperoxy compound. The failure to observe CYP101 in low temperature radiolytic reduction experiments,[41−44] however, cannot be explained by a conventional interpretation.

(36) Koo, L. S.; Immoos, C. E.; Cohen, M. S.; Farmer, P. J.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **2002**, *124*, 5684–5691.
(37) Kwart, H. *Acc. Chem. Res.* **1982**, *15*, 401–408.
(38) Brandi, P.; Galli, C.; Gentili, P. *J. Org. Chem.* **2005**, *70*, 9521–9528.
(39) Pan, Z.; Zhang, R.; Newcomb, M. *J. Inorg. Biochem.* **2006**, *100*, 524–532.
(40) Zhang, R.; Nagraj, N.; Lansakara, D. S. P.; Hager, L. P.; Newcomb, M. *Org. Lett.* **2006**, *8*, 2731–2734.
(41) Davydov, R.; Macdonald, I. D. G.; Makris, T. M.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1999**, *121*, 10654–10655.
(42) Davydov, R.; Makris, T. M.; Kofman, V.; Werst, D. E.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 1403–1415.

Table 2. First-Order Rate Constants and Estimated Activation Energies for P450 Compound I Hydroxylations of C−H Bonds[a]

substrate	P450	temp (°C)	k_{ox} (s^{-1})	E_a[b,c]	BDE[b,d]
lauric acid[e]	CYP119	22	0.8	17.9	98
cyclopropylmethyl[f]	CYP119	22	10.4	16.4	95
	CYP2B4	22	12.4	16.3	95
benzphetamine[g]	CYP119	22	32.5	15.7	89
	CYP2B4	22	48.4	15.5	89
benzyl alcohol	CYP119	−50	0.48	13.7	79

[a] Rate constants from this work and refs 25−27. [b] Energies in kcal/mol. [c] E_a estimated with the assumption that $\Delta S^{\ddagger} = 0$; see text. [d] References for the BDE values are in the text. [e] The unactivated C−H bonds in lauric acid are oxidized. [f] The methyl group in (*S*,*S*)-2-(*p*-trifluoromethylphenyl)cyclopropylmethane is oxidized. [g] The *N*-methylamino group in benzphetamine is oxidized.

A controversial rationalization for the difference is that the first-formed species in the cryoreduction experiments might be an oxidant other than Compound I,[45] an iron(V)-oxo transient[46−48] or iron-complexed hydrogen peroxide.[49−51]

The temperature-dependent kinetic function for the BA-d_2 oxidation reaction permits comparisons with other results and as well as provides a benchmark for computations. The log *A* term in the Arrhenius function is 13.3, which reflects very low entropy demand as expected for a first-order process ($\Delta S^{\ddagger} =$ 0.4 eu at ambient temperature), and the activation energy is E_a = 15.1 kcal/mol ($\Delta H^{\ddagger} = E_a - RT$ = 14.5 kcal/mol) for oxidation of BA-d_2. Until other temperature-dependent functions for Compound I rate constants are available, we suggest that kinetic results at one temperature might be used to calculate activation energies *with the assumption* that entropy demand in the first-order reaction is essentially nil. We caution that this assumption can only be reasonable when the reaction is demonstrated to be a first-order process, that is, when saturation kinetics have been obtained, and both the binding constant and first-order rate constant are known.

Despite the caveat concerning the limitation for measured first-order rate constants, a picture of P450 Compound I reactivity in C−H hydroxylation reactions already is emerging. Table 2 lists first-order rate constants for CYP119 Compound I and CYP2B4 Compound I hydroxylations measured in LFP studies and in this work. Assuming that log A = 13.1 ($\Delta S^{\ddagger} =$ 0), we calculated the activation energy E_a for each reaction. Bond dissociation energies (BDEs) are from a compendium of values[52] and approximations for the C−H BDEs of the methyl group in methylcyclopropane,[53] of the methyl groups in trimethylamine,[54] and of the benzylic bonds in benzyl alcohol.[38] Note that benzphetamine contains various C−H bonds, but the product of the P450 Compound I reaction is from hydroxylation of a methyl group adjacent to a tertiary amine.[27] The activation energies for the hydroxylation reactions are nicely correlated with the bond energies (Figure 6).

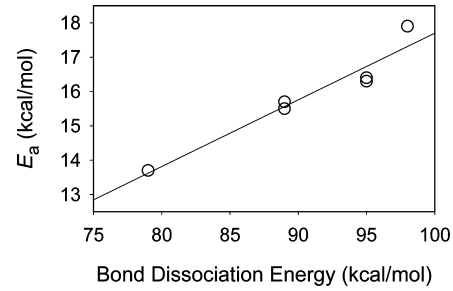


Figure 6. Correlation of activation energies for P450 Compound I hydroxylation reactions with bond dissociation energies of the oxidized C−H bond in the substrates.

Kinetic Isotope Effects and Tunneling. The observed H/D kinetic isotope effect (KIE) for BA-d_0 and BA-d_2 at −50 °C is $k_H/k_D = 11.5 \pm 0.6$. Because kinetic data are available for all three isotopomers at −50 °C, one can separate the observed KIEs into the primary KIE (*P*) and secondary KIE (*S*). The rate constant for reaction of BA-d_0 is defined as twice the rate constant for reaction of an individual hydrogen atom in the substrate (eq 5). The observed rate constant for substrate BA-d_1 is then given by eq 6, where the rate constant for reaction of the hydrogen atom is affected by a secondary KIE, and that for the deuterium atom exhibits a primary KIE. For BA-d_2, the observed rate constant is given by eq 7; both deuterium atoms exhibit a primary KIE and are affected by the neighboring deuterium atom that imparts a secondary KIE. Using this convention, the ratio of rate constants for BA-d_0 and BA-d_2 is equal to PS (eq 8), and the ratio of rate constants for BA-d_1 and BA-d_2 is equal to $(P + S)/2$ (eq 9). Solving the quadratic function generated from eqs 8 and 9 gives P = 11.94 and S = 0.965 at −50 °C.

$$k_{obs(BA\text{-}d_0)} = 2 \times k_H \quad (5)$$

$$k_{obs(BA\text{-}d_1)} = k_H/S + k_H/P \quad (6)$$

$$k_{obs(BA\text{-}d_2)} = 2 \times k_H/PS \quad (7)$$

$$k_{obs(BA\text{-}d_0)}/k_{obs(BA\text{-}d_2)} = PS = 11.52 \ (\text{at } -50\ ^\circ\text{C}) \quad (8)$$

$$k_{obs(BA\text{-}d_1)}/k_{obs(BA\text{-}d_2)} = (P + S)/2 = 6.45 \ (\text{at } -50\ ^\circ\text{C}) \quad (9)$$

The preceding analysis is for equal reactivity of the two prochiral hydrogen atoms in benzyl alcohol. CYP119 is a chiral enzyme with a chiral binding site, of course, and it is possible that the two hydrogen atoms in the substrate react with different rate constants. For BA-d_0 and BA-d_2 substrates, the observed rate constants will be averages of the rate constants for reactions of the two prochiral hydrogen atoms. In the case of BA-d_1, the substrate consists of two enantiomers, and differences in the rate constants for reactions of the enantiomers would be an important factor *if* equilibration of the substrate in the activated

(43) Davydov, R.; Perera, R.; Jin, S. X.; Yang, T. C.; Bryson, T. A.; Sono, M.; Dawson, J. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **2005**, *127*, 1403–1413.
(44) Denisov, I. G.; Makris, T. M.; Sligar, S. G. *J. Biol. Chem.* **2001**, *276*, 11648–11652.
(45) Koppenol, W. H. *J. Am. Chem. Soc.* **2007**, *129*, 9686–9690.
(46) Harischandra, D. N.; Zhang, R.; Newcomb, M. *J. Am. Chem. Soc.* **2005**, *127*, 13776–13777.
(47) Pan, Z.; Zhang, R.; Fung, L. W. M.; Newcomb, M. *Inorg. Chem.* **2007**, *46*, 1517–1519.
(48) Pan, Z.; Wang, Q.; Sheng, X.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **2009**, *131*, 2621–2628.
(49) Newcomb, M.; Hollenberg, P. F.; Coon, M. J. *Arch. Biochem. Biophys.* **2003**, *409*, 72–79.
(50) Chandrasena, R. E. P.; Vatsis, K. P.; Coon, M. J.; Hollenberg, P. F.; Newcomb, M. *J. Am. Chem. Soc.* **2004**, *126*, 115–126.
(51) Newcomb, M.; Chandrasena, R. E. P. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 394–403.
(52) Luo, Y.-R. *Handbook of Bond Dissociation Energies in Organic Compounds*; CRC Press: Boca Raton, FL, 2003.
(53) Halgren, T. A.; Roberts, J. D.; Horner, J. H.; Martinez, F. N.; Tronche, C.; Newcomb, M. *J. Am. Chem. Soc.* **2000**, *122*, 2988–2994.
(54) Wayner, D. D. M.; Clark, K. B.; Rauk, A.; Yu, D.; Armstrong, D. A. *J. Am. Chem. Soc.* **1997**, *119*, 8925–8932.

Compound I species was not fast. With fast equilibration and a large excess of substrate, however, BA-d_1 will behave as if it is a single compound, and both the primary and secondary KIEs found experimentally are the averages for reactions at the two prochiral positions.

One of the reasons for studying benzyl alcohol is that the KIEs for oxidation of this substrate have been measured with several oxidants. Of particular interest for P450 enzymes, very large KIEs were found in oxidations of benzyl alcohol and its deuterated isotopomers by the Compound I analogue from 5,10,15,20-tetramesitylporphyriniron(III) chloride (TMP Compound I), and variable temperature studies indicated a large tunneling component for these C−H oxidation reactions.[28] In a similar manner, large KIEs implicating tunneling were apparent in another stoichiometric TMP Compound I oxidation[55] and in oxidations under catalytic turnover conditions by other porphyrin−iron complexes.[56] Large KIEs have been reported for enzyme-catalyzed C−H oxidations by, for example, lipoxygenase[57] and methane monooxygenases,[58,59] and it seemed possible that tunneling might be important in P450 oxidation reactions.

For the CYP119 Compound I oxidation of benzyl alcohol, a tunneling component appears to be present, although it is not immediately obvious, nor is the evidence conclusive. The KIE measured at −50 °C is consistent with a typical value for a KIE without tunneling; $k_H/k_D = 11.5$, which gives $\Delta\Delta G^{\ddagger} = 1.08$ kcal/mol. At ambient temperature, the same difference in free energies of activation would result in a KIE of $k_H/k_D = 6.3$ if the ratio of pre-exponential terms was unity. In the LFP kinetic study of CYP119 Compound I reactions at 22 °C, however, the experimental KIE value obtained for oxidations of benzyl alcohol and its perdeuterated isotopomer was $k_H/k_D = 2.3$.[25] Such a large temperature dependence of the KIE cannot be accommodated by a classical kinetic model for KIEs and suggests that a tunneling component is involved in the reaction as explained in detail by Kwart.[37]

The conclusion from the TMP model for Compound I study was that tunneling in oxidation of benzyl alcohol was the major reaction at −50 °C,[28] but the chemical reaction for CYP119 Compound I is faster than that for the TMP Compound I. In fact, the tunneling components estimated for the TMP Compound I reaction with BA-d_0 and BA-d_2 at −50 °C are on the same order of magnitude as the rate constants measured in this work for CYP119 Compound I reactions. If a small tunneling component is present in the rate constant for BA-d_2 reaction at −50 °C, then a small error will result in the activation energy,[37] leading to an underestimation of the rate constant for the reaction at ambient temperature as noted earlier.

Another set of KIE studies of P450 Compound I oxidations was measured directly.[26] The substrate was a methylcyclopropane, where the BDEs of the methyl C−H bonds are reduced from those in the methyl group of an alkane by about 3 kcal/mol due to conjugation of the C−H bonds with the adjacent cyclopropyl C−C bonds.[53] For the substrate (*S*,*S*)-2-(*p*-trifluoromethylphenyl)cyclopropylmethane, the rate constants for C−H oxidation of the methyl group by CYP119 and CYP2B4 Compounds I at 22 °C were 10.4 and 12.4 s^{-1},[26] or about 1 order of magnitude greater than the rate constant for CYP119 Compound I oxidation of the unactivated C−H bonds in lauric acid.[25] The observed KIEs for oxidations of RCH_3 and RCD_3 at 22 °C were $k_H/k_D = 11.2$ and 9.8 for the two Compounds I, and the primary KIEs were $P = 9.8$ and 8.9. There was no compelling evidence for tunneling in these oxidation reactions, although the primary KIEs were somewhat larger than the theoretical limit for a classical C−H KIE at 22 °C.

In summary, oxidations of benzyl alcohol and its benzylic monodeuterio and dideuterio isotopomers by the Compound I species from the cytochrome P450 enzyme CYP119 were studied at low temperatures in buffer solution containing 50% glycerol. The oxidations displayed saturation kinetics, which allowed solution of the binding constants and first-order rate constants for the reactions. From studies at −50 °C, both primary and secondary isotope effects were derived, which were seemingly conventional, but the temperature dependence of the KIEs and comparison of results extrapolated to 22 °C with previous LFP studies suggested that a significant tunneling component was present in the low temperature kinetic values. For oxidations of the dideuterio isotopomer conducted between −50 and −25 °C, a van't Hoff function for binding and an Arrhenius function for the first-order oxidation reaction were generated. Extrapolation of the binding constant for benzyl alcohol in CYP119 compound to room temperature gave a value about 1 order of magnitude less favorable than the binding constant for lauric acid. For the first-order oxidation reaction, the entropy of activation for the oxidation reaction was small, and the rate constant was large in comparison to those for Compound I models. The detailed kinetic parameters obtained in this work should serve as benchmarks for computational studies.

Experimental Section

Materials. CYP119 was prepared and purified as previously described.[24,29,30] The samples used had an *R*/*Z* ratio (A_{416}/A_{280}) > 1.5. Basic solutions of peroxynitrite were prepared by the method of Uppu and Pryor.[32] Benzyl alcohol (BA-d_0) was a commercial sample. BA-d_1 and BA-d_2 were prepared by reduction of benzaldehyde and methyl benzoate, respectively, with $LiAlD_4$. The deuterated samples were purified by short path distillation. NMR spectra of the deuterated substrates and mass spectra for all BA samples are in the Supporting Information.

Kinetic Studies. Data were acquired on an Ocean Optics USB4000 diode array spectrometer. The photolysis unit was an EFOS Novacure 2001 spot lamp employing a mercury bulb with a 365 ± 5 nm cutoff filter. The reaction cells were polycarbonate microcuvettes with a 10 mm path length and 2 mm width. The reaction cell was held in a thermally regulated box that contained entries for the fiber optics cables of the spectrometer and the gel optics waveguide of the spot lamp. Control experiments demonstrated good Beer's Law behavior of BA in the buffer−glycerol mixtures to concentrations at least 30% greater than those used in kinetic studies. In a typical experiment, a 200 μL solution of CYP119 (5 μM) in 100 mM phosphate buffer (pH 7.0) containing 50% by volume glycerol and a desired amount of substrate was cooled to −8 °C, and a basic solution (ca. 1 μL) of 60 mM peroxynitrite was added, giving a mixture with pH 7.4. The formation of CYP119 Compound II was followed spectroscopically and judged to be complete when the absorbance at λ_{max} for Compound II ($\lambda_{max} = 430$ nm) was unchanging. When the PN had largely decayed as judged by the intensity of absorbance at 308 nm, the solution was cooled to the desired reaction temperature (−50 to −25 °C). The samples were irradiated with 2.0 J of light delivered in 0.5 s, which converted Compound II to Compound I. Kinetic data were obtained with 0.2 s integration times. The data

(55) Takahashi, A.; Kurahashi, T.; Fujii, H. *Inorg. Chem.* **2007**, *46*, 6227–6229.
(56) Sorokin, A. B.; Khenkin, A. M. *J. Chem. Soc., Chem. Commun.* **1990**, 45–46.
(57) Jonsson, T.; Glickman, M. H.; Sun, S. J.; Klinman, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 10319–10320.
(58) Nesheim, J. C.; Lipscomb, J. D. *Biochemistry* **1996**, *35*, 10240–10247.
(59) Ambundo, E. A.; Friesner, R. A.; Lippard, S. J. *J. Am. Chem. Soc.* **2002**, *124*, 8770–8771.

at 416 nm were solved for double exponential signal growth where the rate of the major process was dependent on the concentration of substrate. Results for individual kinetic runs are in the Supporting Information.

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Supporting Information Available: Detailed kinetic results, NMR spectra of BA-d_1 and BA-d_2, mass spectra for BA isotopomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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