Kinetic Isotope Effects in Hydroxylation Reactions Effected by Cytochrome P450 Compounds I Implicate Multiple Electrophilic Oxidants for P450-Catalyzed Oxidations[†]

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ABSTRACT: Kinetic isotope effects were measured for oxidations of (*S*,*S*)-2-(*p*-trifluoromethylphenyl)cyclopropylmethane containing zero, two, and three deuterium atoms on the methyl group by Compounds I from the cytochrome P450 enzymes CYP119 and CYP2B4 at 22 °C. The oxidations displayed saturation kinetics, which permitted solution of both binding constants (K_{bind}) and first-order oxidation rate constants (k_{ox}) for both enzymes with the three substrates. The binding constant for CYP2B4 Compound I was about 1 order of magnitude greater than that for CYP119 Compound I, but the oxidation rate constants were similar for the two. In oxidations of **1**-d_0, $k_{ox} = 10.4$ s^{-1} for CYP119 Compound I, and $k_{ox} = 12.4$ s^{-1} for CYP2B4 Compound I. Primary kinetic isotope effects (*P*) and secondary kinetic isotope effects (*S*) were obtained from the results with the three isotopomers. The primary KIEs were large, $P = 9.8$ and $P = 8.9$ for CYP119 and CYP2B4 Compounds I, respectively, and the secondary KIEs were small and normal, $S = 1.07$ and $S = 1.05$, respectively. Large intermolecular KIEs for **1**-d_0 and **1**-d_3 of $k_H/k_D = 11.2$ and 9.8 found for the two Compounds I contrast with small intermolecular KIEs obtained previously for the same substrate in P450-catalyzed oxidations; these differences suggest that a second electrophilic oxidant, presumably iron-complexed hydrogen peroxide, is important in cytochrome P450 oxidations under turnover conditions.

The ubiquitous cytochrome P450 enzymes (P450s or CYPs)[1] are heme-containing enzymes that catalyze a wide range of oxidation reactions in nature including hydroxylations of high-energy C−H bonds to give alcohol products (*1*). In humans, P450s catalyze both highly specific oxidation reactions, such as oxidations of androgens to estrogens, and broad spectrum oxidations of drugs, prodrugs, and other xenobiotics in the liver (*2*). In addition, overexpression of P450s is related to disease states in humans including cancer and liver disease. Thus, the details of the reactions of P450s are of considerable interest from pharmacological and medical perspectives.

Detection and study of the active oxidants in P450 enzymes have been the objectives of much research in the four decades since the first reports of P450s. In heme-containing enzymes related to P450s, peroxidases and catalases, reactions of the enzymes with hydrogen peroxide give iron(IV)-oxo porphyrin radical cations that are termed Compounds I (*3, 4*), and Compounds I have long been assumed to be the active oxidants in P450s. The P450s differ structurally from peroxidase and catalase enzymes in that the fifth ligand to iron in the P450s is thiolate from a protein cysteine instead of nitrogen from histidine or oxygen from tyrosine. P450s typically also differ from peroxidases and catalases in the mechanisms of their activation reactions; most P450s are activated by a sequence of reactions (*5*) involving reduction of the ferric enzyme, oxygen binding, a second reduction step, and protonation steps as opposed to reaction of the resting ferric enzyme with hydrogen peroxide. Despite the widespread assumption that the active oxidants in P450s are Compounds I, no P450 Compound I has been detected under natural reaction conditions, and attempts to detect Compound I from reactions of P450s with peroxy compounds, so-called shunt reactions, met with limited success. For example, reactions of $P450_{cam}$ with peroxy acids gave UV−visible spectral changes that were ascribed to a short-lived Compound I (*6, 7*), but freeze-quench studies of $P450_{cam}$ reactions with peroxy acids employing ESR, ENDOR, and Mössbauer spectroscopies provided no evidence for Compound I (*8*−*10*).

In the absence of direct studies of the P450 oxidants, a number of mechanistic probe studies have been conducted to gain information about the active oxidants. Several probe studies led to the conclusion that a single oxidant in P450

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[1] Abbreviations: P450, cytochrome P450; CYP, cytochrome P450; ESR, electron spin resonance; ENDOR, electron nuclear double resonance; KIE, kinetic isotope effect; PN, peroxynitrite; GC, gas chromatography; LFP, laser flash photolysis; PM, photomultiplier; HPLC, high-performance liquid chromatography; *R*/*Z*, ratio of maximum absorbance for the Soret band divided by absorbance at 280 nm; UV, ultraviolet; PCET, proton-coupled electron transfer; BDE, bond dissociation energy; NADPH, nicotinamide adenine dinucleotide phosphate.

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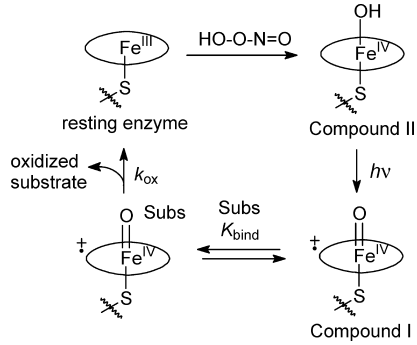
Scheme 1



enzymes was not sufficient to explain observed reactivity patterns (*11*). These results have been interpreted as indicating the presence of multiple electrophilic oxidants (*11*−*13*) or multiple spin states of a single Compound I species (*14*). In the multiple oxidants model, the most likely alternative oxidant is hydrogen peroxide complexed to ferric P450 (i.e., porphyrin-Fe^{III}-HOOH) (*15*−*19*), a requisite intermediate in the so-called uncoupling reaction of P450 enzymes where hydrogen peroxide is released at the expense of productive oxidations as well as in the shunt reactions where P450s are activated by hydrogen peroxide and a species evaluated as a competent oxidant by thermodynamic analyses (*20*). It is important to note that iron-complexed hydrogen peroxide is kinetically indistinguishable from its deprotonated form, a hydroperoxy-iron species (i.e., porphyrin-Fe^{III}OOH), which, for $P450_{cam}$, was the last detectable intermediate in cryogenic reduction studies employing ESR and ENDOR spectroscopic analysis (*21, 22*).

Mechanistic investigations of P450-catalyzed oxidations include kinetic isotope effect (KIE) studies of C−H hydroxylation reactions. Although the results from hydrogen−deuterium KIE studies might appear to be straightforward, their interpretations often are complicated. The oxidation steps in P450 reaction sequences can occur after the rate-determining step (*5*), which can result in intermolecular KIEs from competitive oxidations of undeuterated and deuterated substrates that are masked. Thus, for example, a large *intramolecular* KIE might be found for hydroxylation of a substrate with a reactive $RCHD_2$ group, but the corresponding *intermolecular* KIE for reactions of mixtures of RCH_3 and RCD_3 could be small because the isotopically sensitive step follows the rate-determining step in the oxidation sequence, which is often thought to be the second electron transfer step (*5*). Adding to the difficulty for interpreting results, a masked intermolecular KIE can be partially unmasked when another reaction competes with the isotopically sensitive reaction.

In part, ambiguous results of P450 KIE studies were due to the fact that reactions of the active P450 oxidants could not be studied directly. We recently reported that P450 Compounds I can be produced by photooxidations of the corresponding Compounds II (Scheme 1) (*23*−*25*), which permitted the first kinetic studies of these species. In this work, we report direct kinetic studies of P450 Compound I oxidations of isotopomers of a mechanistic probe substrate that was previously used in studies of oxidations by several P450s under turnover conditions (*18, 26*). The objective was to delineate the isotopically sensitive chemistry due solely to reactions with Compounds I and to compare this to the results found under turnover conditions. The ratios of unrearranged to rearranged alcohol products obtained in the Compound I oxidations indicate that the hydroxylation reactions occur by concerted oxygen insertion in agreement with the results of many hypersensitive probe studies (*27*). The intermolecular KIEs measured in the Compound I oxidations were much different than those previously found under turnover conditions with P450 enzymes (*18, 26*), indicating that Compounds I were not be the only oxidizing species produced under turnover conditions. An especially important observation is that the Compound I hydroxylation reactions occur with equilibration of the substrates in the active sites of P450 Compounds I, whereas previous intermolecular KIEs with the same substrate were largely masked (*18*), which is not possible if fast equilibration of the substrates in the activated enzyme occurs.

MATERIALS AND METHODS

Materials. The expressions in *E. coli* and purifications of CYP119 and CYP2B4 were previously reported (*23, 28, 29*). Solutions of peroxynitrite (PN) were prepared by the method of Uppu and Pryor (*30*). The preparations of (*S*,*S*)-2-(*p*-trifluoromethylphenyl)cyclopropylmethane (**1**) and its dideuteriomethyl and trideuteriomethyl isotopomers were previously described (*18*). The preparations of samples of *trans*-2-(trifluoromethylphenyl)cyclpropylmethanol (**3**) and 1-(trifluoromethyl)phenyl-3-buten-1-ol (**4**) were previously described (*26*).

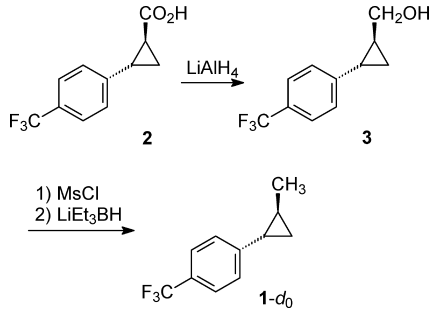
Bulk Photolyses. To a solution of 4 nmol of P450 enzyme and 20 equiv of **1** in 100 mM potassium phosphate buffer (pH 7.0) in a cuvette placed in a temperature-controlled cuvette holder at 0 °C was added 25 equiv of PN. The final volume was 200 μL, and the final pH was 7.4. Approximately 10 s after mixing, the sample was irradiated with 1 pulse of light from a mercury bulb with 390−510 nm cutoff filters (pulse duration = 0.2 s, dose = 2.0 J) delivered from an EFOS Novacure 2001 photolysis unit. The reaction mixture was extracted with methylene chloride, dried over $MgSO_4$, and analyzed by GC (Carbowax).

Kinetic Studies. The methods used are the same as previously described (*24, 25*). In brief, in a typical laser flash photolysis (LFP) study, 10 μM P450 enzyme and a desired amount of substrate in 100 mM potassium phosphate buffer (pH 7.0) were mixed with an equal volume of 0.25 mM PN solution in a stopped-flow mixing unit affixed to an Applied Photophysics LK-60 kinetic spectrometer. The final concentration of P450 was 5 μM in 50 mM potassium phosphate buffer (pH 7.4). Approximately 5 s after mixing, the mixture was irradiated with 355 nm laser light (ca. 10 mJ delivered in 7 ns). Absorbances were monitored with PM tubes using monochromatic light. The data were analyzed with the Applied Photophysics software or with nonlinear regression analyses in the SigmaPlot software program.

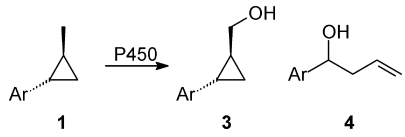
RESULTS

The substrates used in this work were isotopomers of (*S*,*S*)-2-(*p*-trifluoromethylphenyl)cyclopropylmethane (**1**) (Scheme 2) containing zero, two, or three deuterium atoms on the methyl group. The substrates were prepared from the corresponding carboxylic acid, *trans*-2-(*p*-trifluoromethylphenyl)cyclopropanecarboxylic acid (**2**), which was re-

Scheme 2



Scheme 3



solved as previously reported (*26*) to give the (+)-enantiomer that is known to have an absolute configuration of (*S*,*S*) (*18*). The sample of acid (*S*,*S*)-**2** used for preparation of substrates **1** was determined to be 99.3% ee by HPLC analysis of the amide prepared with a sample of (*S*)-1-phenylethylamine (*18*). Reduction of acid **2** with $LiAlH_4$ gave alcohol **3**, which was converted to its mesylate that was reduced with $LiEt_3BH$ to give substrate **1**-d_0 (*26*). A similar reduction sequence using $LiAlD_4$ and $LiEt_3BH$ as reducing agents gave **1**-d_2, and a sequence using $LiAlD_4$ and $LiEt_3BD$ gave **1**-d_3.

The P450 enzymes used in this study were CYP119, a soluble P450 from a thermophile (*31*), and CYP2B4, also known as $P450_{LM2}$, a phenobarbital-induced P450 from rabbit liver that was one of the first P450s isolated and well characterized (*32*). The enzymes were expressed in *Escherichia coli* and purified as previously reported (*28, 33*). They were judged to be high purity from the *R*/*Z* values (ratios of the absorbance at λ_{max} of the Soret band to that at 280 nm), which were >1.5.

The formation of Compounds I by the sequence of reactions shown in Scheme 1 was reported for both CYP119 (*23, 24*) and CYP2B4 (*25*), and the same methods were used in this work. Peroxynitrite (PN) oxidations of the resting enzymes gave Compounds II, iron(IV)-hydroxy species (*34*), and irradiation of Compounds II with 355 nm laser light resulted in photoejections of an electron and concomitant deprotonations to give Compounds I. Earlier studies demonstrated that PN used at the concentrations employed for production of Compounds II did not have measurable effects on the reactivity of the P450 enzymes (*23*−*25*).

The products from oxidations of substrate **1** by P450 Compounds I were determined in bulk oxidation studies. In previous studies of oxidations of **1** by several P450s under turnover conditions, the only products formed in measurable amounts were alcohols **3** and **4** (Scheme 3) (*18, 26*). Both products derive from oxidation of the methyl group, where product **4** can be formed via a radical or cationic intermediate. Authentic samples of **3** and **4** were prepared for GC and GC−mass spectral analysis (*18, 26*).

In the present work, we studied bulk oxidations of substrate **1** by both of the P450 Compounds I. The reactions involved production of Compounds I in the presence of excess **1** in a

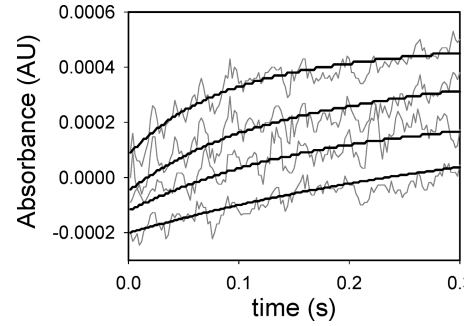


FIGURE 1: Kinetic traces for reactions of CYP2B4 Compound I with substrate **1**-d_0. The concentrations of substrate were (from the bottom) 0.0, 0.26, 1.03, and 2.58 mM.

temperature-controlled reactor containing a cuvette holder that had fiber optics leads for a UV−visible spectrometer and incorporated a light guide from a commercial photolysis unit. With UV−visible spectroscopic monitoring, P450 Compounds II were prepared at 0 °C by reaction of the resting enzymes with PN in the presence of substrate **1**-d_0. The samples were irradiated with 390−510 nm light in a single dose of 2 J delivered in 0.5 s to produce Compounds I. At the conclusions of the photolyses, the UV−visible spectra were similar to those of the resting enzymes. The products were analyzed by GC and GC−mass spectral comparison to authentic samples (*18, 26*), and the only products detected in the bulk photolysis experiments were alcohols **3** and **4**. The yield of **3** plus **4** from the CYP119 Compound I oxidation was 67% based on enzyme, and the ratio of [**3**]/[**4**] was 9.8:1. From CYP2B4 Compound I, the yield of **3** plus **4** was 70%, and the ratio of [**3**]/[**4**] was 8.5:1.

Kinetic studies of oxidations of **1** by P450 Compounds I were accomplished by the laser flash photolysis (LFP) method previously described (*24, 25*). The P450 enzymes were mixed with PN solution in a stopped-flow mixing unit affixed to an LFP kinetic spectrometer. Under these conditions, Compounds II were formed within seconds after mixing. The mixtures were irradiated with 355 nm laser light to generate Compounds I. Reactions of Compounds I were monitored at 430 nm; at this wavelength, the signal intensity increases as Compound I reacts. For studies with substrate present, the substrate concentrations were large such that pseudo-first-order conditions with respect to **1** were maintained. Figure 1 shows representative kinetic traces from reactions of CYP2B4 Compound I with **1**-d_0.

The kinetic traces were solved for double exponential growth where the major reaction (80−90%) was substrate concentration dependent and the minor reaction was independent of substrate concentration. The origin of the constant minor kinetic component is not known with certainty, but it appears to involve a hysteresis. In any event, including the minor kinetic component in the data analysis gave a better fit in terms of errors. Omitting the minor component, i.e., solving for a single exponential, had a small effect on the kinetic values of the major component.

The concentration-dependent rate constants are collected in Table 1. In the absence of substrate, the rate constants were on the order of 5 s^{-1}, and the rate constants increased as a function of the concentration of substrate. Kinetic isotope effects were obvious, and it also was apparent that CYP2B4 Compound I oxidized substrate **1** more efficiently at low concentrations of

Table 1: Rate Constants for Reactions of P450 Compounds I with Isotopomers of Substrate **1**[a]

enzyme	substrate	concn (mM)	k_{obs} (s^{-1})
CYP119	**1**-d_0	0.0	5.78 ± 0.12
		1.03	7.82 ± 0.15
		2.06	9.11 ± 0.18
		4.12	11.1 ± 0.2
		6.18	12.1 ± 0.4
		8.24	12.7 ± 0.6
		9.78	13.0 ± 0.6
	1-d_2	0.0	5.68 ± 0.12
		1.01	6.46 ± 0.15
		2.02	7.01 ± 0.16
		4.04	7.62 ± 0.23
		6.06	8.01 ± 0.24
		8.08	8.21 ± 0.26
		9.60	8.33 ± 0.28
	1-d_3	0.0	5.65 ± 0.12
		1.1	5.88 ± 0.13
		3.3	6.11 ± 0.15
		5.5	6.24 ± 0.14
		7.7	6.27 ± 0.18
		9.9	6.36 ± 0.21
CYP2B4	**1**-d_0	0.0	3.1 ± 0.16
		0.26	6.9 ± 0.8
		0.52	9.4 ± 0.9
		1.03	11.4 ± 1.0
		1.54	12.4 ± 0.8
		2.06	12.9 ± 0.9
		2.58	13.3 ± 0.9
	1-d_2	0.0	5.44 ± 0.16
		0.25	6.9 ± 0.5
		0.50	7.8 ± 0.6
		1.01	8.5 ± 0.6
		1.52	8.9 ± 0.6
		2.02	9.2 ± 0.6
		2.52	9.2 ± 0.6
	1-d_3	0.0	5.20 ± 0.13
		0.28	5.66 ± 0.15
		0.55	5.84 ± 0.16
		1.10	6.05 ± 0.17
		1.65	6.1 ± 0.2
		2.20	6.2 ± 0.2

[a] For reactions at 22 ± 1 °C. Errors are 1σ.

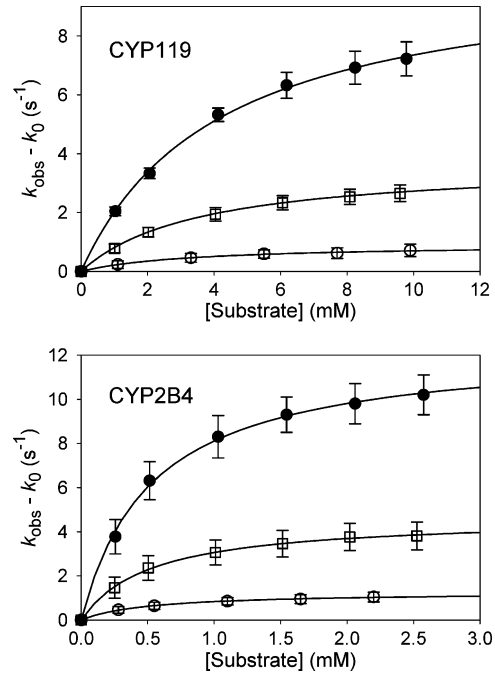


FIGURE 2: Rate constants for reactions of CYP119 Compound I (top) and CYP2B4 Compound I (bottom) with substrate **1**-d_0 (filled circles), **1**-d_2 (squares), and **1**-d_3 (open circles). Error bars are 1σ. The lines are the fits for saturation kinetics calculated with the K_{bind} and k_{ox} values listed in Table 2.

Table 2: Binding Constants and Oxidation Rate Constants for Reactions of Isotopomers of Substrate **1** with Cytochrome P450 Compounds I[a]

enzyme	substrate	K_{bind} (M^{-1})	k_{ox} (s^{-1})	KIE_{app}[b]
CYP119	**1**-d_0	241 ± 15	10.4 ± 0.3	
	1-d_2	280 ± 10	3.7 ± 0.5	
	1-d_3	297 ± 36	0.93 ± 0.04	11.2 ± 0.6
CYP2B4	**1**-d_0	1890 ± 130	12.4 ± 0.3	
	1-d_2	1920 ± 110	4.6 ± 0.1	
	1-d_3	1940 ± 140	1.26 ± 0.03	9.8 ± 0.3

[a] For reactions at 22 °C in 50 mM phosphate buffer (pH 7.4). Errors are 1 standard deviation. [b] Apparent intermolecular kinetic isotope effect (k_H/k_D) for reactions of **1**-d_0 and **1**-d_3.

substrate than CYP119 Compound I, reflecting a larger binding constant for CYP2B4 Compound I.

The rate constants for Compounds I reacting with the three isotopomers of substrate **1** are shown graphically in Figure 2. This presentation clearly demonstrates that the reactions display saturation kinetics with reversible substrate binding as shown in Scheme 1. Such kinetics are described by eq 1, where k_{obs} is the observed rate constant, k_0 is the rate constant in the absence of substrate, K_{bind} is the substrate binding constant, k_{ox} is the first-order rate constant for the oxidation reaction, and [Sub] is the concentration of substrate. Solution of the kinetic values in Table 1 by nonlinear regression analysis according to eq 1 gave the results listed in Table 2, and the values of K_{bind} and k_{ox} in Table 2 were used to generate the lines in Figure 2.

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

The kinetic data in Table 2 is sufficient for calculation of the primary and secondary kinetic isotope effects (KIEs) in the oxidation reactions. Substrate **1**-d_0 reacts with three reactive hydrogen atoms on the methyl group and no KIE. Substrate **1**-d_3 reacts with three reactive deuterium atoms, and these exhibit a primary KIE and two secondary KIEs. Thus, the relative rate constants for reactions of **1**-d_0 and **1**-d_3 give an apparent kinetic isotope effect according to eq 2, where KIE_{app} is the observed ratio of rate constants for the two isotopomers, P is a primary KIE, and S is a secondary KIE, and where we have assumed that the two secondary KIEs are equal and multiplicative. In this context, the rate constant for reaction of **1**-d_2 is given by eq 3, where the first term on the right side of the equation is the rate constant for reaction of the single H-atom that reacts with two secondary KIEs and the second term on the right side of the equation gives the rate constant for reaction of the two D-atoms that react with a primary KIE and one secondary KIE. Simultaneous solution of eqs 2 and 3 for the data kinetic data in Table 2 gave best fits as follows: For CYP119 Compound I, the primary isotope effect is $P = 9.8$, and the secondary isotope effect is $S = 1.07$. For CYP2B4 Compound I, $P = 8.9$, and $S = 1.05$. The errors in these fits are in the range of 1−2%, which compound the experimental errors in measuring the KIEs of 5% (CYP119) and 3% (CYP2B4). One should note that the kinetic values for the **1**-d_2 substrate were used only for the determinations of P and S, and the small relative errors, good internal consistency for the two enzymes, and reasonable values for P and S speak to the high precision of the method we employed. Further-

more, the excellent agreement of the intramolecular KIEs in this work (from comparison of the rate constants for **1**-d_0 and **1**-d_3) with the intermolecular KIEs found with substrate **1**-d_2 demonstrates that there is no KIE masking in the intermolecular studies.

$$\mathrm{KIE_{app}} = k_{\mathbf{1}\text{-}d_0}/k_{\mathbf{1}\text{-}d_3} = k_{\mathbf{1}\text{-}d_0}/(k_{\mathbf{1}\text{-}d_0}/PS^2) = PS^2 \quad (2)$$

$$k_{\mathbf{1}\text{-}d_2} = (1/3)(k_{\mathbf{1}\text{-}d_0}/S^2) + (2/3)(k_{\mathbf{1}\text{-}d_0}/PS) \quad (3)$$

DISCUSSION

The method for kinetic studies of P450 Compounds I employed in this work was developed only recently. Despite considerable efforts, no active oxidant has been observed in a cytochrome P450 enzyme under turnover conditions. Shunt reactions, oxidations of resting P450s with peroxy oxidants such as hydrogen peroxide or a peroxy acid, have been attempted with rapid mixing methods dating back more than 3 decades (*35*). Typically, short-lived species have been observed by UV−visible spectroscopy in stopped-flow studies (*6, 7, 35, 36*), but freeze-quench methods with EPR, ENDOR, and Mössbauer spectroscopic studies demonstrated that transients produced in reactions of $P450_{cam}$ (CYP101) or $P450_{BM3}$ (CYP102) with peroxy acids were not Compounds I (*8*−*10*).

The photooxidation reaction giving Compound I involves either an ejection of an electron from an iron(IV)-oxo neutral porphyrin species or a concomitant ejection of an electron and deprotoantion of an iron(IV)-hydroxy species. In studies of a simple model porphyrin−iron complex and horseradish peroxidase enzyme, irradiation of the iron(IV)-oxo porphyrin species with 355 nm light resulted in photoejection to give the corresponding known iron(IV)-oxo porphyrin radical cations (*37*). This method was extended to P450 enzymes CYP119 (*23, 24*) and CYP2B4 (*25*), by initially oxidizing the enzyme to its Compound II intermediate with peroxynitrite (*34, 38*) and then irradiating with 355 nm light. In the case of CYP119, X-ray absorbance spectroscopy indicated that the Compound II species was an iron(IV) hydroxide as opposed to an iron(IV)-oxo derivative, and therefore, the photochemical process involves a photoejection of an electron and a proton transfer to give the Compound I species. Whether this phenomenon is a proton-coupled electron transfer (PCET) is not yet determined, but the likely sources of the electron (the porphyrin) and the proton (the hydroxy group of the iron(IV) hydroxide) are remote as required for a PCET process as it is often envisioned. In any event, the photochemical generation of Compounds I provides these species in relatively high concentrations such that spectroscopic and kinetic studies are possible, and submillisecond kinetic studies of some Compound I analogues have been demonstrated (*37*). In the applications with the P450 enzymes used in this work, Compounds I decay with lifetimes that are subsecond and well within the capabilities of photomultiplier tubes and modern diode array detectors.

One possibly surprising kinetic result from our work is that the first-order rate constants for Compound I oxidations of the methyl group in substrate **1** are not large. Nonetheless, these values are consistent with the limited number of P450 Compound I rate constants that have been measured. The rate constants for oxidations of an *N*-methyl group in benzphetamine were $k_{ox} = 32\ s^{-1}$ for CYP119 Compound I and $k_{ox} = 48\ s^{-1}$ for CYP2B4 Compound I (*25*), and the rate constant for CYP119 Compound I oxidation of unactivated C−H bonds in lauric acid was $k_{ox} = 0.8\ s^{-1}$ (*24*). Substrate relative reactivities can be anticipated from homolytic bond dissociation energies (BDEs). The BDE of a C−H bond in an *N*-methyl group is smaller than that of a C−H bond in a cyclopropylmethyl group, which is smaller than the BDE of an alkyl C−H bond (*39, 40*). Thus, the rate constants for CYP119 Compound I oxidations are consistent with BDEs.

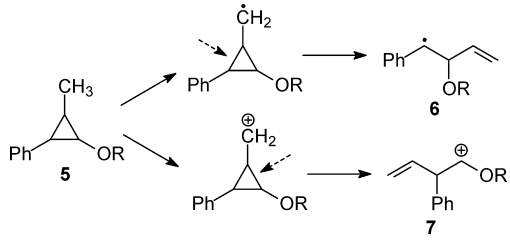
The kinetic values for oxidations of substrate **1** by the P450 Compounds I also appear to be consistent with rate constants for reactions of iron(IV)-oxo porphyrin radical cation models, where unactivated C−H bonds are not oxidized appreciably at ambient temperatures (*41*). The thiolate ligand in P450 Compounds I apparently imparts a modest increase in reactivity of the iron-oxo species. Nonetheless, some studies with P450 enzymes suggested that Compounds I might be much more reactive. For example, the oxidant formed at low temperature in reaction of $P450_{cam}$ appeared to react very rapidly with camphor (*22*). A resolution of this paradox likely will require a direct comparison between a P450 Compound I produced by the photooxidation route with the oxidant produced from the same enzyme via the cryoreduction pathway (*22*) in order to determine if two pathways give the same oxidizing species.

In the bulk reactions of **1**-d_0 with both Compounds I, the only products detected were alcohols **3** and **4**. These results are similar to those found when substrate **1** and its enantiomer were oxidized by several P450 enzymes under turnover conditions (*18, 26*). Alcohol **3** obviously is produced by oxidation of the methyl group in **1**. Alcohol **4** also derives from oxidation of the methyl group followed by either a radical or cationic rearrangement that gives the corresponding benzyl radical or cation. Because substrate **1** reacts only at the methyl position, kinetic isotope effect studies are simplified in that it is not necessary to correct for other reactions of the substrate that compete with the isotopically sensitive reaction.

The results of our work address the following two contemporary questions in regard to P450-catalyzed oxidations: "What is the mechanism of oxidation by P450 Compound I?", and "Is there a single P450 oxidant or are there multiple oxidants?" Because previous mechanistic studies of P450-catalyzed reactions have been conducted under turnover conditions without detection of the active oxidant(s), these two questions have not been separated. Now that P450 Compounds I can be prepared and studied, it is possible to characterize the reactions of these transients to address the first question and to compare the results to those found under turnover conditions to address the second.

The product ratios of alcohols **3** and **4** found in oxidations of **1** provide insight concerning the mechanism of the oxidation reaction. The product ratios were [**3**]/[**4**] = 9.8 for CYP119 and [**3**]/[**4**] = 8.5 for CYP119. The rate constant for ring opening of the corresponding cyclopropylcarbinyl radical is $k = 2.2 \times 10^{11}\ s^{-1}$ at 0 °C (*42*), and the corresponding cyclopropylcarbinyl cation is not expected to be a discrete species (*43*). Accordingly, one calculates that the lifetime at 0 °C of the reacting complex must be 400−500 fs if the transient is radicaloid and even shorter if the transient

Scheme 4



is cationic. A subpicosecond lifetime is too short for any true intermediate, requiring that the small amount of rearrangement occurs within the lifetime of the transition state of an insertion process.

The above conclusion is in agreement with results from second-generation hypersensitive mechanistic probes (*27*). Early mechanistic probe studies of P450-catalyzed oxidations often gave conflicting results regarding radical lifetimes, but many of those studies employed probes that gave the same rearranged product from a radical intermediate or a cationic intermediate. Hypersensitive probes that differentiate between cationic and radical intermediates by providing distinct reaction channels were developed to address that shortcoming. For example, if probe **5** (Scheme 4) was converted to a radical intermediate that rearranged, then products from benzylic radical **6** would be formed, whereas if **5** was converted to a cationic intermediate, then products from oxonium ion **7** would be formed (*44, 45*). When probes such as **5** were used in P450-catalyzed oxidations, both radical- and cation-derived products were found, and when the cation-derived products were removed from consideration, the "radical lifetimes" calculated from the amounts of radical-derived rearrangement products were found to be subpicosecond, or too short for true radical intermediates (*27, 45, 46*).

In oxidations of substrate **1**, it is possible in principle for alcohol **4** to derive from either a radical and a cationic intermediate. However, the formation of cationic intermediates in reactions of P450 Compounds I is unlikely (*24*). When CYP119 Compound I was prepared from peroxynitrite containing ^{18}O-label, the isotopic label was found in the oxidation products styrene oxide (from styrene) and 1-phenylethanol (from ethylbenzene), and when the reactions were conducted with unlabeled PN in ^{18}O-labeled water, no label was obtained in the oxidation products (*24*). The latter results exclude pathways for formation of products via cations that react with water to give oxygenated products.

In summary, then, the product distributions of alcohols **3** and **4** reflect only the reactions of Compounds I, and cations are not formed in these reactions. These product ratios can be compared to the ratios of products found when substrate **1** was oxidized by P450 enzymes under turnover conditions. Specifically, oxidations of substrate **1** and its enantiomer by five P450s gave [**3**]/[**4**] product ratios that varied from 14 to 1.5 (*18, 26*). The differences in product ratios found in the P450-catalyzed reactions and in the reactions of Compounds I suggest that Compounds I were not the only oxidants formed in the P450-catalyzed reactions; that is, multiple oxidants are implicated in P450-catalyzed oxidations.

The KIE results in the present study provide more direct evidence that multiple oxidizing species are formed in P450 enzymes under turnover conditions. KIE studies of oxidations of substrate **1** and its enantiomer were reported for five P450 enzymes reacting under turnover conditions at 10 °C (*18*). In those reactions, KIEs were measured for both products **3** and **4**, and consistent differences in the KIE values for the two products indicated that two oxidation pathways were involved. Moreover, the apparent KIE values for *intermolecular* competition oxidations of **1**-d_0 and **1**-d_3 were smaller (in many cases much smaller) than the KIE values from intramolecular competition in the oxidation of **1**-d_2, requiring that considerable KIE masking occurred in the intermolecular KIE studies (*18*).

In oxidations of **1**-d_2 by CYPΔ2B4 under turnover conditions at 10 °C (*18*), the experimental apparent KIE for oxidation of **1**-d_2 was $k_H/k_D = 9.4$. For the other four P450s studied with **1**-d_2 and for all five P450s oxidizing the enantiomer of **1**-d_2, the apparent intramolecular KIE values were similar. Using the nomenclature in eqs 2 and 3, these values are equal to P/S as discussed previously (*18*). Assuming a value of $S = 1.07$ for the secondary KIE, the primary KIE for CYPΔ2B4 oxidizing **1**-d_2 at 10 °C is $P \approx 10$, which is similar to the value found in this work for CYP2B4 Compound I. Unfortunately, such large values for primary hydrogen KIEs are common, and they provide little insight regarding similarities or disparities of the mechanisms in the two types of reactions. This particular result is permissive for many reaction scenarios but not conclusive for any.

The *intermolecular* KIEs found in oxidations of **1**-d_0 and **1**-d_3 under turnover conditions are much more informative than the *intramolecular* KIEs. In all examples of oxidations of **1** and its enantiomer (10 sets of reactions), the intermolecular KIEs were smaller than the intramolecular KIEs (*18*). For example, for oxidation of **1**-d_0 and **1**-d_3 by CYPΔ2B4, the intermolecular KIE was $k_H/k_D = 2.3$ when the intramolecular KIE was $k_H/k_D = 9.4$ (*18*). The small intermolecular KIE was due to masking, where the isotopically sensitive reaction occurred after the rate-determining step. In the limiting case where all substrates react following a rate-determining step that is not isotopically sensitive, the observed KIE would be $k_H/k_D = 1$. When an alternative reaction pathway for the isotopically sensitive step exists, both occurring after the rate-determining step, then the masked KIE can be partially "unmasked", resulting in KIE values that are larger than 1 but smaller than the true k_H/k_D, and such partially unmasked KIEs were found for all cases of oxidation of **1**-d_0 and **1**-d_3 mixtures studied under turnover conditions (*18*).

The observation of KIE masking in P450-catalyzed oxidations of mixtures of **1**-d_0 and **1**-d_3 requires that the actual oxidation steps were not fully rate controlling (*18*). In the present work, however, substrate **1** equilibrated in the active sites of Compounds I from both CYP119 and CYP2B4 giving saturation kinetics as shown in Figure 2 and discussed above. Thus, in reactions of Compounds I, the isotopically sensitive oxidation reactions were the rate-controlling steps. It follows that another oxidant likely was involved in much of the oxidation chemistry of substrate **1** under turnover conditions.

The conclusion from this work that a second oxidant was involved in P450-catalyzed oxidations of probe **1** under turnover is the same conclusion as reached in previous studies from several lines of reasoning (*18*). A supporting observation in that regard was that the degree of unmasking in

intermolecular KIE studies correlated with the amount of hydrogen peroxide released from the P450 enzymes (*18, 47*). Hydrogen peroxide is released in an uncoupling reaction where NADPH is consumed but oxidized product is not formed, and this reaction would logically compete with reaction of the ferric−H_2O_2 complex as an oxidant (*20*) and provide a pathway for unmasking. Thus, it was proposed that the ferric−H_2O_2 complex could be the major oxidant for probe **1** and that highly masked intermolecular KIEs were obtained with P450s that were efficient oxidants with respect to uncoupling and largely unmasked KIEs were obtained with P450s that were not (*18*).

In the earlier KIE study, the assumption was that oxidations by Compounds I would proceed through sequences where the actual oxidation step followed the rate-determining step. It is often assumed that the second electron transfer step in the P450 activation sequence, the reduction of a ferric−superoxide complex to a ferric−peroxo complex, is the rate-determining step (*5*), although examples of fast substrate exchange in the activated enzyme are known (*48*). In this work, we have shown that the rate-determining steps in P450 Compound I oxidations of substrate **1** are the oxidation reactions and that substrates freely exchange in the activated Compound I species. KIE masking is not possible when the rate-determining step is the isotopically sensitive step, and thus, it follows that the observation of any KIE masking in the turnover reactions of P450s requires that Compound I was not the only oxidant formed in those reactions. For the specific case of CYP2B4, the observed KIE for Compound I oxidizing **1**-d_0 and **1**-d_3 was 9.8, whereas the observed KIE for the same substrate with CYPΔ2B4 reacting under turnover conditions was 2.3 (*18*). A second oxidant is required to explain the results under turnover conditions.

An alternative to the two oxidants model for P450-catalyzed oxidations has been presented. Many computational works have rationalized experimental results as arising from two or more reactive states of Compound I (*14*). Unfortunately, direct observations of Compound I and kinetic studies of its reactions do not support the two-state hypothesis. There is no evidence for distinct spectroscopic signatures from multiple states of Compound I, but more importantly there is no kinetic signature for multiple forms of the oxidant. That is, there is no mixture of two versions of Compounds I as far as direct kinetic measurements are concerned. Multiple states that are in rapid equilibrium are possible, but different states of Compound I that are kinetically distinct, as is often assumed in theoretical models (*14*), are not supported by any direct studies of Compound I reactions performed to date.

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