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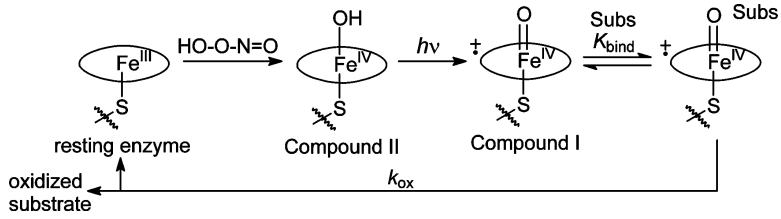


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# Spectra and Kinetic Studies of the Compound I Derivative of Cytochrome P450 119

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***Abstract:*** The Compound I derivative of cytochrome P450 119 (CYP119) was produced by laser flash photolysis of the corresponding Compound II derivative, which was first prepared by reaction of the resting enzyme with peroxynitrite. The UV−vis spectrum of the Compound I species contained an asymmetric Soret band that could be resolved into overlapping transitions centered at ∼367 and ∼416 nm and a Q band with $\lambda_{max} \approx 650$ nm. Reactions of the Compound I derivative with organic substrates gave epoxidized (alkene oxidation) and hydroxylated (C−H oxidation) products, as demonstrated by product studies and oxygen-18 labeling studies. The kinetics of oxidations by CYP119 Compound I were measured directly; the reactions included hydroxylations of benzyl alcohol, ethylbenzene, Tris buffer, lauric acid, and methyl laurate and epoxidations of styrene and 10-undecenoic acid. Apparent second-order rate constants, equal to the product of the equilibrium binding constant ($K_{bind}$) and the first-order oxidation rate constant ($k_{ox}$), were obtained for all of the substrates. The oxidations of lauric acid and methyl laurate displayed saturation kinetic behavior, which permitted the determination of both $K_{bind}$ and $k_{ox}$ for these substrates. The unactivated C−H positions of lauric acid reacted with a rate constant of $k_{ox} = 0.8\ s^{-1}$ at room temperature. The CYP119 Compound I derivative is more reactive than model Compound I species [iron(IV)−oxo porphyrin radical cations] and similar in reactivity to the Compound I derivative of the heme−thiolate enzyme chloroperoxidase. Kinetic isotope effects ($k_H/k_D$) for oxidations of benzyl alcohol and ethylbenzene were small, reflecting the increased reactivity of the Compound I derivative in comparison to models. Nonetheless, CYP119 Compound I apparently is much less reactive than the oxidizing species formed in the $P450_{cam}$ reaction cycle. Studies of competition kinetics employing CYP119 activated by hydrogen peroxide indicated that the same oxidizing transient is formed in the photochemical reaction and in the hydrogen peroxide shunt reaction.

The ubiquitous cytochrome P450 (CYP or P450) enzymes are heme-containing enzymes with thiolate from protein cysteine as the fifth ligand to iron.[1] P450s serve in several catalytic roles in nature, but their major function is to catalyze oxidation reactions, typically via two-electron oxo-transfer processes. In humans, the P450s perform both highly specific reactions, such as oxidation of androgens to estrogens, and broad-spectrum oxidations of drugs, pro-drugs, and xenobiotics in the liver.[2] Much of the interest in P450s derives from the pharmaceutical impacts of these enzymes and their relationships to disease states, including cancers and liver disease, that result from overexpression of P450s.[3−5]

Exceptionally high reactivity is displayed by some P450s in their ability to oxidize unactivated C−H bonds in substrates, and the nature of the P450 transient involved in the oxidation steps has been a subject of study since the initial discoveries of P450s in the 1960s. Other heme-containing enzymes, such as chloroperoxidase (CPO) and horseradish peroxidase, react with hydrogen peroxide to convert the ferric forms of the enzymes to iron(IV)−oxo porphyrin radical cations that are termed "Compound I" derivatives,[6,7] and it has long been assumed that the activated transient in a P450 enzyme that effects oxidation reactions is a Compound I species.[1]

The oxygen-containing transients in P450s typically are formed by a reaction sequence unlike that in the peroxidases (Figure 1).[8−10] Following substrate binding, the ferric enzyme is reduced, and then molecular oxygen binds reversibly; next, a second reduction occurs to give the peroxoiron species. Protonation of this species on the distal oxygen gives the hydroperoxyiron intermediate, which is the last well-characterized transient in the P450 reaction sequence, having been

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observed by electron paramagnetic resonance (EPR) and electron−nuclear double-resonance (ENDOR) spectroscopies when cryogenic conditions were employed.[11,12] A second protonation on the distal oxygen and loss of water by heterolytic fragmentation of the O−O bond would give an iron−oxo species with the iron atom in the formal +5 oxidation state, which is either a transient perferryloxo species that can relax to a Compound I derivative or the transition state for the direct formation of the Compound I derivative. The perferryloxo species or the Compound I derivative can react in two-electron oxo-transfer reactions to return the ferric enzyme.

Peroxidase enzymes form Compound I species by reactions with hydrogen peroxide,[7] and P450s also can react with hydrogen peroxide or other hydroperoxy compounds to give an active oxidant in what is termed a "shunt" reaction (Figure 1). Fast mixing, stopped-flow mixing, and freeze−quench mixing experiments with P450s and peroxy species have been attempted for many years[13] with limited success. A short-lived transient with a calculated spectrum resembling that of CPO Compound I was detected in reactions of $P450_{cam}$ with *m*-chloroperoxybenzoic acid (mCPBA),[14,15] but freeze−quench studies of the same enzyme with either mCPBA or peroxyacetic acid oxidation employing EPR, ENDOR, and Mössbauer spectroscopic analyses indicated that a Compound I derivative did not accumulate to a detectable level.[16−19] Moreover, production of various iron−oxo transients under "cryoreduction" conditions suggested that the reaction of the active oxidant in $P450_{cam}$ with substrate is faster than the rate of formation of this species, which likely precludes its detection in mixing studies.[11,12] Despite the outcome of attempted $P450_{cam}$ oxidations with mCPBA, reactions of another P450 enzyme, cytochrome P450 119 (CYP119), with mCPBA gave promising results in that a short-lived transient with a calculated spectrum similar to that of CPO Compound I was formed;[20] these results are discussed in detail below.

The difficulties in observing a Compound I derivative of a P450 enzyme in fast-mixing studies prompted us to explore an alternative entry to these intermediates that has much shorter temporal resolution than mixing. In principle, photolyses of iron(IV)−oxo neutral porphyrin complexes, so-called Compound II derivatives, could give Compound I derivatives by photoejection of an electron from the porphyrin macrocycle, and laser flash photolysis (LFP) methods with photomultiplier detection would permit submicrosecond temporal resolution. In practice, we found that photolyses of Compound II derivatives of a model

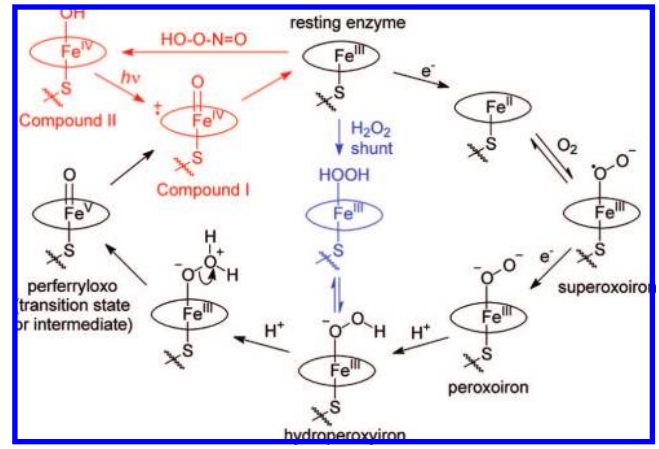


***Figure 1.*** Reaction cycles for P450 enzymes. The normal reaction cycle is shown in black, and the shunt reaction pathway is shown in blue. The sequence of reactions used to produce Compound I in this work is highlighted in red. It should be noted that the Compound II derivative is formulated as a ferrylhydroxy species, on the basis of recent XAS studies of the CYP119 Compound II species (ref 34).

iron−porphyrin complex, horseradish peroxidase, and myoglobin gave Compound I derivatives.[21] Extension of the photooxidation method to the production of a Compound I derivative of the CYP119 enzyme was subsequently reported in a communication.[22] In the present report, we detail the spectra and kinetic studies of the CYP119 Compound I derivative.

## Results

The enzyme used in this study was CYP119, which was expressed in *Escherichia coli* and purified as previously described.[22−24] The CYP119 samples employed were of high purity, as determined by *R*/*Z* values of 1.5 or greater, where *R*/*Z* is the ratio of absorbances at 416 and 280 nm. CYP119 was originally thought to derive from the thermophile *Sulfolobus solfataricus*, but a recent study indicated that it came from the closely related organism *Sulfolobus acidocaldarius* that contaminated an *S. solfataricus* culture.[25]

**Formation of a Compound II Derivative by Peroxynitrite Oxidation of CYP119.** The photooxidation method for production of a Compound I derivative requires that a Compound II derivative first be produced. The only report of the formation of a true Compound II derivative of a P450 enzyme prior to our work was the claim that peroxynitrite (PN) reacted with cytochrome $P450_{BM3}$ (CYP102) to give the corresponding Compound II derivative as a transient and eventually deactivated the enzyme by nitration of a tyrosine residue.[26] Compound II derivatives of other heme-containing enzymes were known to be formed by treatment of the resting ferric enzymes with PN,[27−31] and CPO, which is a heme−thiolate enzyme closely

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related to the P450s, also gives a transient Compound II derivative upon reaction with PN.[22,26,32]

In a communication, we reported that CYP119 was oxidized to the corresponding Compound II derivative by PN.[22] Subsequent to that publication, Green and co-workers[33] reported that CYP102 did not give the Compound II derivative upon reaction with PN but instead gave a nitrosyl complex. The implications in the recent paper[33] were that the UV−vis spectral changes were incorrectly interpreted in the original CYP102 study[26] and that PN does not oxidize P450 enzymes to Compound II derivatives. The dramatically different interpretations[26,33] for the outcome of the PN reaction with CYP102 suggested that further study of this system was in order, especially since a short-lived transient (with a lifetime of a few seconds or less) was detected in both investigations, whereas nitrosyl complexes of heme proteins typically are quite stable.

For the CYP119 enzyme, the formation of the Compound II derivative by reaction with PN was further demonstrated with X-ray absorption spectroscopy (XAS) studies of the Compound II species that gave a detailed picture of the bonding to iron in this derivative.[34] The edge and near-edge features in the XANES spectrum supported an iron(IV) oxidation state, and the EXAFS spectrum gave bond lengths to iron similar to those found for the CPO Compound II species,[35] including a long Fe−O bond (1.82 Å) implicating a protonated ferryl oxygen species, Fe(IV)−OH.[34] In the XAS project,[34] an authentic sample of the nitrosyl complex of CYP119 was prepared and studied for comparison to the Compound II derivative. A long-lived transient (lifetime of hours) was formed by reaction of the resting enzyme with either NO gas or the NO donor diethylamine diazeniumdiolate, and XAS spectra of the NO complex differed from those of Compound II. In the X-ray beam at <140 K, photoreduction of the CYP119−NO complex was observed with multiminute lifetimes, whereas the CYP119 Compound II derivative showed no apparent photoreduction after hours of irradiation under the same conditions, leading us to conclude that the Compound II derivative contained essentially no nitrosyl adduct as a contaminant.[34]

In the kinetic studies reported in this work, CYP119 was treated with 20−25 equiv of PN. Excess PN was required for efficient conversion of the enzyme to the Compound II derivative because PN, which is prepared and stored in basic solutions, is protonated in buffer solutions to give peroxynitrous acid, which rapidly decomposes in an acid-catalyzed process to give nitrite and nitrate.[36−39] The CYP119 enzyme also catalyzes the decay of PN, similar to other heme-containing enzymes.[22,28,31,32] Figure S1A in the Supporting Information shows the spectra of CYP119 resting enzyme and the Compound II derivative, and Figure S1B in Supporting Information shows typical results from monitoring the signal at 429 nm ($\lambda_{max}$ for the Compound II derivative) as a function of time under two types of detection conditions. The Compound II derivative formed in less than 2 s, and PN decayed fully in less than 10 s.

The CYP119 that was present after decay of the Compound II species was spectroscopically indistinguishable from the original enzyme, but it was possible that the enzyme was altered by the PN treatment. For example, PN treatment of CYP102 results in nitration of a tyrosine residue.[26] As a control reaction for our work, we exposed CYP119 to PN at a concentration typical for the LFP studies, allowed the PN to decay, and then conducted a shunt reaction (oxidation of styrene using hydrogen peroxide as a sacrificial oxidant). The yield of styrene oxide in this reaction was the same as when untreated CYP119 was used as discussed later, and thus, the CYP119 behaved the same before and after PN treatment. This study does not prove that CYP119 was unaltered by PN but does show that the reactivity of the enzyme was not affected by the PN treatment at the concentrations used in our studies.

In summary for this section, the reaction of CYP119 with PN at ambient temperature rapidly formed the Compound II derivative. This species persisted when excess PN was present and decayed with a rate constant of ~0.5 $s^{-1}$ at ambient temperature after the PN was depleted. After the PN treatment and decay of the Compound II species back to ferric enzyme, the UV−vis spectrum of enzyme was indistinguishable from that of the original enzyme and the catalytic chemistry of the CYP119 enzyme when activated in a hydrogen peroxide shunt reaction was unaffected.

**Laser Flash Photolysis Generation of CYP119 Compound I.** The apparatus used for the LFP studies was a commercial kinetic unit affixed with a stopped-flow mixing unit that permitted mixing on the millisecond time scale. Samples were delivered to a sample cell that could be irradiated with laser light from the bottom and analyzed by an analyzing beam from one side. The third harmonic of a Nd:YAG laser (355 nm) was used, with a nominal power of 4 mJ at the reaction cell delivered in 7 ns. Spectra were acquired with either a diode-array detector with a minimum integration time of 1.2 ms or photomultiplier tubes (PMTs) with rise times of ~2 ns. The analysis beam was produced by a 150 W xenon lamp at constant power for long detection times or pulsed to high power for short (<0.1 ms) detection times.

The CYP119 Compound II derivative is photolabile, and its decay under constant irradiation from the analysis beam was noticeably faster when it was irradiated with a full spectrum of UV−vis light as opposed to low-intensity monochromatic light. Nonetheless, some diode-array experiments were conducted with a full range of light in order to characterize the spectra of the transients. In all of the studies using PMTs, the monochromator was placed before the sample cell to reduce the exposure of the sample to light.

The dashed line in Figure 2 shows the difference spectrum obtained by irradiating samples containing CYP119 Compound II with 355 nm laser light. This spectrum, which was formed in the 7 ns time span of the laser pulse, contains a bleached

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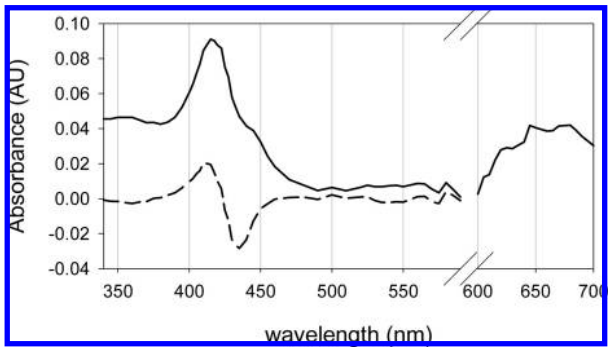


***Figure 2.*** UV−vis spectrum of CYP119 Compound I. The dashed line is the observed difference spectrum obtained by LFP irradiation of the CYP119 Compound II species. A scaled spectrum of CYP119 Compound II was added to the difference spectrum to produce the spectrum for the Compound I derivative (solid line). The Q-band region from 600 to 700 nm is an amplification of an observed difference spectrum with no correction for bleaching.

region in which the absorbance of the Compound II derivative was stronger than the absorbance of the product of the photochemical reaction and a growth region in which the product absorbed more strongly than the Compound II derivative. The difference spectrum is quite similar to those observed when the Compound II derivatives of a model porphyrin−iron complex, horseradish peroxidase, and myoglobin were irradiated with 355 nm light to give the corresponding Compound I derivatives.[21] Specifically, in each of those cases, negative peaks were red-shifted from positive peaks because the absorbance maximum for the Soret band of the Compound II species was at a longer wavelength than that of the Compound I species.

Addition of a normalized spectrum of the CYP119 Compound II derivative to the difference spectrum in Figure 2 compensated for the destroyed Compound II species and gave a spectrum of the Compound I derivative of CYP119 (the solid line in Figure 2). The spectrum consists of a broad Soret band comprising two overlapping peaks, the smaller one at ∼360−370 nm and the larger one at ∼416 nm, and a broad Q band in the range 600−700 nm that is highly characteristic of a porphyrin radical cation.[40]

The position of the Q-band absorbance for the CYP119 Compound I species was expected,[40] but that for the absorbance of the Soret band was surprising. On the basis of the UV−vis spectral results for the heme−thiolate enzyme CPO, P450 Compound I derivatives are predicted to have a Soret band absorbance with $\lambda_{max} = 367$ nm. The UV−vis spectrum of CPO-I, first reported in 1980,[41] has a highly asymmetric Soret band. Egawa et al.[42] later demonstrated that this band can be resolved into two overlapping symmetric peaks.

The second derivative of the Soret-band spectrum provides another demonstration that the "peak" of the Soret band of CPO-I actually comprises overlapping peaks. Figure 3A shows a spectrum of CPO-I obtained in a previous study in our laboratory[43] along with its second-derivative spectrum, in which the two observed minima clearly demonstrate that two peaks

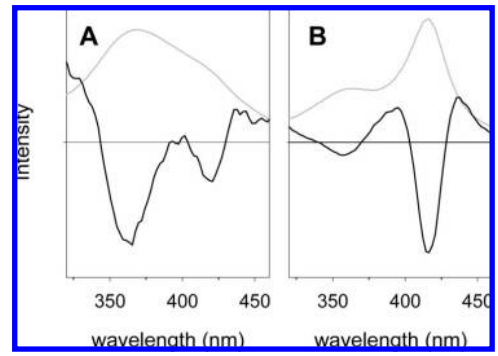


***Figure 3.*** Absorbance spectra (gray lines) and second-derivative spectra (black lines) for the Soret bands of the Compound I derivatives of (A) CPO and (B) CYP119. The scales were arbitrarily adjusted to show the signals. The horizontal lines indicate zero intensity.

are present. The minima of the negative peaks in the second-derivative spectrum are slightly displaced (by ∼2 nm) from the maxima of the individual peaks (the short-wavelength band to shorter wavelength and the long-wavelength band to longer wavelength) from the values that were used by Egawa and co-workers[42] for their simulation; this displacement is an artifact of the differentiation. The CYP119 Compound I Soret band also contains two peaks, which are apparent in the spectrum and even more obvious in the second-derivative spectrum (Figure 3B). The two peaks in the Soret bands of the Compound I derivatives of CPO and CYP119 have similar $\lambda_{max}$ values, but the relative intensities are reversed in the two spectra. From this type of analysis, the Soret bands of CPO Compound I and CYP119 Compound I are seen to be quite similar.

No P450 Compound I spectrum has been measured previously, but P450 Compound I spectra have been calculated from deconvolution of spectral data obtained in rapid-mixing studies. Egawa et al.[14] reported results from rapid mixing of mCPBA and $P450_{cam}$ in 1994, and similar studies were conducted with CYP119 by Sligar and co-workers[20] in 2002. In both studies, the spectra for the Soret bands of the Compound I derivatives calculated from the composite data using global analysis methods contained highly asymmetric peaks with $\lambda_{max} \approx 367$ nm, similar to that of CPO-I. Deconvolution methods often cannot give unique simulations but instead yield answers that depend on the initial guesses for the rate constants. When this occurs, one must select the "correct" spectrum from among the possibilities; in the present case, this involved evaluation of the results using the known spectrum of CPO-I as a guide.[40]

We repeated the stopped-flow mixing study[20] of CYP119 with mCPBA at 4 °C and obtained the results shown in Figure 4. For the first 65 ms of the reaction, the signal centered at ∼367 nm grew slightly and the one centered at ∼416 nm decreased slightly (Figure 4A). After 65 ms, the signals returned to the original absorbances of the resting enzyme (Figure 4B). The spectral file (800 spectra) was deconvoluted by global analysis using a model involving the two steps A + B → C (with a second-order rate constant $k_{AB}$) and C → A (with a first-order rate constant $k_C$), where A is the CYP119 enzyme, B is mCPBA, and C is the Compound I derivative of CYP119. We used the same software and kinetic model as used in the previous study.[20] Examples of simulated spectra of CYP119 Compound I resulting from four deconvolutions with different initial guesses for the rate constants are shown in Figure 4, and 12 additional sets of spectra and their residuals are given in Table S1 in the Supporting Information. We emphasize that no constraints were employed in these deconvolutions.

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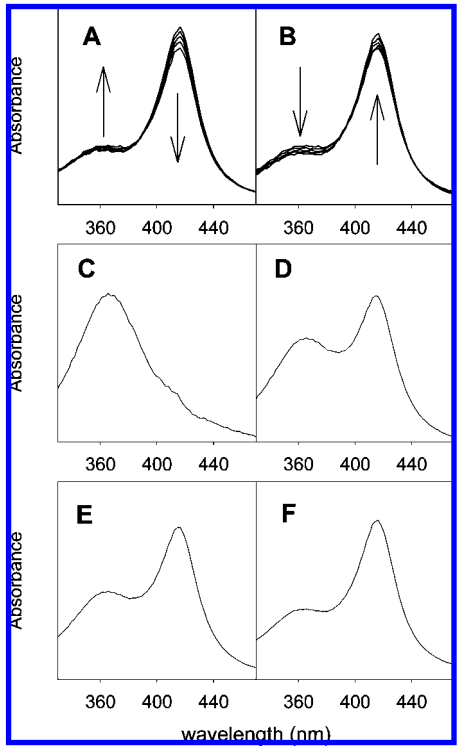



***Figure 4.*** Results from stopped-flow mixing of 14 $\mu$M CYP119 with 8.6 $\mu$M mCPBA at 4 °C. (A) Observed spectra at 1.0, 7.0, 13, 26, and 65 ms after mixing. (B) Observed spectra at 65, 180, 290, 390, and 700 ms after mixing. (C−F) Simulated spectra of the intermediate obtained from unconstrained minimizations using different initial guesses for the rate constants. The program-derived final values for $k_C$ in the deconvolutions were (C) 17.4, (D) 9.2, (E) 6.7, and (F) 2.3 $s^{-1}$. The Supporting Information provides additional information.

As noted earlier, the Soret band of CYP119 Compound I can be resolved into two peaks, one centered at 367 nm and the other at 416 nm. The deconvolutions weight these peaks differently depending on the values for the rate constants. The simulated spectrum associated with $k_C = 17.4\ s^{-1}$ (Figure 4C) is essentially the same as the simulated spectrum for CYP119 Compound I reported previously[20] and similar to the CPO Compound I spectrum.[41] As the value of $k_C$ decreases, the simulated spectra display an increasing component of the 416 nm peak and a decreasing component of the 367 nm peak. Eventually, when $k_C = 2.3\ s^{-1}$ (Figure 4F), the simulated spectrum closely matches the experimental spectrum of the CYP119 Compound I species (Figure 2). Importantly, a $k_C$ value of 2 $s^{-1}$ for decay of the transient at 4 °C is consistent with the measured rate constant for decomposition of the CYP119 Compound I derivative in the absence of substrate, whereas a decay rate constant of 17 $s^{-1}$ is too large (see below).

In summary for this section, laser flash photolysis of the CYP119 Compound II derivative gave the CYP119 Compound I derivative. The experimental spectrum of this species was obtained from the LFP experiments using the difference spectrum (i.e., the difference of spectra recorded before and immediately after the flash). Stopped-flow mixing results for oxidation of CYP119 with mCPBA gave the same calculated UV−vis spectrum for the Soret band of CYP119 Compound I as obtained in the LFP experiments when an appropriate rate constant for decay of this transient was found in the spectral deconvolution. The Soret-band absorbance of CYP119 Compound I contains two overlapping transitions, much like that of CPO Compound I[42] but with a different ratio of the component

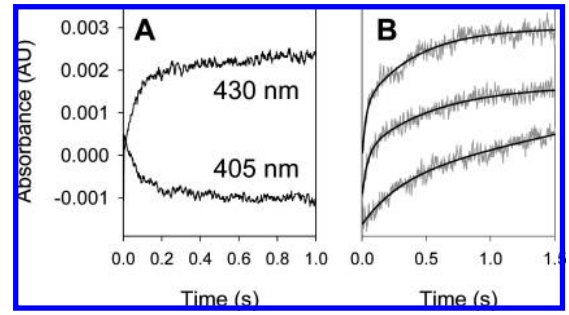
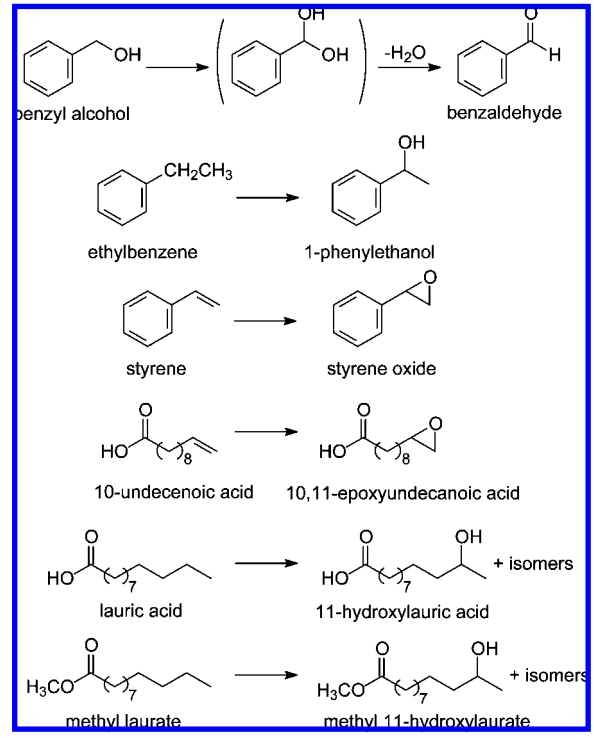



***Figure 5.*** Kinetic traces. (A) Growth at 430 nm and decay at 405 nm for reaction of CYP119 Compound I with 1.5 mM styrene; the rate constants obtained from the two traces were the same. (B) Kinetic traces (gray lines) and fits (black lines) at 430 nm for reactions of CYP119 Compound I with benzyl alcohol at substrate concentrations of (bottom to top) 0.0, 0.6, and 2.2 mM.

***Chart 1***




peaks. The Q band displays a broad, weak absorbance between 600 and 700 nm, as expected for a porphyrin radical cation structure.[40]

**Kinetics of CYP119 Compound I Reactions.** No direct kinetic studies of reactions of a P450 Compound I derivative with substrate have been reported previously, but such studies are now possible. Using 2 ns risetime PMTs, first-order rate constants as large as $2 \times 10^8\ s^{-1}$ can be measured, and diffusion-controlled bimolecular reactions are readily monitored. Decay of CYP119 Compound I to the ferric enzyme resting state resulted in decreasing absorbance at wavelengths in the range 390−420 nm and increasing absorbance in the range 425−450 nm. The results obtained by following the kinetics in the two wavelength ranges were the same (Figure 5A). In practice, we generally followed signal growth at ~430 nm.

The kinetic studies involved the oxidation reactions shown in Chart 1. Characterizations of the products from these oxidation reactions are discussed below. All of the kinetic studies

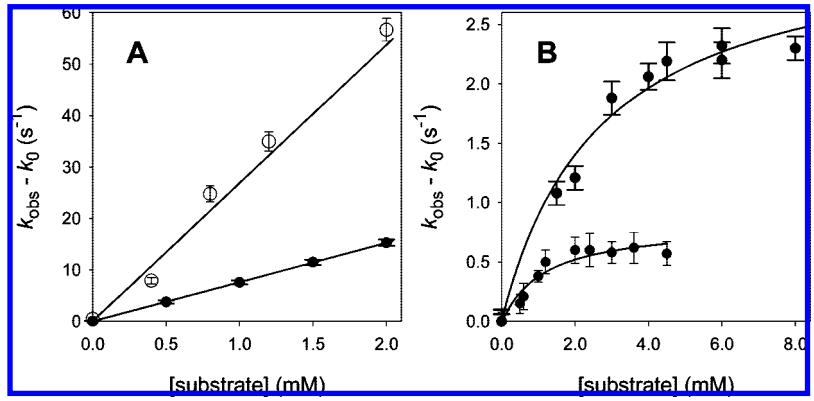



***Figure 6.*** Kinetic results for reactions of CYP119 Compound I in 25 mM phosphate buffer (pH 7.4) at 22 °C: (A) benzyl alcohol (top) and styrene (bottom); (B) methyl laurate (top) and lauric acid (bottom). The lines are regression solutions based on (A) eq 1 and (B) eq 3. The error bars on the pseudo-first-order rate constants represent one standard deviation.

were performed with large excesses of substrate to ensure that secondary oxidations were not important.

For reactions with substrates, the substrate of choice was present in the enzyme solution prior to mixing that solution with the PN solution. A series of reactions was conducted with varying concentrations of substrate while maintaining pseudo-first-order reaction conditions. Figure 5B shows typical results obtained with benzyl alcohol. Each trace was fit using a double-exponential function in which the major process (80−90%) varied with the concentration of substrate and the minor process was constant in successive experiments. Because the CYP119 Compound I derivative reacts with excess PN, the laser flash was delayed until ∼5 s after mixing to permit PN decay. In all cases for a series of studies with a given substrate, the reaction conditions were maintained such that the background rate remained constant. For example, a series of studies with a given substrate employed one solution of enzyme, one solution of PN, and a constant delay time after mixing before the laser flash.

First-order rate constants for reaction of CYP119 Compound I without added substrate varied in the range 2−8 $s^{-1}$ at 20 °C, depending on the amount of excess peroxynitrite present in the solution (with larger rate constants obtained when the concentration of PN was greater). PN is stored in basic solutions and decays rapidly in an acid-catalyzed reaction upon mixing with the buffered solutions of enzyme. Delaying the laser irradiation for 5 s after mixing allowed most of the PN to decay, as determined by UV−vis spectroscopy, yielding small, relatively consistent rate constants for decay of CYP119 Compound I. Details of the background decay reaction for the Compound I derivative should be revealed with further study, and this "reaction" might actually be a sum of several reactions, including reaction with PN, reaction of the Compound I derivative with nitrite, which is a major product of PN decay,[36,38] and self-reaction of the Compound I moiety with an oxidizable portion of the protein. In any event, the first-order decay reaction of Compound I introduced a constant kinetic effect that was subtracted from the observed rate constants.

For reactions of CYP119 Compound I with most of the substrates studied, we observed a linear relationship between the pseudo-first-order rate constant for reaction of Compound I and the concentration of substrate.[44] Figure 6A shows typical results for this group of substrates. The rate constants are described by eq 1:

$$k_{obs} - k_0 = k_{app}[\text{substrate}] \quad (1)$$

where $k_{obs}$ is the observed pseudo-first-order rate constant, $k_0$ is the background first-order rate constant, $k_{app}$ is an apparent second-order rate constant for reaction with the substrate, and [substrate] is the substrate concentration. Plots of ($k_{obs}$ − $k_0$) versus [substrate] had slopes of $k_{app}$, as shown in Figure 6A, and values of $k_{app}$ determined for the reactive substrates are listed in Table 1. We note that the apparent second-order rate constant for reaction in an enzyme's active site is a composite value consisting of two factors, an equilibrium constant for binding substrate ($K_{bind}$, units of $M^{-1}$) and a first-order rate constant for the oxidation reaction of the bound substrate ($k_{ox}$, units of $s^{-1}$), as shown in eq 2:

$$k_{app} = K_{bind}k_{ox} \quad (2)$$

For reactions of lauric acid and methyl laurate with CYP119 Compound I, the kinetic behavior was different than that found with other substrates. We observed saturation kinetics, with the rate of reaction approaching a limiting value at high substrate concentrations (Figure 6B). When saturation kinetics is observed, the rate constants are described by eq 3:

***Table 1.*** Apparent Second-Order Rate Constants for Reactions of CYP119 Compound I with Organic Substrates[a]

| substrate | $k_{app}$ ($M^{-1}$ $s^{-1}$) | CPO-I $k_{app}$ ($M^{-1}$ $s^{-1}$)[b] |
|---|---|---|
| benzyl alcohol | $(2.70 \pm 0.07) \times 10^4$ | |
| benzyl alcohol (pH 6.4)[c] | $(1.97 \pm 0.08) \times 10^4$ | |
| benzyl alcohol-$d_7$ | $(1.17 \pm 0.06) \times 10^4$ | |
| ethylbenzene | $(2.54 \pm 0.12) \times 10^3$ | $9.6 \times 10^2$ |
| ethylbenzene-$d_{10}$ | $(1.6 \pm 0.4) \times 10^3$ | $3.7 \times 10^2$ |
| styrene | $(7.64 \pm 0.18) \times 10^3$ | $6.1 \times 10^4$ |
| 10-undecenoic acid | $(9.5 \pm 0.3) \times 10^3$ | $6.4 \times 10^3$ |
| Tris buffer (pH 7.4)[d] | $(2.5 \pm 0.2) \times 10^2$ | |
| lauric acid[e] | $7.2 \times 10^2$ | |
| methyl laurate[e] | $1.2 \times 10^3$ | |

[a] Reactions conducted at 22 ± 1 °C in 25 mM phosphate buffer (pH 7.4) unless otherwise noted. Errors are 1$\sigma$. [b] Apparent second-order rate constants for reactions of CPO Compound I derivative, from ref 43. [c] This reaction was run in 25 mM phosphate buffer (pH ∼6.4). [d] The reaction was conducted in 25 mM phosphate buffer (pH 7.4) using Tris buffer as the substrate; Tris stands for tris(hydroxymethyl)-aminomethane, $(HOCH_2)_3CNH_2$, which is mainly protonated at pH 7.4. [e] For lauric acid and methyl laurate, the apparent second-order rate constants were calculated from the measured values of $K_{bind}$ and $k_{ox}$ discussed in the text.

(44) In the initial communication of this work (ref 22), we reported that lauric acid and styrene had no effect on the kinetics of CYP119 Compound I decay. The substrate concentrations used in those studies were too small to give measurable kinetic effects.

$$k_{obs} - k_0 = \frac{K_{bind} k_{ox}[substrate]}{K_{bind}[substrate] + 1} \quad (3)$$

Nonlinear regression analysis of the data according to eq 3 gave values for both $K_{bind}$ and $k_{ox}$. For reactions at 22 °C in 25 mM phosphate buffer (pH 7.4), the results for lauric acid were $K_{bind} = 900 \pm 300\ M^{-1}$ and $k_{ox} = 0.8 \pm 0.1\ s^{-1}$ and those for methyl laurate were $K_{bind} = 370 \pm 90\ M^{-1}$ and $k_{ox} = 3.3 \pm 0.3\ s^{-1}$.

Because $k_{app}$ is the product of $K_{bind}$ and $k_{ox}$ (eq 2), we were able to calculate $k_{app}$ for lauric acid and methyl laurate; the values are included in Table 1 for comparison to those for the other substrates studied. Table 1 also contains some rate constants measured for reactions of the Compound I derivative of the heme−thiolate enzyme CPO.[43]

Perhaps the most noteworthy kinetic result is the small rate constant for oxidation of unactivated C−H bonds in lauric acid ($k_{ox} = 0.8\ s^{-1}$). If the binding constant for lauric acid were not large enough to result in saturation of the activated enzyme, then we would not have been able to measure the kinetics of this reaction. Nonetheless, the measured rate constant is consistent with the observed reactivities of a number of iron(IV)−oxo porphyrin radical cations, which are known to be too low in activity to oxidize unactivated C−H bonds in hydrocarbons efficiently.[8,45] P450 enzymes do oxidize unactivated C−H bonds rapidly under natural conditions, of course, and it is commonly assumed that the requisite reactivity arises from the electron-donating effect of the cysteinate ligand.[7,10] For CYP119-I, the cysteinate ligand does appear to increase reactivity somewhat (see below) but not enough to match the apparent reactivity for C−H oxidations displayed by some P450s in nature.

A paradox involving Compound I reactivities and P450 oxidations is that Compound I derivatives in models are not reactive enough to explain the oxidation strength of P450 enzymes. When $P450_{cam}$ with substrate present in the active site was allowed to react with radiolytically generated electrons and oxygen under cryogenic conditions, the last intermediate in the sequence observable by EPR and ENDOR spectroscopy was the hydroperoxyiron species (see Figure 1).[11,12] Extrapolation of the low-temperature results to ambient temperature suggests that the hydroperoxyiron species would decay at room temperature with a rate constant of $k \approx 1000\ s^{-1}$. Because no subsequent iron−oxo transient was observed, the transient that effected the oxidation must have reacted with the unactivated C(5)−H bond of camphor with a rate constant greater than that of its formation. Thus, if the estimate for the decay of the hydroperoxyiron species is correct, then the oxidation reaction at ambient temperature would have a rate constant of $k_{ox} > 1000\ s^{-1}$; in turn, if that approximation is correct, then camphor C(5)−H oxidation by activated $P450_{cam}$ is more than 3 orders of magnitude faster than the lauric acid C−H oxidation by CYP119 Compound I. In principle, the large difference in reactivities might be due to very poor "tuning" of the reaction in CYP119, which displays maximal activity at ~70 °C.[25,46,47] Alternatively, it is possible that P450s can produce a more reactive transient than Compound I, a perferryloxo species (see Figure 1) that has a finite lifetime and can effect oxidations before it relaxes to a Compound I species by internal electron transfer. This controversial conjecture is supported by recent evidence for the production of perferryloxo porphyrins and corroles that are several orders of magnitude more reactive in oxidation reactions than their iron(IV)−oxo ligand radical cation isomers[48,49] and by results of thermodynamic cycles indicating that a perferryloxo intermediate in a P450 enzyme is energetically accessible.[50]

The rate constants found for oxidations of ethylbenzene, styrene, and 10-undecenoic acid by CYP119-I are in general agreement with those obtained for reactions of the same substrates with the Compound I derivative of CPO. CPO-I has long been available from hydrogen peroxide oxidation of the enzyme and has served as a model for P450 Compound I in a variety of spectroscopic studies.[35,41,42,51,52] Apparent second-order rate constants for two-electron oxo-transfer reactions of CPO-I also are available.[43,53] The similarity in the rate constants for CYP119-I and CPO-I oxidations of common substrates reinforces the conclusion that Compound I derivatives of P450 enzymes are well modeled by CPO-I, but it must be remembered that the observed rate constants for oxidations by CYP119-I and CPO-I are apparent second-order rate constants that contain both equilibrium binding constants and first-order oxidation rate constants. More meaningful comparisons can be made when these composite rate constants are separated into their constituent parts.

It is possible to crudely estimate the $K_{bind}$ and $k_{ox}$ values for substrates that gave only apparent second-order rate constants. The binding constant for lauric acid by CYP119-I measured in this work was $900\ M^{-1}$. For CYP119 resting enzyme, the lauric acid binding constant is nearly 400 times larger than the styrene binding constant.[54] The assumption that the same differential exists for complexation of these two substrates by CYP119-I yields an estimate of $K_{bind} \approx 2\ M^{-1}$ for styrene, and the value for the oxidation rate constant would be $k_{ox} \approx 4000\ s^{-1}$. This estimated first-order rate constant for styrene oxidation at ambient temperature gives $\Delta G^{\ddagger} = 12.4$ kcal/mol, which appears to be in reasonable agreement with $\Delta G^{\ddagger}$ values of ~15 kcal/mol for styrene oxidations by simple Compound I models that should be less reactive than P450 Compound I species[45] if one factors in an expected accelerating effect for the thiolate ligand in CYP119-I. If similarly small $K_{bind}$ values are assumed for the other aromatic substrates studied here, namely, ethylbenzene and benzyl alcohol, then the $k_{ox}$ values for these substrates are in the 1000 and 20 000 $s^{-1}$ ranges, respectively, which again appear to be in line with rate constants for reactions of simple Compound I models.[45]

Another manifestation of the kinetic acceleration afforded by the thiolate ligand in CYP119-I is seen in the kinetic isotope effect (KIE) results. For benzyl alcohol and ethylbenzene, $k_H/k_D = 2.3$ and 1.6, respectively, under the assumption that the substrate binding constants were unaffected by isotopic substitu-

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tion. These KIE values are relatively small; for example, an H/D KIE value of 4.4 for ethylbenzene-$d_0$ and -$d_{10}$ was found for oxidation by the Compound I derivative of 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin−iron.[45] The small KIE values for CYP119-I suggest a large, negative value of $\Delta G°$ for oxidation of these substrates relative to the value in the models, leading to an early transition state and a small difference in the $\Delta G^{‡}$ values (and small KIEs) for reactions of the nondeuterated and perdeuterated substrates.

We performed some experiments to crudely evaluate the effect of the buffer on the Compound I reaction kinetics. Most of the oxidations in Table 1 were conducted in phosphate buffer at pH 7.4. For benzyl alcohol, the value of $k_{app}$ was reduced by ~25% when the reaction was conducted in phosphate buffer at a pH of ~6.4. Until it is possible to separate the constituent factors in $k_{app}$, we are not able to determine whether the kinetic pH effect is due to reduced binding or to a change in the oxidation rate constant (or both), but the noteworthy point is that one should take care to monitor the pH closely in kinetic studies of P450 Compound I oxidations.

We also performed a cursory study using tris(hydroxymethyl)-aminomethane (Tris) buffer as a substrate for CYP119-I because the hydroxymethyl groups of Tris were expected to be reactive and some oxidations by CYP119 have been conducted with bis-Tris buffer.[54,55] We did not attempt to isolate products from the oxidation of Tris because they undoubtedly are highly water-soluble, but it is most likely that the oxidation gave a geminal diol at one carbon that could dehydrate to an aldehyde group. The point of the study was to determine if Tris would react rapidly with CYP119 Compound I, and this was accomplished by using a phosphate buffer system and adding Tris in varying amounts as if it were a typical substrate. When the reaction was studied in this manner, we obtained a reasonably large $k_{app}$ value of 250 $M^{-1}$ $s^{-1}$ for Tris. This result indicates the need for caution with respect to the use of Tris or bis-Tris buffers in Compound I oxidation studies. For example, bis-Tris buffer has been used at a concentration of 50 mM in studies of styrene oxidation by CYP119-I under $H_2O_2$ shunt conditions.[55] If bis-Tris reacts with the same rate constant as Tris, then the reaction of CYP119-I with the buffer had a pseudo-first-order rate constant of $k \approx 12$ $s^{-1}$, which is ~6 times as large as that for the decay in the presence of phosphate buffer.

**Comparisons of CYP119 Shunt Oxidation Reactions to CYP119-I Reactions.** For most P450 enzymes, the sequence of reactions that produces active oxidant involves reduction of the ferric enzyme, reversible binding of oxygen, a second reduction step, and protonation reactions (see Figure 1). Many P450 enzymes can be "shunted" with peroxides such as hydrogen peroxide in reactions that likely resemble those of the peroxidase enzymes. Although there is limited evidence for the formation of a Compound I derivative in these shunt reactions, P450 enzymes have been known for many years to catalyze oxidizations of substrates under shunt conditions,[56] and the presence of a Compound I transient generally has been assumed. In the specific case of CYP119, hydrogen peroxide and alkyl hydroperoxide shunt reactions are known to give oxidized substrates.[25,46,47,54,55]

***Table 2.*** Results of Competition Shunt Oxidations by CYP119[a]

| substrate A | substrate B | $k_A/k_B$[b] | LFP ratio[c] |
|---|---|---|---|
| benzyl alcohol | benzyl alcohol-$d_7$ | $2.65 \pm 0.14$ | $2.3 \pm 0.3$ |
| ethylbenzene | ethylbenzene-$d_{10}$ | $1.95 \pm 0.14$ | $1.6 \pm 0.8$ |
| benzyl alcohol | styrene | $3.25 \pm 0.14$ | $3.5 \pm 0.2$ |
| styrene | lauric acid | $0.16 \pm 0.01$ | 10.6 |
| benzyl alcohol | lauric acid | $0.52 \pm 0.04$ | 38 |

[a] Competition reactions between substrates A and B at equal initial concentrations in 50 mM phosphate buffer (pH 7.0) at 22 ± 1 °C. [b] Ratio of rate constants from eq 4; errors are for 95% confidence. [c] Ratio of rate constants from LFP studies (Table 1); errors are for ~95% confidence.

We performed CYP119−hydrogen peroxide shunt reactions for comparison to the LFP kinetic studies. In the shunt reactions, CYP119 in buffer with a mixture of two substrates was treated with aliquots of hydrogen peroxide. Following the reactions, the products were extracted into an organic solvent, after which a standard was added and the mixtures were analyzed by GC for quantification and GC−MS for product identification. The relative rate constants for reactions of the two substrates were determined using eq 4, which is the equation for competitive kinetics in reactions of substrates A and B when the competing reactions are second-order processes:[57]

$$\frac{k_A}{k_B} = \frac{\ln([A]_0/[A]_F)}{\ln([B]_0/[B]_F)} \quad (4)$$

In eq 4, $[X]_0$ is the initial concentration of substrate X and $[X]_F$ is the concentration of substrate X at the end of the reaction.

Table 2 lists the results of the competition kinetics studies. The ratios of rate constants for the competing reactions in the shunt experiments are listed as $k_A/k_B$ in column 3. Column 4 in the table contains the ratios of rate constants obtained in the LFP studies for the same substrates. The ratios of rate constants typically should *not* be the same for the two types of studies because the shunt reactions studied here involve competitions, and a substrate with a large binding constant might be a competitive inhibitor of a substrate with a small binding constant. Nonetheless, if there is an equality in the equilibrium binding constants for the two substrates, then the ratios of rate constants for the two types of studies should be equal. That condition can be assumed to hold for the KIE studies because isotopic substitution should have at most only a minor effect on substrate binding. For both benzyl alcohol and ethylbenzene, we found the same KIE values in the competition studies as in the LFP studies, indicating that the oxidizing species is the Compound I derivative in both types of oxidation experiments.

The ratio of rate constants for the competition study involving benzyl alcohol and styrene and the corresponding ratio of LFP rate constants for these substrates also were the same. This suggests either that the two aromatic substrates have similar equilibrium binding constants or that the binding constants for both were too small to saturate the enzyme. In either case, the $k_{app}$ values found in the LFP studies would control the relative reactivities in the competition studies.

The competition results with lauric acid were much different than those involving two aromatic substrates. In the LFP studies, lauric acid reacted with a $k_{app}$ value that was much smaller than those measured for benzyl alcohol and styrene (see Table 1), but lauric acid was oxidized more efficiently than either of the aromatic substrates in the competition study. Thus, lauric acid

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was a competitive inhibitor of the aromatic substrates in the competition studies. The binding constant for lauric acid found in the LFP studies, $K_{bind} = 900\ M^{-1}$, is the equilibrium constant for complexation in the active site of the Compound I derivative. In the competition studies, where the substrate concentrations were 0.2 mM, a $K_{bind}$ value of 900 $M^{-1}$ would not result in saturation of the activated enzyme. For the resting CYP119 enzyme, however, the reported binding constant for lauric acid is $\sim 8 \times 10^5\ M^{-1}$,[54] which would result in saturation of the resting enzyme with 0.2 mM lauric acid. More detailed studies are needed to understand the kinetic effects fully, but the tentative conclusion is that CYP119 saturation by lauric acid before activation of the enzyme by reaction with $H_2O_2$ resulted in limited access of the aromatic substrates to the active site of the activated enzyme.

**Products from Oxidation Reactions.** For the substrates studied in this work, the products of oxidations by P450 enzymes in general are known to be epoxidation products for alkenes and hydroxylation products for hydrocarbons.[10] In the specific case of CYP119, oxidation reactions of lauric acid and styrene have been studied under shunt conditions as well as under a more natural reaction sequence.[25,46,47,54,55] The major product from lauric acid oxidation is 11-hydroxylauric acid, and the 12-, 10-, and 9-hydroxylated products also are formed. The major product from styrene oxidation is styrene oxide.

In the present work, shunt reactions similar to the competition kinetics studies were conducted, and the products were analyzed using GC and GC−MS. The oxidation products were identified by comparison of their GC retention times and mass spectral fragmentation patterns to those of authentic samples of the products benzaldehyde (from benzyl alcohol), styrene oxide (from styrene), 1-phenylethanol (from ethylbenzene), and 10,11-epoxyundecanoic acid (from 10-undecenoic acid). For lauric acid, the product mixture from reaction with CYP119 is known to contain 11-hydroxylauric acid as the major product, 12-hydroxylauric acid as the second most abundant product, and other hydroxylauric acids as minor products.[54] We converted the lauric acid products to the corresponding methyl esters by reaction with diazomethane. The mixture of products from this sequence was compared to the mixture obtained from oxidation of methyl laurate in order to identify the methyl laurate oxidation products; these were found to be the same as those from oxidation and subsequent methylation of lauric acid, although the ratios of products differed slightly.

The yields of products from some of the shunt reactions were determined using GC by addition of a calibrated internal standard to the product mixture from the oxidations. The yields of oxidation products from styrene and ethylbenzene were 8−10% based on hydrogen peroxide as the limiting reagent. Similar yields for hydrogen peroxide shunt reactions with CYP119 were reported previously.[54]

In order to evaluate the effect of PN on the CYP119 enzyme, we conducted a shunt reaction identical to the one with styrene except that the CYP119 enzyme was first treated with 20 equiv of PN. The yield of styrene oxide formed in this shunt reaction was the same as that found with untreated CYP119. Thus, the PN reaction using 20 equiv of PN appears to have no effect on the CYP119 enzyme.

The LFP study involved production of subnanomole amounts of reactive transients, but we also performed bulk photolysis reactions that were designed to simulate the LFP studies on a somewhat larger scale. In these reactions, 200 $\mu$M PN was added to a mixture of 10 $\mu$M CYP119 and 100 $\mu$M substrate (final concentrations are listed) in a vessel held in a 0 °C bath. After 10 s, the reaction vessel was removed from the bath, placed in a photochemical reactor at room temperature, and irradiated for ~1 min with five 15 W bulbs emitting light centered at 350 nm. The reaction mixture was then worked up and analyzed using GC and GC−MS to quantify and identify the products. For styrene and ethylbenzene as substrates, we found styrene oxide and 1-phenylethanol in ~70 and ~65% yield, respectively, based on enzyme. We estimate that under the conditions employed for production of the Compound II species in these experiments, 80−90% of the enzyme was converted to the Compound II derivative. Thus, the total efficiency for the two processes (photochemical production of Compound I and its reaction with substrate) was very high (in the 70−90% range), given that "self-decay" of the CYP119 Compound I derivative occurred at a measurable rate on the time scale of the bulk photolysis studies. In a series of control reactions with styrene as the substrate, we found no oxidization product when PN, CYP119, or light was omitted from experiments otherwise identical to the styrene reaction described above.

The origin of the oxygen atoms in the oxidation products was established by performing two sets of bulk photochemical oxidation reactions with $^{18}O$-labeled species. In the first set, we used doubly labeled peroxynitrite ($NaN^{16}O^{18}O_2$) synthesized from $H_2{}^{18}O_2$ that had previously been prepared from $^{18}O_2$. In the second set, we used unlabeled peroxynitrite in a 1:1 mixture of unlabeled and $^{18}O$-labeled water ($H_2{}^{16}O/H_2{}^{18}O = 1:1$). The reactions were conducted by addition of the substrate to the enzyme followed by addition of PN and then photolysis. The products were analyzed by GC−MS using single-ion monitoring (SIM) for the molecular ions. In the case of styrene, the molecular ion of the unlabeled styrene oxide product appears at *m*/*z* 120; in the study conducted with doubly labeled PN, the ratio of integrals for the *m*/*z* 120 and 122 peaks was 1:2, while the integral ratio using unlabeled PN in labeled water was >99:1. Similar results were obtained for oxidation of ethylbenzene, in which the unlabeled product alcohol has a molecular ion at *m*/*z* 122; for the reaction with doubly labeled PN, the ratio of integrals for *m*/*z* 122 and 124 was 1:2, whereas as the ratio found in the reaction with unlabeled PN in labeled water was >99:1.

The labeling results demonstrate that the Compound I oxidations involved reaction via insertion of the oxygen from the iron−oxo species rather than electron transfer followed by water capture of the radical cations. The formation of oxidation products via radical cations had already been judged to be highly unlikely because it is known that styrene radical cation reacts largely to give benzaldehyde[58] and electron-transfer oxidations of ethylbenzene and other alkylarenes give good yields of phenol products,[59] but neither benzaldehyde nor ethylphenols were found in our product mixtures.

## Conclusions

Photoejection of an electron from the P450 119 Compound II derivative produced the P450 Compound I species (CYP119-I), which is widely regarded as the active transient in P450-catalyzed oxidation reactions. The UV−vis spectrum of CYP119-I was thus directly observed (as opposed to calculated) and found to be similar to the UV−vis spectrum of chloroperoxidase

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Compound I, consisting of two overlapping peaks in the Soret-band region and a long-wavelength Q-band absorbance.

The LFP method for production of CYP119-I permitted direct kinetic studies of reactions of this transient with a variety of organic substrates. The measured rate constants for many substrates were apparent second-order rate constants, which are products of a binding constant times a first-order rate constant for the oxidation reaction, that were similar in magnitude to those for oxidations of the same substrates by the Compound I derivative of chloroperoxidase. For lauric acid and methyl laurate, saturation kinetics were observed, and values of both $K_{bind}$ and $k_{ox}$ were determined. The rate constant for oxidations of unactivated C−H positions in lauric acid was 0.8 $s^{-1}$ at ambient temperature, which is more than 3 orders of magnitude smaller than the $k_{ox}$ value estimated for oxidation of the unactivated C(5)−H bond in camphor by the oxidizing transient formed in $P450_{cam}$. Possible explanations for the differences in reactivities are that CYP119, which is from a thermophile, is not optimized for reactions at ambient temperature and that a different, more highly reactive type of oxidant can be formed in the natural reaction sequence of P450 enzymes.

By comparing relative reactivities in competition studies involving CYP119 and hydrogen peroxide with the ratios of rate constants from the LFP studies of the same substrates, we conclude that the same transient is produced in both types of experiments. Furthermore, product studies, including oxygen-18 labeling studies, demonstrated that the known products of P450-catalyzed oxidations were formed from photochemically generated CYP119-I by insertion of the oxygen atom of the iron−oxo group, the expected reaction pathway for Compound I derivatives.

The methods developed in this work are expected to be useful for studies of Compound I derivatives of other P450 enzymes. Further insights into the nature and reactivity of the active oxidants in P450s should be forthcoming.

## Experimental Section

The preparation and isolation of CYP119 followed the methods previously described.[22−24] The enzyme used in these studies had an *R*/*Z* ratio ($A_{416}/A_{280}$) of >1.5. All of the substrates used in the oxidation studies were commercial samples of nominally high purity that were checked by NMR spectroscopy or GC for purity. Basic solutions containing PN were prepared by the method of Uppu and Pryor.[60]

**LFP Studies.** The spectroscopic and kinetic studies of CYP119 were conducted in a similar manner using a stopped-flow mixing unit (SX-18, Applied Photophysics) affixed to an LKS-60 laser flash photolysis kinetics unit (Applied Photophysics). The laser light used was the third harmonic (355 nm) from a Quantel Brilliant B Nd:YAG laser that delivered ~4 mJ to the base of a 2 mm × 10 mm quartz reaction flow cell that was charged by the mixing unit. A 150 W xenon lamp under continuous irradiation conditions was used for most studies; the lamp was pulsed during acquisition of the data used for the CYP119 Compound I spectrum. PMTs with monochromatic light or a PDA 1 diode-array detector (Applied Photophysics) with broad spectrum light were used for detection. When monochromatic light was used, the monochromator was placed before the reaction cell to ensure that low-intensity light impinged on the sample.

In a typical LFP study, 10 μM CYP119 in 50 mM phosphate buffer (pH 7.0) was placed in one syringe of the mixing unit, and 0.25 mM PN solution was placed in a second syringe. Equal volumes of the two syringes were mixed in the reaction cell, and the resulting mixture had a pH of 7.4. After a delay of 5 s, the laser was fired. For kinetic studies, the substrate at the desired concentration was added to the CYP119 solution.

**Competition Shunt Reactions.** A 2 mL solution containing 20 μM CYP119 and 0.2 mM of the desired substrates in phosphate buffer (pH 7.0) was stirred at room temperature as aliquots of $H_2O_2$ were added over 0.5 h (total addition of 0.4 mM). The mixture was extracted with methylene chloride, and the organic phase was washed with brine, dried ($MgSO_4$), filtered, and analyzed by GC and/or GC−MS on low-polarity columns (DB-5 or equivalent). Relative rate constants were determined using eq 4.

**Product Identifications and Yields in Shunt Reactions.** Reactions were conducted as described above for shunt reactions with the exception that one substrate was employed. Following the reaction, the products were analyzed by GC and GC−MS using low-polarity columns (DB-5 or equivalent) for all of the substrates except lauric acid and methyl laurate. Products were identified by comparison of GC retention times and mass spectral fragmentation patterns to those of authentic samples, most of which were commercial samples. A sample of 10,11-epoxyundecanoic acid was prepared by mCPBA oxidation of 11-undecenoic acid as previously reported.[61] Yields were determined relative to an internal standard added after the product workup. For lauric acid, the product mixture was converted to a mixture of methyl esters by reaction with diazomethane, and the mixture was analyzed on high-polarity columns (Carbowax or equivalent). Products from lauric acid oxidation by CYP119 and $H_2O_2$ were reported,[54] and a commercial sample of 12-hydroxylauric acid was available for comparison. The methyl laurate product mixture also was analyzed on high-polarity columns, and the products were identified by comparison to the products from the lauric acid oxidations.

**Product Yields in Bulk Photolyses.** A 1.0 mL solution containing 10 μM CYP119 and 0.1 mM substrate (styrene or ethylbenzene) in 50 mM phosphate buffer (pH 7.0) was cooled in a 0 °C bath. To this solution was added 5 μL of 100 mM PN solution. After 10 s, the mixture was removed from the ice bath and irradiated in a photochemical reactor containing five 15 W fluorescent bulbs (300−400 nm irradiation band) for 1 min. The reaction mixture was worked up by extraction into $CHCl_3$, and an internal standard was added, after which the mixture was analyzed by GC for yield as described above. In a series of control reactions for the styrene oxidation, the reaction was repeated with the exception that the CYP119 enzyme, the PN, or the irradiation step was omitted; no styrene oxide was detected in these reactions.

**Oxygen-18 Labeling Studies.** Doubly labeled hydrogen peroxide, $H_2{}^{18}O_2$, was prepared from $^{18}O_2$ as previously described.[62,63] The $H_2{}^{18}O_2$ was then used for the preparation of doubly labeled sodium peroxynitrite, $NaN^{16}O^{18}O_2$, from isoamyl nitrite by the method of Uppu and Pryor.[60] Experiments were performed with doubly labeled PN in unlabeled water and with unlabeled PN in labeled water (1:1 $H_2{}^{16}O/H_2{}^{18}O$). In these studies, solutions of CYP119 in 50 mM phosphate buffer (pH 7.0) were prepared in a cell placed in a temperature-regulated chamber cooled to 0 °C. Substrate followed by PN solution was added, and the UV−vis spectrum was measured with a fiber-optic diode-array UV−vis spectrometer. The final concentrations were 10 μM CYP119, 0.1 mM substrate, and 0.5 mM PN. The samples were irradiated with 15 pulses of 320−490 light (1 J delivered in 0.1 s per pulse). UV−vis spectroscopy confirmed that the Compound II species was depleted by the photolysis. The samples were worked up as above and analyzed by GC−MS in SIM mode.

**mCPBA Mixing Study.** The experiment followed the procedure reported by Kellner et al.[20] Thus, thermostatted (4 °C) solutions of CYP119 and mCPBA were mixed in the stopped-flow kinetic unit to give 14 μM CYP119 and 8.6 μM mCPBA. Data acquisition over

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2 s gave 800 spectra. Deconvolution was achieved with global-analysis software from Applied Photophysics for the two-step model $A + B \rightarrow C$ and $C \rightarrow A$. The results from the global analysis program were functions of the initial guesses of the rate constants.

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**Supporting Information Available:** Results of PN mixing studies (Figure S1) and additional examples of deconvolutions of spectra from stopped-flow mixing of CYP119 with mCPBA (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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