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Biochemical and Biophysical Research Communications 338 (2005) 394–403
BBRC
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Review

Highly reactive electrophilic oxidants in cytochrome P450 catalysis

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Received 24 August 2005
Available online 6 September 2005

Abstract

The cytochrome P450 enzymes effect a wide range of oxidations in nature including difficult hydroxylation reactions of unactivated C—H. Most of the high energy reactions of these catalysts appear to involve highly electrophilic active species. Attempts to detect the reactive transients in the enzymes have met with limited success, but evidence has accumulated that two distinct electrophilic oxidants are produced in the P450 enzymes. The consensus electrophilic oxidant termed ''iron-oxo'' is usually thought to be an analogue of Compound I, an iron(IV)-oxo porphyrin radical cation species, but it is possible that a higher energy electronic isomer of Compound I is required to account for the facility of the C—H oxidation reactions. The second electrophilic oxidant of P450 is speculative; circumstantial evidence suggests that this species is iron-complexed hydrogen peroxide, but this oxidant might be a second spin state of iron-oxo. This overview discusses recent studies directed at detection of the electrophilic oxidants in P450 enzymes and the accumulated evidence for two distinct species.
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Keywords: Cytochrome P450 enzymes; Oxidation; Compound I; Iron-oxo

The cytochrome P450 enzymes (P450s) are responsible for a vast number of oxidations in nature including the very difficult oxidations of unactivated C—H bonds to produce alcohols. Since the initial reports of P450s in the late 1960s [1–3], chemists and biochemists have been intrigued with the nature of the active oxidants and the mechanisms of oxidation reactions catalyzed by these heme-containing enzymes. The interest reflects not only scientific curiosity but also the potential for commercial applications, and many homogeneous oxidation catalysts are metal-porphyrin mimics of P450 enzymes [4–6]. In the past decade, various studies increased understanding of the oxidants in P450 enzymes even though the highly reactive electrophilic oxidants have thus far eluded direct detection both in the natural reaction sequence and in rapid mixing experiments with sacrificial oxidants. This overview highlights recent studies aimed at detection of the oxidants in P450 enzymes, indirect mechanistic studies that provided evidence for multiple electrophilic P450 oxidant species, and models for the electrophilic P450 oxidants. Recent reviews addressed mechanistic implications and structural aspects of some works in more detail [7–13].

The P450 enzymes contain protoporphyrin IX (heme) with thiolate of a protein cysteine serving as the fifth ligand to iron. The catalytic cycle of the P450 enzymes has been studied for years, and many of the steps are well understood [14]. The known and putative iron-oxygen intermediates are shown in Fig. 1. The resting enzyme, with iron in the +3 valence state, binds substrate and is reduced. Reversible oxygen binding gives the superoxide complex, which is the last observable species in the normal reaction sequence. A second reduction step is the rate-limiting process for the overall reaction; it produces a peroxo-iron species that apparently serves as a nucleophilic oxidant in reactions that are similar to Baeyer–Villiger oxidations [15,16]. Protonation of the peroxo-iron species on the distal oxygen atom gives a hydroperoxy-iron species that has been detected in recent low temperature studies [17,18] as discussed below. A second protonation on the distal oxygen would give a hydrated iron-oxo intermediate, that

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doi:10.1016/j.bbrc.2005.08.208

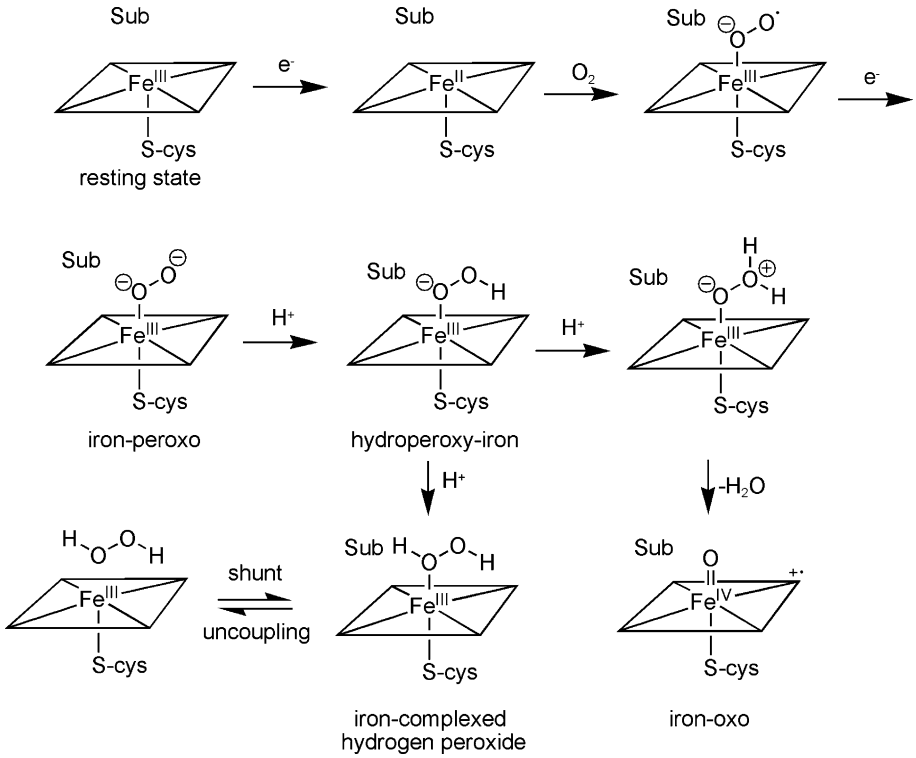


Fig. 1. The various known and putative iron–oxygen complexes in cytochrome P450 enzymes. The parallelogram represents a heme macrocycle, and sub is a substrate molecule.

either subsequently or concomitantly [19] eliminates a water molecule in a heterolytic cleavage of the O—O bond to give an iron-oxo species. Alternatively, if the second protonation reaction occurs on the proximal oxygen atom, iron-complexed hydrogen peroxide would be formed. The latter species is a requisite intermediate (or transition structure) for the uncoupling reaction of P450 enzymes, the unproductive reaction where hydrogen peroxide is released, and for the shunt reaction, where a P450 enzyme is activated by reaction with hydrogen peroxide [20].

The iron-oxo species is generally thought to be an electrophilic oxidant in the P450 enzymes. This transient has not yet been observed in the reaction cycle of P450s, but a related species has been detected in peroxidase and catalase enzymes where it is formed by reaction of the ferric enzyme with hydrogen peroxide [21,22]. In the peroxidase enzymes, the transient is known as Compound I, and it has been characterized as an iron(IV)-oxo porphyrin radical cation. In model chemistry, related iron(IV)-oxo porphyrin radical cations can be prepared by oxidation of porphyrin iron(III) salts with oxidants such as *m*-chloroperoxybenzoic acid (mCPBA) [23]. The one-electron reduction product of Compound I is an iron(IV)-oxo neutral porphyrin species known as Compound II, another species that has been characterized in peroxidase enzymes [21].

Attempts to detect electrophilic oxidants in P450 enzymes

For many years, chemists attempted to detect the P450 oxidants in rapid mixing experiments, typically focusing on the strong Soret band absorbance in the UV–visible spectrum of the enzyme. In these studies, a sacrificial oxidant such as mCPBA was mixed with the enzyme in a stopped-flow mixing apparatus that had millisecond temporal resolution. Changes in the enzyme's absorbance bands can be observed in fast mixing experiments providing evidence for transient oxidants (see below), but the oxidants cannot be detected under natural reaction conditions, at least in the case of the most commonly studied P450 enzyme, $P450_{cam}$. This conclusion results from low temperature studies performed by Hoffman's and Sligar's groups, where the oxidant(s) in $P450_{cam}$ with substrate present must have reacted faster than they were formed.

In Hoffman's cryoreduction method [17,18], the resting enzyme was reduced at low or ultra-low temperatures by electrons produced by γ-radiolysis. The reduced enzyme then reacted with oxygen to give the iron-superoxide species that was further reduced to give the peroxo-iron intermediate. At very low temperatures, the peroxo-iron species was stable and could be characterized by ESR and ENDOR spectroscopies. Annealing the samples at somewhat warmer temperatures yielded the hydroperoxy-iron species, which also could be characterized by ESR and ENDOR spectroscopies. When the samples were annealed at even warmer temperatures, however, the only new species observed from the wild-type enzyme was the alcohol product tightly bound to the iron atom of the enzyme. Upon further warming, this product complex relaxed to give the same alcohol product bound to resting enzyme as is formed by treating the resting enzyme with alcohol product.

When the $P450_{cam}$ T252A mutant was studied in similar cryoreduction experiments [17,18], the oxidized product 5-hydroxycamphor was not formed. That result was consistent with previous studies with the same site-directed mutant that are discussed below. As with the wild-type enzyme, the hydroperoxy-iron species could be observed in the mutant at low temperatures. In this case, however, annealing at warmer temperatures gave the resting enzyme as the only observable product. Apparently, the hydroperoxy-iron species was protonated on the proximal oxygen, and hydrogen peroxide was released.

Low temperature studies with $P450_{cam}$ also were performed by introduction of electrons from decay of phosphorous-32 [24]. In these studies, UV–visible spectroscopy was used to monitor the reacting species. Again, there was no evidence for accumulation of a reactive transient after the hydroperoxy-iron species, which eventually gave resting enzyme when warmed.

The failure to detect a new transient formed after the hydroperoxy-iron species indicates that the oxidizing reactions in the presence of substrate are at least 1 order of magnitude faster than the reactions that form the oxidant. If the transient reacted less rapidly than that, then it would accumulate at the beginning of the reaction. Extrapolation of Hoffman's low temperature results [18] suggests that the hydroperoxy-iron species reacts at ambient temperature with a rate constant on the order of 1000 s^{-1}. Thus, the oxidant in $P450_{cam}$ appears to effect the hydroxylation reaction of camphor at ambient temperature with a pseudo-first-order rate constant that is larger than 1000 s^{-1}.

Rapid mixing shunt reactions have been attempted with P450 enzymes with limited success. These experiments often are performed without substrate present, which removes a natural protection mechanism for the P450 enzymes, where the first reduction reaction is not possible in the absence of substrate. Without substrate present, it is possible that the activated form of the enzyme will react with itself, and this apparently occurs with $P450_{cam}$.

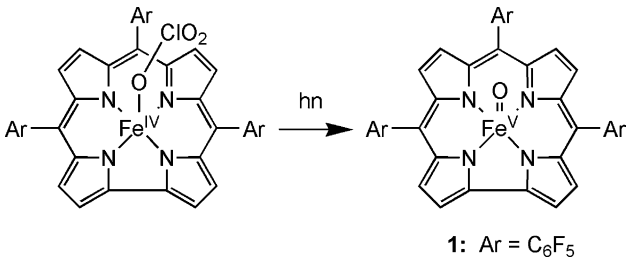
Rapid mixing of $P450_{cam}$ with mCPBA was reported to give a short-lived transient identified as a Compound I species [25]. Rapid mixing freeze-quench studies described below, however, indicated that a Compound I species did not accumulate in the reaction of this enzyme with peroxyacetic acid. A recent study appears to resolve the contradictory reports [26]. Reaction of $P450_{cam}$ with mCPBA again gave a short-lived species, and this transient displayed the expected Q-band absorbance for a porphyrin radical cation, suggesting that it was a Compound I species [26]. In addition, a Compound II protein radical also apparently was produced, consistent with the species observed in freeze-quench studies discussed below. The amounts of transients that accumulated were functions of the peroxy acid employed, the pH, and the reaction temperature [26]. In the presence of one-electron reductants with excess oxidant, multiple turnover conditions obtained.

A Compound I transient also was observed fleetingly in reaction of another P450 enzyme with a peroxy acid. CYP119 is a P450 enzyme from a thermophile that has unknown function in nature. Reaction of resting CYP119 with mCPBA gave a species identified as a Compound I transient by its absorption maximum in the Soret band region (370 nm) and the Q-band region (610, 690 nm) [27]. The transient decayed with a first-order rate constant of about 30 s^{-1} at 4 °C to give unknown products [27], and reactions of the transient with substrate were not attempted.

Despite the recent UV–visible spectroscopy studies, the transient formed in the shunt reaction of $P450_{cam}$ with peroxyacetic acid was too reactive to detect in rapid freeze-quench mixing studies [28–30]. In this approach, the enzyme was mixed with a sacrificial oxidant rapidly, quenched by low temperature trapping, and analyzed by various techniques. Reaction times were as short as a few milliseconds. When the enzyme was mixed with peroxyacetic acid in the absence of substrate, iron(IV)-oxo species analogous to Compound II were observed, and these species also contained a radical center in the protein indicating that the activated enzyme had oxidized itself to give, ultimately, a radical center at an amino acid position [30]. Similar fast reductions of other iron-oxo species are known; for example, the initial species formed by reaction of myoglobin with hydrogen peroxide reacts too fast to detect, and the observed species in this reaction is an iron(IV)-oxo protein peroxyl radical formed by electron transfer from the protein and reaction of the protein radical with oxygen [31,32].

An alternative approach for forming the P450 oxidants is to employ photochemical methods. Photophysical processes are typically very fast, giving products on the nanosecond timescale, and sensitive nanosecond-resolution photomultipliers and diodes are available for detection. Two photochemical entries to high valent iron-oxo species are currently being developed in our group, photo-oxidation (or photo-ejection) from a lower oxidation state of the iron-oxo species and photo-induced cleavage of metal ligand bonds, and both of these have been shown to produce metal-oxo species at the proper valence level for the P450 oxidants.

In photo-oxidation reactions, an electron is ejected from the compound into the surrounding environment. Photo-oxidations of the porphyrins in Compound II and its analogues to give Compound I and analogues were demonstrated for a porphyrin-iron(IV)-oxo model compound, Compound II of horseradish peroxidase, and a Compound II analogue of myoglobin [33]. The kinetic resolution of the method was excellent. For the myoglobin Compound I analogue, one electron transfer oxidation of *N*,*N*-dimethylbenzylamine was found to have a second-order rate constant of 6×10^5 M^{-1} s^{-1}. In the presence of 9 mM amine, the pseudo-first-order rate constant for decay of the Compound I species was 9000 s^{-1}, or the reaction was essentially completed within 0.2 ms, but the kinetics were readily followed. In fact, the kinetic method can handle rate constants that are more than 1 order of magnitude greater than those in this study.



Scheme 1.

Photochemical cleavages of the transition metal ligand complexes also can give metal-oxo species. The method was demonstrated by the production of porphyrin-manganese(V)-oxo [34,35], porphyrin-manganese(IV)-oxo [35], and corrole-manganese(V)-oxo species [36]. More importantly in regard to the P450 enzymes, the method was extended to the production of a corrole-iron-oxo intermediate that appears to be the iron(V)-oxo species **1** (Scheme 1) [37]. Transient **1** was highly reactive, oxidizing ethylbenzene at ambient temperature with a second-order rate constant of 570 M^{-1} s^{-1}. The high reactivity of this species is discussed later in the context of the reactivities of the P450 oxidants.

Evidence for multiple electrophilic oxidants in P450s

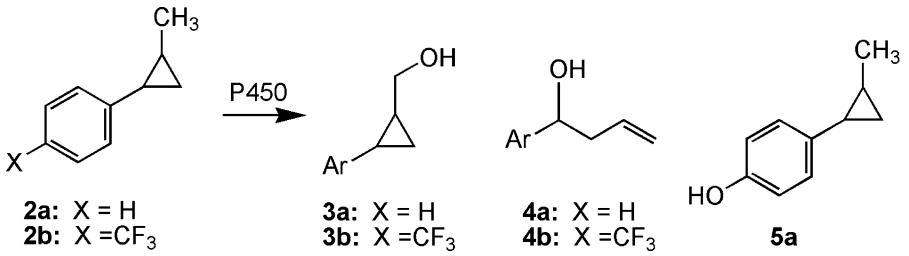
Although direct detection of the electrophilic P450 oxidants remains elusive, several studies in the past decade provided strong evidence that multiple electrophilic oxidants exist in these enzymes. This possibility was presented earlier based on, for example, changes in product ratios when shunt reactions were compared with natural reactions [38], but those types of studies are complicated by the possibility that different reactions can be observed when the oxidant is formed in the presence and absence of substrate. The results of site-directed mutagenesis studies provide more convincing evidence because reactions can be compared under natural turnover conditions with substrate present, with the reductase enzymes complexed to the P450s, and with electrons delivered sequentially.

The site-directed mutagenesis studies that were important for providing evidence for multiple electrophilic oxidants were performed with P450 enzymes and their mutants in which a conserved threonine was replaced with another amino acid. Crystal structures of soluble P450 enzymes available by the mid-1990s [39–42] displayed a highly conserved threonine in the oxygen binding pocket, and a similarly positioned threonine also could be located in other P450 enzymes by sequence alignments [43]. It was generally assumed that this threonine was involved in the protonation reactions of the peroxo-iron species or at least in the organization of a hydrogen-bonded water network. A logical supposition was that mutation of the conserved threonine would affect the rates of formation of transients or equilibrium constants.

Mutation of threonine-252 in $P450_{cam}$ to alanine [44,45] or valine [45] resulted in much reduced rates of hydroxylation of camphor and low yields of alcohol product relative to the amount of NADH consumed, whereas mutation of threonine-252 to serine had only a minor effect [45]. In a similar manner, mutation of threonine-268 in P450 BM3 to alanine resulted in a mutant in which NADPH consumption was uncoupled from product formation [46]. These mutation studies supported the idea that the conserved threonine was involved in formation of the active oxidant(s) in P450 enzymes.

In 1998, Coon's group reported competitive oxidation studies using two pairs of wild-type and mutant P450 enzymes in which the conserved threonines were changed to alanines. The enzymes studied were expressed forms of two hepatic enzymes, P450 Δ2B4 [16] (phenobarbital induced) and P450 Δ2E1 [47] (ethanol-inducible). These enzymes contain a short deletion on the N-terminal ends of the proteins. In the initial studies, the wild-type and mutant pairs of P450s were compared in oxidation reactions of simple alkenes: styrene, cyclohexene, *cis*-2-butene, and *trans*-2-butene [47]. Styrene was epoxidized to styrene oxide, and the other substrates reacted to give both epoxides and allylic alcohols. For the P450 Δ2B4 pair, the rates of oxidations of all substrates tested decreased for the T302A mutant in comparison to the wild-type enzyme. For P450 Δ2E1 enzymes, however, the rates of allylic hydroxylation decreased for the T303A mutant, but the rates of epoxidation reactions increased. The unusual kinetic behavior for the P450 Δ2E1 pair seems only to be consistent with two distinct oxidants, one of which was a preferential hydroxylating agent and the other a preferential epoxidizing agent, with different amounts of the two oxidants formed in the two enzymes.

The same pairs of enzymes were later used in oxidations of racemic mixtures of the two mechanistic probes (**2**) (Scheme 2) [48]. Oxidation of the probes at the methyl position can give either unrearranged alcohol (**3**) or rearranged alcohol (**4**) products, the rearranged product arising from either a radical or cationic intermediate. Probe **2a** also can be oxidized on the phenyl ring to give product **5a** and other phenols, and it behaves like the simple alkenes in that it provides two positions for reactions. In probe **2b**, the trifluoromethyl group reduces aromatic ring oxidation reactions to undetectable levels. Oxidations of probe **2a** by the pairs of wild-type and mutant P450 gave results similar in direction but more dramatic than those with simple alkenes. Methyl group oxidation was by far the major reaction pathway (81–85%) for the wild-type enzymes, but both mutants gave higher relative yields of phenols **5a** (44% to 55%). Mechanistically, the phenol-producing reaction apparently resembles an epoxidation, and the results with **2a** indicate that there are two distinct electrophilic oxidizing species formed in different amounts when the threonine is present or removed. Again, one oxidant appears to be a preferential hydroxylating agent and the other a preferential epoxidizing agent.

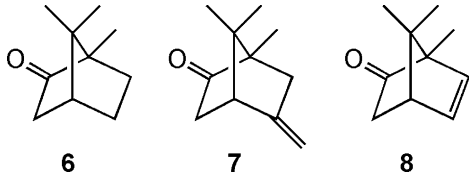


Scheme 2.

Although the regioselectivity with probe **2a** changed considerably for the wild-type and mutant P450s, the ratio of unrearranged (**3a**) to rearranged (**4a**) products from methyl group oxidation was relatively constant for each pair of enzymes. This suggests that the two oxidants showed relatively high fidelity in their reactions. For probe **2b**, however, where aryl group oxidation was prevented by the electron-withdrawing trifluoromethyl group, the ratios of products **3b**–**4b** changed dramatically between the wild-type and mutant enzymes [48]. Assuming that the change in production of the two oxidants indicated from the results with probe **2a** was the same in the oxidation of probe **2b**, one concludes that both oxidants are capable of hydroxylating the methyl group in the probes. That is, in the absence of an alternative epoxidation reaction channel, the oxidant that is a preferential epoxidizing agent can effect hydroxylation reactions at the weakly activated C—H bond adjacent to the cyclopropyl ring [49].

The same pairs of wild-type and mutant P450 enzymes also were studied as catalysts for *ipso*-substitution reactions that convert *p*-substituted phenols to hydroquinones [50]. The results were similar to those found with other substrates in that the P450 Δ2E1 T303A mutant was more active than the wild-type enzyme in several substitution reactions. In fact, some substitution reactions were catalyzed by the mutant but not by the wild-type enzyme. Given that the rates of allylic hydroxylation by the mutant enzyme were smaller than the rates of hydroxylation by the wild-type enzyme [47], the increased activity of the mutant in the *ipso*-substitution reactions indicates that the electrophilic oxidant assigned as the preferential epoxidizing agent is highly electrophilic and the major species effecting the substitutions.

In recent studies with $P450_{cam}$ and its T252A mutant, changes in reactivities clearly implicated multiple electrophilic oxidants [51]. The wild-type and mutant enzymes were compared in their reactions with the natural substrate camphor (**6**) and the structurally related alkenes **7** and **8**. The T252A mutant was a poor hydroxylating agent, as noted earlier, with the rate of hydroxylation of camphor reduced to less than 1% of the rate observed for the wild-type enzyme. For the expoxidation reactions of the two alkenes, however, the T252A mutant rates were 21% and 14%, respectively, those found with the wild-type enzyme. The conclusion was that the epoxidation reaction must be accomplished at least in part by an oxidant that cannot hydroxylate camphor.



A similar competitive study was performed with P450 BM3 and its T268A mutant [52] Reaction of *N*,*N*,-dimethyl-*p*-thioanisole results in either oxidation of sulfur to give the sulfoxide or oxidation of a methyl group to give, ultimately, N-dealkylation. For the wild-type enzyme, the ratio of sulfoxide formation to N-dealkylation was 15, whereas the ratio in the mutant was 60. The results indicate that two oxidants exist, and that the species responsible for preferential sulfur oxidation was formed to a greater extent in the mutant. Furthermore, a study of kinetic isotope effects (KIEs) in oxidation of this substrate demonstrated that the change in product ratio was due to two distinct oxidants and not to differential substrate binding. Specifically, no isotopically sensitive branching was found when the methyl groups of the substrate were substituted with deuterium. This phenomenon was demonstrated not to result from a small intrinsic KIE for the methyl group oxidation nor from slow interchange of the positions in the substrate, and the only explanation that remained was that two distinct oxidants were involved in the reactions.

Kinetic isotope effect studies of oxidations of probes **2a** and **2b** also support the conclusion that two distinct electrophilic oxidants are formed in P450 enzymes. In those studies, the methyl groups of the probe substrates were substituted with two deuterium atoms to permit intramolecular KIE studies [53–55]. In addition, mixtures of probe **2b** containing no deuterium or tri-deuterated at the methyl group were studied to determine intermolecular KIEs [55]. The KIEs for the rearranged and unrearranged products from **2b** differed by a relatively constant amount, requiring that at least two isotopically sensitive reactions be involved in the reactions; these could be two competitive reactions or consecutive isotopically sensitive reactions [55]. For probe **2a**, the differences in KIEs for the rearranged and unrearranged products appeared to require three isotoptically sensitive reactions,

which could be explained by different KIEs in two competing reactions and a consecutive KIE in a sequential reaction [54]. Importantly, in the intermolecular KIE studies with probe **2b**, some type of KIE unmasking reaction was required, and the extent of unmasking correlated with rates of release of hydrogen peroxide for the enzymes [55]; the significance of this observation is discussed below.

Whereas differences in reactivities of shunted versus natural P450 reactions must be evaluated with care, one recent study found several variations in reactivities that strongly implicate multiple electrophilic oxidants [56]. For example, the use of buffered carbonate solutions instead of phosphate solutions resulted in reductions in the rates of O-dealkylation reactions but did not reduce rates of N-dealkylation reactions, and shunted reactions displayed much larger intramolecular kinetic isotope effects in an N-demethylation reaction than found for NADPH-supported reactions.

Studies designed to probe the mechanisms of P450-catalyzed oxidation reactions also have provided circumstantial evidence that a second electrophilic P450 oxidant exists. Mechanistic probes are substrates that provide evidence for production of a transient by rearrangements that are observed in the products of the reactions. Probes were designed to distinguish between radical and cationic intermediates, and oxidation reactions of these probes by several hepatic enzymes were studied, including the P450 Δ2B4 and P450 Δ2E1 wild-type and mutant pairs discussed above [57–60]. Evidence for production of some type of cationic intermediate was found in all of these studies as determined by the formation of cationic rearrangement products. The cationic intermediates were thought to be protonated alcohols, the products that would be formed by insertion of OH^+ into a C—H bond. Such a reaction would not be possible for the iron-oxo species, but it could occur if hydroperoxy-iron or iron-complexed hydrogen peroxide was an alternate oxidant.

What are the electrophilic oxidants in P450 enzymes?

The consensus electrophilic oxidant, iron-oxo

For many years, the consensus view has been that one electrophilic oxidant in P450 enzymes is a species similar in character to Compound I of the peroxidase enzymes, an iron(IV)-oxo porphyrin radical cation species; this is the species that is usually called "iron-oxo" in the P450 community. In peroxidase and catalase enzymes, Compound I can be observed [21], and it is possible to generate similar species from model compounds [23]. It seems highly likely that one of the P450 oxidants has the molecular formula of a Compound I species, but it is possible that the electronic structure is not similar to that of Compound I.

Although the active oxidant reacted too rapidly for detection in the $P450_{cam}$ cryoreduction studies [17,18], one can deduce that cleavage of the O—O bond occurred before the hydroxylation step. In the reaction of the wild-type enzyme, the first-observed product state was alcohol product tightly bound to the iron atom, and this product state relaxed to a more loosely bound alcohol on further warming [18]. The production of tightly bound alcohol requires that the O—O bond in the activated complex, cleaved before the oxidation reaction occurred, and this establishes the composition of the iron-oxo species as being the same as that of Compound I. Nonetheless, the reactivity of the iron-oxo species appears to be much greater than that of peroxidase Compound I and its analogues. The oxidation of camphor to 5-hydroxycamphor is an energetically difficult reaction involving functionalization of an unactivated C—H bond. If the rate constant for decay of the $P450_{cam}$ oxidant is greater than $1000\ s^{-1}$ at ambient temperature as indicated from the cryoreduction studies [17,18], then the activation energy for the reaction would be of the order of 12 kcal/mol assuming a small entropy term for the pseudo-first-order reaction. In contrast, an authentic Compound I species generated by the photo-oxidation method reacted with ethylbenzene at ambient temperature with an activation energy that was greater than 11 kcal/mol in the second-order reaction [33]. Given that the aliphatic C—H bond in camphor will be about 12 kcal/mol stronger than the benzylic C—H bond in ethylbenzene, the oxidant in P450 appears to be much more reactive than the Compound I species.

One possible explanation for the paradox is that the iron-oxo species in the P450 enzyme is actually a high energy electronic isomer of Compound I, an iron(V)-oxo species, that reacts in oxidation reactions before it relaxes to the ground state. The first-formed species in the O—O cleavage reaction should be an iron(V)-oxo neutral porphyrin species, which is a high energy isomer of an iron(IV)-oxo porphyrin radical cation [61]. If an intrinsic barrier for the electron transfer isomerization reaction exists, then this species might react as an oxidant before it isomerizes to the lower energy Compound I species. Indeed, it appears as if our new photochemical ligand cleavage approach produced a corrole-iron(V)-oxo species that reacted before it isomerized to an iron(IV)-oxo corrole radical cation [37]. Because a corrole ligand is a trianion as opposed to a porphyrin dianion, corrole complexes should be less reactive than the corresponding porphyrin complex, but the corrole-iron-oxo species formed photochemically reacted with ethylbenezene more than 100 times faster than the corresponding Compound I analogue [37]. Our estimate is that the corrole-iron-oxo was about six orders of magnitude more reactive than expected for an iron(IV)-oxo corrole radical cation, or the activation energies for its oxidation reactions were about 8 kcal/mol less than what one might predict for the ground state species.

The other electrophilic oxidant

Many researchers believe that the second electrophilic oxidant in the P450 enzymes is the hydroperoxy-iron spe-

cies or, more likely, iron-complexed hydrogen peroxide, and various lines of evidence support this conclusion [10]. Perhaps, the most compelling is that this model provides for distinct oxidants that are not likely to equilibrate rapidly and likely cannot equilibrate at all. Computational studies suggest that heterolytic cleavage of the O—O bond in the hydroperoxy-iron species is concomitant with protonation on the distal oxygen and that the energy release in the cleavage reaction is quite large [19].

The importance of the energy barrier for hydration of the iron-oxo species, a process that would regenerate the hydroperoxy-iron species, cannot be overemphasized. If the computational result [19] is correct, then it is not possible for the iron-oxo species to return to hydroperoxy-iron or to participate in the uncoupling reaction that releases hydrogen peroxide. That feature provides strong support for hydroperoxy-iron or iron-complexed hydrogen peroxide acting as an oxidant. For example, the $P450_{cam}$ T252A mutant releases hydrogen peroxide essentially exclusively when camphor is the bound substrate [17,18,45,51], which indicates that no iron-oxo species was produced in the mutant. Nonetheless, the mutant epoxidized alkene substrates **7** and **8** with good efficiency, even though it did not hydroxylate camphor [51].

A similar situation exists for intermolecular KIE studies. If an isotopically sensitive reaction follows the slow step in an enzyme-catalyzed process, then the KIE in an intermolecular KIE study can be completely masked. In that case, the enzyme is committed to complete the reaction after the slow step even if the isotopically sensitive reaction is slowed by deuterium substitution, and no kinetic isotope effect would be observed (i.e., $k_H/k_D = 1$). In the P450 enzymes, the second electron transfer step is slow and irreversible, and this step will display little if any kinetic sensitivity to the isotopic substitution of the substrate. Therefore, a P450-catalyzed oxidation of substrate with deuterium atoms substituted for reactive hydrogen atoms might be expected to show no experimental KIE, or the KIE could be fully "masked". However, if a different partitioning reaction competes with the isotopically sensitive reaction, then the partitioning reaction can partially "unmask" a masked KIE, even if the reaction experiencing the KIE is fast relative to another step in the overall process.

In the KIE studies with probe **2b** [55], the intrinsic KIEs could be determined from intramolecular KIE experiments, and the degree of unmasking in intermolecular KIE studies could be determined. Five P450 enzymes were studied in that work including the wild-type and mutant pairs of P450 Δ2B4 and P450 Δ2E1. With probe **2b**, the only products detected were those from oxidation of the methyl group. Therefore, the observed unmasking in intermolecular KIE studies (competitions between methyl-d_0 and methyl-d_3 substrates) requires that some alternate reaction of the oxidant be competitive with the oxidation reaction, and the extent of unmasking was found to correlate at the qualitative level with the relative amounts of hydrogen peroxide released by the same enzymes when they were activated in the absence of substrates [55]. If hydrogen peroxide release was the unmasking reaction, then the oxidant apparently must have been iron-complexed hydrogen peroxide.

Mechanistic probe studies that implicated the formation of cationic intermediates in P450-catalyzed hydroxylation reactions [57–60] also implicate hydroperoxy-iron or iron-complexed hydrogen peroxide as an intermediate because the insertion of OH^+ into a C—H bond is the most likely source of the cationic intermediate. It is important to note that initial production of a radical followed by oxidation of the radical to a cation can be excluded in some of these mechanistic probe studies because radical intermediates would have been diverted to different rearranged products via ultra-fast radical rearrangements [57–60].

Evidence for iron-complexed hydrogen peroxide as an oxidant remains circumstantial, but protonated hydrogen peroxide has been known to be a powerful oxidizing agent for nearly half a century [62]. The hydroperoxy-iron is computed not to be a powerful oxidant [63], but computational investigation of oxidation reactions by protonated hydrogen peroxide suggests that Lewis acid-catalyzed oxidations by hydrogen peroxide will be facile [64]. Effectively, the iron complex of hydrogen peroxide can be seen as a complex of OH^+, and such complexes have been shown to be extremely powerful oxidants in the gas phase [65]. Hydroperoxy-iron or iron-complexed hydrogen peroxide is known to be the oxidant in heme oxygenase, the enzyme that degrades porphyrin rings by oxidation of the macrocycle [66], providing a good model for the P450 enzymes. Future studies directed at the production, detection, and kinetic studies of iron-complexed hydrogen peroxide will be important for evaluating the potential for the species to serve as a P450 oxidant.

Alternative models for the second electrophilic oxidant exist. It is possible in principle that two spin states of the iron(IV)-oxo radical cation species (or Compound I) are similar in energy and react differently in various reactions [67], and this model has been presented to rationalize various experimental observations in P450 chemistry [68]. Unfortunately, there is no universal agreement that two spin states of Compound I are close in energy, with other contemporary computational approaches concluding that they are not [69–71]. In addition, no experimental evidence for two reactive states is available from studies of enzymes and models where Compound I can be detected. The two-state model also does not provide a reasonable pathway for formation of cation-derived products from mechanistic probe studies. Finally, the evidence for distinct oxidants that do not equilibrate on the time scale of the oxidation reactions is overwhelming, and it is not clear that two spin states of Compound I should have a significant barrier for interconversion, which requires an electron spin flip.

Another computational alternative for the second electrophilic oxidant is a species best described as the iron complex of water oxide. Water oxide (H_2O—O) is a tautomer of hydrogen peroxide and possible oxidant [72], and iron-

complexed water oxide ($Fe—H_2O—O$) is predicted to be a powerful oxidant that should react with low selectivity and small kinetic isotope effects [73]. The main objections to this model describing the second electrophilic oxidant in P450 would be the inherent high energy necessary to access the complex and the fact that it would insert an oxygen atom instead of OH^+.

In conclusion, the highly reactive electrophilic oxidants in cytochrome P450 enzymes have eluded detection for many years but continue to attract attention for scientific and practical purposes. Strong evidence supports the contention that two distinct types of electrophilic oxidants are formed in P450 enzymes. It is possible that the reactivity of the P450s towards specific substrates can be tuned by the populations of these distinct oxidants, and this feature might be an important point in regard to drug metabolism [74]. It seems likely that new experimental methods currently being developed will reveal more detailed information about these oxidants within the next few years.

Acknowledgment

Studies in the authors' laboratory were supported by a grant from the National Institutes of Health (GM-48722).

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