

Oxidizing Intermediates in P450 Catalysis: A Case for Multiple Oxidants

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Abstract

Cytochrome P450 (P450 or CYP) catalysis involves the oxygenation of organic compounds via a series of catalytic intermediates, namely, the ferric-peroxo, ferric-hydroperoxo, Compound I (Cpd I) and $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ intermediates. Now that the structures of P450 enzymes have been well established, a major focus of current research in the P450 area has been unraveling the intimate details and activities of these reactive intermediates. The general consensus is that the Cpd I intermediate is the most reactive species in the reaction cycle, especially when the reaction involves hydrocarbon hydroxylation. Cpd I has recently been characterized experimentally. Other than Cpd I, there is a multitude of evidence, both experimental as well as theoretical, supporting the involvement of other intermediates in various types of oxidation reactions. The involvement of these multiple oxidants has been experimentally demonstrated using P450 active-site mutants in epoxidation, heteroatom oxidation and dealkylation reactions. In this chapter, we will review the P450 reaction cycle and each of the reactive intermediates to discuss their role in oxidation reactions.

Keywords

Cytochrome P450 • Reaction cycle • Compound I • Ferric-peroxo • Ferric-hydroperoxo • Reactive intermediates • Multiple oxidants

Abbreviations: Cpd I or Cpd II compound I or II of a heme enzyme, an $\text{Fe}^{\text{IV}}=\text{O}$ radical cation species, CPO chloroperoxidase, CYP or P450 cytochrome P450, CYP119A1 orphan P450 from *Sulfolobus acidocaldarius*, CYP2B4 phenobarbital-inducible rabbit liver microsomal P450 enzyme (P450LM2), CYP2E1 alcohol-inducible rabbit liver microsomal P450 enzyme (P450LM3), CYP51A1 lanosterol 14 α -demethylase, ENDOR electron-

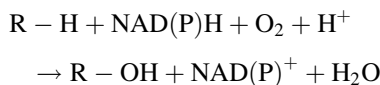
nuclear double resonance, EPR electron paramagnetic resonance, ES enzyme-substrate complex, FAD flavin adenine dinucleotide, FMN flavin mononucleotide, heme iron protoporphyrin IX (heme-b), KIE kinetic isotope effect, NOS nitric oxide synthase enzyme, P450BM3 fatty acid hydroxylating P450 enzyme from *Bacillus megaterium*, P450CAM camphor-hydroxylating P450 enzyme from *Pseudomonas putida*, RH substrate, ROH oxidized substrate.

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2.1 Introduction

Cytochrome P450 (P450 or CYP) enzymes are heme-thiolate ligated monooxygenases that are ubiquitous in the biological kingdom and catalyze a variety of oxidation reactions covering a wide range of substrates [1, 2]. Hemeproteins are classified as P450s when their $\text{Fe}^{\text{II}}\text{--CO}$ complex has a maximum Soret absorbance at 450 nm [3]. P450s were discovered five decades ago because of their important role in xenobiotic clearance from the human body, but the interesting nature of their chemistry has attracted attention from chemists, biochemists, biophysicists, structural biologists and now even biotechnologists. Oxygen activation is central to life as spin forbiddance makes ground-state triplet molecular oxygen by itself inert toward organic molecules [4]. Living beings therefore use enzyme systems for oxygen activation to perform biologically important reduction-oxidation (redox) reactions. P450s are one of the metal-containing oxygenases that utilize molecular oxygen to stereo- and regio-selectively oxygenate substrates under physiological conditions. While P450s are capable of diverse reactions, they are in fact mostly known for their ability to catalyze the oxidation of inert substrate C–H bonds under physiological conditions. To put into perspective, the bond strength of a typical secondary C–H bond is about $101 \text{ kcal mol}^{-1}$ [5]. Present understanding of the P450 catalytic mechanism has been developed over the course of the last four decades by advances in genomics, molecular biology and spectroscopy [6]. Comparison with analogous heme oxygen activation systems has also greatly contributed to our current understanding of its mechanism. Knowledge of the P450 intermediates is now being used for development of efficient inorganic catalysts for laboratory and commercial use [7]. Alternatively, in biotechnological setups, P450s are being modified to catalyze stereoselective oxidation reactions [8]. Oxidation of substrates by P450 enzymes can be summarized by the following equation:



The catalytic mechanism of P450s occurs in a cyclic fashion involving systematic generation of intermediates, some of which are transient [9]. Electrons for this oxidation reaction are provided by NAD(P)H and are shuttled to the P450 active site with the aid of reductase enzymes. Water molecules in the active site donate protons. Based on associated redox systems, P450s can be classified as type I or type II as shown in Fig. 2.1 [10]. Type I P450s are mainly the mammalian mitochondrial and bacterial P450s, which utilize a flavoprotein to transfer electrons to P450s via an intervening iron-sulfur cluster protein (Fe_2S_2). Type II P450s are the mammalian xenobiotic-metabolizing enzymes that receive electrons via FAD- and FMN-containing reductases. There are certain exceptions such as P450BM3 in which the heme and FMN/FAD-containing reductase domains are part of a single polypeptide that functions as a self-sufficient unit [11, 12].

2.2 The P450 Catalytic Cycle

P450CAM (CYP101A1) is a bacterial P450 enzyme from *Pseudomonas putida* that converts 1R-(+)-camphor to 5-*exo*-hydroxycamphor. This enzyme is soluble, thus easy to purify and was the first P450 whose crystal structure was solved [13]. Since then, its structure has been extensively studied and it has served as a prototype for structure-function studies of the entire P450

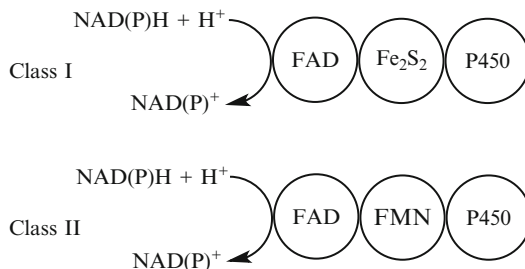


Fig. 2.1 Electron transfer chains in class I and class II of P450 enzymes (See Ref. [10])

family. The reaction cycle for P450CAM also holds true for the entire P450 family. The putative catalytic mechanism of P450s in which the substrate RH is oxidized to R-OH in a series of steps is shown in Fig. 2.2. The catalytic cycle begins with the reversible substrate binding to the water-coordinated low-spin ($S = 1/2$) resting state of the ferric enzyme (Fig. 2.2, I). Substrate binding causes displacement of water as the sixth ligand to the heme with formation of the high-spin ($S = 5/2$) pentacoordinate enzyme-substrate adduct (Fig. 2.2, 2), resulting in the shift of the midpoint redox potential of the heme to a more positive value (from -330 to -173 mV) [14]. This sharp shift in the reduction potential enables electrons to flow from NAD(P)H to the P450 enzyme via an associated reductase. The first electron generates the reduced ferrous-substrate adduct (Fig. 2.2, 3). Subsequent binding of dioxygen generates the oxyferrous complex or a resonance-stabilized ferric-superoxide complex $\text{Fe}^{+3}\text{-OO}\cdot^-$, a η^1 superoxide radical anion coordinated to the ferric heme center with an unpaired electron on the terminal oxygen atom (Fig. 2.2, 4) [15]. The second electron from NAD(P)H then reduces the oxyferrous complex resulting in the $\text{Fe}^{+3}\text{-OO}^-$ (Fig. 2.2, 5) ferric-peroxo intermediate. This is also the rate-limiting step. Protonation of this intermediate leads to the $\text{Fe}^{+3}\text{-OOH}$ (Fig. 2.2, 6) ferric-hydroperoxo intermediate, also known as Compound 0 (Cpd 0). A second protonation of this intermediate leads to O-O bond heterolysis forming the transient and highly reactive porphyrin π radical cation ferryl complex (Fig. 2.2, 7) known as Cpd I. Cpd I derives its name from the analogous high-valent Cpd I species of heme peroxidases [16, 17]. According to the now well-accepted mechanism for hydrocarbon hydroxylation, Cpd I abstracts a H atom from the substrate resulting in a ferryl hydroxyl intermediate (Fig. 2.2, 8) known as protonated Compound II (Cpd II) and a substrate radical. In what is known as the oxygen rebound, the hydroxyl moiety on the iron combines with the substrate radical to give the hydroxylated product, while the enzyme returns to its resting ferric state.

In addition to the normal catalytic pathway, there are three uncoupling reactions within the cycle that lead back to the enzyme-substrate adduct without any product formation. The first is the auto-oxidation of the oxyferrous enzyme with simultaneous generation of a superoxide anion (Fig. 2.2, I). In the second shunt pathway, the hydroperoxo anion dissociates from the ferric-hydroperoxo intermediate (Fig. 2.2, II). Heterolytic cleavage of the O-O bond is critical for Cpd I formation. Incorrect protonation at the distal oxygen generates the $\text{Fe}^{+3}\text{-(H}_2\text{O}_2\text{)}$ intermediate (Fig. 2.2, 9) followed by dissociation of hydrogen peroxide without substrate turnover. This pathway is often seen in the active-site alcohol-alanine mutant [18]. In the oxidase shunt (Fig. 2.2, III), the ferryl intermediate is reduced to water in lieu of substrate oxidation. In an alternative pathway to the normal reaction cycle, the enzyme can be turned over without the nucleotide-reducing equivalents via the peroxide shunt (Fig. 2.2, IV). Cpd I can be generated from this pathway using oxygen atom donors such as peracids, peroxides and iodosobenzene [19–21].

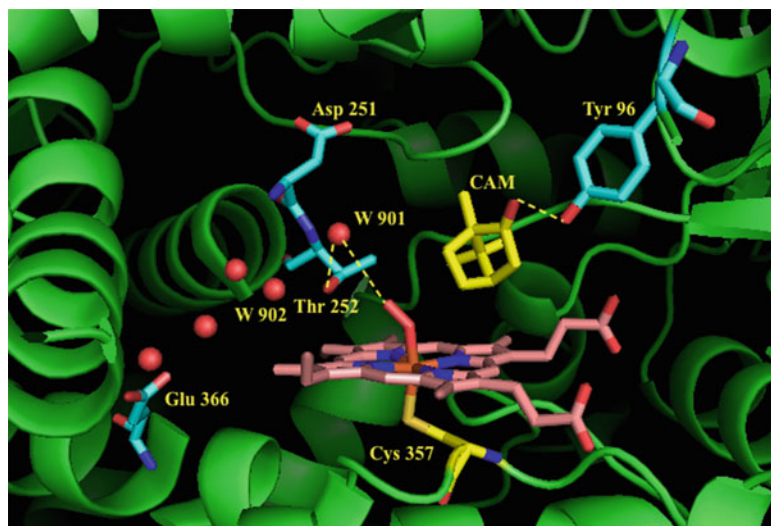
2.3 Nature of the P450 Active Site

Currently, there are over 20,000 known CYP genes, a summary of which can be found here, (<http://dmelison.uthsc.edu/CytochromeP450.html>). Notably, P450 enzymes share their catalytic capabilities with certain heme-containing enzymes such as catalases, peroxidases, oxygenases, etc. but all these enzymes have remarkably different structures. The architecture of the active site in P450s plays a crucial role in the sequential generation of intermediates in the P450 catalytic cycle. For a complete understanding of P450 monooxygenation chemistry, the majority of research has focused on the factors influencing electron delivery and dioxygen binding to the heme iron, proton addition to the bound dioxygen in the distal pocket of the heme and cleavage of the O-O bond. Residues most important for oxygen activation are the heme proximal cysteine ligand (Cys357 in P450CAM) and the acid-alcohol pair

group of camphor to orient the site of oxygenation above the heme. The water molecules Wat901 and Wat902 seen in the active site are implicated as the source of protons required for formation of the active $\text{Fe}^{\text{IV}}=\text{O}$ species. Accordingly, the Thr252 H-bonds to Wat901, which serves as the H-bond donor to the distal oxygen atom of the heme-bound dioxygen in P450CAM.

The heme in P450 enzymes is of the heme-b type, where the iron protoporphyrin-IX is covalently linked to the protein backbone via a Fe-S

Fig. 2.3 Active site of camphor-bound oxy-P450CAM constructed using PDB file 1DZ8 [9]



bond to cysteine. In the case of P450CAM, Cys357 serves as the cysteine residue as seen in Fig. 2.3. The proximal cysteine thiolate ligand is indispensable for P450 catalytic activity and mutation of the cysteine residue leads to loss of activity [23]. In the P450 catalytic cycle, one electron reduction of the oxyferrous state followed by protonation of the distal oxygen leads to the ferric-hydroperoxo intermediate. A second protonation of the ferric-hydroperoxo intermediate followed by heterolytic cleavage of the O—O bond leads to formation of Cpd I, which is the primary oxidant in the cycle. Maintaining the cysteine as a thiolate anion on the proximal side of the heme at the same time as the iron in the ferrous state is crucial for Cpd I generation [24]. The thiolate anion is stabilized by H bonds from the protons of the adjacent residues, Leu358 (3.5 Å), Gly359 (3.3 Å) and Gln360 (3.3 Å). Mutation of these residues led to distortion in H-bonding and an increase in the uncoupling of the ferric-hydroperoxo intermediate [25, 26].

Dawson and coworkers suggested that the polarizable nature of the cysteine thiolate anion ligand provides a strong ‘push’ of electron density via the heme onto the O—O bond of the ferric-hydroperoxo intermediate, thus promoting

heterolytic O—O bond cleavage [27, 28]. Furthermore, the electron-donating nature of the thiolate ligand also helps to stabilize the resulting Cpd I intermediate. This result is similar to the effect seen in cytochrome *c* peroxidase that contains a partially deprotonated proximal histidine ligand, wherein the imidazolate ‘push’ in concert with a ‘pull’ from the conserved distal His-Arg amino acids lead to heterolytic cleavage of the O—O bond to generate Cpd I [29].

2.3.2 Role of the Acid-Alcohol Pair in Oxygen Activation

An acid-alcohol pair that is highly conserved in almost all P450 enzymes aids oxygen activation in the distal heme pocket. The alcohol in most cases is threonine or serine and the acid can be aspartate or glutamate. In the case of P450CAM, these residues are Asp251-Thr252. Given their highly conserved nature and proximity to the heme-dioxygen binding site, the role of this acid-alcohol pair in catalysis has been examined in several mutagenesis studies. Specifically, the role of Thr was investigated by changing the residue to Ala. In P450CAM, the Thr252Ala

mutant was almost completely uncoupled, leading to normal NADH and O₂ consumption but essentially no product formation [18, 30]. Based on this result in P450CAM and other P450s as well [31, 32], the alcohol residue is thought to stabilize water molecules in the active site by H-bonding during substrate oxygenation [18, 30, 33]. Ishimura and coworkers demonstrated that the uncoupling reaction is promoted when the Thr in P450CAM is mutated to a Ser or Asn, thereby ascertaining the role of Thr in stabilizing the H-bonding network in the distal pocket and controlling proton delivery to the distal oxygen of bound dioxygen [22]. The Thr252Ser and Thr252Asn mutant enzymes retained more than half of the hydroxylating capability of the enzyme. The Thr252 residue also participated in H-bonding with the distal oxygen of the oxyferrous-P450 complex in P450CAM [9]. The high resolution crystal structure of the P450CAM Thr252Ala mutant showed a clearly perturbed H-bonding network and excess water molecules in the active site [33]. It is thought that this perturbation leads to uncoupling due to incorrect delivery of the second proton to the proximal oxygen [34]. Just as in P450CAM, the Thr268 in P450BM3 has been shown to play an important role in sustaining the proton delivery pathway from the bulk solvent to the dioxy-bound heme. Mutation of Thr268 to Ala also leads to uncoupling followed by reduced substrate oxidation [31, 35].

Unlike the alcohol residue, the acid residue has an important role in electron transfer following oxyferrous intermediate formation. In P450CAM, the mutagenesis of Asp251 to Asn leads to decreased turnover in the mutant enzyme rather than uncoupling [36, 37]. The Asp251Asn mutant displays an increased kinetic solvent isotope effect compared to the wild-type enzyme and a directly linear correlation to NADH consumption on bulk proton concentration, indicating that the proton delivery pathway has been modified in the Asp251Asn mutant [36]. Structural analysis of the Asp251Asn mutant reveals significant changes in the active site. The Asn251 and Lys178 side chains rotate away from the active site and the

Asn251 H-bonds to Asp182, causing open access to the heme [36]. The flexibility of the Asp251 side chain stabilized by electrostatic bonding plays an important role in dioxygen scission in P450CAM and suggests a similar role for the conserved acid functionality in other P450 enzymes.

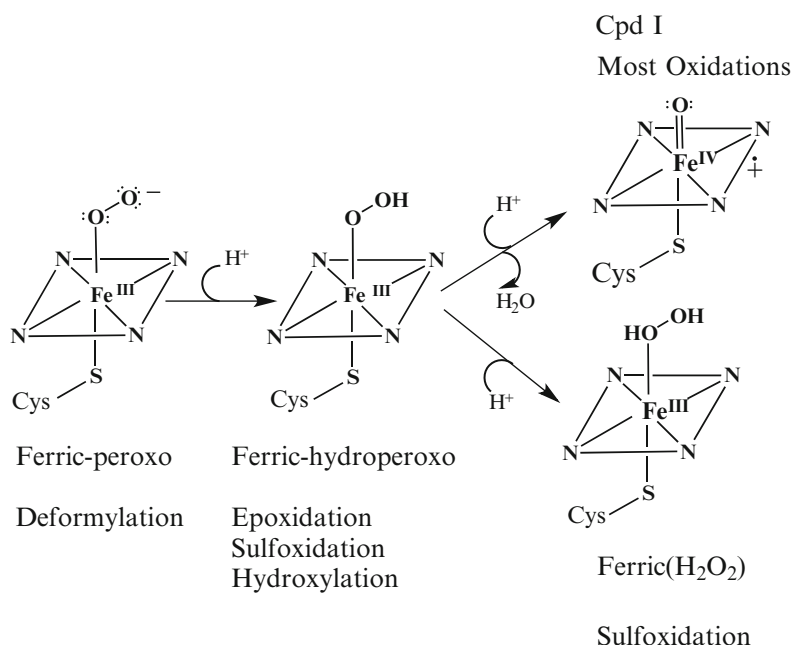
2.4 Multiple Oxidants in P450 Catalysis

Although the P450s are a single family of enzymes, the wide variety of substrates oxidized by P450 is quite astounding. The catalytic cycle of P450s has been well established based on P450CAM as the prototype. The key catalytic intermediates have been detected and well characterized. The ferryl Cpd I intermediate, initially thought to be too short lived to detect, has now been well characterized [38]. Despite the fact that this intermediate has not been detected in the normal P450 catalytic cycle, it has been observed in the peroxide shunt pathway and there is little doubt about its involvement in substrate oxidation. While the ferryl Cpd I intermediate is thought to be the oxidant of choice in most oxidation reactions, the nature of certain catalytic intermediates and comparison with analogous reactions catalyzed in other enzyme systems make it difficult to deny the existence of multiple oxidizing species in the catalytic cycle. As seen in Fig. 2.4, several reaction intermediates other than Cpd I are thought to be capable of catalyzing some oxidation reactions depending on the type of substrate.

2.4.1 The P450 Dioxygen Complex

In P450 enzymes, binding of dioxygen to the ferrous heme traps O₂ for substrate oxidation. This generates the ferrous-dioxygen complex (Fe⁺²-OO), which is in resonance with the ferric-superoxide complex (Fe⁺³-OO^{•-}) (Fig. 2.2, 4). The binding constant of dioxygen to P450CAM is $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at 4 °C [39]. The oxyferrous complex of P450s is not as

Fig. 2.4 Multiple P450 oxidants and types of reactions catalyzed



stable as that of oxygen carrier proteins. In P450CAM, it is moderately stable in the presence of camphor and auto-oxidizes back to the ferric state at the rate of 0.01 s^{-1} at room temperature [39, 40]. The oxyferrous-P450 complex is similar to that generated in many analogous heme proteins such as myoglobin, hemoglobin, CPO, NOS, etc. [41–44]. The oxyferrous stretching band of oxyferrous P450CAM as determined by resonance Raman spectroscopy is $1,141 \text{ cm}^{-1}$, which is typical for superoxide complexes [45]. Using cryocrystallization, the oxyferrous complex of P450CAM was determined at atomic resolution. A representative figure is shown in Fig. 2.5 [9].

The oxygen is coordinated to the heme iron in a slightly bent fashion with the Fe-O-O angle being 142° . The oxyferrous complex is stabilized with the aid of a H bond between the distal oxygen and hydroxyl of the nearby Thr252 residue. While the oxyferrous complex by itself is not known to catalyze any oxidation reaction, its formation is necessary to generate the subsequent catalytic intermediates in the reaction cycle.

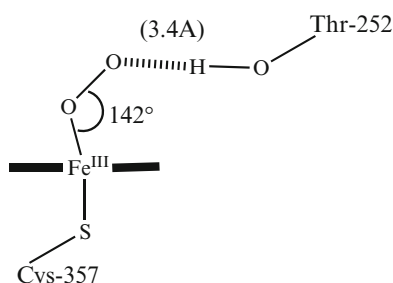
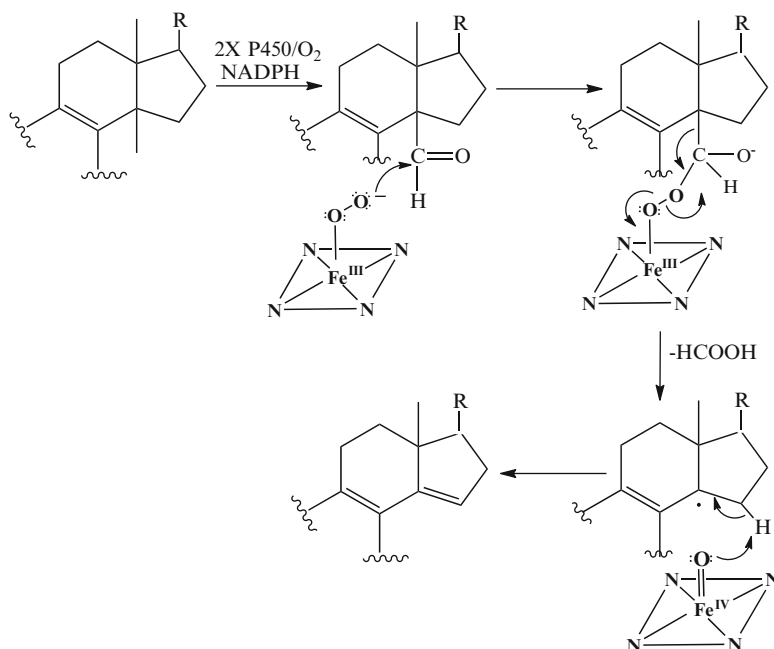


Fig. 2.5 Oxyferrous complex of P450-CAM determined using PDB file 1DZ8 [9]

2.4.2 Ferric-Peroxo Intermediate as a Nucleophilic Oxidant

Akhtar and coworkers first proposed a role for the ferric-peroxo intermediate in the final step of oxidative deformylation catalyzed by lanosterol 14α -demethylase [46, 47]. The enzyme catalyzes the oxidative deformylation of lanosterol, concomitantly forming olefin in three oxidative steps, and each step utilizing a single equivalent of NADPH and O_2 as seen in Fig. 2.6. The final

Fig. 2.6 Mechanism of oxidative deformylation catalyzed by lanosterol 14 α -demethylase (See Ref. [47])



step results in cleavage of the C14–C32 bond with stereoselective removal of 15 α -H, resulting in the formation of a 14,15 double bond and release of formic acid. The proposed mechanism involves homolytic cleavage of the O–O bond in a peroxy-aldehyde adduct to give an alkoxy free radical that decays to the olefin as a result of H abstraction by the simultaneously-created ferryl species.

Similar mechanisms have also been proposed for demethylation in estrogen formation by aromatase (CYP19A1) and in the CYP17A1-catalyzed C–C bond scission of 17 α -hydroxyprogesterone [47, 48]. The formic acid formed in these P450-catalyzed oxidative deformylations has been shown to retain the original carbonyl oxygen and hydrogen as well as an atom from molecular oxygen, clearly pointing to the involvement of the ferric-peroxy intermediate in the mechanism. In the CYP17A1 (17 α -hydroxylase-17,20-lyase)-catalyzed reaction, oxygen labeling experiments also point to homolytic scission of the O–O bond in the peroxy-substrate adduct [49]. Vaz and coworkers analyzed the elimination reaction of the aliphatic

aldehyde in the rabbit drug-metabolizing CYP2B4 enzyme. These reactions also seem to corroborate the involvement of peroxy anion-supported homolytic scission, followed by fragmentation of the adduct into a carbon radical and a formyl species that yields olefin products [50]. Further evidence supporting this mechanism is found when the carbon radical formed during the reaction inactivates the heme in P450 [51, 52].

The electrophilic nature of aldehydes makes them easily susceptible to attack from the nucleophilic peroxy anion. Such an example of nucleophilic attack is also seen in nitric acid synthase (NOS) (Fig. 2.7). NOS is a heme-containing enzyme that catalyzes the conversion of arginine to *N*-hydroxyarginine and then to citrulline and nitric oxide. The second step of this reaction has been proposed to involve nucleophilic addition of the ferric-peroxy species to the –C=NOH bond of the substrate [53]. Inorganic metalloporphyrins mimicking the ferric-peroxy intermediate have also been shown to catalyze the deformylation, as well as epoxidation, of α , γ -unsaturated carbonyl groups [54, 55].

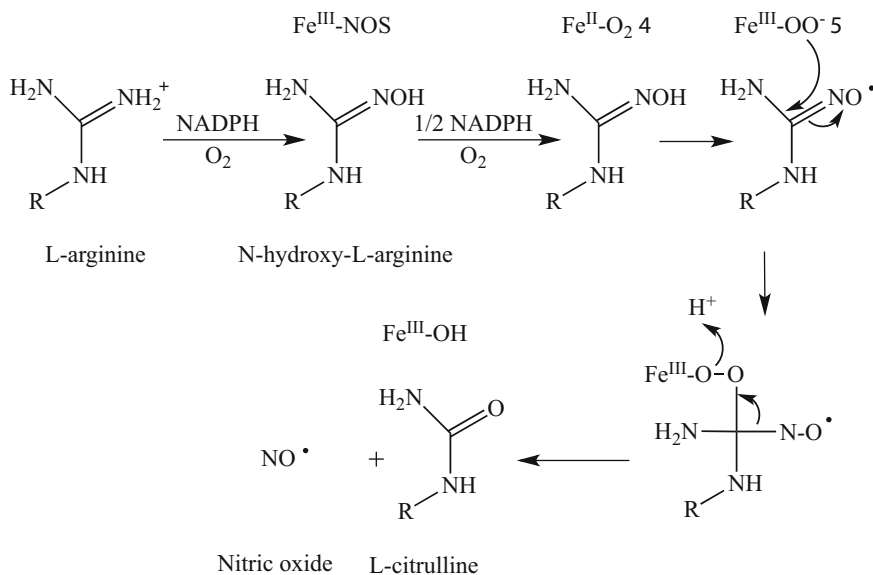


Fig. 2.7 Mechanism of oxidative deformylation catalyzed by NOS (See Ref. [53])

2.4.3 The Ferric-Hydroperoxo Intermediate as an Electrophilic Oxidant

The ferric-hydroperoxo intermediate has been proposed as an oxidant in catalysis involving nucleophilic substrates. However, unlike in the case of the ferric-peroxo intermediate, the hypothetical involvement of the ferric-hydroperoxo species in oxidative catalysis is not supported by solid evidence. Given the electrophilic nature of most substrates oxidized by P450s, Cpd I is the clear favorite oxidant in these cases due to its high reactivity. The most compelling evidence for involvement of the ferric-hydroperoxo intermediate was demonstrated via substrate oxidation by active-site mutants in P450s. In P450CAM, the conserved Thr252 alcohol side chain was mutated to Ala and the resulting Thr252Ala mutant was unable to catalyze the hydroxylation of camphor. Instead, the mutant was highly capable of accepting electrons from the nucleotide cofactor to convert dioxygen to hydrogen peroxide, due to improper protonation to the proximal oxygen of the ferric-peroxo intermediate, thus leading to uncoupling (Fig. 2.2, II) [18, 30]. This mutant is, therefore, unable to form Cpd I but generates both

the ferric-peroxo and ferric-hydroperoxo intermediates. ENDOR spectroscopic analysis of the cryoreduced Thr252Ala mutant shows a buildup of the ferric-hydroperoxo intermediate at 77 K, annealing at high temperatures yield the ferric enzyme but no hydroxylated product [56, 57]. As such, the Thr mutant of several P450s has been used in the study of a number of electrophilic oxidation reactions.

Vaz, Coon and coworkers were the first to study the effects of the active-site Thr to Ala mutation in rabbit drug-metabolizing CYP2B4 and CYP2E1 enzymes using various alkene substrates (Fig. 2.8) [58]. The researchers observed a decrease in allylic oxidation of the alkenes. In contrast, the Thr303Ala mutation in CYP2E1 significantly increased the rates of epoxidation compared to the wild-type enzyme.

On the other hand, the corresponding Thr302Ala mutant of CYP2B4 demonstrated reduced rates of both allylic hydroxylation and epoxidation. Increased epoxidation versus allylic hydroxylation was observed in the CYP2E1 Thr303Ala mutant and was construed as evidence that epoxidation could be catalyzed by the ferric-hydroperoxo intermediate, while decreased Cpd I formation led to decreased

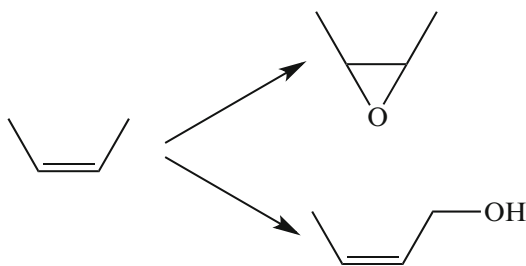


Fig. 2.8 Possible epoxidation reaction products in P450 enzymes

hydroxylation. However, failure to observe similar results with CYP2B4 along with high quantities of hydroxylated product made the data somewhat less reliable, apparently because Cpd I was still being generated to a significant extent.

Dawson and coworkers, in collaboration with the Sligar laboratory, studied the reactivity of the ferric-hydroperoxo intermediate in the Thr252Ala mutant of P450CAM using the alkene epoxidation reaction. Unlike the CYP2B4 and CYP2E1 enzymes, the Thr252Ala P450CAM mutant catalyzed the formation of less than 1 % of the hydroxylated product, thus providing a robust system to analyze the presence of a second oxidant, in this case the ferric-hydroperoxo intermediate. Both substrates were easily oxidized to epoxides (Fig. 2.9) at a rate that was ~15–20 % compared to that of wild-type P450CAM [59]. These results substantiated the work of Vaz and coworkers regarding the involvement of a second electrophilic oxidant.

Shaik and coworkers examined alkene epoxidation in the context of the two-state reactivity theory involving Cpd I [60, 61] and proposed that the ferric-hydroperoxo species is a sluggish oxidant compared to the highly reactive Cpd I species. The researchers concluded that the ferric-hydroperoxo species has a large energy barrier to overcome, whereas ferric-hydroperoxo conversion to Cpd I is barrierless [62]. P450 reactivity also appears to be influenced by H-bonding to the proximal thiolate ligand and polarity changes in the vicinity [62–64].

P450 reactivity is also proposed to be influenced by changes in the relative amounts

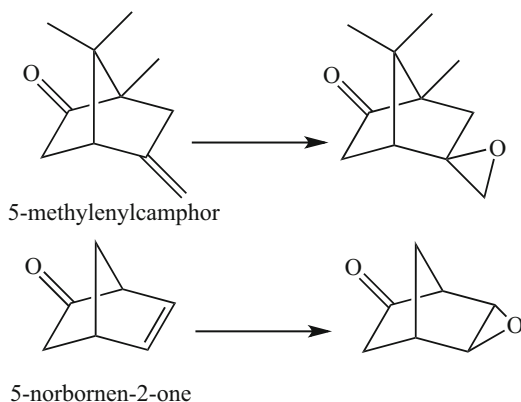


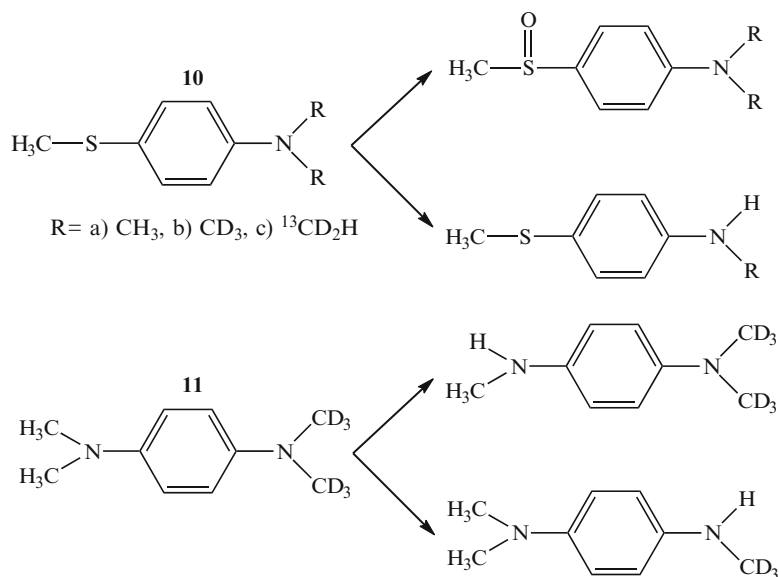
Fig. 2.9 Olefin epoxidation by P450 CAM (See Ref. [59])

of high-spin and low-spin Cpd I, rather than amounts of the ferric-hydroperoxo and Cpd I species [62]. However, the ‘two-state’ reactivity theory cannot clearly explain why the Thr252Ala mutant does not hydroxylate camphor. If the Cpd I oxidizing species only displays variation in the amounts of high-spin and low-spin states, the mutant enzyme should also have displayed significant hydroxylation activity.

The ferric-hydroperoxo intermediate has also been investigated as a potential oxidant in heteroatom oxidations. Jones and coworkers have looked particularly at sulfoxidation and *N*-dealkylation reactions utilizing P450BM3. Using clever substrate design and the Thr268Ala mutant, the researchers sought to test whether the two products originated from the same oxidant species (Fig. 2.10) [65]. Thus, substrate **10a** showed four times increased sulfoxidation activity compared to *N*-dealkylation activity. Next, the investigators used an isotopically sensitive *N*-dealkylation substrate, **10b**, to test the premise that due to a large kinetic isotope effect (KIE), sulfoxidation activity would be higher than *N*-dealkylation activity if both products arose from a single oxidant.

However, a negligible KIE was observed leading to several possible conclusions: (1) both products arose from different oxidants, (2) binding of substrate to the P450BM3 enzyme caused an interchange in the position of substituents, thereby changing their position in the catalytic

Fig. 2.10 Heteroatom oxidation by P450BM3
(See Ref. [65])



site, and (3) the inherent KIE of *N*-dealkylation was very small. Using substrate **11**, the researchers were able to demonstrate the rapid interchange of substituents at the end of the molecule. This result combined with an intramolecular KIE for substrate **10c** eliminated the last two possibilities, leading to the proposal that both the *N*-dealkylation and sulfoxidation products arose from two different oxidants. The authors suggested that *N*-dealkylation was a product of Cpd I-mediated oxidation while sulfoxidation likely involved the ferric-hydroperoxo intermediate, without eliminating the possibility that the data could result from different forms of the same active oxygen species, i.e. Cpd I. In accordance with the ‘two-state’ reactivity theory, where the low-spin and high-spin states form different enzyme-substrate (ES) complexes, the *N*-dealkylation and sulfoxidation reaction products can result from two non-interchangeable ES complexes [63, 65]. Watanabe has also proposed that the modified reactivity in the active-site threonine to alanine mutants may be a result of the altered water molecule network in the active site, which affects the H bonding of the Cpd I-ES complex [66]. This alteration can skew the ratios of the

low-spin and high-spin state of Cpd I, thereby affecting the mutant reactivity.

The ferric-hydroperoxo species has also been implicated as an oxidant in hydrocarbon hydroxylation reactions. Catalysis by the ferric-hydroperoxo species was proposed to involve a cationic protonated alcohol intermediate [67] (Fig. 2.11) as opposed to a radical intermediate formed in the radical rebound pathway (Fig. 2.2).

Newcomb and coworkers used ‘radical clock’ experiments to provide evidence for involvement of a cationic intermediate [67, 69–72]. In the first of such studies, the oxidation of *trans*-1-methyl-2-(4-trifluoromethyl)-phenylcyclopropane was examined. The substrate could be oxygenated either on the methyl group yielding methyl alcohol as an unrearranged product, or on the phenyl ring giving a ring-opened alcohol as a rearranged product. Using CYP2B1 as a biocatalyst, the substrate **12b** was shown to generate a ring-opened product, characteristic of a cationic rearrangement pathway (Fig. 2.12) [73].

Reaction of substrate **12a** with the CYP2B4 Thr302Ala mutant enzyme showed a mixture of products, both unrearranged and rearranged methyl oxidation as well as phenyl ring oxidation products. There was little difference in the ratio

Fig. 2.11 Proposed hydrocarbon hydroxylation mechanism by the ferric-hydroperoxo species (See Refs. [67, 68])

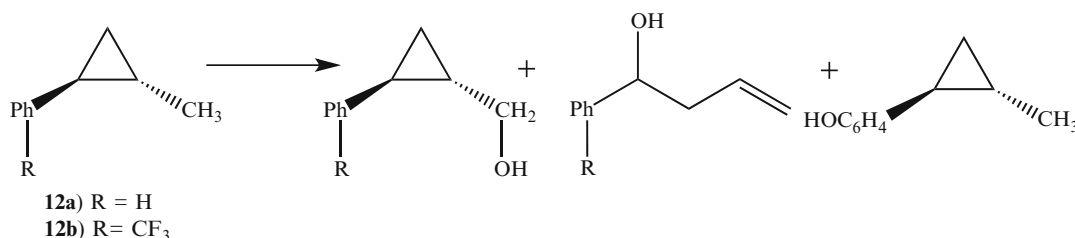
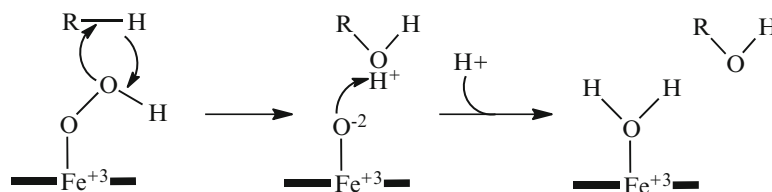


Fig. 2.12 Radical clock probes for mechanistic elucidation in CYP2B4 (See Ref. [73])

of rearranged to unrearranged product between the wild-type and mutant enzyme. However, a higher ratio of phenyl oxidation was seen in the mutant enzyme. The authors suggested that this result clearly indicated an alternative oxidant at play in the mutant enzyme that preferred the easier phenyl ring oxidation. To suppress phenyl ring oxidation in substrate **12b**, the phenyl ring was replaced with an electron withdrawing—CF₃ group, which produced an altered ratio between the ring-opened and ring-closed products. This intimated a change in the oxidant in the hydroxylation reaction for that substrate. While these results satisfyingly conveyed involvement of the ferric-hydroperoxo species in the hydroxylation of certain substrates, the species is indeed a sluggish oxidant whereas Cpd I appears to be the oxidant of choice in hydrocarbon hydroxylations [61]. While the ferric-hydroperoxo species appears in almost all heme-based oxygen activation enzymes, there are a few examples where it plays a primary role in substrate oxidation. For example, heme oxygenase catalyzes the oxidation of heme to biliverdin [74] and the first step of this oxidation involves an α -meso-hydroxylation of the heme group that is thought to be catalyzed by an electrophilic oxidant, most likely, the ferric-hydroperoxo intermediate [75–77].

2.4.4 Cpd I as the Most Powerful Oxidant

The mechanism of oxidation in P450s has been established by comparison with other heme-based oxygen activating enzymes as well as spectroscopic characterization of the reaction intermediates. P450s are similar to other metalloenzymes such as NOS and chloroperoxidase (CPO) in that they all have heme coordinated to a cysteine thiolate ligand. P450s and NOS are oxidoreductases that activate molecular oxygen [78, 79]. The P450s have long been presumed to oxidize substrates via a reactive porphyrin radical cation ferryl species known as Cpd I. Additional evidence for reactive intermediates was also collected by direct observation through a combination of various spectroscopic techniques [80]. Based on the observed activation of P450s by hydrogen peroxide, alkyl hydroperoxides, periodate and iodosobenzene, oxygen activation was assumed to occur by a two-electron reduction of dioxygen to the level of H₂O₂ followed by formation of the ferryl intermediate as seen in heme-containing peroxidases [81]. Synthetic metalloporphyrins could form a porphyrin radical cation ferryl species at low temperature on reaction with peroxy acids and this intermediate had the ability to

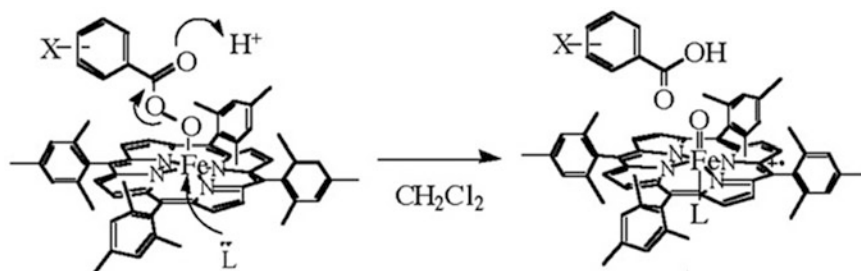


Fig. 2.13 Cpd I formation in synthetic metalloporphyrins by peroxy acids (Reproduced with permission from Groves [81], Copyright 2003 National Academy of Sciences, USA)

insert an oxygen atom into hydrocarbon substrates (Fig. 2.13) [82].

When the transfer of an oxygen atom from the peroxy acid to produce the ferryl intermediate occurs, then the substrate is referred to as an ‘oxygen-rebound’ substrate [83]. Cpd I has been well characterized in CPO [84] and was thought to be elusive in P450 until recently. In 2010, Green and coworkers were successfully able to directly observe Cpd I in CYP119A1 for the first time [38]. Cpd I was formed in about 75 % yield by the reaction of ferric CYP119A1 with *m*-chloroperbenzoic acid. The resulting Cpd I species could then hydroxylate C–H bonds in lauric acid with an apparent rate constant of $k_{app} = 1.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The Mossbauer spectrum of this Cpd I species was similar to that seen using Cpd I of CPO. The mechanism of oxygen atom transfer from Cpd I to form the hydroxylated product has been a hotly debated topic. The initially proposed concerted mechanism of oxygen insertion [85] fell aside in favor of the two-step H atom abstraction/oxygen rebound mechanism [83]. As explained in Sect. 2.4.3, the ferric-hydroperoxo intermediate has also been implicated as an oxidant in a few hydrocarbon hydroxylations. ENDOR spectroscopic studies with cryoreduced wild-type P450CAM and its active-site mutants provided compelling evidence in favor of H atom abstraction/hydroxyl rebound [57]. Active oxidant species of P450CAM were prepared by cryoreduction at 77 K of the oxyferrous intermediate in the P450CAM-camphor complex. The ferric-peroxo and ferric-hydroperoxo intermediates were observed upon slowly

warming to 119 K and were subsequently characterized by EPR and ENDOR spectroscopy. Around 200 K, the ferric-hydroperoxo species was quantitatively converted to 5-*exo*-hydroxycamphor, the natural product of camphor hydroxylation. While the ferryl intermediate was not observed directly, this oxidation was assumed to proceed through the hydroxyl intermediate due to the following observations in the experiment. After formation of the ferric-hydroperoxo species upon slowly warming the sample, the first species observed had the hydroxyl group bound to the heme iron, as was expected for the H atom abstraction in the ferryl-mediated mechanism. Had the ferric-hydroperoxo species been involved in the oxidation, it would have initially formed hydroxycamphor via hydroxy insertion of the distal oxygen atom of the ferric-hydroperoxo species. Hydroxycamphor would be required to displace the hydroxyl/water that was bound to heme, but this displacement reaction was implausible to occur at 200 K. Furthermore, ENDOR spectroscopy showed that the hydrogen attached to the hydroxyl oxygen in the hydroxycamphor product originated from the C-5 position of camphor, further supporting the ferryl mechanism. Involvement of the ferric-hydroperoxo species would have required this H atom to originate from the surrounding solvent.

Shaik and coworkers examined the mechanism of hydrocarbon hydroxylation using theoretical calculations and proposed a two-state reactivity instead of two-oxidant reactivity [86–89]. The researchers proposed that the porphyrin radical cation ferryl species exists in two

spin states, a quartet spin state and a doublet spin state that are close in energy. Both species initiate the reaction by nearly identical H atom abstraction transition states. The species in the doublet state can quickly collapse to the product in a barrierless reaction with no formation of an intermediate. This almost-concerted mechanism is aided by the increased interaction of the cysteine thiolate ligand with the heme iron, the push effect. On the other hand, the quartet state must overcome a significant energy barrier to form the product, thus allowing formation and rearrangements of radicals if any. The two-state reactivity model has provided a good explanation for the stereochemical scrambling and structural rearrangement resulting from the radical clock experiments. Direct observation and characterization of Cpd I has cemented the H atom abstraction/hydroxyl rebound mechanism of P450 enzymes [90].

2.4.5 $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ as an Oxidant in Sulfoxidation Reactions

As described earlier, Jones and coworkers, based on the oxidation of a substrate with both amine and thioether functional groups in P450BM3, proposed that two different oxidants can be responsible for the oxidation of the two classes of substrates. The ferric-hydroperoxo species was proposed to be the oxygenating species

responsible for sulfur oxidation [65]. In the same vein, De Voss and coworkers analyzed the oxidation of thia fatty acids using P450BM3 as the biocatalyst, O_2 as the oxidant and NADPH as the cofactor (Fig. 2.14) [91].

Analysis of the products indicated that presence of the thioether functionality dramatically shifted the regiochemistry of the reaction. With substrates **13** and **14**, the oxidation was distributed across the last three methylene groups. However, replacement of the second methylene group with sulfur resulted in the oxidation in **15** and **16** occurring exclusively at the sulfur. Interestingly, the sulfoxides were S enantiomers whereas the alcohols were R enantiomers. While it was speculated that thioethers undergo unusual binding to yield S sulfoxides, it has been shown in the past that modified fatty acids undergo R oxidation exclusively [91–93]. Substrates were also reacted with the P450BM3 Thr268Ala active-site mutant, based on previous studies showing that the mutant enzyme formed very little Cpd I and was able to accumulate the ferric-hydroperoxo species [18, 30, 31]. Despite the low turnover in the mutant for substrate **14**, product distribution and enantioselectivity remained unchanged between the wild-type and mutant enzyme. This demonstrated that there occurred reduced Cpd I formation in the mutant. For the thia fatty acid substrates, negligible change was observed in the turnover, product distribution and

Fig. 2.14 Fatty acid oxidation by P450BM3, arrows indicate sites of oxidation and stereochemistry of products formed (See Ref. [91])

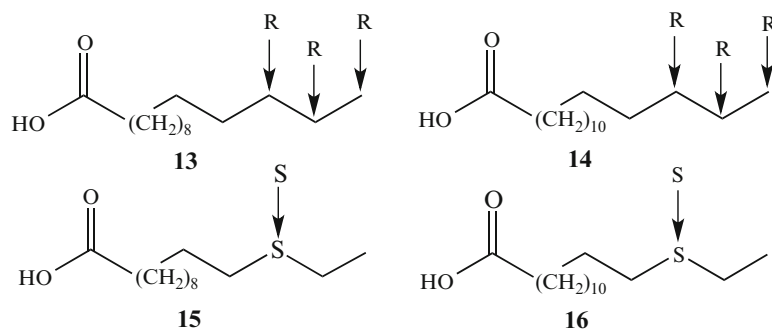
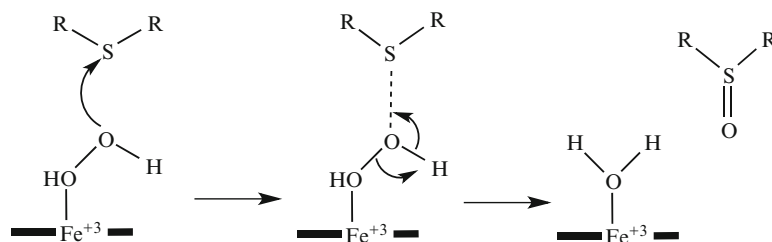


Fig. 2.15 Proposed mechanism of sulfoxidation by the $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ intermediate (See Ref. [94])



enantioselectivity of the products between the products of the wild-type and mutant enzyme. The authors proposed that sulfur oxidation must be easily catalyzed by the ferric-hydroperoxo species, thereby enabling the mutant enzyme to form comparable amounts of product compared to that of the wild-type enzyme.

However, Shaik and coworkers have recently used theoretical calculations to show that the $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ complex (Fig. 2.2, 9) is a very efficient oxidant for sulfoxidation reactions in P450s and iron corrolazine compounds [94]. The $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ complex was shown to undergo a nucleophilic attack from the distal oxygen atom of the peroxo complex, resulting in heterolytic O–O bond scission that is coupled to proton transfer (Fig. 2.15). The $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ complex could also catalyze the oxidation on sulfur much faster than could Cpd I. The ferric-hydroperoxo intermediate, in contrast, had a high barrier via the homolysis pathway of oxygen insertion [94]. This finding offers a new paradigm for sulfoxidation reactions in P450s and their synthetic monologues.

2.5 Conclusions

The mechanistic complexity of P450 enzymes has been intensely debated for the last few decades. The recent direct observation of P450 Cpd I and resulting studies of its reactivity have provided strong support for the validity of the H atom abstraction/radical rebound mechanism in hydrocarbon hydroxylation reactions. The role of the ferric-peroxo intermediate as a nucleophilic oxidant is also well established with experimental evidence. The proposed role of the ferric-hydroperoxo intermediate as an electrophilic

oxidant remains to be established. Its role as an oxidant has been proposed mainly based on turnover studies in P450 mutants with impaired ability to form Cpd I. A recent addition to this oxidant puzzle is the $\text{Fe}^{\text{III}}-(\text{H}_2\text{O}_2)$ intermediate, which has been proposed to be more active than Cpd I in thio-ether oxidation reactions. An alternative explanation for the multiple oxidant hypothesis is provided by the theoretical two-state reactivity hypothesis involving Cpd I, which has advanced some explanations for the disparate experimental data. However, additional experimental and theoretical data are still needed to provide further insights into the mechanisms of P450 catalysis.

Acknowledgment The NIH (GM-26730) has supported cytochrome P450 research in the Dawson laboratory. We would like to thank Dr. Masanori Sono for pertinent advice.

References

1. Poulos TL, Johnson EF (2005) Structures of cytochrome P450 enzymes. In: Ortiz de Montellano PR (ed) *Cytochrome P450: structure, mechanism, and biochemistry*, 3rd edn. Kluwer Academic/Plenum Publishers, New York, pp 87–114
2. Sono M, Roach MP, Coulter ED, Dawson JH (1996) Heme-containing oxygenases. *Chem Rev* 96:2841–2888
3. Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. *J Biol Chem* 239:2370–2378
4. Filatov M, Reckien W, Peyerimhoff SD, Shaik S (2000) What are the reasons for the kinetic stability of a mixture of H_2 and O_2 ? *J Phys Chem A* 104:12014–12020
5. Blanksby SJ, Ellison GB (2003) Bond dissociation energies of organic molecules. *Acc Chem Res* 36:255–263

6. Sligar SG, Makris TM, Denisov IG (2005) Thirty years of microbial P450 monooxygenase research: peroxo-heme intermediates—the central bus station in heme oxygenase catalysis. *Biochem Biophys Res Commun* 338:346–354
7. Meunier B (1992) Metalloporphyrins as versatile catalysts for oxidation reactions and oxidative DNA cleavage. *Chem Rev* 92:1411–1456
8. Glieder A, Farinas ET, Arnold FH (2002) Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase. *Nat Biotechnol* 20:1135–1139
9. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG (2000) The catalytic pathway of cytochrome P450cam at atomic resolution. *Science* 287:1615–1622
10. Nebert DW, Gonzalez FJ (1987) P450 genes: structure, evolution, and regulation. *Annu Rev Biochem* 56:945–993
11. Narhi LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J Biol Chem* 261:7160–7169
12. Ruettinger RT, Wen LP, Fulco AJ (1989) Coding nucleotide, 5' regulatory, and deduced amino acid sequences of P-450BM-3, a single peptide cytochrome P-450:NADPH-P-450 reductase from *Bacillus megaterium*. *J Biol Chem* 264:10987–10995
13. Poulos TL, Finzel BC, Gunsalus IC, Wagner GC, Kraut J (1985) The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J Biol Chem* 260:16122–16130
14. Gunsalus IC, Pederson TC, Sligar SG (1975) Oxygenase-catalyzed biological hydroxylations. *Annu Rev Biochem* 44:377–407
15. Meunier B, de Visser SP, Shaik S (2004) Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. *Chem Rev* 104:3947–3980
16. Winfield ME (1965) Mechanisms of oxygen uptake: the autoxidation of myoglobin and of reduced cyanocobaltates and their significance to oxidase reactions. In: King TE, Mason HS, Morrison M (eds) *Oxidases and related redox systems*, vol 1. Wiley, New York, pp 115–130
17. Dunford HB, Stillman JS (1976) On the function and mechanism of action of peroxidases. *Coord Chem Rev* 19:187–251
18. Imai M, Shimada H, Watanabe Y, Matsushima-Hibiya Y, Makino R, Koga H, Horiuchi T, Ishimura Y (1989) Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: possible role of the hydroxy amino acid in oxygen activation. *Proc Natl Acad Sci U S A* 86:7823–7827
19. Egawa T, Shimada H, Ishimura Y (1994) Evidence for compound I formation in the reaction of cytochrome P450cam with m-chloroperbenzoic acid. *Biochem Biophys Res Commun* 201:1464–1469
20. Schünemann V, Lendzian F, Jung C, Contzen J, Barra AL, Sligar SG, Trautwein AX (2004) Tyrosine radical formation in the reaction of wild type and mutant cytochrome P450cam with peroxy acids: a multifrequency EPR study of intermediates on the millisecond time scale. *J Biol Chem* 279:10919–10930
21. Spolitat T, Dawson JH, Ballou DP (2005) Reaction of ferric cytochrome P450cam with peracids: kinetic characterization of intermediates on the reaction pathway. *J Biol Chem* 280:20300–20309
22. Shimada H, Watanabe Y, Imai M, Makino R, Koga H, Horiuchi T, Ishimura Y (1991) The role of threonine 252 in the oxygen activation by cytochrome P-450 cam: mechanistic studies by site-directed mutagenesis. In: Simandi LI (ed) *Dioxygen activation and homogeneous catalytic oxidation*. Elsevier, Amsterdam, pp 3136–3319
23. Auclair K, Moënné-Loccoz P, Ortiz de Montellano PR (2001) Roles of the proximal heme thiolate ligand in cytochrome P450cam. *J Am Chem Soc* 123:4877–4885
24. Perera R, Sono M, Sigman JA, Pfister TD, Lu Y, Dawson JH (2003) Neutral thiol as a proximal ligand to ferrous heme iron: implications for heme proteins that lose cysteine thiolate ligation on reduction. *Proc Natl Acad Sci U S A* 100:3641–3646
25. Yoshioka S, Takahashi S, Ishimori K, Morishima I (2000) Roles of the axial push effect in cytochrome P450cam studied with the site-directed mutagenesis at the heme proximal site. *J Inorg Biochem* 81:141–151
26. Yoshioka S, Tosha T, Takahashi S, Ishimori K, Hori H, Morishima I (2002) Roles of the proximal hydrogen bonding network in cytochrome P450cam-catalyzed oxygenation. *J Am Chem Soc* 124:14571–14579
27. Dawson JH (1988) Probing structure-function relations in heme-containing oxygenases and peroxidases. *Science* 240:433–439
28. Dawson JH, Holm RH, Trudell JR, Barth G, Linder RE, Bunnenberg E, Djerassi C, Tang SC (1976) Oxidized cytochrome P-450. Magnetic circular dichroism evidence for thiolate ligation in the substrate-bound form. Implications for the catalytic mechanism. *J Am Chem Soc* 98:3707–3709
29. Poulos TL, Kraut J (1980) The stereochemistry of peroxidase catalysis. *J Biol Chem* 255:8199–8205
30. Martinis SA, Atkins WM, Stayton PS, Sligar SG (1989) A conserved residue of cytochrome P-450 is involved in heme-oxygen stability and activation. *J Am Chem Soc* 111:9252–9253
31. Yeom H, Sligar SG, Li H, Poulos TL, Fulco AJ (1995) The role of Thr268 in oxygen activation of cytochrome P450BM-3. *Biochemistry* 34:14733–14740
32. Imai Y, Nakamura M (1988) The importance of threonine-301 from cytochromes P-450 (laurate (ω -1)-hydroxylase and testosterone 16 α -hydroxylase) in substrate binding as demonstrated by site-directed mutagenesis. *FEBS Lett* 234:313–315
33. Raag R, Martinis SA, Sligar SG, Poulos TL (1991) Crystal structure of the cytochrome P-450CAM

- active site mutant Thr252Ala. *Biochemistry* 30:11420–11429
34. Harris DL, Loew GH (1996) Investigation of the proton-assisted pathway to formation of the catalytically active, ferryl species of P450s by molecular dynamics studies of P450eryF. *J Am Chem Soc* 118:6377–6387
35. Truan G, Peterson JA (1998) Thr268 in substrate binding and catalysis in P450BM-3. *Arch Biochem Biophys* 349:53–64
36. Vidakovic M, Sligar SG, Li H, Poulos TL (1998) Understanding the role of the essential Asp251 in cytochrome P450cam using site-directed mutagenesis: crystallography, and kinetic solvent isotope effect. *Biochemistry* 37:9211–9219
37. Gerber NC, Sligar SG (1994) A role for Asp-251 in cytochrome P-450cam oxygen activation. *J Biol Chem* 269:4260–4266
38. Rittle J, Green MT (2010) Cytochrome P450 compound I: capture, characterization, and C-H bond activation kinetics. *Science* 330:933–937
39. Loida PJ, Sligar SG (1993) Molecular recognition in cytochrome P-450: mechanism for the control of uncoupling reactions. *Biochemistry* 32:11530–11538
40. Eisenstein L, Debey P, Douzou P (1977) P450cam: oxygenated complexes stabilized at low temperature. *Biochem Biophys Res Commun* 77:1377–1383
41. Antonini E, Brunori M (1971) Hemoglobin and myoglobin in their reactions with ligands. North-Holland, Amsterdam
42. Couture M, Stuehr DJ, Rousseau DL (2000) The ferrous dioxygen complex of the oxygenase domain of neuronal nitric-oxide synthase. *J Biol Chem* 275:3201–3205
43. Macdonald IDG, Sligar SG, Christian JF, Unno M, Champion PM (1998) Identification of the Fe–O–O bending mode in oxycytochrome P450cam by resonance Raman spectroscopy. *J Am Chem Soc* 121:376–380
44. Sono M, Eble KS, Dawson JH, Hager LP (1985) Preparation and properties of ferrous chloroperoxidase complexes with dioxygen, nitric oxide, and an alkyl isocyanide. Spectroscopic dissimilarities between the oxygenated forms of chloroperoxidase and cytochrome P-450. *J Biol Chem* 260:15530–15535
45. Bangcharoenpaupong O, Rizos AK, Champion PM, Jollie D, Sligar SG (1986) Resonance Raman detection of bound dioxygen in cytochrome P-450cam. *J Biol Chem* 261:8089–8092
46. Fischer RT, Trzaskos JM, Magolda RL, Ko SS, Brosz CS, Larsen B (1991) Lanosterol 14 α -methyl demethylase. Isolation and characterization of the third metabolically generated oxidative demethylation intermediate. *J Biol Chem* 266:6124–6132
47. Akhtar M, Corina D, Miller S, Shyadehi AZ, Wright JN (1994) Mechanism of the acyl-carbon cleavage and related reactions catalyzed by multifunctional P-450s: studies on cytochrome P-450_{17 α} . *Biochemistry* 33:4410–4418
48. Akhtar M, Alexander K, Boar RB, McGhie JF, Barton DH (1978) Chemical and enzymic studies on the characterization of intermediates during the removal of the 14 α -methyl group in cholesterol biosynthesis. The use of 32-functionalized lanostane derivatives. *Biochem J* 169:449–463
49. Corina DL, Miller SL, Wright JN, Akhtar M (1991) The mechanism of cytochrome P-450 dependent C-C bond cleavage: studies on 17 α -hydroxylase-17,20-lyase. *J Chem Soc Chem Commun* 782–783
50. Roberts ES, Vaz AD, Coon MJ (1991) Catalysis by cytochrome P-450 of an oxidative reaction in xenobiotic aldehyde metabolism: deformylation with olefin formation. *Proc Natl Acad Sci U S A* 88:8963–8966
51. Kuo CL, Raner GM, Vaz ADN, Coon MJ (1999) Discrete species of activated oxygen yield different cytochrome P450 heme adducts from aldehydes. *Biochemistry* 38:10511–10518
52. Bestervelt LL, Vaz AD, Coon MJ (1995) Inactivation of ethanol-inducible cytochrome P450 and other microsomal P450 isozymes by trans-4-hydroxy-2-nonenal, a major product of membrane lipid peroxidation. *Proc Natl Acad Sci U S A* 92:3764–3768
53. Korth HG, Sustmann R, Thater C, Butler AR, Ingold KU (1994) On the mechanism of the nitric oxide synthase-catalyzed conversion of N^ω-hydroxy-L-arginine to citrulline and nitric oxide. *J Biol Chem* 269:17776–17779
54. Wertz DL, Sisemore MF, Selke M, Driscoll J, Valentine JS (1998) Mimicking cytochrome P-450 2B4 and aromatase: aromatization of a substrate analogue by a peroxo Fe(III) porphyrin complex. *J Am Chem Soc* 120:5331–5332
55. Sisemore MF, Burstyn JN, Valentine JS (1996) Epoxidation of electron-deficient olefins by a nucleophilic iron(III) peroxo porphyrinato complex, peroxo (tetramesitylporphyrinato)ferrate(1–). *Angew Chem Int Ed* 35:206–208
56. Davydov R, Macdonald IDG, Makris TM, Sligar SG, Hoffman BM (1999) EPR and ENDOR of catalytic intermediates in cryoreduced native and mutant oxy-cytochromes P450cam: mutation-induced changes in the proton delivery system. *J Am Chem Soc* 121:10654–10655
57. Davydov R, Makris TM, Kofman V, Werst DE, Sligar SG, Hoffman BM (2001) Hydroxylation of camphor by reduced oxy-cytochrome P450cam: mechanistic implications of EPR and ENDOR studies of catalytic intermediates in native and mutant enzymes. *J Am Chem Soc* 123:1403–1415
58. Vaz ADN, McGinnity DF, Coon MJ (1998) Epoxidation of olefins by cytochrome P450: evidence from site-specific mutagenesis for hydroperoxo-iron as an electrophilic oxidant. *Proc Natl Acad Sci U S A* 95:3555–3560
59. Jin S, Makris TM, Bryson TA, Sligar SG, Dawson JH (2003) Epoxidation of olefins by hydroperoxo–ferric cytochrome P450. *J Am Chem Soc* 125:3406–3407
60. de Visser SP, Ogliaro F, Harris N, Shaik S (2001) Multi-state epoxidation of ethene by cytochrome

- P450: a quantum chemical study. *J Am Chem Soc* 123:3037–3047
61. de Visser SP, Ogliaro F, Sharma PK, Shaik S (2002) What factors affect the regioselectivity of oxidation by cytochrome P450? A DFT study of allylic hydroxylation and double bond epoxidation in a model reaction. *J Am Chem Soc* 124:11809–11826
 62. Ogliaro F, Cohen S, de Visser SP, Shaik S (2000) Medium polarization and hydrogen bonding effects on compound I of cytochrome P450: what kind of a radical is it really? *J Am Chem Soc* 122:12892–12893
 63. Ogliaro F, de Visser SP, Cohen S, Sharma PK, Shaik S (2002) Searching for the second oxidant in the catalytic cycle of cytochrome P450: a theoretical investigation of the iron(III)-hydroperoxo species and its epoxidation pathways. *J Am Chem Soc* 124:2806–2817
 64. de Visser SP, Ogliaro F, Sharma PK, Shaik S (2002) Hydrogen bonding modulates the selectivity of enzymatic oxidation by P450: chameleon oxidant behavior by compound I. *Angew Chem Int Ed* 41:1947–1951
 65. Volz TJ, Rock DA, Jones JP (2002) Evidence for two different active oxygen species in cytochrome P450 BM3 mediated sulfoxidation and *N*-dealkylation reactions. *J Am Chem Soc* 124:9724–9725
 66. Watanabe Y (2001) Alternatives to the oxoferryl porphyrin cation radical as the proposed reactive intermediate of cytochrome P450: two-electron oxidized Fe(III) porphyrin derivatives. *J Biol Inorg Chem* 6:846–856
 67. Newcomb M, Shen R, Choi SY, Toy PH, Hollenberg PF, Vaz ADN, Coon MJ (2000) Cytochrome P450-catalyzed hydroxylation of mechanistic probes that distinguish between radicals and cations. Evidence for cationic but not for radical intermediates. *J Am Chem Soc* 122:2677–2686
 68. Jin S, Bryson T, Dawson J (2004) Hydroperoxoferric heme intermediate as a second electrophilic oxidant in cytochrome P450-catalyzed reactions. *J Biol Inorg Chem* 9:644–653
 69. Toy PH, Newcomb M, Coon MJ, Vaz ADN (1998) Two distinct electrophilic oxidants effect hydroxylation in cytochrome P-450-catalyzed reactions. *J Am Chem Soc* 120:9718–9719
 70. Newcomb M, Le Tadic-Biadatti MH, Chestney DL, Roberts ES, Hollenberg PF (1995) A nonsynchronous concerted mechanism for cytochrome P-450 catalyzed hydroxylation. *J Am Chem Soc* 117:12085–12091
 71. Newcomb M, Aebischer D, Shen R, Chandrasena REP, Hollenberg PF, Coon MJ (2003) Kinetic isotope effects implicate two electrophilic oxidants in cytochrome P450-catalyzed hydroxylations. *J Am Chem Soc* 125:6064–6065
 72. Chandrasena REP, Vatsis KP, Coon MJ, Hollenberg PF, Newcomb M (2003) Hydroxylation by the hydroperoxy-iron species in cytochrome P450 enzymes. *J Am Chem Soc* 126:115–126
 73. Toy PH, Dhanabalasingam B, Newcomb M, Hanna IH, Hollenberg PF (1997) A substituted hypersensitive radical probe for enzyme-catalyzed hydroxylations: synthesis of racemic and enantiomerically enriched forms and application in a cytochrome P450-catalyzed oxidation. *J Org Chem* 62:9114–9122
 74. Ortiz de Montellano PR, Wilks A (2000) Advances in inorganic chemistry. In: Sykes G, Mauk AG (eds) *Iron porphyrins*, vol 51. Academic, San Diego, pp 359–407
 75. Tenhunen R, Marver H, Pinstone NR, Trager WF, Cooper DY, Schmid R (1972) Enzymic degradation of heme. Oxygenative cleavage requiring cytochrome P-450. *Biochemistry* 11:1716–1720
 76. Wilks A, Ortiz de Montellano PR (1993) Rat liver heme oxygenase. High level expression of a truncated soluble form and nature of the meso-hydroxylating species. *J Biol Chem* 268:22357–22362
 77. Wilks A, Torpey J, Ortiz de Montellano PR (1994) Heme oxygenase (HO-1). Evidence for electrophilic oxygen addition to the porphyrin ring in the formation of α -meso-hydroxyheme. *J Biol Chem* 269:29553–29556
 78. Sundaramoorthy M, Terner J, Poulos TL (1995) The crystal structure of chloroperoxidase: a heme peroxidase–cytochrome P450 functional hybrid. *Structure* 3:1367–1378
 79. Groves JT, Wang CCY (2000) Nitric oxide synthase: models and mechanisms. *Curr Opin Chem Biol* 4:687–695
 80. Makris TM, Schlichting I, Sligar SG (2005) Activation of molecular oxygen by cytochrome P450. In: Ortiz de Montellano PR (ed) *Cytochrome P450: structure, mechanism, and biochemistry*, 2nd edn. Plenum Press, New York, pp 149–182
 81. Groves JT (2003) The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies. *Proc Natl Acad Sci U S A* 100:3569–3574
 82. Groves JT, Haushalter RC, Nakamura M, Nemo TE, Evans BJ (1981) High-valent iron-porphyrin complexes related to peroxidase and cytochrome P-450. *J Am Chem Soc* 103:2884–2886
 83. Groves JT, McClusky GA (1976) Aliphatic hydroxylation via oxygen rebound. Oxygen transfer catalyzed by iron. *J Am Chem Soc* 98:859–861
 84. Groves JT, Watanabe Y (1988) Reactive iron porphyrin derivatives related to the catalytic cycles of cytochrome P-450 and peroxidase. Studies of the mechanism of oxygen activation. *J Am Chem Soc* 110:8443–8452
 85. Shapiro S, Piper JU, Caspi E (1982) Steric course of hydroxylation at primary carbon atoms. Biosynthesis of 1-octanol from (1R)- and (1S)-[1-3H, 2H, 1H; 1-14C]octane by rat liver microsomes. *J Am Chem Soc* 104:2301–2305
 86. Shaik S, Filatov M, Schröder D, Schwarz H (1998) Electronic structure makes a difference: cytochrome P-450 mediated hydroxylations of hydrocarbons as a two-state reactivity paradigm. *Chem Eur J* 4:193–199
 87. Harris N, Cohen S, Filatov M, Ogliaro F, Shaik S (2000) Two-state reactivity in the rebound step of alkane hydroxylation by cytochrome P-450: origins of free radicals with finite lifetimes. *Angew Chem Int Ed* 39:2003–2007
 88. Ogliaro F, de Visser SP, Groves JT, Shaik S (2001) Chameleon states: high-valent metal-oxo species of

- cytochrome P450 and its ruthenium analogue. *Angew Chem Int Ed* 40:2874–2878
89. Ogliaro F, Harris N, Cohen S, Filatov M, de Visser SP, Shaik S (2000) A model “rebound” mechanism of hydroxylation by cytochrome P450: stepwise and effectively concerted pathways, and their reactivity patterns. *J Am Chem Soc* 122:8977–8989
90. Krest CM, Onderko EL, Yosca TH, Calixto JC, Karp RF, Livada J, Rittle J, Green MT (2013) Reactive intermediates in cytochrome P450 catalysis. *J Biol Chem* 288:17074–17081
91. Cryle MJ, De Voss JJ (2006) Is the ferric hydroperoxy species responsible for sulfur oxidation in cytochrome P450s? *Angew Chem Int Ed* 45:8221–8223
92. Truan G, Komandla MR, Falck JR, Peterson JA (1999) P450BM-3: absolute configuration of the primary metabolites of palmitic acid. *Arch Biochem Biophys* 366:192–198
93. Capdevila JH, Wei S, Helvig C, Falck JR, Belosludtsev Y, Truan G, Graham-Lorence SE, Peterson JA (1996) The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *J Biol Chem* 271:22663–22671
94. Wang B, Li C, Cho KB, Nam W, Shaik S (2013) The $\text{Fe}^{\text{III}}(\text{H}_2\text{O}_2)$ complex as a highly efficient oxidant in sulfoxidation reactions: revival of an underrated oxidant in cytochrome P450. *J Chem Theory Comput* 9:2519–2525

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Hrycay, E.G.; Bandiera, S.M. (Eds.)

2015, XV, 368 p. 150 illus., 59 illus. in color., Hardcover

ISBN: 978-3-319-16008-5