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Review

Omega Oxygenases: Nonheme-iron enzymes and P450 cytochromes

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Abstract

Enzymes that effect with ease one of the most difficult chemical reactions, hydroxylation of an unfunctionalized alkyl group, are of particular interest because highly reactive intermediates must be produced. A typical example, the hydroxylation of fatty acids in the ω position, is now known to occur widely in nature. The catalysts, which can be called "ω-oxygenases," also insert molecular oxygen into a variety of other substrates at positions removed from activating functional groups, as in steroids, eicosanoids, and numerous drugs and other xenobiotics. Progress in the characterization of bacterial nonheme-iron enzymes, and plant, bacterial, and mammalian P450 cytochromes that catalyze fatty acid ω-oxidation, and evidence for multiple functional oxidants are summarized.

Keywords: ω-Oxygenases; Fatty acid and alkane hydroxylation; Nonheme-iron oxygenases; P450 mechanism

ω-Oxidation was discovered over seventy years ago by Verkade et al. [1] in the Netherlands when they fed fatty acids of intermediate chain length (or their esters or glycerides) to their colleagues or to dogs and isolated the resulting urinary dicarboxylic acids. Whereas α- and β-oxidation of fatty acids have long been known and occur at positions activated by the adjacent carboxyl group, ω-oxidation at the particularly unreactive terminal methyl group was completely unexpected. Because studies on reactions that occur in living systems but defy chemical prediction have often led to the discovery of interesting new coenzymes and enzymes, we undertook a biochemical investigation of ω-oxidation. Our early studies showed that a ^{14}C-labeled fatty acid underwent terminal oxidation in liver tissue [2], but the instability and insolubility of the liver microsomal enzyme system prevented further progress. We then focused on a more tractable bacterial system, and, upon returning to study hepatic ω-oxidation almost 10 years later, eventually solubilized and characterized cytochrome P450 as the mammalian catalyst for ω-oxygenation [3].

This brief review is concerned with the widespread occurrence and mechanism of fatty acid ω-oxidation, now known to occur in numerous microorganisms, all animals examined as well as the human, and more recently in plants. Cytochrome P450 is the predominant catalyst, with nonheme-iron-containing enzymes in bacteria as other examples. In a more general sense, "ω-oxygenases" can be defined as enzymes that insert molecular oxygen into a variety of substrates at positions removed from activating functional groups. Microbial fatty acid and alkane oxygenases containing nonheme-iron are widespread, and, like cytochrome P450s, capable of ω-oxidative attack on compounds of physiological importance such as steroids, eicosanoids, lipid hydroperoxides, fat-soluble vitamins, and retinoids, as well as an almost unlimited array of xenobiotics encompassing drugs, isoprenoids such as camphor, solvents, anesthetics, pesticides, antioxidants, dyes, and even plant products such as flavorants and odorants. The oxygenation of such a variety of substrates by P450s may seem

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indiscriminate, but in many instances the modification is positionally and even stereochemically specific [4,5].

Bacterial nonheme-iron ω-oxygenases

Assuming that hydrocarbons might be even more challenging substrates than fatty acids for biological ω-oxidation, we isolated from soil samples a bacterium that grew well on alkanes such as hexane. This ''gasoline bug'' turned out to be a strain of *Pseudomonas oleovorans*, cell-free extracts of which required NADH for the aerobic conversion of octane to *n*-octanol [6,7]. Of special interest, fatty acids were converted to their ω-hydroxy derivatives [8]. As is now well known, the enzyme system was resolved into three fractions by Peterson et al. [9], and reconstitution in the presence of NADH under aerobic conditions provided the functional enzyme complex needed for the ω-oxidation of substrates. The components were purified and characterized as follows. A red nonheme-iron protein was identified as rubredoxin [10], previously only known to occur in anaerobic bacteria, in which at that time it had no known function. The homogeneous 1Fe protein could readily be converted to a 2Fe rubredoxin by the addition of ferrous ammonium sulfate under reducing conditions [11]. A yellow flavoprotein with a typical spectrum was also purified to homogeneity and shown to contain one molecule of FAD and to function as an NADH-rubredoxin reductase [12,13]. The third enzyme was the faintly yellow ω-hydroxylase, which was activated when ferrous ions were added [14,15], and contained about one mole of iron per mole of partially purified protein. However, the properties of this nonheme-iron enzyme, including extensive aggregation, made it difficult for further detailed characterization. As described below, our laboratory then turned its attention mainly to the cytochrome P450 system in liver microsomes, but in ensuing years the *Ps. oleovorans* system continued to be examined by other investigators, who have made excellent progress in characterizing the genetic, catalytic, and physical properties, a few examples of which are given below.

Rubredoxins have been thoroughly studied in recent years and are now recognized as a family of nonheme-iron proteins with an active site containing an iron atom tetrahedrally coordinated to four cysteine residues [16]. This redox protein in *Ps. oleovorans* is over three times the size of a typical rubredoxin, and sequence alignments suggest that the corresponding gene (Alk-G) [17] encodes two rubredoxin-type domains separated by a linker of about 70 amino acid residues. Most rubredoxins have two Cys-X-X-Cys-Gly motifs for binding a single iron atom. This is found in the C-terminal domain of the *oleovorans* protein (C-Rb), and also in the N-terminal domain (N-Rb), where Gly is replaced by other amino acid residues. Perry et al. [18] recently examined the solution structure of the 2Fe rubredoxin by NMR spectroscopy and solution X-ray scattering, and also studied the interactions with rubredoxin reductase. C-Rb together with part of the linker region was purified after expression in *Escherichia coli*, and the structure was determined by high-resolution NMR. The structure of the C-Rb domain was found to be similar to those of conventional 1Fe rubredoxins from other organisms, whereas the linker region had no discernible structure. These features were confirmed by X-ray scattering methods, which also showed that N-Rb has a similar molecular conformation to that of C-Rb. The authors stated that their results, taken with data published previously by Lee et al. [19] on the full-length Alk-G protein, indicate that the C-Rb domain is the primary acceptor of electrons from the reductase. In contrast, the N-Rb domain apparently does not receive electrons at a significant rate from the reductase, but becomes reduced by rapid electron transfer from the C-domain. C-Rb and Alk-G stabilize the bound FAD in the reductase and quench the emission of FAD fluorescence, suggesting a close approach of the N-terminal iron domain of rubredoxin to the flavin in the electron–transfer complex. The reductive half-reaction of the reductase occurs by a simple one-step mechanism in which the oxidized enzyme is reduced to an enzyme-NAD^+ charge transfer species, and it is believed that the reoxidation of the reductase by 2Fe rubredoxin is similar in kinetic terms [19].

The bacterial hydroxylase is an integral-membrane oxygenase now often referred to as ω- or alkane-hydroxylase (AlkB). It has been shown to catalyze not only the energetically demanding hydroxylation of unactivated aliphatic methyl groups, but also a number of other characteristic reactions [20–22]. These are consistent with the formation of high-valent electrophilic catalytic intermediates comparable to those found in cytochrome P450 [23] and also in a number of nonheme-iron proteins [24]. In a study of the topology of the ω-hydroxylase, van Beilin et al. [25] obtained data that predicted the occurrence of six transmembrane segments and, in the cytoplasm, the amino terminus, two hydrophilic loops, and a large carboxyl-terminal domain, and only three very short loops exposed to the periplasm. Several years ago, the Münck laboratory [26] carried out important studies that provided evidence for a diiron cluster in the ω-hydroxylase. The corresponding gene was expressed in *E. coli*, and the enzyme was purified as nearly homogeneous protein vesicles by differential centrifugation and HPLC cation exchange chromatography, without the detergent solubilization step often included for membrane proteins. Mössbauer determinations revealed that it has an exchange-coupled dinuclear iron cluster previously found in several soluble diiron proteins. The cluster contains an antiferromagnetically coupled pair of high-spin (FeIII) sites, with an occupancy of up to 0.9 cluster per AlkB. The authors reported that the diferric cluster could be reduced by dithionite and that the resulting diferrous state was air-stable. In the presence of O_2 and octane as substrate, the diferrous cluster was quantitatively reoxidized, providing evidence for the diiron cluster as the active site. The data were consistent with a cluster coordination rich in nitrogen-containing ligands. Sequence analyses in

several laboratories have indicated that at least 11 nonheme integral-membrane enzymes, including AlkB, contain the 8-histidine motif required for catalytic activity in stearoyl-CoA desaturase. The presence of diiron clusters in both soluble and integral-membrane protein families, each with a distinct consensus motif, greatly extends the role of diiron centers in oxygen activation biochemistry.

More recently, Shanklin and Whittle [27] have provided additional evidence linking the *Ps. oleovorans* alkane ω-hydroxylase and the fatty acid desaturase family. They point out that these two systems share a requirement for iron and oxygen for activity in a manner similar to that of the nonheme integral-membrane epoxidases, acetylenases, conjugases, ketolases, decarbonylases, and methyl oxidases. Despite having no overall sequence similarity, AlkB and the desaturase-like enzymes contain a series of histidine residues in similar positioning with respect to hydrophobic regions thought to be transmembrane domains. These investigators [27] tested by scanning alanine mutagenesis whether the conserved His residues in the ω-hydroxylase are functionally equivalent to those of the desaturase-like enzymes. Alanine substitution for any of the eight conserved histidines in AlkB resulted in complete inactivation, whereas substitution for three non-conserved nearby histidines caused only partial inactivation. Their results led to the conclusion that the histidine motif in the hydroxylase is equivalent to those in the desaturases, and that histidines play a vital role such as coordination of the Fe ions of the diiron active site. The reactions catalyzed by the three enzyme components of the bacterial ω-hydroxylation system are shown in Fig. 1. Current literature and reviews by Coulter and Ballou [28] and Costas et al. [29] show that the family of known oxygen-activating nonheme-iron enzymes continues to grow.

ω-Oxygenation in plants

Cuticle, the thin continuous waxy film on the external surface of many higher plants, consists chiefly of cutin in the aerial parts, and a counterpart called suberin occurs in the roots. They serve as structural components of the permeability barrier protecting plants against water loss and pathogen, insect, and mechanical damage. Kolattukudy [30] was the first to study the biosynthesis of cutin, which was known to consist of a meshwork of polymerized cross-esterified hydroxy fatty acids. The formation of 16-hydroxy palmitic acid upon incubation of ^{14}C-labeled palmitic acid with leaf discs thus established the importance of fatty acid ω-oxidation in plants. Derived from cellular lipids, cutin, and suberin are now known to be polymeric networks of oxygenated C16 and C18 fatty acids cross-linked by ester bonds, with the primary or secondary hydroxyl group of one fatty acid linked to the carboxyl group of another [31].

Cytochrome P450 is the major ω-oxygenating catalyst in plants, where many more of the cytochromes are being found than in mammalian species [32]. Duan and Schuler [33] have recently published an excellent summary of differential expression and evolution of the *Arabidopsis* CYP86A subfamily with a phylogenetic comparison of plant, mammalian, and fungal ω-hydroxylases. The phylogenetic tree of potentially orthologous fatty acid ω-hydroxylases from different organisms shown in Fig. 2 is taken from their article. They included all such hydroxylases known to occur in plants, some representatives from the mammalian CYP4 family (human 4A11, 4B1, and 4F2, and rat 4a1), yeast (52A1 and 52A2 in *Candida tropicalis*), as well as a bacterial fatty acid hydroxylase, P450 BM-3 (CYP102), and four plant P450s known to be involved in reactions other than fatty acid hydroxylation. To summarize their results, phylogenetic analysis of all functionally defined plant, mammalian, and fungal fatty acid ω-hydroxylases could be divided into two groups. A well-characterized group (CYP86A, CYP94A, and CYP94B) has a common ancestor with mammalian and fungal sequences, and another less well-characterized group (CYP78A, CYP92B, and CYP703A) has a common ancestor with the plant-specific group involved in the synthesis and metabolism of many secondary metabolites. The proteins in the latter group have low activities for lauric acid ω-hydroxylation, possibly because they have other physiological substrates.

There is substantial evidence that all P450s derive from a common ancestor, with even the most distant P450s sharing more homology among themselves than with other hemoproteins, such as hemoglobins, peroxidases, catalases, and nitric oxide syntheses, etc. [34]. More recent progress

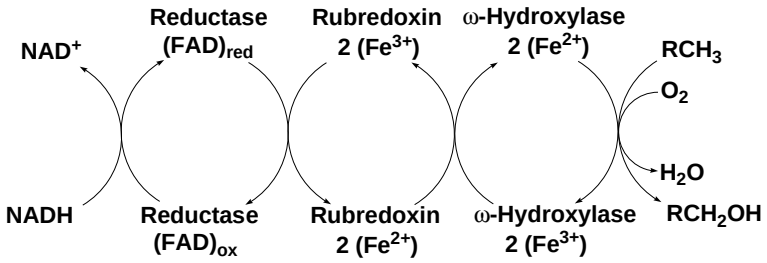

Fig. 1. Conversion of octane to octanol or of laurate to ω-hydroxylaurate in the reconstituted enzyme system from *Ps. oleovorans* under aerobic conditions in the presence of NADH. Each of the iron atoms in the rubredoxin is tetrahedrally coordinated to four cysteine residues [16]. The diiron cluster in the hydroxylase is coordinated to nitrogen-containing ligands, presumably histidine residues [27].

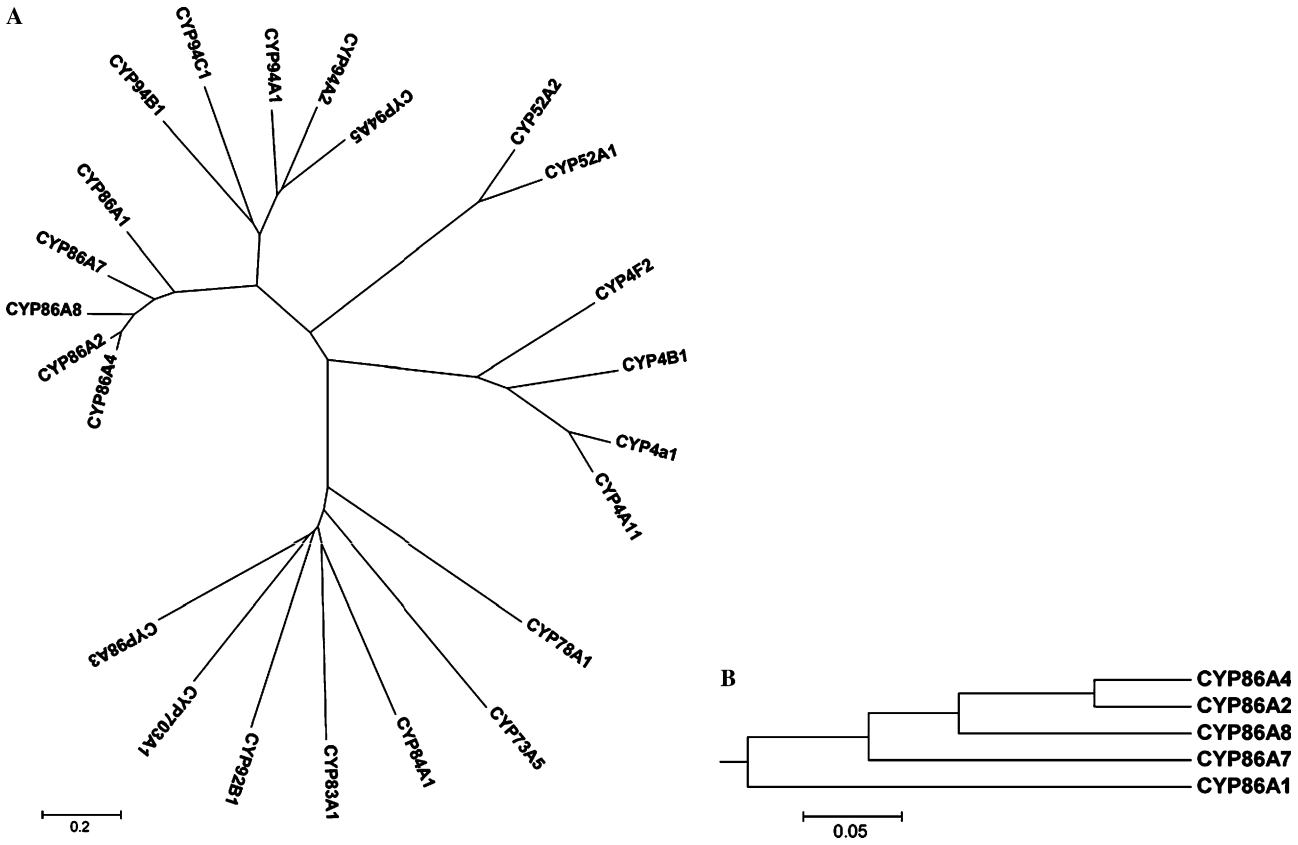

Fig. 2. Phylogenetic trees for fatty acid ω-hydroxylases. (A) An uprooted phylogenetic tree, developed using MEGA version 2.1, includes the fatty acid ω-hydroxylases from *Arabidopsis* (CYP94B1, CYP94C1, and CYP86A subfamily), *V. sativa* (CYP94A1, CYP94A2), *Nicotiana tabacum* (CYP94A5), *Ps. hybrida* (CYP92B1, CYP703A1), *Zea mays* (CYP78A1), *C. tropicalis* (CYP52A1, CYP52A2), rat (CYP4A 1), human (CYP4A11, CYP4B1, and CYP4F2), and a number of *Arabidopsis* P450s not known for metabolizing fatty acids (CYP73A5, CYP84A1, CYP83A1, and CYP98A3). (B) The phylogenetic tree for the CYP86A subfamily is drawn proportional to branch lengths calculated using maximum parsimony analysis with the proportional bootstrap value shown below (figure and legend taken from [33]).

has led to an understanding of the diversity and evolution of related plant P450s and their reductases [35]. In addition to the role of fatty acids in cuticle synthesis, they are involved in the formation of signaling molecules [36–39]. Furthermore, ω-hydroxylases are responsible for preventing the accumulation of high, possibly toxic levels of free fatty acids in plant cells liberated by phospholipases in response to stress [40]. For example, CYP94A1, which catalyzes fatty acid ω-oxidation, is selectively induced by chemical stress in *Vicia sativa* seedlings [41]. In *Arabidopsis*, ω-hydroxylation is apparently involved in development to control trichome differentiation, the establishment of apical dominance, and senescence in plants [42].

Mammalian and bacterial ω-oxygenation by cytochrome P450

So many mammalian P450 cytochromes in various species and tissues are now known to be capable of oxidizing saturated fatty acids in the ω- or nearby positions that only a few examples will be cited. Lauric acid ω-hydroxylation by phenobarbital-inducible P450 2B4 was the reaction that demonstrated functional reconstitution of the hepatic microsomal enzyme system [43]. Particularly significant are some of the kidney cytochromes [44], including CYP4A7 [45] and human P450 2E1, according to a compilation by Rendic [46]. Guengerich et al. [47] in an accompanying article list human CYPs 2J2, 4A11, 4B1, and 4F12 as playing a major role in the ω-oxidation of saturated fatty acids. In 1981, arachidonic acid joined the list of substrates for these P450-mediated reactions when the 19- and 20-OH derivatives were isolated from reaction mixtures containing NADPH and rabbit kidney cortex microsomes [48–50]. Capdevila et al. [51] have recently reviewed the role of cytochrome P450 and the metabolism of this physiologically important polyunsaturated fatty acid and oxygenated eicosanoids. Again, numerous isoforms of the cytochromes are involved in such reactions. Among the 57 cytochromes recognized in the human genome, Guengerich et al. [47] include the following as particularly significant in catalyzing arachidonic acid oxidation: 4F2, 4F3, 4F8, 5A1, and 8A1.

Our understanding of ω-oxidation was greatly aided by the availability of P450 BM-3 (CYP102), resulting from the discovery by the Fulco group of several cytochromes that were produced in *Bacillus megaterium* grown in the

presence of phenobarbital and shown to be capable of catalyzing this reaction [52]. BM-3 is unique among P450s in that it is a self-sufficient monooxygenase with both the P450 heme and reductase domains on a single polypeptide chain. The products were ω-1, ω-2, and ω-3 hydroxy fatty acids, in a ratio dependent on the length of the fatty acid [53]. The homogeneous, recombinant enzyme purified from *E. coli* was shown by Boddupalli et al. [54] to catalyze the reaction in a tightly coupled manner to give one mole of product per mole of oxygen, fatty acid, and NADPH consumed. Of major importance, the crystal structure of the hemoprotein domain of P450 BM-3 was determined as a useful prototype for microsomal P450s [55]. The review article by Peterson and Graham-Lorence [56] summarizes elegant structural studies on the substrate access channel and other important features of catalysis by P450 BM-3 and other bacterial P450s.

Multiple oxidants and multiple mechanisms in P450 catalysis

As already stated, curiosity about the ability of living cells to catalyze a particularly difficult chemical reaction, hydroxylation of an isolated alkyl group, led us to study biological fatty acid and alkane ω-oxidation. What do we know now about the mechanism, considering that such reactions have attracted the interest of biochemists, chemists, and biophysicists for several decades? From the beginning, the reactions were presumed to involve a highly reactive intermediate produced in the enzymes by a process called oxygen activation, resulting in ambient temperature conversions.

The early mechanistic model for P450-catalyzed hydroxylation was an oxygen insertion reaction, or "oxene" reaction, effected by the consensus electrophilic oxidant, a high-valent iron oxygen complex termed iron-oxo. That

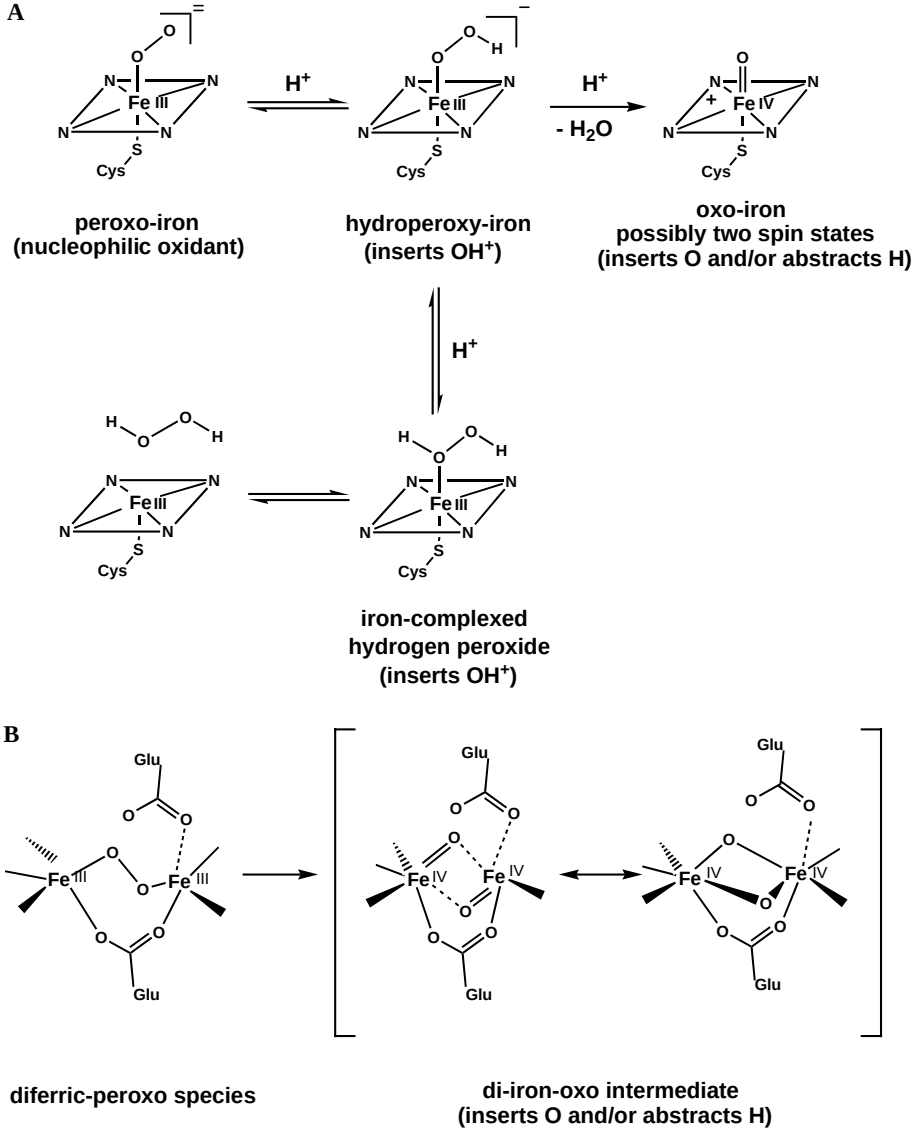

Fig. 3. (A) Versatility in P450 oxygenating species, with the iron-oxygen intermediates in P450 catalysis and their proposed roles as oxidants. Modified from [59,68]. (B) Similar intermediates postulated for the di-iron systems [28,29].

model was replaced by the hydrogen abstraction-oxygen rebound pathway proposed by Groves and Han [57,58]. However, the complexity of oxygenation reactions by P450 cannot be attributed to a single pathway [59]. Readers are referred to recent extensive articles on oxygen activation [60], computational approaches [61], and substrate oxidation, including hydrocarbon hydroxylation [62], and the work in our own laboratory will be summarized only briefly. Important studies with bacterial cytochromes in several laboratories provided details that made our own investigations with purified liver microsomal P450 isoforms possible. In particular, a decreased rate of camphor hydroxylation observed upon mutation of a highly conserved threonine to alanine at the oxygen-binding pocket of P450cam was shown by the Sligar group at Illinois [63] and the Ishimura group in Tokyo [64]. Furthermore, similar effects seen with the BM-3 enzyme were also attributed to disruption of proton delivery to the active site [65]. We took advantage of such information to examine the effect of mutation of the corresponding essential threonine residue in truncated P450 2B4 [66]. The results obtained indicated that the block in proton delivery to the active site led to enhancement of the peroxo-iron species over the oxenoid, as judged by striking alterations in the rates of product formation with several substrates. Thus, evidence was obtained for two distinct electrophilic oxidants, which was supported by additional experiments on olefin epoxidation by 2B4 T302A and by the corresponding mutant of the alcohol-inducible isoform, 2E1 T303A [67].

As shown in Fig. 3A, our findings support the concept that three distinct oxidants are functional in P450 catalysis, including the nucleophilic peroxo-iron species that has been implicated in aldehyde deformylation [69,70]. Of much interest, as indicated in Fig. 3B, similar intermediates have been proposed for the di-iron systems [28,29]. Recent attempts to measure the lifetime of the P450 intermediates by mechanistic probes, or "radical clocks" have confirmed the existence of the two reactive electrophilic oxidants, hydroperoxy-iron and oxo-iron [59,68]. Presumably this reaction scheme with three functional forms of "active oxygen," generated from molecular oxygen by reduction by two electron equivalents and subsequent delivery of two protons, and with a requirement for the thiolate ligand [71], is generally applicable to cytochrome P450, regardless of the source. Thus, although bacterial, plant, mammalian, and other forms of P450 differ in many properties, including their ability to bind different substrates, they are believed to generate the same three functional oxygen species from molecular oxygen.

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