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Oxygen Activation by Cytochrome P450 Monooxygenase

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Abstract

Unlike photosystem II (PSII) that catalyzes formation of the O-O bond, the cytochromes P450 (P450), members of a superfamily of hemoproteins, catalyze the scission of the O-O bond of dioxygen molecules and insert a single oxygen atom into unactivated hydrocarbons through a hydrogen abstraction-oxygen rebound mechanism. Hydroxylation of the unactivated hydrocarbons at physiological temperatures is vital for many cellular processes such as the biosynthesis of many endogenous compounds and the detoxification of xenobiotics in humans and plants. Even though it carries out the opposite of the water splitting reaction, P450 may share similarities to PSII in proton delivery networks, oxygen and water access channels, and consecutive electron transfer processes. In this article, we review recent advances in understanding the molecular mechanisms by which P450 activates dioxygen.

Keywords

P450; oxygen intermediate; oxygen splitting; hydroxylation; P450 reductase

Introduction

Dioxygen appeared in the Earth's atmosphere in the Paleoproterozoic era approximately two billion years ago thanks to photosynthetic organisms that utilize sunlight to split water molecules into dioxygen via photosystem II (PSII). Accumulation of dioxygen in the atmosphere brought about fundamental changes in life on Earth. To harness the oxidative power of oxygen, nature has evolved various enzymes, mainly iron-sulfur and heme-containing proteins, to transport, store, and activate dioxygen for vital cellular functions such as respiration, biosynthesis, detoxification, etc.

The cytochromes P450 (P450) are members of a superfamily of hemoproteins that activate dioxygen to catalyze the oxidation of unactivated hydrocarbons (C-H). The general reaction for P450-catalyzed reactions is expressed as:

, where R-H is substrate. In both P450 and PSII, water and oxygen are essential components. This oxidative reaction occurs in living cells at physiological temperatures and is vitally important for the biosynthesis of many endogenous compounds such as fatty acids, steroid hormones, eicosanoids and for the detoxification of pollutants, pesticides, drugs, carcinogens, etc. in humans and plants. Understanding the structure and function of P450 has been of great interest to pharmacologists, toxicologists, chemists, and biophysicists in the past four decades.

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Much of the enthusiasm has been driven by the fact that P450s play a central role in drug metabolism in humans and by the potential use of P450s as biocatalysts. In this article we review recent advances in understanding the molecular mechanisms by which P450s activate dioxygen in the context that lessons may be learned from this extraordinary class of enzymes. Literature on P450s is vast and it is an insurmountable task to cover all of it. Readers are referred to several excellent monographs and reviews for more details on P450s (Guengerich, 2001; Denisov et al., 2005; Ortiz de Montellano, 2005; Hollenberg et al., 2008).

Classification and nomenclature

P450 is ubiquitous and exists in all organisms. The number of distinct P450 sequences that have been sequenced has increased sharply in recent years due to the completion of several genomic projects. More than 7700 P450 sequences in 866 families have been identified, of which 2740 sequences are found in animals and 2675 sequences in plants (<http://drnelson.utmem.edu/p450stats.2007.htm>). The name P450 is derived from the fact that the carbonmonoxy P450 complex has a maximum absorption at 450 nm in the optical spectrum.

At the recommendation of a nomenclature committee P450s are named on the basis of their amino acid sequence identity. For the P450s found in plants, their phylogenetic origins are also taken into account. P450s within a specific family share 40% sequence identity and within a subfamily the sequence identity is 55% or greater (Nelson, 2006). The sequence identity across different species is low (~15%).

P450s can be classified into four categories based on their redox partners. Class I P450s found in bacterial and eukaryotic mitochondrial membranes receive electrons from ferredoxin, an iron-sulfur protein, which is in turn reduced by ferredoxin reductase. Class II P450s found in the endoplasmic reticulum membranes of eukaryotes receive electrons directly from NADPH-dependent cytochrome P450 reductase (CPR), a diflavoprotein that contains both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Class III P450s are self-sufficient such that P450 and CPR genes are fused into a single polypeptide. Class IV P450s are capable of accepting electrons directly from NADPH.

Overall mechanism and architecture

P450s catalyze the oxidation of a vast number of substrates with substantially different coupling efficiency (referred to as percentage of electrons from NAD(P)H used to produce products), ranging from a few percent to nearly 100%, as observed in the hydroxylation of camphor by P450cam. However, all P450s follow a generally accepted reaction cycle as shown in Figure 1. The resting state of P450 (1) has a hexacoordinated low spin (6c-LS) heme. The sixth axial ligand is a weakly bound H₂O molecule. Substrate binding displaces the aqua ligand to yield a pentacoordinated high spin (5c-HS) heme (2). The LS-to-HS shift usually results in an increase in the redox potential of the heme to facilitate electron transfer from its redox partner to the P450. The 5c-HS ferric P450 is then reduced by its redox partner to give the ferrous heme (3) to which oxygen binds at a bimolecular rate of $\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$ to generate the oxyferrous intermediate (4). Oxyferrous P450 is the first of a series of oxy-intermediates on the pathway leading to dioxygen activation. The second electron is then transferred from the redox partner to the oxyferrous intermediate to yield the peroxo intermediate (5) followed by protonation to give a hydroperoxo intermediate (6) and ultimately leading to heterolytic cleavage of the O-O bond with formation of the highly reactive oxyferryl intermediate (7), referred to as Compound I (Cpd I) by analogy to the heme peroxidases.

It is estimated that the redox potential of oxyferryl species is 1.4 V (Koppenol, 2007) and it is believed to be the oxygenating species that inserts a single oxygen atom into substrates. The last step is product release, returning the P450 to the resting ferric state. As shown in Figure

1, the P450 reaction can be uncoupled to give byproducts such as superoxide and hydrogen peroxide that are potentially harmful to cells. The vast majority of the P450 reactions are not 100% coupled, particularly in the mammalian P450s.

Despite the low sequence homology, the polypeptide chain of P450 is folded in a surprisingly conserved fashion. From the known crystal structures of all species, the secondary structure of P450s consists of approximately twelve α -helices, of which the I and L helices that are in direct contact with the heme are highly conserved as shown in Figure 2. The I helix also contains the critical residues involved in proton delivery to peroxo- and hydroperoxo intermediates. The most conserved amino acid residue in all P450s is a cysteinyl residue that is bound to the heme iron as the fifth axial ligand. The thiolate-heme ligand plays a critical role in activating O-O cleavage through a “push-pull” mechanism (Sono et al., 1996). Because of this unique Fe-S chemistry, the thiolate ligand is located in a conserved region, referred to as β -bulge, that has the signature sequence of F-XX-G-K(R)-XX-C-X-G.

Electron transfer to P450s

Dioxygen activation by P450s requires sequential addition of two one-electrons to the P450 from NAD(P)H through the redox partners. The first electron reduces ferric P450 to ferrous P450 to facilitate dioxygen binding, whereas the second electron reduces oxyferrous P450 toward further dioxygen activation. Class II P450s receive electrons from NADPH via CPR, whereas the electrons from NADH or NADPH are sequentially transferred to ferredoxin reductase, ferredoxin, and P450 in Class I P450s. Electron transfer (ET) between P450cam and its redox partner, putidaredoxin (Pdx), has been extensively studied. The first ET from Pdx to ferric P450cam can be readily measured in the presence of CO, P450cam and reduced Pdx and occurs at $\sim 30\text{--}40\text{ s}^{-1}$ at 25 °C only in the presence of a substrate like camphor (Hintz and Peterson, 1981; Kuznetsov et al., 2006). Substrate binding to P450 is normally a fast process with rate constants in the order of $10^4 \sim 10^6\text{ M}^{-1}\text{s}^{-1}$. The substrates of P450s are typically lipophilic and readily bind to the hydrophobic binding pocket in the active site of the P450s. Therefore, substrate binding to the active site of P450 is energetically favored. A beneficial consequence of substrate binding towards oxygen activation is the displacement of the water ligand, resulting in a LS-to-HS spin shift of the heme. Concomitant with the spin shift, the redox potential may be increased by $\sim 50\text{--}100\text{ mV}$. In P450cam and P450 BM3, the substrates induce increases in redox potentials by $\geq 100\text{ mV}$ (Sligar, 1976; Sligar and Gunsalus, 1976; Daff et al., 1997), whereas in the microsomal P450 2B4 the redox potential increases from -320 to -245 mV upon benzphetamine binding (Zhang et al., 2003). This spin-induced increase in the redox potential results in a more efficient reduction of ferric P450 by the redox partner. For example, an increase by 130 mV in the redox potential in the F393A mutant of P450 BM3 accelerated the reduction of ferric P450 BM3 by 3.4 fold in the presence of arachidonate (Ost et al., 2001).

It was originally thought that the second ET from Pdx to oxyferrous P450cam was rate-limiting in P450cam. However, Brewer and Peterson determined that under single turnover conditions reduced Pdx transfers one electron to oxyferrous P450cam at $\sim 60\text{--}100\text{ s}^{-1}$ at 4 °C (Brewer and Peterson, 1988). Recent studies using double-mixing stopped-flow spectrophotometry determined the rate of the second electron transfer is 118 s^{-1} at 4 °C, significantly faster than the rate of the first ET to P450cam. It appears that the rate-limiting step is actually the reduction of ferric P450cam by Pdx (Kuznetsov et al., 2006).

Studies involving site-directed mutagenesis, computational modeling, and biophysical spectroscopies such as EPR, Raman, and NMR demonstrated that Pdx interacts with the proximal surface of P450cam through the electrostatic interaction and suggested that Arg-112 at the putative Pdx binding site forms the ET pathway in the P450cam•Pdx complex. Substitution of Arg112 with neutral residues such as Cys, Met, or Tyr reduces the redox

potentials of P450cam and diminished the rate of ET from Rdx to P450cam (Unno et al., 1996). It was also revealed that Pdx acts as the specific electron donor for the turnover reaction of P450cam. Another important feature of the P450cam•Pdx complex is that Pdx induces conformational changes of P450cam upon complex formation. EPR and resonance Raman studies clearly exhibited that the complex formation with Pdx converts the spin-state of ferric P450cam from the high to low spin state (Lipscomb, 1980; Unno et al., 1996). Recent multidimensional NMR study on the complex of P450cam with Pdx showed that the binding of Pdx structurally perturbs the several regions involving the substrate access channel in P450cam (Pochapsky et al., 2003). These structural changes of P450cam upon the binding of Pdx are supposed to be essential for the enzymatic activity of P450cam. It was found that the mutations at the putative Pdx binding site, Arg-109 or Arg-112, inhibit the conformational changes in P450cam and suppress the hydroxylation activity to 1–500 $\mu\text{M}/\text{min}/\mu\text{M}$ heme corresponding to 1/1000 to 1/3 of that for the wild-type enzyme (Shimada et al., 1999; Shimada et al., 2001). Although the Pdx-induced structural changes of P450cam have been supposed to be crucial for the P450cam catalysis, it is still unclear how the conformational changes in the active site of P450cam promote the turnover reaction.

Compared with bacterial P450s, microsomal P450s in general have much lower turnover rates in catalysis. This is in part due to sluggish ET from CPR to microsomal P450s in addition to an increase in the partition leading to the uncoupling reaction. The ET between CPR and microsomal P450s usually requires pre-incubation to form an active P450•CPR complex as the association between these two heterogeneous proteins is a relatively slow process. The kinetics of reduction of the ferric P450s by CPR is generally multiphasic and the fast phase occurs at $\sim 2\text{--}10\text{ s}^{-1}$ with amplitudes of $\sim 60\text{--}80\%$ (Pompon and Coon, 1984; Guengerich et al., 2004; Zhang et al., 2008). The presence of substrates may enhance the rate significantly in some cases, but may have marginal or no effect in others. For instance, the presence of the substrate benzphetamine at a concentration of 1 mM enhances the rate of the first ET from CPR to P450 2B4 by $\sim$ twofold. This variation may depend on whether substrate binding induces changes in the redox potentials of the microsomal P450s. Measurement of the second ET event from CPR to oxyferrous P450s has been challenging due to existence of multiple intermediate chromophores from CPR such that the absorbance changes from the kinetic measurement make it very difficult to decipher the kinetics. However, the second ET rate has been successfully determined using a 5-deazaFAD-substituted mutant CPR to reduce oxyferrous P450 2B4. As expected, the kinetics of reduction are biphasic; the fast phase with an relative amplitude of 30% has a rate of 8 s^{-1} at $15\text{ }^{\circ}\text{C}$ and the slow phase occurs at 0.37 s^{-1} (Zhang et al., 2003). The overall ET rate from CPR to oxyferrous P450 2B4 is slower than that of the first ET. Unlike Class I P450s, microsomal oxyferrous P450s are capable of accepting electron from cytochrome b5 (cyt b5), another hemoprotein ($\sim 15\text{ kDa}$) bound to the endoplasmic reticulum. Cyt b5 is a better electron donor to oxyferrous P450s exhibiting a rate of $\sim 10\text{--}15\text{ s}^{-1}$. There are numerous reports that cyt b5 stimulates P450 catalysis to various extents ranging from a few percents to nearly 10 fold (Porter, 2002; Zhang et al., 2005). The precise mechanism by which microsomal P450s interact with CPR and cyt b5 is poorly understood and certainly merits further investigation.

Oxygen Intermediates

The first oxy-intermediate in the P450 reaction cycle is oxyferrous P450 (**4**) forming after the transfer of the first electron. To split the O-O bond, the transfer of the second electron with the concomitant delivery of two protons is required. To elucidate the mechanism of dioxygen activation, extensive studies have been carried out to characterize each step of the reaction by biochemical, site-directed mutagenic, and biophysical techniques. P450cam serves as a paradigm for the mechanistic study of these P450 mechanisms due to the fact that it is a soluble protein, its reaction is highly coupled and its crystal structure was the first to be solved.

Reduction of ferric P450 to the ferrous state is a prerequisite for O₂ binding. In solution O₂ binds to ferrous heme with a rate of $\sim 105 - 107 \text{ M}^{-1} \text{ s}^{-1}$ (Zhang et al., 2003; Denisov et al., 2006). Oxyferrous P450 is the only oxy-intermediate that is stable enough for biophysical characterization above cryogenic temperatures. The stability of oxyferrous P450 varies substantially among different isoforms of P450s and can be affected by the binding of substrate. Oxyferrous P450cam decays at 0.005 s^{-1} ($t_{1/2}=2.3 \text{ min}$) at 20 °C (Lipscomb et al., 1976) and its stability is not affected by camphor. In contrast, oxyferrous P450 3A4 decays significantly faster with $t_{1/2}$ of 30 ms at 5 °C in the absence of substrate (Denisov et al., 2006). Binding of testosterone and bromocriptine stabilizes oxyferrous P450 3A4 increasing the k to 0.12–2.5 s^{-1} . It is unclear why substrates stabilize the oxyferrous intermediate in some isoforms of P450 but have no effect in others. There are a few lines of evidence suggesting that hydrogen bonding to the oxygen atom of the oxyferrous P450 may play a role. A thermodynamic analysis estimates the redox potential of the oxyferrous P450, $\text{Fe(III)-OO}^-/\text{Fe(II)}$, is 10 mV, indicating that binding of oxygen to ferrous P450 raises the redox potential by $\sim 200 \text{ mV}$ above the resting state. Formation of the oxyferrous P450 provides a further driving force towards dioxygen activation.

Except for oxyferrous P450, all other oxy-intermediates are relatively unstable and have yet to be observed at ambient temperature. In recent years progress has been made to find spectroscopic evidence for the existence of the activated oxygen species at cryogenic temperature. A novel approach involves the use of γ -irradiation to provide the second electron to oxyferrous P450 at 77 K. Subsequent annealing of the radiolytically reduced oxyferrous P450 allows accumulation of the cryotrapped intermediates for studies by EPR, ENDOR, resonance Raman spectroscopy, and X-ray crystallography. The Q-band EPR spectrum of cryoreduced oxyferrous P450cam WT shows g -tensors at 2.29, 2.16 and 1.96, which is consistent with an end-on hydroperoxide intermediate (**6**) as previously observed in myoglobin (Davydov et al., 1999; Davydov et al., 2001). The peroxo intermediate (**5**) was not observed in cryoreduced oxyferrous P450cam WT, but it was observed with g -tensors at $g = 2.25, 2.16$, and 1.96 in a D251N mutant where proton delivery is disrupted. These g -values demonstrate that the peroxoanion is bound to the iron end-on, not side-on where it would give a rhombic high spin at $g=4.3$. These results not only provide direct evidence for the existence of peroxo- and hydroperoxide intermediates and the geometry of peroxo ligand, but they also suggest that proton delivery to the peroxo intermediate occurs even at 77 K. The optical absorption spectrum of the hydroperoxo intermediate (**6**) of P450cam was obtained in frozen aqueous/glycerol glass at 77 K using ^{32}P -enriched phosphate as the electron source (Denisov et al., 2001, 2001). The optical spectrum of the hydroperoxo intermediate shows a pronounced split Soret band at ~ 360 and 440 nm and the Soret band is shifted to red by $\sim 23 \text{ nm}$ compared with that of the oxyferrous intermediate. These spectral features are similar to a calculated spectrum of the reduced oxyferrous intermediate based on the density functional theory (DFT) (Harris et al., 1998). According to Harris et al., the split Soret band is due to mixing of the sulfur p orbitals with the porphyrin π orbitals modulated by the oxygen ligand. The hydroperoxo intermediate is stable at temperatures up to 180 K, above which spectral changes occur indicative of decay leading to product formation. The first direct observation of the vibrational modes of Fe-OOH of the hydroperoxo intermediate was reported by Mak et al using resonance Raman spectroscopy (Mak et al., 2007). At 77 K a strong ν (O-O) band of Fe-OOH was observed at 1130 cm^{-1} and the ν (Fe-OOH) at 559 cm^{-1} . None of these studies revealed evidence for Cpd I.

It is well established that Cpd I in horseradish and many other peroxidases is a ferryl/porphyrin π -radical cation ($\text{Fe(IV)por}^\bullet$) with two oxidizing equivalents above the resting state (Dolphin et al., 1971). It has long been postulated that Cpd I is the oxygenating species in P450 catalysis. It has been of great interest for mechanistic studies of P450s to trap and identify this oxygenating species. Several studies reported transient optical spectra that might be attributed to Cpd I of P450cam as observed in stopped-flow spectrometers following mixing ferric

P450cam with peracids (Egawa et al., 1994; Spolidakis et al., 2006). Theoretically the reaction of P450 with peracids, the so-called peroxide shunt, should produce an intermediate which is two oxidizing equivalents above the resting ferric state like Cpd I. Due to the non-selective nature of optical spectroscopy, the presence of Cpd I in these studies needs more definitive proof by other spectroscopic studies. In fact Schunemann et al reported a Fe(IV)/Tyr• species, but not Cpd I, with freeze quench EPR and Mössbauer when ferric P450 reacted with peracetic acid (Schunemann et al., 2000). A subsequent study by the same group showed that only trace amount of tyrosyl radical is formed and no iron(IV)-oxo species is detected in the presence of substrate camphor (Schunemann et al., 2002). A relatively stable Cpd II ($t_{1/2} \approx 10$ s at 23 °C), referred to as the iron(IV)-oxo species without the protein radical, was prepared from thermophilic P450 119 from *Sulfolobus Solfataricus* by reacting ferric P450 119 with peroxyxynitrite (Newcomb et al., 2008). X-ray absorption spectroscopy (XAS) shows that the length of the Fe-O bond and the Fe-S bond are 1.82 Å and 2.24 Å, respectively. The bond length is consistent with a single bonded Fe-OH moiety. Newcomb and coworkers also reported the formation of Cpd I in P450 119 through photo-excitation of Cpd II with a ~ 5 mJ laser pulse at 355 nm (Newcomb et al., 2006). Due to the low conversion yield (~5%), only a difference spectrum could be obtained that showed an increase in the absorbance in the range of 400–410 and 640–670 nm. Surprisingly, this transient species decays with a lifetime of ~ 200 ms at 20 °C and lacks reactivity to the substrate laurate. This observation contradicts the prediction based on EPR and ENDOR studies of P450cam that the rate of hydroxylation of camphor may well exceed 1000 s^{-1} at ambient temperature as Cpd I was not observed even at 77 K after annealing the cryoreduced P450cam for 1 minute at ~200 K (Davydov et al., 1999; Davydov et al., 2001). Further studies are required to validate the identification of this photo-induced intermediate species.

Proton delivery network and the role of water in P450 catalysis

Generation of the highly reactive Cpd I requires efficient and precise delivery of two protons at the distal oxygen of the peroxo intermediate once the oxyferrous P450 has been reduced by a second electron from its redox partner. It is known from EPR and ENDOR studies of cytotrapped oxyferrous P450 that the proton is delivered to the distal oxygen of the peroxoanion even at 77 K. Protonation of the proximal oxygen atom of the hydroperoxo intermediate (**6**) will likely lead to the uncoupling reaction which produces hydrogen peroxide. Due to the high reactivity of the activated oxy-intermediates, water in the active site must be tightly regulated to avoid the uncoupling reaction that generates a reactive oxygen species like hydrogen peroxide.

When the first crystal structure of P450 was solved for P450cam in 1985 (Poulos et al., 1985), it was not clear how protons were delivered to the oxy-intermediates in the active site as the heme of P450cam was found to be completely buried in the interior of protein and not solvent accessible. The aqua ligand was absent from the camphor-bound ferric P450cam and there were no other water molecules observed in the active site. Subsequent site-directed mutagenesis and biophysical studies established that the proton delivery system in P450s consists of critical amino acid residues, ordered water molecules, and in some cases, it also involves the substrates. The elaborate proton delivery network of P450s is best illustrated in the elegant work by Schlichting and coworkers using trapping techniques and cryocrystallography (Schlichting et al., 2000). As reported earlier by Poulos et al (Raag et al., 1991), the camphor-bound P450cam has no water molecules in the active site. The side chain of Thr252 is 5.2 Å away from the heme Fe. Analysis of the structure of oxyferrous P450cam, however, revealed some very interesting changes induced by dioxygen binding. A new ordered water molecule (WAT901) appears in the so-called “groove” of the distal I helix above the porphyrin ring and at the same time Asp251 and Thr252 undergo conformational changes such that WAT901 forms a hydrogen bond with the hydroxyl group and the amide nitrogen of

Thr252. The sequestered WAT901 is connected through an internal water channel consisting of WAT902, WAT687, WAT566, WAT523, and this extends to Glu366 as shown in Figure 3. This internal water chain may constitute the proton delivery network. The proposed proton delivery network is supported by site-directed mutagenic studies and studies of kinetic solvent isotope effects. Mutation of Asp251 to Asn decreases the catalytic activity by 2 orders of magnitude and the turnover rates of the D251N mutant in protium/deuterium mixtures show a large kinetic isotope effect of 10 compared with 1.8 for the P450cam WT (Gerber and Sligar, 1992; Vidakovic et al., 1998). Mutation of the highly conserved Thr252 to Ala in P450cam diminishes the camphor hydroxylation activity without significantly affecting NADH consumption. As a result, the T252A mutant promotes hydrogen peroxide/water production (Martinis et al., 1989). The X-ray structure of ferric T252A mutant of P450cam shows that the internal water channel is present prior to dioxygen binding, demonstrating that the active site of T252A is more accessible to solvent molecules (Raag et al., 1991). The ready access of water molecules to the active site is likely the cause for hydrogen peroxide production. It appears that Thr252 functions like a “water gate” in addition to its role in proton delivery. The importance of hydration of the distal pocket is illustrated in the studies by Makris et al (Makris et al., 2007) where the effect of dehydration of the distal pocket was investigated by the introduction of bulkier residues like valine and threonine at the Gly248 position. Gly248 is located inside the I-helical groove where WAT901 is anchored by hydrogen bonding to the hydroxyl group and the nitrogen of the amide group of Thr252, as well as to the carbonyl oxygen of the amide group of Gly248. The groove was originally proposed to be a binding pocket for dioxygen. It was later established that the groove is in fact a binding pocket for a water molecule (WAT901) forming part of the proton delivery channel. Replacement of Gly248 with a bulky residue expels WAT901 from the groove and results in a substantial decrease in turnover rates and an inability to deliver the first proton to produce the hydroperoxo intermediate upon radiolytic reduction at 77 K as was observed in the D251N mutant.

The involvement of the hydroxyl group of the substrate in proton delivery was reported for P450c21, P450 107A, and P450 158A2 (Nagano et al., 2005; Zhao et al., 2005; Tosha et al., 2008). P450c21 catalyzes the hydroxylation of progesterone and 17 α -hydroxyprogesterone at C-21 position and the reaction is better coupled with 17 α -hydroxyprogesterone by 11%. The studies of the vibrational mode of the oxyferrous P450c21 by Raman spectroscopy demonstrated that ν_{O-O} is sensitive to the substrate; progesterone gave a single ν_{O-O} band at 1137 cm^{-1} while 17 α -hydroxyprogesterone split ν_{O-O} into two bands at 1124 and 1138 cm^{-1} (Tosha et al., 2008). It was postulated that 17 α -hydroxyprogesterone is hydrogen bonded to the iron-linked oxygen and participates in proton delivery.

P450 107A and P450 158A2 lack the highly conserved threonine that participates in proton delivery and dioxygen activation in most other P450s. In place of this highly conserved threonine is an alanine residue. The X-ray structure of dioxygen-bound P450 107A demonstrated that the 5-OH of the substrate 6-deoxyerythronolide B, a precursor for the biosynthesis of erythromycin, donates a hydrogen bond to the iron-linked dioxygen, and the active site water molecule is absent. The internal water channel connecting the I-helical cleft still exists as in P450cam (Nagano et al., 2005). The results suggest direct involvement of the 5-OH of 6-deoxyerythronolide B in the proton transfer process. A similar role has been proposed for the 5-OH/7-OH of the substrate flaviolin of P450158A2 by Waterman's group (Zhao et al., 2005). A 70-fold decrease in catalytic activity was observed when a substrate analogue (2-hydroxy-1,4-naphthoquinone) lacking the 5-OH and 7-OH groups was substituted for flaviolin. The X-ray structure showed that two flaviolin molecules are bound to the active site of P450158A2. Binding of the two flaviolin molecules not only closes the substrate access channel through the FG loop, but also stabilize three ordered water molecules in the active site through hydrogen bonding as shown in Figure 3. What is so revealing is that an ordered water chain connecting the iron-linked dioxygen to the bulk solvent of the distal surface of protein

is present in the X-ray structure of dioxygen-bound P450158A2. This is the only report showing a complete hydrogen bonding network extending from the active site to the protein surface in any P450. It appears that there are two distinct proton delivery pathways. Contrary to P450cam that uses an internal water channel on the proximal side of the heme for proton delivery, the postulated proton delivery channel of P450158A2 is located on the distal side and may be shared with the substrate access channel. Both of the pathways require the participation of ordered water molecules.

Similar to P450, the reaction center of PSII is highly oxidizing with a redox potential of ~ 1.3 eV (Rappaport et al., 2002). It is conceivable that the access of water and other small molecules to the active site must be regulated to avoid unwanted reactions. Recently putative water access channels have been reported for PSII. Based on solvent accessibility simulations and structural analysis of the crystal structure of PSII, water access channels were identified to be located at the luminal side of the oxygen evolving center (OEC) involving CP43 and probably other protein components like D1 and D2 (Murray and Barber, 2007; Ho and Styring, 2008).

Water molecules associated within proteins are involved in a variety of functional roles, some of which are specific to a given system, whereas others are general to all proteins. The protein-associated water molecules are in constant exchange with the bulk solvent and this dynamic component is crucial to protein function in several ways: 1) water is unlikely to be rate limiting in biological processes; 2) rotational entropy contributes to the binding of highly localized water molecules; 3) the rapid exchange of surface water molecules is key to protein motion; and 4) water is an important mediator for protein adaptability to changes in the environment. In the cases of Photosystem II (PSII) and P450, a water molecule is special since it is the substrate and product, respectively. All these functionalities demand the access of water molecules to the active sites to be highly regulated in order to avoid unwanted reactions.

It is of note that water molecules other than in the active site can also have effects on the P450 function. Studies of hydrostatic pressure and osmotic pressure on the spin transition of P450cam showed that the volume changes induced by hydrostatic pressure and osmotic pressure are 29 and -350 ml/mol respectively. It was thus deduced that a total of 19 water molecules affect the spin equilibrium and these must include water molecules outside the active site (Di Primo et al., 1995). The effect of water molecules on the interaction of P450 with Pdx was reported by Morishima's group based on an osmotic pressure study (Furukawa and Morishima, 2001). The interaction of P450cam with oxidized Pdx results in the uptake of 13 water molecules at the protein interface with a K_a of $0.058 \mu\text{M}^{-1}$, whereas there are 25 water molecules at the protein interface with a K_a of $0.83 \mu\text{M}^{-1}$ when Pdx is reduced. Apparently the additional water molecules enhances the binding of P450cam with reduced Pdx by 14-fold.

Oxygen transfer

In spite of decades of efforts to obtain spectroscopic evidence for the existence of Cpd I in P450 catalysis, its identification remains elusive. However, it is widely assumed that Cpd I is the oxygenating species that transfers the activated oxygen atom to P450 substrates. Our current understanding of the oxygen insertion mechanism through an iron-oxo species is largely ascribed to the work of Groves et al (Groves and McClusky, 1976). It was proposed that oxygen insertion occurs through abstraction of one hydrogen atom from the substrate to give a radical intermediate ($\text{R}\bullet$) followed by oxygen rebound to form C-OH as shown in Scheme I. The results from numerous studies of kinetics, stereoselectivity, and isotope effects for the hydroxylation reactions catalyzed by P450s conform to this proposed oxygen rebound mechanism. The most convincing evidence supporting the hydrogen abstraction-oxygen rebound mechanism probably came from the studies on the oxidation of a special group of substrates termed "radical clocks" whose the radical form has a known rate constant for rearrangement. Oxidation of bicyclo[2.1.0]pentane by microsomal P450s yielded both rearranged and unarranged products

and the ratio of rearranged to unarranged products implicated a lifetime of 50 ps for the radical intermediate, or a rate constant of $\sim 10^{10} \text{ s}^{-1}$ for oxygen rebound (Ortiz de Montellano and Stearns, 1987). Similar rate constants of $0.2\text{--}2.8 \mu 10^{10} \text{ s}^{-1}$ were reported for the oxidation of thujone by P450cam and P450 BM3 (He and de Montellano, 2004). As more radical clock compounds were tested, rate constants in the order of $10^{12} - 10^{13} \text{ s}^{-1}$ were reported, which imply a transition state rather than a true radical intermediate. This raises questions as to whether there exists alternative mechanism.

Taking into account the number of substrates and the complex reactions P450s catalyze, it is conceivable that other oxy intermediates, such as peroxo-iron, hydroperoxo iron or H_2O_2 coordinated iron, may also be involved in the reaction cycle. Thermodynamic calculation indicates that the redox potentials of both species are $\sim 1.0 \text{ V}$ (Koppenol, 2007) and thus their electrophilic attack on nucleophiles would be thermodynamically feasible. There is inferential evidence suggesting that the hydroperoxo (**6**) and peroxo (**5**) intermediates are capable of catalyzing the oxidation of hydrocarbons. As previously mentioned, mutation of the critical Thr252 to Ala in P450cam led to excessive hydrogen peroxide production, presumably due to disruption of proton delivery required to form Cpd I. Nonetheless, the hydroperoxo intermediate (**6**) of T252A of P450cam was formed even at 77 K (Davydov et al., 1999; Davydov et al., 2001). Mutation of the equivalent threonine residue, Thr303 in P450 2E1 and Thr302 in P450 2B4, decreased hydroxylation while enhancing the epoxidation of olefins (Vaz et al., 1998, 1998). The product ratios for epoxidation vs hydroxylation in WT and the T303A mutant differ by as much as 5–10 fold in the presence of *cis*- or *trans*-butene. Therefore, it was postulated that the hydroperoxo intermediate (**6**) is responsible for epoxidation of olefin compounds. Surprisingly, the T252A mutant of P450cam that shows no hydroxylation activity is able to catalyze epoxidation of an olefin substrate, providing support for the reactivity of the hydroperoxo intermediate in P450 catalysis (Jin et al., 2003, 2003). Involvement of a peroxo-iron intermediate (**5**) in P450 catalysis has also been postulated based on deformylation of cyclohexane carboxaldehyde catalyzed by the T302A mutant of P450 2B4 and the steroid deformylation reaction by P450 aromatase (Akhtar et al., 1982; Vaz et al., 1996).

The existence of multiple oxidants in P450 catalysis has not gained support from DFT calculations that concur with the hydrogen abstraction-oxygen rebound mechanism involving Cpd I. Based on these theoretic calculations Shaik and co-workers proposed a two-state reactivity theory where the low spin ($S=1/2$) iron-oxo species has no barrier for rebound while the high spin ($S=3/2$) iron-oxo species has a significant barrier for rebound and thus has a significant lifetime (Ogliaro et al., 2000). Variations in the spin state may account for the wide range of rate constants reported for oxygen rebound. Based on these calculations, the hydroperoxo-iron species is a strong base and protonation of its distal oxygen leads to barrier-free formation of Cpd I. Compared with Cpd I, a higher energy barrier is expected for epoxidation of olefins by hydroperoxo-iron species (Harris and Loew, 1998; Ogliaro et al., 2002). To what extent these theoretic calculations reflect the real environment for the dioxygen activation in the P450 catalysis is an important question.

Concluding remarks

The last decade has seen tremendous progress in understanding the molecular mechanism of dioxygen activation by the versatile P450 monooxygenases, particularly in the area of X-ray structural analysis, identification of activated oxygen intermediates, and density function theoretic calculations. Kinetic, biophysical and theoretical studies converged to validate the hydrogen abstraction-oxygen rebound mechanism by an iron-oxo species in the P450 catalysis even though spectroscopic evidence for the existence of Cpd I is still lacking. The participation of other oxidants such as the hydroperoxo- and peroxo intermediates in the P450 catalysis, though controversial, is a possibility that cannot be ruled out. EPR and ENDOR studies of

cryoreduced oxyferrous P450cam have provided conclusive evidence for the existence of peroxo- and hydroperoxo intermediates. Studies by site-directed mutagenesis, kinetic isotope effects, X-ray structural analysis and protein dynamic simulations have provided detailed mechanistic information about proton delivery networks. Two distinct proton delivery networks have been postulated. These studies reveal that the interplay of critical residues, ordered water molecules, and the substrate is crucial for protonation of dioxygen leading to activation. Efficient protonation of the iron-linked oxygen is a key step for the overall efficacy of the P450 catalysis. The variation in coupling efficiency among different isoforms of P450s may well reflect the efficiency of the proton delivery system. In a totally opposite way from PSII that oxidizes water molecules to dioxygen through sequential accumulation of oxidizing equivalents by photo-excitation of a special pair of chlorophylls, nature has worked its wonders in P450 to activate dioxygen through a sequential series of reductions for the vital cellular processes.

Abbreviation

P450	cytochrome P450
cyt b5	cytochrome b5
CPR	cytochrome P450 reductase
Pdx	putidaredoxin
Cpd I	compound I, NADP, nicotinamide adenine dinucleotide phosphate, PSII, photosystem II

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Figure 1.
Catalytic cycle of P450s.

Figure 2.

X-ray structure of P450cam showing the secondary structure and prosthetic heme moiety. The backbone is colored rainbow from blue at the N-terminus to red at the C-terminus. The heme and the thiolate ligand are represented as magenta and gray sticks, respectively.

Figure 3.

Examples of two different proton delivery pathways observed in P450s. (A) an internal proton delivery network as observed in P450cam, consisting of ordered water molecules, E366 and the critical residues of T252, D251 and G248 located in the groove of the I helix; (B) proton delivery network that involves substrates and extends to the distal surface of P450158A2. Water molecules are colored blue, dioxygen is red, the heme is magenta and the heme iron is orange sphere.

Scheme I.
Hydrogen abstraction-oxygen rebound mechanism