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Biochemical and Biophysical Research Communications 338 (2005) 346–354
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Review

Thirty years of microbial P450 monooxygenase research: Peroxo-heme intermediates—The central bus station in heme oxygenase catalysis

Stephen G. Sligar [a,b,c,*], Thomas M. Makris [a], Ilia G. Denisov [a]

[a] Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA
[b] Department of Chemistry, University of Illinois, Urbana, IL 61801, USA
[c] The College of Medicine, Univeristy of Illinois, Urbana, IL 61801, USA

Received 25 July 2005
Available online 24 August 2005

Abstract

Oxygen has always been recognized as an essential element of many life forms, initially through its role as a terminal electron acceptor for the energy-generating pathways of oxidative phosphorylation. In 1955, Hayaishi et al. [Mechanism of the pyrocatechase reaction, J. Am. Chem. Soc. 77 (1955) 5450–5451] presented the most important discovery that changed this simplistic view of how Nature uses atmospheric dioxygen. His discovery, the naming and mechanistic understanding of the first "oxygenase" enzyme, has provided a wonderful opportunity and scientific impetus for four decades of researchers. This volume provides an opportunity to recognize the breakthroughs of the "Hayaishi School." Notable have been the prolific contributions of Professor Ishimura et al. [Oxygen and life. Oxygenases, Oxidases and Lipid Mediators, International Congress Series, Elsevier, Amsterdam, 2002], a first-generation Hayaishi product, to characterization of the cytochrome P450 monooxygenases.

Keywords: Oxygenase catalysis; Heme-peroxo intermediate; Cytochrome P450

Oxygenases can be classed as mono- or di- depending on whether one or both atoms of O_2 are incorporated into the substrate [1,2]. The monooxygenases represent a sort of "half-way" point on the pathway for the full four-electron reduction of dioxygen to generate two water molecules, which is the reaction stoichiometry of the cytochrome oxidases. Monooxygenases require only two electrons and two protons to reductively cleave atmospheric dioxygen, producing only a single water molecule in the process while saving the second atom for substrate functionalization and formal oxidation. Hence, the cytochrome P450s have also been referred to as "mixed-function oxidases" since they catalyzed both an oxygenase and oxidase function. The electron transfer functionality of the P450s earned them the right to share the title of "cytochrome." It is the mechanistic enzymology of the cytochromes P450 that will be the focus of this chapter. A historical accounting of the discovery of these ubiquitous P450s is the subject of other excellent reviews in this volume dedicated to Professor Hayaishi and will not be repeated herein.

A word about nomenclature for the uninitiated

By the early 1970s, several P450 monooxygenases had been identified. These included the membrane-bound human, rat, and rabbit enzymes involved in drug and steroid metabolism as well as a single bacterial P450 heme system. It was widely thought that there were but a couple dozen P450s, and they were either labeled by their presumed substrate (e.g., P450cam for camphor), by electrophoretic mobility (e.g., LM-2 for liver microsomal fraction two) or by other shortened rubric. When it was clear that there existed a large superfamily of P450 isozymes, more than a single substrate could be metabolized by a given isoform and there was a complex regulatory mechanism that controlled the expression, a systematic nomenclature based on gene se-

* Corresponding author. Fax: +1 217 265 4073.
E-mail address: s-sligar@uiuc.edu (S.G. Sligar).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.094

quence was introduced [3]. For this chapter, P450cam is also referred to as CYP101 following this nomenclature.

There are many ways to organize a review of microbial P450 research. One is to attempt a historical chronology of contributions following Professor Hayaishi's initial discovery. A second might be a "walk around the wheel," describing the intermediate states of heme, oxygen, and substrate as well as the electrons/protons needed to link these states into a reaction cycle. A third might be to provide a myopic and biased list of the major discoveries related to cytochrome P450 mechanism over the past 30 years. All of these formats have been used in recent reviews [2,4,5].

For the sake of brevity, we begin the historical mechanistic discussion of cytochrome P450 from the mid-1970s. At this point, an adrenal mitochondrial system had been functionally reconstituted [6], and the bacterial P450cam CYP101 from *Pseudomonas putida* had been purified to homogeneity and reconstituted with its iron–sulfur and flavin electron transfer proteins to efficiently hydroxylate camphor with absolute regio- and stereo-specificity. We knew that the bound substrate displaced a water coordinated to the open axial ligand position thereby moving the system to a high-spin ferric manifold, and the first electron transfer formed a ferrous heme that could bind oxygen [7]. Though, pioneering work in the Gunsalus et al. [8] and Peterson et al. [9] laboratories, spectral evidence was obtained for the ferrous-oxygenated state, analogous to those well-studied species in the oxygen transport proteins, hemoglobin and myoglobin. This oxy–ferrous state was only quasi-stable, decaying back to ferric, with the release of superoxide, with a 90 s half-life at room temperature. Nevertheless, the existence of the oxy–ferrous intermediate was well documented, stabilized, and later isolated by the low-temperature methods pioneered by Douzou and coworkers [10]. P450cam was indeed a gift from Nature, as to date, this P450 isozyme displays the most stable ferrous–oxy state, which has allowed a more recent generation of subsequent intermediates in the reaction cycle. Thus, in the mid-1970s, the bioinorganic understanding of P450 catalysis could be summarized by the reaction cycle reproduced as Fig. 1A from the period graphics. The intervening 25 years have been spent attempting to: (1) structurally define these species and (2) observe and characterize the intermediate states of heme–iron, oxygen, and carbon substrate that make up the encircled question mark.

Since the early 1970s, it has also been appreciated that the P450s could use hydrogen peroxide and other exogenous oxidants to produce oxygenated products [11]. Enzymatic peroxidase mechanisms were well known at the time [12], and hence it was natural to think of the two enzymes as having a common set of intermediate states. A schematic describing this connectivity is shown in Fig. 1B. Since the oxygen atom remaining after water production is at the redox level of an "oxene" (with six electrons in its outer valence shell), the P450 enzymes are referred to as "oxene transferases." Thus, conceptually the balance of reducing equivalents, oxygen nuclei, and protons suggested that the question mark of Fig. 1A contained successive events of second electron transfer to make a peroxo anion, a single protonation to form a hydroperoxo, and finally a second protonation of the distal oxygen to effect the heterolytic scission of the O–O bond, forming an iron-bound "oxene" or "compound I–ferryl porphyrin cation radical" found in the peroxidases.

A focused view of the heme–iron–oxygen states of the peroxidases, P450 monooxygenases, heme oxygenases, and oxidases is provided by Fig. 2. This schematic places the heme-bound peroxo states as central and common players in these varied systems. The remainder of this chapter will review and summarize the documented observation of the peroxo states, as well as provide new data to contribute a spectroscopic characterization of this important central intermediate. From these schematic representations of

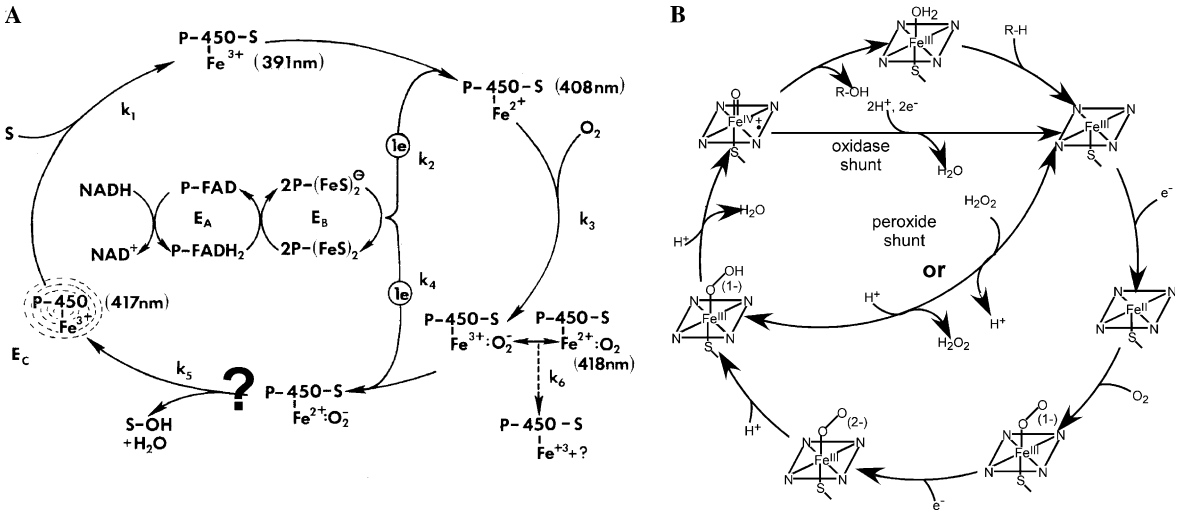

Fig. 1. Early (ca. 1975) views of the cytochrome P450 reaction mechanism. (A) Recognizes the importance of the ferrous-dioxygen intermediate and its intermediate position between two one-electron transfer events. (B) The conceptual merging of monooxygenase and peroxidase heme–oxygen chemistries, with the peroxidases bringing dioxygen, and two protons and two reducing equivalents to the heme reactive center. Obvious from this schematic is the potential of the P450 monooxygenases to utilize peroxide or any other exogenous oxidants to drive substrate oxygenation.

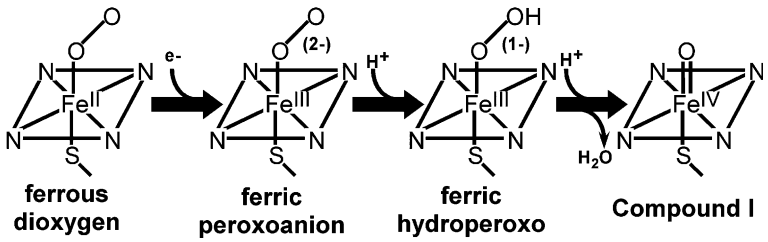

Fig. 2. Heme–oxygen states in the reaction cycle of cytochrome P450.

the cytochrome P450 "wheel," the overall goal of research in this field is clearly twofold. First to actually observe and spectroscopically characterize the intermediate states of oxygenated, peroxo anion, hydroperoxo, and the higher valent metal–oxo complex. The second is to define what, if any, chemical reactivities and physiological function can be assigned to each of these oxygenase intermediates.

Access to the peroxo states through radiolytic reduction of oxygenated heme proteins

Even though it was appreciated in the mid-1970s that a peroxo state existed following a second electron transfer, it was not until the pioneering work of Davydov, Huttermann, Peterson, and others who had introduced pulse radiolysis and low-temperature radiolytic reduction of frozen heme proteins together with electron paramagnetic resonance characterization to define a doubly reduced dioxygen state [13–18].

Radiolytic reduction at cryogenic temperatures is an approach similar to matrix isolation chemistry or low-temperature photolysis [19], wherein a desired transient species is generated by high-energy irradiation and stabilized by the solid solvent matrix. Since 1974 after the work on cytochrome *c* and hemoglobin [20], cryogenic radiolysis has been utilized as a method for immobilization of unstable heme protein intermediates. Using γ- or X-ray irradiation of frozen solutions, hemoglobin, myoglobin, cytochrome P450, peroxidases, and cytochrome *c* in different ligation states were reduced at 77 K and the stabilized intermediates were later characterized by optical absorption spectroscopy, magnetic circular dichroism, and EPR [12–17].

Importantly, the peroxo– and hydroperoxo–ferric intermediates in hemoglobin, myoglobin, HRP, and cytochrome P450 were isolated and assigned by their characteristic low-spin EPR signal with *g*-factors 2.27, 2.17, and 1.94 [5,21]. The wealth of early data has demonstrated that the radiolytically reduced intermediates formed at 77 K remained stable for many months as long as they were kept below the solvent glass transition temperature, typically 180–190 K. Using single crystal EPR on isotopically enriched ^{17}O and ^{57}Fe oxymyoglobin [16] and proton ENDOR [15], Huttermann and co-workers demonstrated that the excess spin density in reduced oxymyoglobin is located on iron and distal oxygen atoms, giving an early explanation for the EPR spectra observed subsequently for ferric-peroxo intermediates in heme proteins with different proximal ligands [14,17,21]. Later using optical absorption spectroscopy and ^{32}P irradiation, our laboratory [22] was able to show the direct conversion of oxy–ferrous CYP101 into the ferric-hydroperoxo intermediate as a result of one-electron reduction at 77 K, and to assign the Soret maximum at 440 nm to the same intermediate previously characterized by EPR [21,23]. The Q-band EPR and ENDOR investigations beautifully applied by Hoffman and colleagues will be described in detail in a subsequent section.

Spectroscopic characterization of a hydroperoxo state in P450 catalysis

Following the initial observation of an EPR signal consistent with a peroxo oxygen bound to the P450 heme via low-temperature radiolysis, there ensued a concerted effort to precisely define the electronic structure of the peroxo states by a variety of spectroscopic tools. Low-temperature stabilization of the ferrous–oxy complex, followed by introduction of the second electron via radiolysis, has allowed a detailed characterization of these intermediate states in P450cam CYP101. Although ^{60}Co provides an intense source of γ-rays and the most efficient method to generate the ~4 Mrad necessary radiolysis, access to high intensity ^{60}Co is sometimes difficult. The use of common [^{32}P]phosphate buffer solutions offers a convenient, albeit slow, path to these doubly reduced states [22,24].

An important tool for documenting peroxo states is UV–visible spectroscopy since this allows connectivity to ambient temperature rapid mix experiments. Table 1 shows our UV–Vis spectroscopic characterization of the hydroperoxo states as generated by low-temperature radiolysis of the ferrous–oxy complex in a variety of systems.

From Table 1, it is clear that the reduction of oxy–ferrous complexes in all heme protein systems results in an observed red-shift of the Soret absorption band. This shift is much more pronounced in the cytochromes P450 and chloroperoxidase (CPO), which have thiolate as a proximal ligand, than in the other heme proteins, which are ligated with a proximal histidine. The Soret bands of ferric-hydroperoxo intermediates in P450 and CPO resemble those observed in the bis-thiolate complexes by Dawson et al. [29] in their systematic overview of normal and hyperporphyrin spectra of P450 and CPO. The similarity is a result of

Table 1
Optical characterization of heme–peroxo states

Heme protein[a]	Fe^{2+}–O_2 cm^{-1} (nm)	Fe^{3+}–OOH cm^{-1} (nm)	Difference cm^{-1}
CYP101	23,980 (417)	22,700 (440.5)	1280
CYP101 (–S)	23,980 (417)	22,730 (440)	1250
CYP101 D251N	23,810 (420)	22,675 (441)	1135
CYP101 D251N–T252A	24,070 (415.5)	22,780 (439)	1290
CYP119 (–S)	24,040 (416)	22,780 (439)	1260
CPO (–S)	23,420 (427)	22,270 (449)	1150
HO heme complex	24,040 (416)	23,810 (420)	230
HRP	24,070 (415.5)	23,870 (419)	200
Mb	23,840 (419.5)	23,360 (428)	520

[a] *Abbreviations:* CYP101, P450 with the substrate camphor bound; (–S) without substrate; D251N–T252A the indicated site-directed mutants of CYP101 with camphor bound; CPO, chloroperoxidase; HO, heme oxygenase; HRP, horseradish peroxidase; Mb, sperm whale myoglobin; data cited from [22,25–28] and an unpublished work.

significant perturbation of porphyrin orbitals caused by strong electron donation from thiolate and peroxide axial ligands. We have previously reported a split-Soret band in the ferric–hydroperoxo complex formed with P450cam (CYP101) [22]. In Table 1, we present a similar spectral characterization of this state in chloroperoxidase measured at 77–120 K in frozen aqueous glycerol solutions. In contrast to a large red-shift in the Soret position for the thiolate ligated heme systems, in proteins with histidine coordination, the orbital mixing is not as extensive and the resultant shift in optical maxima upon introduction of the second electron is not as dramatic.

While optical methods provide a clear delineation of a peroxo intermediate in cytochrome P450, a detailed understanding of the chemical activity of such states as well as the structural definition of the hydrogen bonding and proton-donating entities demands a more precise understanding of the corresponding heme–ligand electronic structures.

EPR and ENDOR definition of P450 peroxo intermediates

Continued pioneering work by Davydov in collaboration with the EPR and ENDOR expertise of the Hoffman laboratory has provided an unprecedented look at the proton configuration associated with the heme-bound peroxide states in cytochrome P450 [21,23,30]. The application of ^1H ENDOR to the cryogenically irradiated wild-type and mutant CYP101 oxy-complexes has enabled the visualization of proton(s) coordinated to ferric-peroxo species. This has allowed the delineation between a proton that is bound covalently to the complex, as is the typical case for wild-type and the Thr252Ala mutant of CYP101, or one that is simply hydrogen bonded to the peroxo anion adduct, such as in Asp251Asn. A characteristic EPR fingerprint of the protonation process in P450s, and other heme enzymes (HRP, HO), in conjunction with mutant enzymes that deviate from the normal reaction coordinate, has enabled the assessment of the role of active-site residues and sequestered water in the first protonation step (the conversion of the peroxoanion to hydroperoxo) and the basis for peroxide uncoupling.

An important extension was the use of systematic thermal annealing studies to probe the chemical reactivity of peroxide-bound states in the P450 cytochromes. Above the glass transition temperature, one can visualize the evolution of peroxide complexes, eventually into the corresponding monooxygenation reaction products (hydroxycamphor ligated to the ferriheme in the case of CYP101) [23]. This technique has not only confirmed the chemical competency of the radiolytically prepared intermediates, but also provides a basis for addressing the chemical reactivity of heme-peroxide states. While a high-valent iron-oxo state proved impossible to detect in the dioxygen reaction with CYP101, the ^1H ENDOR profile of product ligated states in isotopically exchanged media is clearly consistent with hydroxylation via a higher valent species, in agreement with earlier computational results and isotope scrambling experiments [23]. Continuing work across the heme enzyme, active-site mutant, and substrate landscape by Davydov, Hoffman, and colleagues continues to address the chemical competency of heme-peroxide intermediates in metabolizing more electron-rich substrates.

X-ray absorption spectroscopy to define P450 peroxo structures

The EPR and optical characterization of radiolytically prepared peroxo–ferriheme states have clearly provided an indispensable fingerprint of these intermediates in the P450 monooxygenases. However, despite an ever-growing database, detailed information about the peroxo geometry in heme enzymes has trailed behind that obtained for the non-heme iron inorganic counterparts [31]. Clearly required is a precise structural definition of the heme iron coordination environment and associated changes as the cytochrome P450 steps through its catalytic cycle.

While the coordination geometry of the P450 ferric–peroxo complexes are clearly "end-on" with the iron which is in the low-spin configuration, it is difficult to infer any detailed ligand geometry from only the cryoradiolytic EPR/ENDOR studies outlined. Difficulties in controlling radiolysis in X-ray crystallography, and the overall issue of resolution, also make this approach difficult. Yet, there is clear need for such precise structural information since clear

differences in EPR *g*-tensor and optical spectra are evident across the landscape of peroxo–heme enzyme systems. These include the finite changes associated with the protonation state of the peroxo moiety, differences due to the identity of the axial ligand, the more prevalent histidine versus the thiolate of P450s, NO-synthase, and chloroperoxidase. Significant spectral changes are even observed with various P450 isozymes and substrates.

A powerful tool for defining coordination geometry and high-resolution distance determinations is X-ray absorption spectroscopy (XAS) [32]. In order to realize a precise understanding of the P450 peroxo–hydroperoxo structure and function, we have used XAS to quantitate the ligand environment of these radiolytically generated states in CYP101 [33]. Two distinct tools of XAS are used. First is a detailed analysis of the near-edge of the X-ray absorption cross-section of the heme iron, termed XANES. The second involves transforming the oscillations of the scattering cross-section at energies slightly higher than the edge wavelength. This is termed extended X-ray absorption fine structure spectroscopy (EXAFS). The reader is referred to many excellent reviews on XAS [32,34].

EXAFS was used successfully many decades ago to help identify cysteine as the axial ligand to the heme iron as well as monitor structural changes induced in CYP101 upon substrate and oxygen/carbon monoxide binding [35,36]. We have recently applied XAS to the CYP101 peroxo states. When we compared the near-edge features of oxyferrous and low-temperature radiolytically reduced form of wild-type CYP101, a shift to higher edge energy was observed, consistent with a metal centered oxidation of the iron. This clearly indicates a major structural change concomitant with a redistribution of electron density upon input of the second reducing equivalent required for monooxygenase catalysis. In addition, an observed enhancement of the pre-edge intensity clearly shows an enhanced decrease in centrosymmetry of the first coordination sphere of the heme iron. In a subsequent fitting of the EXAFS region, the decrease in symmetry is mostly attributable to an elongation of the proximal thiolate bond distance. This lengthening of the iron–cysteine bond matches that predicted by density functional theory (DFT) calculations using inorganic models of cytochrome P450 [37,38], and provides an initial understanding of the electronic and geometric changes associated with oxyferrous reduction and subsequent protonation and/or hydrogen bond formation to the distal oxygen of atmospheric dioxygen bound to the heme. These results are given in Fig. 3.

Raman spectroscopic characterization of heme–peroxo intermediates

While EPR/ENDOR can illuminate the existence of nearby protons potentially involved in oxygen activation and substrate functionalization, and XAS can precisely measure iron–nuclei distances, these techniques cannot define the electrostatics of the bound peroxo and separate the subtle differences between a short, strong hydrogen bond from a full covalent protonation event. Clearly needed are spectroscopic measurements of the relevant vibrational modes and energies of these intermediates. We have used resonance Raman (RR) spectroscopy to examine the vibrational structure of peroxo–heme complexes.

The first RR study of the ferric–hydroperoxo intermediate in any heme protein used myoglobin as a model system [28]. The ferric–peroxo intermediate was prepared using cryoradiolytic reduction of oxy-myoglobin precursor in frozen 50% glycerol-buffer solution at 77 K. The one-electron reduced products of radiolysis were assigned as mixtures of unprotonated ferric–peroxo and protonated ferric–hydroperoxo heme complexes using the EPR spectra obtained on the same samples that were used for RR spectroscopy. EPR spectra of the Fe^{3+}-OOH^- intermediate were in good agreement with those published earlier [15,16]. The main new result was the observation of a vibrational ν(Fe–O) stretch frequency identified at 617 cm^{-1} by a 25 cm^{-1} isotope shift when $^{16}O_2$ was replaced by $^{18}O_2$ and by a 4–5 cm^{-1} downshift in the deuterated solvent. Not surprisingly, the ν(O–O) stretch band was not observed, as it is not enhanced in the RR spectra of oxy–ferrous complexes in heme proteins, except those with proximal thiolate ligand, and the bacterial hemoglobins reported by Das et al. [39]. The higher ν(Fe–O) in ferric–hydroperoxo complex in Mb is in agreement with RR spectroscopic studies of similar complexes in non-heme systems [31]. RR studies of several mononuclear non-heme complexes together with DFT calculations [32,40–42] have established a correlation between the frequencies of ν(Fe–O) and ν(O–O) stretch bands and the spin state of the iron atom. For high-spin ferric–hydroperoxo complexes, the ν(Fe–O) band is observed at 440–470 cm^{-1}, i.e., lower than

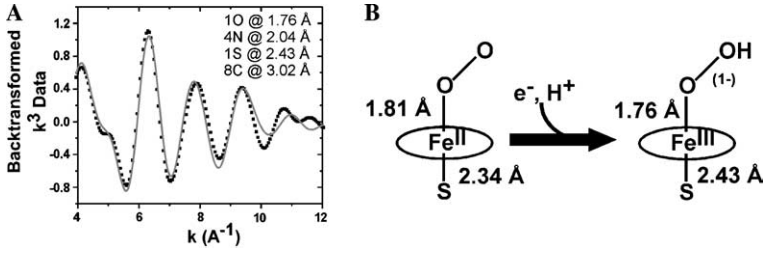

Fig. 3. X-ray absorption spectroscopic characterization of P450 peroxo intermediates. (A) Fit of observed EXAFS data with the indicated coordination model of the P450 peroxo states. (B) Measured iron–oxygen and iron–sulfur distances for the ferrous oxygenated and peroxo intermediates of CYP101.

530–580 cm^{-1}, typical for the same band in ferrous–oxy complexes, and the ν(O–O) band is usually at 820–880 cm^{-1}. Contrary, in low-spin ferric–hydroperoxo complexes the ν(Fe–O) is at 600–640 cm^{-1} and ν(O–O) is at 765–790 cm^{-1}. This means that the strength of the Fe–O bond increases in heme proteins as a result of reduction of the oxy–ferrous complex, and the O–O bond weakens to a larger extent than in similar high-spin complexes in non-heme systems. Hence, in heme proteins, where all known ferric–hydroperoxo complexes are in the low-spin state, the O–O bond is weak and prepared for heterolytic scission, which is the salient point of formation of the ferryl-oxo intermediate from its ferric–hydroperoxo precursor, catalyzed by monooxygenases and peroxidases. The work on the RR characterization of ferric–peroxo and ferric–hydroperoxo intermediates in cytochrome P450, which will provide the direct measurements on both ν(Fe–O) and the ν(O–O) bands as a function of protonation state of this key intermediate, is currently in progress.

Defining distal pocket hydrogen bonding and proton delivery

The unambiguous observation and spectroscopic characterization of the two-electron reduced peroxo–heme states in cytochrome P450 immediately raise the question as to the detailed three-dimensional structures of these states and an understanding of the distal pocket hydrogen bonding and proton-donating residues which contribute to the key steps in monooxygenase catalysis.

A major step forward in understanding P450 catalysis came through the use of site-directed mutagenesis of the bacterial P450 CYP101. In looking back at the vast field of P450 research, one is struck by how often P450 investigators have led or immediately capitalized on new technologies. For instance, the isolation of the P450cam gene by Dr. Koga, a second-generation Hayaishi product through Professor Horiuchi and working with the Gunsalus laboratory, led to its expression in *Escherichia coli* [43] and the first site-directed mutant of an enzyme.

Conserved residues in the P450 active site

By the mid-1980s, there were several amino sequences of P450 cytochromes known, and the realization that this class of oxygenases was indeed only the tip of a very large superfamily. Currently, over 5000 nucleotide sequences have been postulated to code for cytochromes P450. Hence, for some time, it was natural to examine the alignment of predicted protein sequences to shed light on important catalytic residues. Earlier alignment of these sequences revealed two residues that were conserved amongst most of the P450s known. This "acid–alcohol" an adjacent pair of residues, aspartate251-threonine252 in P450cam (CYP101), was of unknown function.

The first experimental focus was the alcohol side chain. The Ishimura and Sligar laboratories independently mutated the P450cam Thr252 to a variety of side chains [44,45]. Most interestingly, the loss of this hydrogen bonding alcoholic side chain had very little impact on the overall activity of the enzyme, as measured by the rate of pyridine nucleotide oxidation in a reconstituted system of putidaredoxin and reductase, yet the production of hydroxycamphor was essentially eliminated. Instead of productive hydroxylation, oxygen is reduced with the release of hydrogen peroxide, and hence the mutant protein is not able to effectively cleave the O–O bond. This uncoupling is thought to be the result of a failed specific delivery of a proton to the distal oxygen to effectively reduce the O–O bond order and generate the "Compound I" state. Initially, the threonine side chain was thought to be the proximal proton relay vehicle. Subsequent work, again following a pioneering and difficult set of experiments from the Ishimura laboratory where in vitro expression was used to place a methoxy group in the P450cam sequence at position 252 [46], suggested the existence of an intervening water molecule.

Mutagenesis of the highly conserved aspartate residue resulted in a different phenotype. The overall pyridine nucleotide oxidation rate was dramatically lowered, yet the mutant enzyme was still able to produce hydroxycamphor at a lower rate [47,48]. Together, these mutants have provided a tool set to use in a variety of physical biochemistry investigations designed to define the electronic structure and proton delivery mechanisms operating in the P450 monooxygenases.

Structural characterization of distal pocket hydrogen bonding and catalytic processing

A major breakthrough in the understanding of P450 catalysis resulted from the X-ray structure determination of the ferrous-oxygenated state by Schlichting et al. [49]. Of critical importance were the structural changes that occurred upon oxygenation of the ferrous heme, Fig. 4. Immediately obvious was a "flip" in the backbone amide linkage between the pseudo-conserved acid (D251) and alcohol (T252) residues. This relocation of the carbonyl, now providing a hydrogen bond with N255, provides a stabilized coordination for a new active site water molecule that is hydrogen bonded to the distal oxygen atom of the O_2 complex. This is made possible by the anchoring of the aspartate side chain to surface residues, allowing for the constrained rotation of the ϕ–ψ angles of this D251–T252 linkage. These results thus provided structural support for the Ishimura proposal of an active site water delivery of the first proton required for dioxygen cleavage.

Understanding the uncoupling of electron flow in P450 oxygenases

The reaction cycle of Fig. 2 identifies several places where the channeling of reducing equivalents toward the cleavage of the heme-bound dioxygen can go astray. An obvious site for electron leakage to air is at the

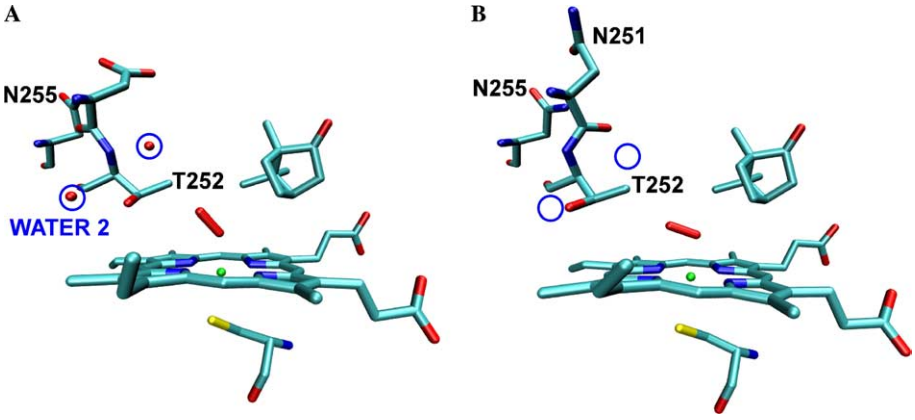

Fig. 4. Structural changes in the active site of P450 CYP101 upon oxygenation. The determination of the three-dimensional structure of the ferrous dioxygen-bound intermediate of CYP101 [49] reveals an important change in the hydrogen bonding configuration in the distal pocket. (A) The ϕ–ψ configuration of the D251–T252 amide linkage and the observed new water that provides a critical hydrogen bond to the distal oxygen atom of the bound dioxygen. (B) The structure of the D251N mutant oxygenated form. Immediately obvious is the lack of a stabilized water through the backbone configuration [33].

protein–protein interfaces between reductase, redoxin, and cytochrome P4450. Indeed, the autoxidation of flavin and iron–sulfur proteins has been well documented. Following transfer into the heme center, there remain three places where uncoupling can occur. The first is through autoxidation of the ferrous dioxygen adduct, releasing superoxide and regenerating ferric P450 [50]. Such heme autoxidations have been studied by Caughey and others for a variety of hemoproteins, and the nucleophilic displacement reactions of oxy P450cam documented [10]. However, the rate for one-electron autoxidation of P450cam is slow compared to the input of the second electron from putidaredoxin, and hence in the reconstituted system this channel is not populated to a great extent.

Two remaining branch points for uncoupling exist and play important physiological roles. The first follows second electron transfer and effects the release of hydrogen peroxide without oxygen–oxygen bond scission. The second is carried out through an "oxidase" stoichiometry wherein two additional electrons are thought to reduce a higher valent iron-oxo state to produce a second water molecule [51]. Only the first of these will be discussed here, as this channel provides an interesting probe of the proton delivery pathways that control the chemistry of oxygenase catalysis.

An obvious point for the peroxide uncoupling pathway to operate is at the level of the peroxo intermediates through a failure in the fidelity of proton transfer. One can consider two potential pathways. First could be a failure in the first proton delivery to the distal oxygen atom with instead delivery of this proton to the oxygen atom proximal to the coordinated heme. While the pK for the proximal oxygen is most certainly less basic than the distal oxygen due to electron push from the axial thiolate and "lone pair" effect of the intervening oxygen, control of pro-

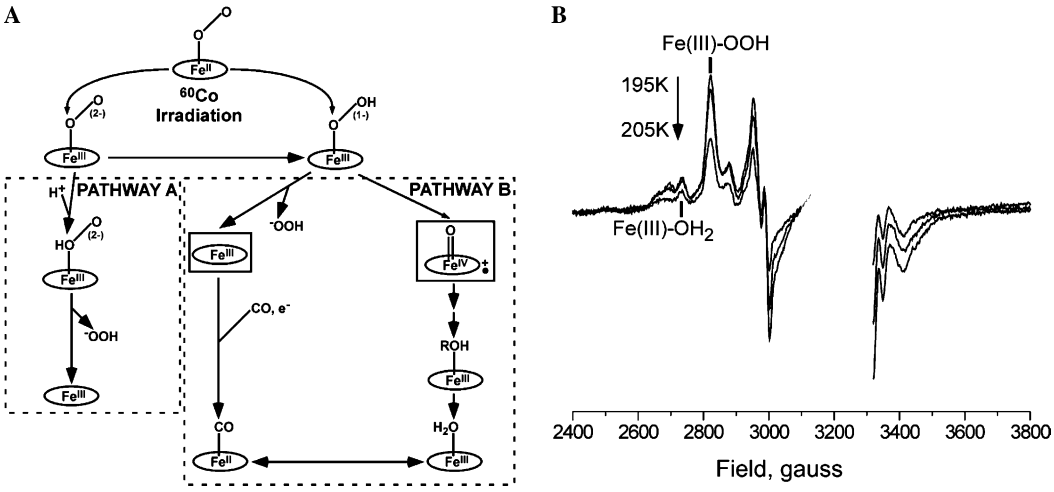

Fig. 5. Uncoupling pathways from the peroxo–heme states. The non-productive release of hydrogen peroxide presumably results from a protonation of the proximal oxygen. Two major pathways are illustrated in (A) which differ on whether this protonation occurs from a peroxo anion (pathway A) or a hydroperoxo state (pathway B), which follows protonation of the distal oxygen. (B) Stepwise annealing of the D251N–T252A mutant while monitoring by EPR and optical spectroscopy, clearly shows that hydrogen peroxide release occurs from the already formed hydroperoxo state [33].

ton transfer events is something most enzymes do very well. Alternatively, one can imagine that hydrogen peroxide release follows distal oxygen protonation from the hydroperoxo state. With the ability to stabilize both the peroxo and hydroperoxo intermediates via low-temperature cryoradiolysis, one has to adopt an experimental approach to verify theoretical predictions [37]. These pathways of heme peroxo decay are schematically illustrated in Fig. 5.

In order to understand the uncoupling reaction of CYP101 in detail, we made use of the previously described mutants T252A and D251N. The former resulted in complete (un)coupling of reducing equivalents in forming oxygenated product, releasing the two-electron reduced hydrogen peroxide. The latter results in a dramatic slowing of the overall enzymatic turnover, but retains the ability to produce hydroxy camphor, albeit at a much reduced rate. It is this D251N mutation that allowed the clear observation of the first proton transfer to bound peroxoanion to form the hydroperoxo state. Could we use this slow aspartate mutant background to reveal which of the alternate pathways for uncoupling occurs? The answer is yes. With reference to Fig. 5, the slowing of the overall enzymatic reaction with the D251N mutation allows the sequential observation of both peroxo and hydroperoxo states. The optical and EPR spectra obtained in the annealing of a cryoreduced D251N–T252A mutant clearly demonstrate that a first proton transfer to form the hydroperoxo state occurs before the events related to the uncoupled production of hydrogen peroxide take place [33].

Summary

Thirty years of microbial cytochrome P450 work have dramatically increased our fundamental chemical and physical understanding of oxygenase function. Despite these advances, much still remains to be understood as to how Nature controls the formation and ultimate chemical reactivity of the intermediate states of metal, oxygen, and substrate to effect efficient catalytic processing. We look forward to summarizing additional advances on the celebration of the next decade of Professor Hayaishi's long and illustrious career as a Physician–Scholar.

Acknowledgments

Our work over the past 30 years on the mechanistic enzymology and bioinorganic chemistry of P450 oxygenase catalysis has been generously supported by the National Institutes of Health. We are particularly thankful for a MERIT award (GM31756) that continues to provide the funds to define ever more precisely the functioning of P450 oxygenase systems. XAS measurements were made possible by the close collaboration of the Dupont-Northwestern-Dow CAT at the Advanced Photon Source and supported by the Department of Energy. We are particularly grateful to Dr. Ilme Schlichting for many illuminating discussions and the X-ray structure results on the oxygenated forms of wild-type and mutant cytochromes P450.

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