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Multiple oxidants and multiple mechanisms in cytochrome P450 catalysis☆

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Although I was familiar with Gunny's earlier groundbreaking studies on pyridoxal phosphate and lipoic acid, I do not recall meeting him until June of 1955. The occasion was a one-day symposium at the University of Illinois held in honor of Professor William C. Rose, who had been my Ph.D. thesis mentor and who was being honored on his retirement after 33 years as head of the Biochemistry Division in the Chemistry Department. Several friends had told me I would enjoy getting acquainted with Gunny because of our common interest in enzyme reaction mechanisms and especially because of his infectious enthusiasm and outgoing personality. On the contrary, he appeared on that occasion to be painfully shy and barely replied to questions that arose, even about his own work. I began to wonder whether, as the next head of biochemistry, he had sufficient vigor and intellectual drive to lead his academic colleagues into the future. Later I learned that he wanted Will Rose, who had a somewhat formal and quiet demeanor, to be completely in the limelight on that occasion. I admired Gunny for his consideration for his predecessor, and soon became aware that he had more than enough talent and drive to pursue his own career and provide leadership at Illinois with great success.

Little did Gunny and I guess that our research interests would later overlap closely and that we would become close personal and scientific friends over many decades, continuing to the present. In 1968 Anthony Lu and I described the role of a solubilized cytochrome P450 from an enzyme system in liver microsomes of phenobarbital-treated rabbits capable of catalyzing fatty acid ω-hydroxylation [1], and Katagiri, Ganguli, and Gunsalus reported finding a soluble cytochrome P450 as part of an enzyme complex in *Pseudomonas putida* that was functional in camphor hydroxylation [2]. In the years ahead, I read with great admiration papers from the Illinois group describing the rapid progress made with the bacterial system as we struggled with the purification of the mammalian membrane-bound system and with the added complexity of many similar, but distinct, P450LM isozymes.[1] At various national and international meetings, Gunny and I had a good-natured competition in describing our respective enzyme systems. When he called bacterial P450cam "selective" or "fastidious" in its substrate preference, I pointed out that it was actually "catalytically restricted" or "camphor-limited." Then when I touted mammalian P450LM as being "multifaceted" and the various isoforms as "the most versatile biological catalysts known," he used uncomplimentary terms like "promiscuous" or "indiscriminate."

The bacterial and microsomal systems were found to differ in several important respects despite having the same heme-centered P450 component as the oxygenating catalyst. In the former, NADH is required as the primary electron donor with the flavoprotein NADH-putidaredoxin reductase and the nonheme iron protein

☆ *Dedication.* Gunny has been an exceptionally fine model to several generations of biochemists as a scientist and as a teacher, and this article is written in recognition of his pioneering contributions to the field of cytochrome P450 and his friendship.

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[1] P450cam is the trivial name for the camphor-inducible enzyme in *Pseudomonas putida*, designated CYP101 (or P450 101) in the current systematic nomenclature based on divergent evolution. P450LM represents a large family of liver microsomal enzymes; the major phenobarbital-inducible isoform in rabbits, called $P450_{LM_2}$ on the basis of its electrophoretic behavior and now designated CYP2B4 (or P450 2B4), is the mammalian enzyme widely used in mechanistic studies. Similarly, the major ethanol-inducible isoform, initially called $P450_{LM_{3a}}$, is currently designated CYP2E1 (or P450 2E1).

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putidaredoxin as redox partners, whereas in the latter NADPH serves as the electron donor with the solubilized flavoprotein NADPH–cytochrome P450 reductase as well as a phospholipid such as phosphatidylcholine for maximal activity. To determine whether any of the components of the two systems were interchangeable, at Gunny's invitation I took my preparations to Urbana, and we carried out the necessary experiments with various combinations. His students watched intently (or apprehensively?) as we pipetted samples into the various reaction mixtures. By the end of a long Saturday afternoon it was clear that each system was highly specific and could not utilize components of the other. Thus, Nature had devised quite different methods of enabling these biologically unrelated P450 oxygenases to accomplish chemically difficult oxidations. In the years ahead the Urbana and Ann Arbor laboratories shared chemicals, enzymes, unpublished data, ideas, and even visiting scientists from around the world. The result was more rapid progress on these remarkable catalysts that did not reveal their secrets easily, as well as the pleasure and stimulation of open scientific exchange. Thanks to Gunny and his associates for their generous approach to research, we all benefited. The cytochrome P450 field is often described as one of the areas of biochemistry in which cooperation is typical. In no small measure this is a reflection of the Gunsalus example.

P450 reaction mechanism

Studies in our own and other laboratories on the mechanistic details of microsomal P450-catalyzed reactions and on the bacterial system in the Gunsalus laboratory, in conjunction with the known behavior of other heme proteins and model systems, led to formulation of a scheme for substrate hydroxylation [3]. The steps, still recognized as part of the overall cycle, are in accord with the stoichiometry of such reactions and are as follows: (1) substrate binding, (2) first electron transfer, (3) dioxygen binding, (4) second electron transfer, (5) splitting of the oxygen-oxygen bond with generation of iron-oxene, (6) proposed formation of a substrate radical as a transient intermediate based on studies with norbornane and P450$_{LM_2}$ [4], (7) oxygen insertion into substrate, and (8) product dissociation. Much has since been learned about the details of proton uptake (as discussed below), the probable identity of the "activated oxygen" shown here as $(FeO)^{3+}$, and even the possibility that species other than iron-complexed oxene may contribute to substrate oxygenation. This scheme, which soon reached the textbooks and has since appeared in numerous slightly altered versions, may now be mainly of historical interest, but applies to P450s occurring throughout Nature.

Diversity of reactions catalyzed

Numerous substrates and reactions are involved in catalysis by microsomal P450 [5], as indicated in Fig. 1. Oxygen reduction not only leads to substrate hydroxylation but also results in the release of superoxide and hydrogen peroxide as well as of water in a 4-electron NADPH oxidase reaction. The well-known peroxide shunt, in which hydrogen peroxide or an organic peroxy compound such as an alkyl hydroperoxide or peracid donates the oxygen atom to the substrate with no requirement for O_2 or of NADPH as an electron donor, is shown. Also depicted in the scheme is the ability of ferrous P450 to donate electrons in a stepwise fashion to bring about reductive reactions under anaerobic conditions. Many compounds, including dyes, *N*-oxides, and epoxides, undergo stepwise 2-electron reduction. Another example is the reductive cleavage of xenobiotic hydroperoxides and lipid hydroperoxides (shown as R'LOOH in the figure) with hydrocarbon formation [5]. The reaction is believed to involve stepwise 1-electron transfer, resulting in homolysis of the peroxide oxygen–oxygen bond and generation of an alkoxy radical, with β-scission of the latter and subsequent reduction of the secondary radical to the hydrocarbon.

Of particular interest to the search for "active oxygen" species in P450 function is the conversion of cyclohexane carboxaldehyde to cyclohexene with loss of the aldehyde carbon as formate [6], as shown on the lower left of the figure. This reaction was explored as a model for the demethylation reaction catalyzed by the steroidogenic P450s, aromatase and lanosterol demeth-

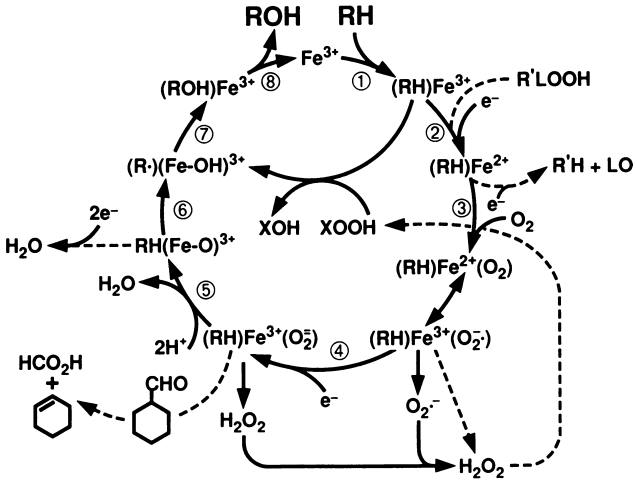

Fig. 1. Overall scheme for mechanism of action of microsomal P450. XOOH represents a peroxy compound that serves as an alternative oxygen donor to molecular oxygen, R′LOOH a lipid hydroperoxide, and R′H and LO the reduction products (alkane and oxo acid, respectively). RH represents a more typical substrate (fatty acid, hydrocarbon, steroid, or drug, for example) and ROH the corresponding product. (Taken from Ref. [5].)

ylase, in which an olefinic product and formate are formed. In our model reaction, a P450LM isozyme is required together with NADPH–cytochrome P450 reductase, NADPH, and O_2. Moreover, externally added H_2O_2 was found to be active with P450 in the deformylation reaction in the absence of the flavoprotein and NADPH. Along with additional evidence described below, these results indicate that a peroxy-hemiacetal-like adduct may be formed between the substrate and molecular oxygen-derived hydrogen peroxide. A role for peroxide in the P450-catalyzed demethylation of steroids has been proposed by Akhtar et al. [7] and several other research groups as reviewed elsewhere [5].

As is now widely known, the reactions catalyzed by microsomal P450s include epoxidation, peroxygenation, deamination, desulfuration, and dehalogenation and additional reactions attributable to P450 continue to be discovered [8]. The substrates attacked encompass physiologically important substances such as steroids, eicosanoids, fatty acids, and retinoids as well as numerous xenobiotics—drugs, alcohols, procarcinogens, antioxidants, organic solvents, anesthetics, dyes, pesticides, odorants, and flavorants. It is not surprising that such a versatile catalyst might use multiple species of "active oxygen" in attacking such an array of organic compounds.

Multiple activated oxygen species in P450 catalysis

Elegant studies in several laboratories with bacterial P450s in recent years have provided important new insights into the details of proton uptake and made possible our own investigations with P450LM isoforms on the involvement of several species of functional active oxygen. To summarize briefly, assuming that proton delivery occurs sequentially rather than in a concerted manner, the steps could be pictured as follows. Two-electron reduction of O_2 gives peroxo-iron, which upon protonation yields hydroperoxo-iron. Uptake of a second proton would then lead irreversibly to the putative oxene species and water or, alternatively, to free hydrogen peroxide and back to the ferric enzyme. The crystal structures of bacterial P450s cam [9], BM-3 [10], terp [11], and eryF [12], as well as sequence alignments [13,14], revealed a highly conserved threonine in the helix at the oxygen-binding pocket and within hydrogen bonding distance of the peroxo-iron complex. Of particular interest, significantly diminished camphor hydroxylation upon mutation of this residue to alanine was demonstrated with the P450 cam enzyme by both the Sligar group at Illinois [15] and the Ishimura group in Tokyo [16], and similar results were obtained for fatty acid hydroxylation with the BM-3 enzyme [17]. The observed effects were attributed to disruption of proton delivery to the active site.

We took advantage of such findings to explore the effect of mutation of the corresponding crucial threonine residue to alanine in a microsomal cytochrome, truncated P450 2B4. The results were dramatic, with an increase of about 10-fold in cyclohexane–carboxaldehyde deformylation to give cyclohexene and formate, and a decrease of about 9-fold in benzphetamine *N*-demethylation to give norbenzphetamine and formaldehyde [18]. Presumably the block in proton delivery to the active site leads to enhancement of the peroxo-iron species at the expense of the oxenoid-iron species. That is, the mutation had caused switching between the two different oxygenating agents. Furthermore, additional experiments on olefin epoxidation by 2B4 T302A and by the corresponding mutant of the alcohol-inducible cytochrome, 2E1 T303A, provided evidence for hydroperoxo-iron as well as oxenoid-iron as electrophilic oxidants [19]. As shown in Fig. 2, such results support the concept that three distinct oxidants are functional in P450 catalysis [20].

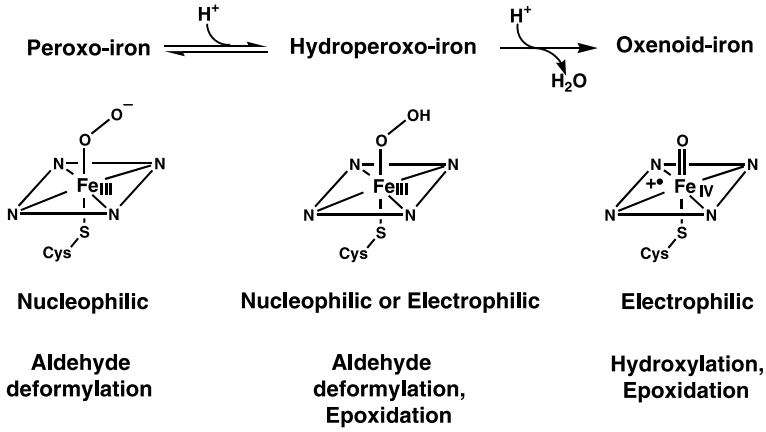

Fig. 2. Versatility in P450 oxygenating species. The nucleophilic or electrophilic properties and some typical reactions catalyzed are indicated under the structures of the peroxo, hydroperoxo, and oxenoid species. (Taken from Ref. [20].)

Insights gained with mechanistic probes

Among the many oxidations catalyzed by P450 enzymes are the remarkable hydroxylations of unactivated C–H bonds in hydrocarbons and related compounds. For more than two decades after several of the purified cytochromes became available, the mechanism of such reactions was thought to involve the initial production of an "iron-oxo" species similar to the known Compound I of peroxidase chemistry. As indicated above, evidence was provided from studies with mutant cytochromes that were blocked in proton delivery for multiple functional oxidants. However, further characterization of oxygenating species has been difficult because of their instability and perhaps also competing reactions. The iron-oxo species is best described as an iron(IV)-porphyrin radical cation, and the spin state of the complex is important [21]. The peroxo-iron and hydroperoxo-iron species have been detected by ESR following radiolytic reduction [22]. However, the iron-oxo species could not be detected, apparently due to its high reactivity, and its identification in transient X-ray crystallography was tentative [23].

Recent attempts to determine the lifetime of such intermediates by use of highly reactive "radical clocks" have shown that the mechanistic paradigm for P450-catalyzed reactions is not complete, but has confirmed the existence of two reactive electrophilic oxidants that can effect hydroxylation [24]. Oxidation of the mechanistic probes *trans*,*trans*-2-methoxy-3-phenylmethylcyclopropane and methylcubane by six isozymes of P450 has been described [25]. These probes were selected to differentiate between cationic and radical species because for these two kinds of intermediates different structural rearrangements occur. The cytochromes used were full-length 2B1 and 2B4 (phenobarbital-inducible), as well as NH_2-terminal-truncated 2B4 and 2E1 together with the corresponding mutants with threonine replaced by alanine. These mutants were those previously alluded to that led to the finding of three functional oxidants [18,19]. The results from oxidations of probes were consistent with previous evidence for two electrophilic species, with the hydroperoxo-iron complex and the iron-oxo complex as effectors of hydroxylation reactions. Furthermore, it was concluded that both species involve insertion processes (with no radical intermediates formed), that hydroxylation by the iron-oxo species gives a neutral alcohol product directly, and that hydroxylation by the hydroperoxo species involves insertion of the elements of OH^+, with a protonated alcohol as the first-formed product.

We have recently reviewed current knowledge of the cytochrome P450-catalyzed hydroxylation reaction, which is complex, involving multiple oxidants and multiple mechanisms [26], as summarized in Fig. 3. In addition to the commonly regarded iron-oxo species, another electrophilic oxidant is believed to exist, either the hydroperoxo-iron species that precedes iron-oxo or the iron-complexed hydrogen peroxide formed by protonation of the hydroperoxo-iron species on the proximal oxygen. This "second" electrophilic oxidant appears to react by insertion of OH^+ into a carbon–hydrogen bond to give a protonated alcohol, as already stated. In contrast, insertion of oxene gives the unprotonated alcohol directly. The scheme takes such findings into account as well as computational work by Shaik et al. [27]

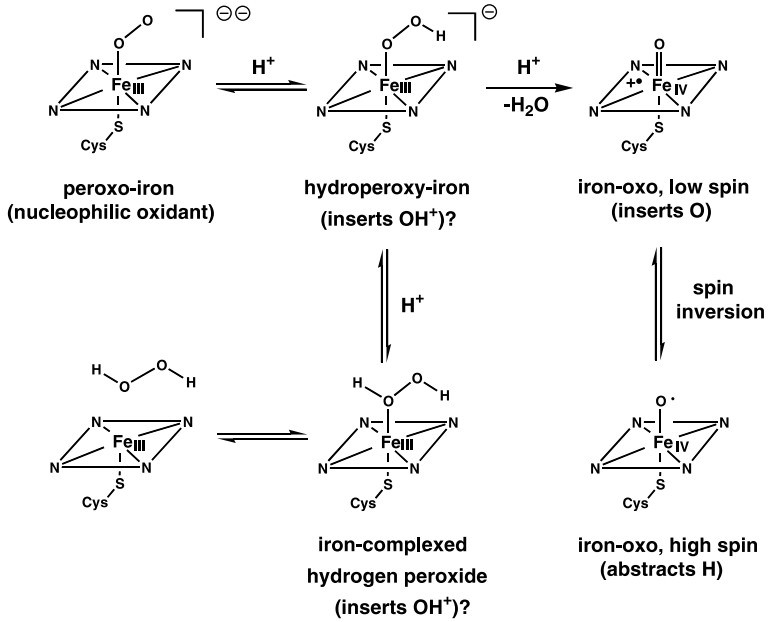

Fig. 3. The iron–oxygen intermediates in P450 catalysis and their possible roles as oxidants. (Taken from Ref. [26].)

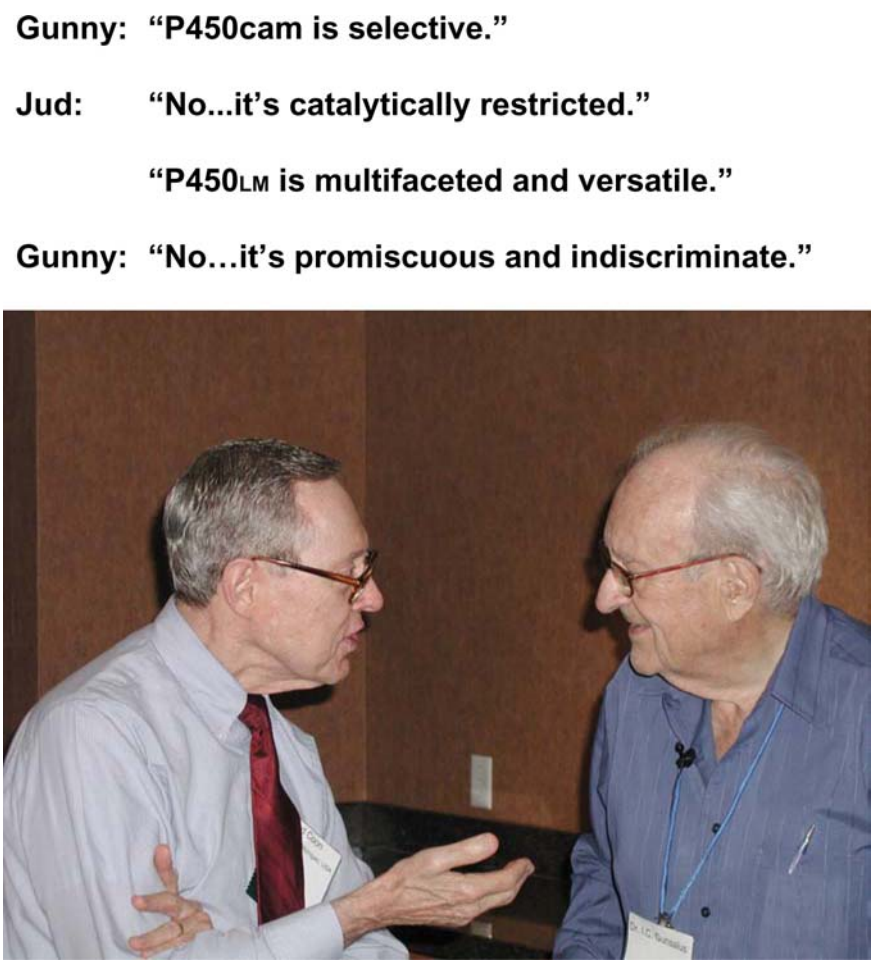

Fig. 4. The photograph, taken at the Sixth International Symposium on Cytochrome P450 Diversity held in Los Angeles in August 2002, and caption were provided by Steve Sligar.

and Yoshizawa et al. [28] that suggests iron-oxo can function in multiple spin states, low-spin and high-spin ensembles that react by oxygen insertion and by hydrogen abstraction to yield a radical, respectively. The proposed low-spin ensemble would insert oxygen by the pathway envisioned in the very early days of P450 mechanistic research [29,30], and the proposed high-spin ensemble would, via hydrogen abstraction, give a radical intermediate, thus resembling the oxygen-rebound pathway put forth some years later [4].

Finally, it is envisioned that cryogenic spectroscopic studies, kinetic measurements with mechanistic probes and other substrates, and models derived from computational work will all provide further information on the identity of multiple functional oxidants in P450 chemistry. P450cam will continue to be an outstanding model in such investigations, being more soluble, more stable, and more readily purified and having a higher catalytic rate than the P450 isozymes of liver microsomes. In contrast, the various P450LM isozymes are exceedingly more versatile (or promiscuous; see photograph in Fig. 4 provided by Steve Sligar), allowing almost any organic compound of mechanistic interest to be studied as a substrate. Both have been highly useful and will continue to be studied as remaining problems of oxygen activation are solved.

Acknowledgments

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