Structure and Chemistry of Cytochrome P450

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1. Introduction

The title to a seminar presentation by I. C. Gunsalus in 1973 was "Oxygen: An essential toxin", referring to the complex role that atmospheric dioxygen has in biology. The relatively simple function as terminal oxidant for aerobic life was dramatically augmented by Osamu Hayaishi with his identification of an enzyme that catalyzes the conversion of catechol to muconic acid by oxidative cleavage.[1] He named this biological catalyst "pyrocatechase", which proved to be the landmark discovery of an enzyme that incorporated atmospheric dioxygen into the carbon chain of the substrate, thereby initiating cleavage of the benzene ring. This review of the oxygenase cytochrome P450 is dedicated to Dr. Hayaishi and his pioneering discovery in what is now the 50th anniversary of his work!

We now realize that Nature has found many ways to utilize atmospheric dioxygen to functionalize molecules through the use of a diverse set of cofactors. Flavin, non-heme iron, copper, and metalloporphyrin complexes have all been conscripted to metabolize atmospheric dioxygen in an oxygenase catalytic cycle, resulting in the incorporation of one or both oxygen atoms into a substrate. This review focuses on one of the heme-containing classes, termed cytochrome P450s and abbreviated CYP. Although but one member in the large group of oxygenases, the cytochrome P450s play a variety of critical roles in biology.

Many members of the cytochrome P450 superfamily of hemoproteins are currently known, and the numbers continue to grow as more genomes are sequenced. There are almost 4000 identified P450 genes at the date of this writing, and they are collected and annotated in a variety of web sites, such as that maintained by Nelson (http://drnelson.utmem.edu/CytochromeP450.html). The cytochrome P450s have been found in all branches of the "tree of life" that catalogs the diversity of life forms. In the broadest terms, there are two main functional roles for these oxygenases. One is the metabolism of xenobiotics (compounds exogenous to the organism) as a protective role of degradation or provision of polar handles for solubilization in preparation for excretion. A second broad functional role is in the biosynthesis of critical signaling molecules used for control of development and homeostasis. In mammalian tissues the P450s play these roles through the metabolism of drugs and xenobiotics and the synthesis of steroid hormones and fat-soluble vitamin metabolism and the conversion of polyunsaturated fatty acids to biologically active molecules, respectively. Similar roles are fulfilled in plants (hormone biosynthesis and herbicide degradation) and insects (control of development via hormone biosynthesis or provision of insecticide resistance). For instance, plants have an unusually large number of P450 genes. A reason is their sessile nature: for example,

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Ilia Denisov graduated in molecular physics and spectroscopy from the Department of Physics, Leningrad State University (now Sankt-Petersburg) in 1981. In 1991 he received his Ph.D. degree in Polymer Physics from the Institute of Macromolecular Compounds, Russian Academy of Sciences. He was a visiting scientist at the University of Milan, Italy, where he worked on hemoglobin ligand binding intermediates with Professor M. Perrella. In 1996 he moved to Washington University to continue his work on hemoglobin with Professor G. K. Ackers. Since 1999 he has been in Professor S. G. Sligar's laboratory, currently as a research scientist. His main interests are in the field of spectroscopic studies of reaction intermediates in heme enzymes and of the structure and stability of proteins in complexes with other biological molecules.

Thomas Makris graduated from the Department of Biology at the University of Pennsylvania in 1996. He received his Ph.D. degree at the University of Illinois in 2003 in the Department of Biophysics and Computational Biology in the laboratory of Professor Dr. Stephen Sligar. His research interests during his Ph.D. work and since then as a postdoctoral researcher in the Sligar laboratory have included the biophysical characterization, chemistry, and protein-mediated transformation of heme–oxygen intermediates in the cytochrome P450s.

plants defend themselves through breakdown of herbicides by catalyzing the synthesis of a large number of secondary metabolites or by synthesizing defense molecules such as DIMBOA.[2,3] In addition, the biosynthesis of critical metabolic regulators is also often carried out by the cytochrome P450s.

The important metabolic role together with the unique chemistry and physical properties of the cytochrome P450s provide a strong attraction for scientists in many disciplines. Relevance to human health was the initial focus of pharmacologists and toxicologists. The role of metal centers and their associated unique spectral properties in the cytochrome P450s is a magnet for bioinorganic chemists and biophysicists. The difficult conversion of unactivated hydrocarbons attracted the bioorganic chemist. With the genome revolution and insights into the complex process of transcriptional and translational regulation, biochemists and molecular biologists found exciting problems in the study of CYPs.

Stephen G. Sligar received his Ph.D. degree in Physics from the University of Illinois in 1975. He served on the faculty in the Department of Molecular Biophysics and Biochemistry at Yale University and returned to the University of Illinois in 1982, where he now holds the I. C. Gunsalus endowed chair in Biochemistry. He is also a member of the Department of Chemistry, the Center for Biophysics and Computational Biology, and the College of Medicine. He holds affiliate appointments in the Beckman Institute for Advanced Science and Technology and the Institute for Genome Biology on the Illinois campus. Awards he has received include a Fulbright Research Scholarship, a Senior Fellowship from the Japan Society for the Promotion of Science, and the Bert L. and Kuggie Vallee Visiting Professorship in inorganic chemistry at Oxford. He is a fellow in the Jerome Karle Nobel Laureate World Innovation Foundation. His research is supported by a Merit Award from the National Institutes of Health as well as grants from the National Science Foundation and National Institutes of General Medical Sciences and centers on understanding the structure and mechanistic function of metalloenzymes and membrane-bound receptors and transporters.

Ilme Schlichting studied biology and physics in Heidelberg, Germany, and received her Ph.D. degree in Biology from the Ruprecht Karls University in Heidelberg in 1990. After a postdoctoral stay with Professor Gregory A. Petsko, Brandeis University, she became a group leader in Roger S. Goody's Department at the Max Planck Institute for Molecular Physiology in Dortmund, Germany. Since 2002 she has been leading the Department of Biomolecular Mechanisms at the Max Planck Institute for Medical Research in Heidelberg, Germany. She is interested in understanding the structural basis of the function and mechanism of proteins. Her current interests include heme- and flavin-containing proteins.

A continuing challenge is to understand how the diverse set of substrate specificities and metabolic transformations are determined by the precise nature of the heme–iron oxygen and protein structure. The structure and electronic configuration of the "active

oxygen" intermediates which serve as efficient catalysts remains an area of active research. Complicating this richness in metabolic potential is the importance of genetic differences, including single nucleotide polymorphisms, which can alter the physiological responses of the cytochrome P450s. Thus, over the past five-plus decades one has seen the evolution from a whole-organ and animal pharmacology approach to a quest for the molecular details necessary for precise understanding of structure and function of the P450 systems in maintaining cellular homeostasis. The P450s are now recognized to occupy a great variety of phylogenetically distributed isoform activities, and these variations in metabolic profile and substrate specificity are ultimately dictated by the bioinorganic chemistry of heme iron and oxygen as controlled by the protein environment.

With the elucidation of precise structures for many P450 hemoproteins as well as the application of varied biochemical and biophysical methodologies, this diverse class of oxygenases is beginning to yield its secrets. Much remains to be learned, however, as many of the fundamental chemical entities and catalytic details, though perhaps described in textbooks, are in fact still poorly understood. The focus of this review is to place the current knowledge base of cytochrome P450 structure−function in context with the general aspects of metalloenzyme function. In 2006 Dr. Hayaishi, the founder of this broad field of oxygen metabolism, will celebrate an important birthday. Hopefully, in reading this review, he will be struck with the outstanding progress that has been realized with this one particular oxygenase and at the same time perhaps provide some important suggestions as to pathways for solving the remaining problems.[4]

Cytochrome P450 has benefited from the attention of inorganic, organic, and physical chemists since its discovery due to its unique spectral properties as well as its ability to efficiently catalyze a variety of difficult biotransformations. With the discovery of P450 involvement in steroid biosynthesis in the 1970s, joined with its central function in drug metabolism, with its role in a variety of other pharmaceutical applications, P450 became one of the most intensively investigated biochemical systems. Multiple monographs, printed conference proceedings, and thematic books have been published as well as special *Methods in Enzymology* volumes, only a few of which can be referenced here.[5−12]

The cytochrome P450s became most known for their efficiency in hydroxylation of unactivated alkanes as only a select few oxygenases possess the requisite "active oxygen" state. With equal efficacy, P450s can carry out a wide variety of biotransformations. The list in ref 13 includes more than 20 different chemical reactions. Some more unusual reactions catalyzed by P450 were recently reviewed by Guengerich.[14]

The mechanism of P450 is a complex cascade of individual steps involving the interaction of protein redox partners and consumption of reducing equivalents, most commonly in the form of NAD(P)H. It is somewhat humbling that the earliest versions of the enzymatic cycle published over 30 years ago had much of the important steps characterized by physical and chemical methods.[15] Continual refinement has led to more detailed versions and the direct observation and structural characterization of new adducts of iron and oxygen. The current version contains eight intermediates, including highly transient caged radical pairs, and has been reviewed from various perspectives.[11,12,16−19]

While the basic concepts central to P450 catalysis were appreciated by early 1970, notable progress in the detailed understanding of these mechanisms has been made in the past decade. This has been possible due to the accumulation of exciting data generated through application of a wide set of new methodologies, including systematic directed mutagenesis, high-resolution X-ray crystal structure determination, multiparametric spectroscopic characterization of intermediates, isolation of critical steps using cryogenic or fast kinetic techniques, and many excellent quantum chemical and molecular dynamics computational studies. The current view of the oxygen activation mechanisms, catalyzed by metal centers in heme enzymes (as well as in non-heme enzymes, which lie outside the scope of this review), ensures one with a much better opportunity to see the common mechanistic picture than was possible earlier.[20] Successful mechanistic studies of other heme enzymes which use different forms of so-called 'active oxygen intermediates', such as peroxidases,[21,22] heme oxygenases (HO),[23−25] catalases,[26] nitric oxide synthases (NOS),[27,28] peroxygenases,[29,30] provide a vision of a highly diverse cofactor. Mechanistic insight from each of these various systems has provided important complementary insight into cytochrome P450 mechanism. A fundamental question remaining is how the protein controls efficient performance of such different functions using similar highly reactive heme−oxygen complexes. The comparison of similar reactive intermediates in different enzymes helps to distinguish between the essential features of each of the enzymes and so provides additional clues to the revelation of the active role of the protein in heme−enzyme catalysis. The recent progress in isolation and cryogenic stabilization of some of these intermediates makes possible direct spectroscopic and structural studies of this type.

An exhaustive review of all achievements in oxygen activation chemistry is clearly difficult, even if the field is limited to the processes directly relevant to P450 catalysis. Discussion of the P450 catalytic mechanism typically focuses on the state of heme iron and oxygen. These steps are (i) oxygen binding to the reduced heme iron and formation of an oxygenated heme $Fe^{2+}-OO$ or $Fe^{3+}-OO^{-}$, (ii) one-electron reduction of this complex to a ferric peroxo state $Fe^{3+}-OO^{2-}$, which is easily protonated to form the hydroperoxo $Fe^{3+}-OOH^{-}$, (iii) second protonation of the latter $Fe^{3+}-OOH^{-}$ complex at the distal oxygen atom to form an unstable transient $Fe-OOH_2$, which is followed by heterolytic scission of the O−O bond and release of the water molecule, and (iv) the various reactions of the remaining higher valent porphyrin metal−oxo complex, often described as a ferryl−oxo

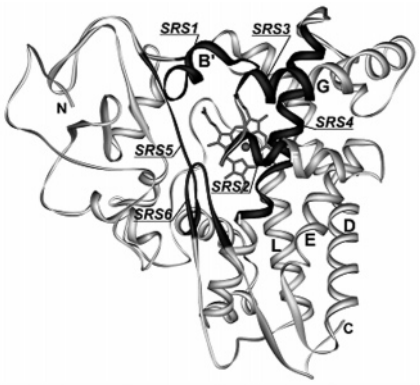

Figure 1. The fold of cytochrome P450s is highly conserved and shown in a ribbon representation (distal face). Substrate recognition sequence (SRS) regions are shown in black and labeled. α-Helixes mentioned in the text are labeled with capital letters.

π-cation porphyrin radical and referred to as "Compound I", following the terminology commonly used after pioneering discoveries made by Theorell, Keilin, and Chance.[31,32] The intermediates formed in reactions ii−iv have common features in all cytochrome P450s, peroxidase, cytochrome oxidase, and heme oxygenase enzymes. Similar iron−oxygen states are thought to play a key role in non-heme oxygen activation. Since it is impossible to cite and mention all relevant studies in the current review due to space limitations, we focus on the critical steps that are specific for P450 function and mechanism. Hence, neither the oxygen activation in non-heme metalloenzymes reviewed in refs 33−35 nor the structure and properties of relevant porphyrin complexes, including models of cytochrome P450 and other oxygen-activating enzymes, as reviewed in a recent edition of the *Porphyrin Handbook*,[36−41] will be discussed in detail. Two Internet sites devoted to the cytochrome P450 field provide the reader with a lot of useful information and links to other valuable sites and to the vast literature that is available for these systems: http://drnelson.utmem.edu/CytochromeP450.html and http://www.icgeb.org/~p450srv/.

2. Active-Site Structure of P450 Enzymes

P450 enzymes share a common overall fold and topology (Figure 1) despite less than 20% sequence identity across the gene superfamily.[42] In past years significant progress has been made in determining the crystal structures of mammalian P450s,[43−50] complementing the information available on the bacterial and fungal cytochromes. The conserved P450 structural core is formed by a four-helix bundle composed of three parallel helices labeled D, L, and I and one antiparallel helix E.[51] The prosthetic heme group is confined between the distal I helix and proximal L helix and bound to the adjacent Cys−heme−ligand loop containing the P450 signature amino acid sequence FxxGx(H/R)xCxG. The absolutely conserved cysteine is the proximal or "fifth" ligand to the heme iron. This sulfur ligand is a thiolate[52] and is the origin of the characteristic name-giving 450-nm Soret absorbance observed for the ferrous−CO complex. Typically, the proximal Cys forms two hydrogen bonds with neighboring backbone amides. A further interaction with a side chain is observed in some P450s, for example, with a Gln in CYP152A1 or a Trp in nitric oxide synthase. Mutations of these and similarly placed residues, e.g., Phe393 in CYP102 (P450 BM3), have pronounced effects on the reduction potential[53−55] or the catalytic activity and the stability of the bond between the heme iron and its fifth and sixth ligand in NOS.[56−59] The long I helix forms a wall of the heme pocket and contains the signature amino acid sequence (A/G)-Gx(E/D)T which is centered at a kink in the middle of the helix. The highly conserved threonine preceded by an acidic residue is positioned in the active site and believed to be involved in catalysis,[60−63] as described in detail in other sections of this review.

Although the P450 fold is highly conserved, there is enough structural diversity to allow for the binding of substrates of significantly different sizes to different P450s (e.g., from ethanol in CYP2E1 to the large peptide antibiotics in CYP165B1 (OxyB) and CYP165C1 (OxyC))[64,65] and with varying degrees of specificity. Some P450s are highly regio- and stereospecific in their oxygenation of substrate, whereas others such as the human liver CYP3A4 metabolize over 50% of the current marketed pharmaceuticals. At the simplest structural level substrate recognition/binding is provided by six "substrate recognition sites" (SRS):[66] the B′ helix region (SRS1), parts of the F and G helices (SRS2 and SRS3), a part of the I helix (SRS4), the K helix β2 connecting region (SRS6), and the β4 hairpin (SRS5) line the P450 active site (see Figure 1). In particular, the SRS predetermine P450 substrate specificity, and point mutations within the SRSs significantly affect substrate specificities.[66] The SRSs are considered to be flexible protein regions, moving upon substrate binding in an induced-fit mechanism so as to favor substrate binding and subsequently the catalytic reaction.[67]

3. Enzymatic Reaction Cycle of Cytochrome P450

A common catalytic cycle of the cytochrome P450s proposed in 1968 (for a review, see refs 17 and 68) still provides the core description of the iron, protein, and oxygen roles and is now generally accepted in an updated form (Figure 2).[16,69,70]

The sequential two-electron reduction of cytochrome P450 and existence of multiple intermediates was first discovered in bacterial CYP101 (P450cam)[71−73] and microsomal systems[74] in the late 1960s and early 1970s. The substrate binding to a resting state of the low-spin (LS) ferric enzyme (**1**) usually perturbs the water coordinated as the sixth ligand of the heme iron and changes the spin state (often partially) to the high-spin (HS) substrate-bound complex (**2**). The HS Fe^{3+} has a more positive reduction potential and thus in CYP101 is much easier reduced to a ferrous state (**3**).[75] In other systems it was observed that the spin shift is not an obligatory part of this cycle, and hence the detailed kinetics of the various cytochrome P450s are difficult to categorize with a common general set of simple rules.[76] Oxygen binding leads to an oxy−P450 com-

plex (**4**), which is the last relatively stable intermediate in this cycle. The reduction of this complex, sequential formation of a peroxo−ferric intermediate (**5a**), its protonated form hydroperoxo−ferric intermediate (**5b**), second protonation at the distal oxygen atom with subsequent heterolysis of O−O bond and formation of Compound I (**6**) and water, and oxygenation of the substrate to form a product complex (**7**) have been addressed by many experimental methods, but very few direct measurements have been possible due to the high reactivity and lack of significant accumulation of these intermediates in kinetic studies.

In addition to having multiple distinct intermediate states, each of which can display its own rich chemistry, the P450 reaction cycle contains at least three branch points, where multiple side reactions are possible and often occur under physiological conditions.[77] The three major abortive reactions are (i) autoxidation of the oxy−ferrous enzyme (**4**) with concomitant production of a superoxide anion and return of the enzyme to its resting state (**2**), (ii) a peroxide shunt, where the coordinated peroxide or hydroperoxide anion (**5a**,**b**) dissociates from the iron forming hydrogen peroxide, thus completing the unproductive (in terms of substrate turnover) two-electron reduction of oxygen, and (iii) an oxidase uncoupling wherein the ferryl−oxo intermediate (**6**) is oxidized to water instead of oxygenation of the substrate, which results effectively in four-electron reduction of dioxygen molecule with the net formation of two molecules of water. These processes are often categorized together and referred to as uncoupling.

3.1. Substrate Binding

In general, the substrates for cytochrome P450 metabolism are hydrophobic and poorly soluble in water, although the metabolism of alcohols, phenols, detergents, and many other organic substances also occurs. Extensive lists of the various substrates and corresponding P450 systems involved have been published.[78,79] Substrate binding to cytochrome P450s is a complex process and could easily be the sole topic of a review, since this biomolecular recognition event can trigger the change of the spin state from LS to HS in the heme iron and concomitantly induce a change in the reduction potential from ca. −300 to ca. 100 mV more positive. In the resting state, or in equilibrium with aerobic media, cytochrome P450s appear in the ferric Fe^{3+} form because of a relatively low reduction potential of the Fe^{3+}/Fe^{2+} couple, usually in the range between −400 and −170 mV.[80−84] This low redox potential is maintained by the presence of the negatively charged proximal thiolate ligand, and the same negative shift of reduction potential can be induced in other heme proteins such as myoglobin[85] by replacement of the proximal His by Cys. The LS−HS thermodynamic equilibria for the Fe^{3+} and Fe^{2+} states of the enzyme are coupled with the sixth ligand binding equilibrium, as described previously.[75,86] As a result, the experimentally measured midpoint potential of the heme enzyme depends on the ability of the substrate to change the heme ligand binding equilibria in both ferric and ferrous enzyme.[87] In purified P450 systems, in the absence of other compounds which can serve as stronger ligands, water coordination at the sixth distal ligand position can stabilize the LS state of the ferric iron.[88] The reduced ferrous cytochrome P450s are predominantly in the HS five-coordinated state because water is a much weaker ligand for the Fe^{2+} heme. This difference in ligation state of ferric and ferrous P450 in the absence of substrates is responsible for the additional stabilization of the ferric state and for the lower midpoint potentials of substrate-free cytochrome P450s.[75]

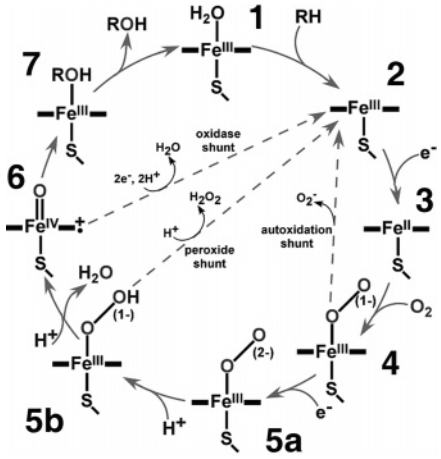

Figure 2. P450 catalytic cycle.

As a result of the substrate binding the water molecule coordinated to Fe^{3+} is usually displaced,[88,89] which is indicated by the shift of the spin state of five-coordinated heme iron to HS, as seen in the case of camphor binding to CYP101.[75] The loss of the sixth ligand of the heme iron thermodynamically destabilizes the ferric state of cytochrome P450 with respect to the Fe^{2+} state, and the midpoint potential of the heme shifts to positive values. The stronger the ability of the substrate to perturb the water ligation to the ferric heme, the more pronounced is the resulting positive shift of the redox potential. These same factors affect the reduced enzyme. In the presence of CO or O_2, which do not bind to ferric iron porphyrins but are strong ligands for the Fe^{2+} heme, the directly measured reduction potential shifts to more positive values as compared with those measured under inert atmosphere.[86,90] One more indication of this coupling between substrate binding and change in reduction potential is the difference between substrate binding free energy in the ferric and ferrous state, which is the direct consequence of a closed thermodynamic cycle as described.[75,86] Other conditions, including temperature and pH as well as the presence of cosolvents, may also change the parameters of the observed high-spin−low-spin equilibrium via perturbation of the sixth ligand binding and at the end significantly change the reduction potential of cytochrome P450.

These changes represent one specific regulatory mechanism, common for this class of enzymes. Such a linkage between substrate binding and redox potential changes explains why the first reduction of the enzyme is shown in Figure 2 as following the

association of substrate with enzyme. In most P450 systems the ultimate reducing agent for the catalytic cycle is NAD(P)H, which has a midpoint potential of −320 mV, and the reduction potentials of the protein's redox partners are roughly in the same range.[91,92] This means that cytochrome P450 should be reduced only slowly before substrate binds. The presence of this mechanism was recognized as an advantageous safeguard against unproductive turnover of the enzyme with the waste of NAD(P)H and formation of toxic superoxide and peroxides which is rendered more likely due to the fast autoxidation of thiolate ligated heme proteins. More complex and finely tuned control of substrate turnover kinetics apparently works in mammalian cytochrome CYP3A4, which is capable of binding two or three substrate molecules.[93,94] In this system the spin shift caused by the cooperative substrate binding can serve as an allosteric switch from slow turnover at low substrate concentration to the faster turnover at high substrate concentrations. As a result, the observed cooperativity of product formation is sometimes higher than the cooperativity of substrate binding,[93] consistent with recent cooperative models of CYP3A4 kinetics.[95]

Many of the cytochrome P450s have a broad spectrum of possible substrates for each individual P450. In some sense this feature seems to render the cytochrome P450s an exception to the classic "lock and key" concept.[96] Instead, they seem to represent a striking embodiment of the "induced fit" model by Koshland in which the enzyme may accommodate very different substrates in the active center by virtue of high flexibility and ability to undergo appropriate conformational changes. Several examples of such specific conformational changes can be visualized by comparison of the same or similar cytochrome P450s without substrate in the active site and with bound substrates.[45,97] Reviews of structural plasticity in cytochrome P450s related to substrate access and product dissociation pathways have appeared in the literature.[67,98]

Large structural rearrangements on the scale of more than 10 Å induced by substrate binding were observed by comparison of X-ray crystal structures of cytochrome P450s crystallized in substrate-free and substrate-bound forms.[67] Analysis of these structures suggests that the aforementioned substrate recognition sites are flexible and provide substrate access to the heme, which is otherwise buried in the protein globule. In the absence of charged and hydrogen-bonding groups on substrate molecules as well as in the active sites of most of P450 enzymes, such binding mechanisms provide an alternative means for substrate stabilization at the active center. As a result, in many cases different substrate analogues bind tightly to P450 enzymes simply because of their poor solubility in water and not because of strong interactions at the active site.[99] One of the most revealing examples is the binding of 'wired substrates'[100,101] in which the natural substrate camphor is tethered to the fluorescent reporter group by hydrophobic links of different length. They bind to CYP101 much tighter than the original unmodified camphor (K_d = 0.8 μM for camphor and 0.02 μM for the most hydrophobic modified substrate[100]) because of the favorable effect of dehydration of the hydrocarbon tether. The X-ray crystal structures of these "wired substrates" bound to CYP101 clearly demonstrate that the long hydrophobic tail is extended through the apparent substrate binding channel in the protein globule up to the surface, where the fluorescent group is partly exposed to the solvent.[102] The same large-scale conformational changes were also observed with substrates linked to a Ru complex.[103] Interestingly, these tethered modified substrates do not displace water coordinated to the heme iron and do not shift the spin state to high spin, as do camphor and adamantanone.[103] This reflects the sensitivity of the spin shift regulatory mechanism to the structure of the bound substrate or analogue, which may be used by the enzyme as a way to recognize the optimal substrates in the situation when there are almost no specific functional groups at the active center which may control the substrate binding specificity.

3.2. Iron Spin Shift and the Heme Redox Potential

Some substrates of the cytochrome P450s bind with very high affinity but do not display the marked shift in the spin state of the ferric heme.[100] For example, a recent crystal structure shows no water at the sixth ligand position for the "wired substrate" but does contain water for another analogue.[102]

Spin state and electronic structure of hexacoordinated low-spin ferric porphyrin models of cytochromes have been extensively discussed by Walker.[104,105] The coupling of the spin state transition with redox potential changes is placed in context with a more general description of the ligand-dependent redox potential.[104,105] The redox potential as the Fe^{3+}/Fe^{2+} equilibrium is perturbed by the changes in ligation state or the changes in ligation strength in the course of reduction or oxidation. In the presence of strong ligands for the ferric heme the redox potential is lower, whereas strong ligands for the ferrous state will increase the redox potential of the heme enzyme (see ref 104 for detailed analysis). This general concept is valid even without notable changes in the spin state, caused by ligand replacement, although the equilibrium constants for the spin state equilibrium change for different ligands.

Other factors may significantly change the heme reduction potential in cytochrome P450, especially if it interacts with other proteins. Resonance Raman[106] and NMR[107,108] studies of ferric CYP101 revealed specific conformational changes of heme, proximal thiolate, and key distal pocket residues caused by formation of complex with putidaredoxin (Pdr). Interaction with adrenodoxin induces the high-spin shift in CYP11A1.[109] The spin state of the heme iron in CYP101 in complex with Pdr is also coupled with specific changes in the ESR spectrum of reduced Pdr,[110] and this coupling may be significant for the function, as noted in a recent theoretical study.[111] Interaction with Pdr also perturbs the CO stretch band in Fe−CO complex in CYP101 and promotes the electron donation to the heme iron from the axial

sulfur ligand of Cys357.[112] The conformational changes in P450 reductase may also play an important role in reductase catalysis, which does not depend solely on the redox potential of the flavin.[113]

The shift of the heme iron spin state from low spin to high spin caused by substrate binding and the resulting change in reduction potential of cytochrome P450 are different manifestations of the same process, the experimentally observed shift of thermodynamic equilibrium in the system with the coupled microscopic equilibria, as a result of the change of one extensive parameter, the substrate concentration.[75,114] Other parameters, such as ligand concentration or temperature, can also change the position of this equilibrium.[86] The same correlation of the spin shift caused by the substrate binding with the changes of redox potential was reported in other cytochrome P450s[87] and related heme enzymes.[115]

The redox potential is a thermodynamic measure of the equilibrium between different oxidation states, and although it often correlates with biologically observed reduction kinetics,[116] it does not solely determine the rate of the heme reduction. In fact, the kinetics of reduction also depends on the spin state change because of the reorganization energy difference involved in changes from a six-coordinated to five-coordinated state. This mechanism of kinetic regulation via spin change has been rationalized on the basis of the Marcus equation.[86] If the reduction is accompanied by changes in coordination state and spin state, the activation barrier is higher and the rate of such a reaction is slower. For example, the spin shift alone was reported to be responsible for the 200-fold increase of the reduction rate in substrate-bound wild-type CYP102 as compared to the substrate-free mutants F393A and F393H of the same protein, although all three enzymes have similar reduction potentials.[117] Despite the positive shift of the reduction potential in these two mutants, the overall catalytic turnover rate is higher in the wild-type enzyme, presumably because of the kinetic control of the later steps in the kinetic cycle, primarily the second electron transfer to the ferrous−oxy complex. On the basis of resonance Raman spectroscopic studies, the large changes of the heme iron reduction potential were attributed partly to the conformational changes of propionate and vinyl groups.[53] Other factors also may be important in reduction kinetics, such as specific conformational changes caused by substrate binding, as well as by formation of the complex with electron-transfer partners.[118] For example, tight binding of steroidal P450 with its redox partner adrenodoxin was found to increase the rate of P450 reduction and product formation.[109] In addition, substrate binding was suggested to be a rate-limiting step in some P450 systems in vivo, partly because the concentration of the substrate is low.[119]

3.3. Oxygen Complex

Oxygen binding to reduced P450 gives the species **4**, the $Fe^{2+}-OO$ (ferrous dioxygen) complex, or $Fe^{3+}-OO^{-}$ (ferric superoxide) complex.[120] The gross structure of oxy−P450 is similar to analogous complexes in oxygen carrier heme proteins (Mb, Hb) and heme enzymes (HRP, HO, etc.). In P450 this complex is diamagnetic and EPR silent, like a ferrous $Fe^{2+}-OO$ complex, but shows Mössbauer quadrupole splitting for iron in oxy−CYP101[121] consistent with Fe^{3+}, similar to those observed for Compound III (oxy−ferrous complex) in HRP[122] and oxy−Hb.[123] The low O−O stretch frequency (1140 cm^{-1}) in the resonance Raman spectra of oxy−CYP101[124,125] is also typical for a ferric superoxide complex. This brought up the continuing discussion about the correct assignment of these complexes in terms of iron's formal oxidation state, Fe^{2+} or Fe^{3+},[126−129] and gave rise to more sophisticated models, like a three-centered four-electron, ozone-like bond.[130,131] Recently the same model dioxygen−iron−porphyrin complexes with different proximal ligands were studied using modern methods of quantum chemistry[132,133] and confirmed the earlier conclusions on the mixed character of dioxygen and iron in these complexes, with only partial electron density transfer from iron to oxygen as averaged over multiple electronic configurations.[111,132,134] Several reviews of iron−dioxygen complexes in oxygen binding proteins[135] and model porphyrin complexes[120,136−142] have been published.

The electronic properties of oxy−P450 were characterized for CYP101 and some other isozymes using optical absorption,[143−146] resonance Raman,[124,125,147,148] EXAFS/XANES,[149,150] and Mössbauer[121] spectroscopies. Structure and spectroscopy of P450 and chloroperoxidase (CPO) complexes with O_2, CO, and CN^{-} have also been reviewed.[13,151] On the theoretical side, a molecular orbital description of dioxygen binding to the ferrous heme has been given.[132,135,152] A theoretical explanation of the specific split Soret band for complexes of the ferrous P450 and CPO with diatomic ligands was first noted in refs153 and 154 for the case of CO and later was extended for other electron-rich ligands with a strong back-donation (e.g., dioxygen, cyanide, thiolate).[13,151]

Absorption spectra and autoxidation properties of oxygen complexes for several other cytochrome P450s[54,55,117,145,146,155−157] and the related thiolate-ligated enzymes NOS[158−160] and CPO[161] have been reported. All are less stable and autoxidize much faster in the substrate-free state, but even in the presence of substrate the autoxidation rates for P450, CPO, and NOS are significantly higher than those for the oxygen carriers Mb and Hb. This may be explained, at least in the case of the monooxygenases which protonate the distal oxygen as part of their natural activity, by general acid catalysis as easy protonation of the coordinated oxygen promotes autoxidation.[162] The active proton delivery to the bound oxygen or peroxide ligand is a salient feature of the P450 mechanism of oxygen activation, and the same mechanism can facilitate autoxidation in these enzymes. In addition, the strong dependence of the autoxidation rate on the reduction potential in mutants of CYP102 also stresses the important role of electronic structure and equilibrium thermodynamic properties of the heme.[117]

Despite significant efforts, the X-ray crystal structure of the $Fe^{2+}-OO$ complex of CYP101 was determined relatively recently[69] and found to be similar

to analogous complexes of other heme enzymes. Oxygen is coordinated in the bent 'end-on' mode with the angle Fe−O−O 142°, indicating no steric conflict with the bound substrate molecule. This Fe−O−O angle is similar to that of 110−123° observed in myoglobins,[163−167] 135−160° in hemoglobins,[168−172] 131° in cytochrome *c* peroxidase,[173] 126° in Cpd III in HRP,[174] 114−134° in guanylate cyclase,[175] and 101−114° in heme oxygenase.[176]

3.4. Formation of the Peroxo/Hydroperoxo Complex

As noted above, the stability of the peroxo state $Fe^{3+}-OO(H)^-$ is marginal, at least in heme enzymes, where the presence of a strong proximal ligand (His, Cys, or Tyr) and aqueous solution near neutral pH defines the structure of most of these complexes as η^1-Fe or end-on and low-spin state. Numerous attempts to isolate such complexes obtained in reactions of hydrogen peroxide with heme enzymes at ambient conditions failed because of their low stability[177,178] and fast conversion to ferryl−oxo species.[179]

Chemical models of $Fe^{3+}-OOH^-$ and $Fe^{3+}-OOR^-$ complexes were prepared using reactions of metalloporphyrins with peroxides at low temperature (200−230 K in solution or freeze-quenched at <120 K) and were studied by EPR, NMR, and optical spectroscopic methods.[180−183] Similar results were obtained with heme proteins,[184] including P450[185] and likely chloroperoxidase.[186] These studies turned out to be in a good agreement with the results obtained using cryogenic radiolysis. Specifically, the characteristic low-spin EPR spectra with narrow *g*-span and the red shift of the Soret band (as compared to the spectrum of oxy−ferrous precursor) were found to be the main spectroscopic features of $Fe^{3+}-OOH^-$ complexes. The direct reactions of peroxide dianion with free Fe^{3+}−porphyrins usually afford the high-spin $Fe^{3+}-OO^{2-}$ complexes with the side-on-bound peroxide and iron displaced out of the porphyrin plane toward the bound ligand.[183,187,188] To realize such structure in the heme protein it would be necessary to break the proximal ligand bond to the iron. It turns out that the presence of the strong proximal ligand, which favors the low-spin state in hexacoordinated $Fe^{3+}-OOH^-$ complexes, is an important restriction on the chemistry of oxygen activation, characteristic of the heme enzymes, as compared to non-heme metalloproteins.

The $Fe^{3+}-OOH^-$ complexes in heme systems have been extensively investigated theoretically by Loew and co-workers[189−194] and other groups.[195−197] These works, together with analogous studies on heme[19] and non-heme[34,35] enzymes, played a definitive role in establishing the currently accepted view on the importance of the second protonation step of the distal oxygen atom of the peroxide ligand for the fast heterolytic cleavage of the O−O bond with Compound I formation.

Many attempts to create and stabilize the reduced oxy−ferrous complex in a cytochrome P450 date to the earliest days of P450 mechanistic studies.[198] These included the steady-state and stopped-flow studies of reconstituted systems,[199−203] replacing dioxygen by superoxide or peroxides as oxygen donors, use of alternative chemical, photochemical,[204] and pulse radiolytic[205,206] methods for fast and efficient reduction of the preformed oxyferrous P450 for spectroscopic characterization. However, only in recent years has the reproducible stable preparation of $Fe^{3+}-OOH^-$ complexes with high yield in P450 and other heme enzymes been achieved using the methods of radiolytic reduction of oxy−ferrous precursors in frozen solutions at 77 K.[207−212] Irradiation with high-energy photons from ^{60}Co γ-sources or using ^{32}P-enriched phosphate as an internal source of high-energy electrons[210,213] generates radiolytic electrons, which can reduce a preformed oxy−ferrous heme protein complex. It was demonstrated that these $Fe^{3+}-OOH^-$ complexes are only stable enough to be characterized in the low-temperature solid state, where translational and rotational motions are severely limited. This approach has long been used in solid-state physics and in matrix isolation chemistry of highly reactive intermediates, formed by photolysis or radiolysis from the precursors frozen in the neutral matrix, usually solid Ar, Kr, or Ne.[214,215] Indeed, the experimental and theoretical methods for selective annealing of point defects in the solid state have provided important guidance in these investigations.

Cryogenic radiolysis in biochemistry and biophysics was first used as a tool for spectroscopic studies of the nonequilibrium intermediates in heme proteins.[211,216] Radiolysis of several ferric proteins with different ligands in frozen aqueous−organic solutions at 77 K was shown to produce the corresponding ferrous species, which then could be annealed at elevated temperatures, and the conformational and chemical relaxation processes were monitored by EPR and optical spectroscopic methods.[216−221] The first reports on cryoradiolysis of oxy−ferrous complexes in heme proteins and formation of $Fe^{3+}-OOH^-$ complex in Hb, Mb, and HRP[222−229] have established the characteristic EPR spectrum of $Fe^{3+}-OOH^-$ complexes with a signature narrow span of *g* values (2.3−2.25, 2.2−2.14, and 1.94−1.97). Optical absorption[225,226] and Mössbauer[123] spectra were also reported in these studies. Similar EPR spectra were obtained using cryoradiolytic reduction of CYP101,[207,230] see Table 1.

Recently, this approach was used systematically by Davydov, Hoffman, Sligar, and collaborators to study the structure and stability of peroxo−ferric intermediates in heme enzymes[156,208,211−213,231−233,236−240] and non-heme systems.[241−248] As a result, direct spectral identification of intermediates **5a** and **5b** was achieved in cytochrome P450s, HO, HRP, and NOS. Interestingly, the EPR spectra are sensitive to the protonation state of the peroxide ligand but do not depend significantly on the nature of the trans proximal ligand, His or Cys. UV−vis spectra have shown a much lower sensitivity to the protonation state of the peroxide. For example, the Soret band and Q-bands display shifts by 3 nm with protonation of the peroxide in the mutant D251N of CYP101 (Makris et al., unpublished data). On the other hand, these bands are sensitive to the nature of the proximal ligand—the Soret band of intermediate **5b** is at

Table 1. EPR Spectra of Peroxoferric and Hydroperoxoferric Enzymatic Intermediates

enzyme	ligands	O_2/e^- donor	g-value	assignment	refs
nitric oxide synthase	Cys	O_2, γ	2.26, 2.16, 1.95	FeOO (η_1)	231
CYP101	Cys	O_2, γ	2.25, 2.16, 1.96	FeOO (η_1)	207, 208, 232
			2.30, 2.17, 1.96	FeOOH (η_1)	
CYP119	Cys	O_2, γ	2.29, 2.20, 1.95	FeOOH (η_1)	156
rat microsomal P450	Cys	BuOOH	2.29, 2.24, 1.96	FeOOR	185
horseradish peroxidase	His	O_2, γ	2.27, 2.18, 1.90	FeOO (η_1)	210
			2.32, 2.18, 1.90	FeOOH (η_1)	
heme oxygenase	His	O_2, γ	2.25, 2.17, 1.91	FeOO (η_1)	209, 233
			2.37, 2.19, 1.92	FeOOH (η_1)	
hemoglobin	His	O_2, γ	2.25, 2.15, 1.97	FeOO(η_1)	223, 228
			2.31, 2.18, 1.94	FeOOH (η_1)	
myoglobin	His	O_2, γ	2.23, 2.13, 1.97	FeOO (η_1)	228, 229
			2.31, 2.18, 1.93	FeOOH (η_1)	
myoglobin (H64N,V)	His	H_2O_2	2.29, 2.16, 1.91	FeOOH (η_1)	234
myoglobin	His	H_2O_2	2.32, 2.19, 1.94	FeOOH (η_1)	235

Table 2. Parameters of Optical Absorption Spectra for Oxyferrous and Hydroperoxoferric Heme Enzyme Complexes

enzyme	proximal ligand	distal ligand	Soret band, nm	refs
chloroperoxidase	Cys	O_2	427	*a*
		OOH^-	449	
CYP101	Cys	O_2	417	213
		OOH^-	440	
CYP119	Cys	O_2	417	156
		OOH^-	440	
horseradish peroxidase	His	O_2	416	210
		OOH^-	419	
heme oxygenase	His	O_2	416	238
		OOH^-	421	
hemoglobin	His	O_2	414	226
		OOH^-	421	
myoglobin	His	O_2	420	212
		OOH^-	428	
myoglobin (H64N,V)	His	OOH^-	430	234

[a] Denisov et al. Unpublished results.

440 nm in P450 but at 420 nm in HRP and HO, see Table 2.

The radiolytic reduction of **4** at 77 K yielded in most cases a protonated hydroperoxo−ferric complex **5b**, although in several proteins, where this protonation was not expected (oxy−Mb, HO mutant, mutant D251N in CYP101), it was possible to observe the unprotonated species **5a**. Nevertheless, irradiation of oxy−P450 at 4 K in liquid helium yielded the unprotonated **5a**.[208] The annealing of **5a** at higher temperatures affords a single protonation at first transition to **5b**, which subsequently undergoes the second protonation and catalytic conversion of the substrate to a product complex.[208] Alternatively, an uncoupling reaction as the direct transition from **5b** to the resting state of the enzyme is observed if the system fails to implement a second protonation at the distal oxygen site to promote O−O cleavage. The lack of Compound I formation on this oxidase/oxygenase pathway in HRP[211] is explained by the inability of this enzyme to deliver the second proton, despite the facile formation of Compound I from hydrogen peroxide. EPR spectra of $Fe^{3+}-OOH^-$ heme complexes were also analyzed in refs 229 and 249. The signature EPR spectrum for the $Fe^{3+}-OOH^-$ intermediate in activated bleomycin, the same as for heme enzymes, was calculated;[250,251] the analysis of the electronic structure of this complex provides detailed insight into the chemical stability of the $Fe^{3+}-OOH^-$ moiety. Neese also noted that the side-on-coordinated iron−peroxo bond is not activated for O−O cleavage.[252]

Thus, during the past decade the method of cryoradiolytic reduction emerged as a new tool in studies of redox systems. It was used mainly as a convenient way to generate the unstable intermediates, which are immobilized in the cryogenic frozen solution and can be studied by spectroscopic methods.[212,236,238−240,253−255] Recently our laboratories were able to collect high-resolution EXAFS and XANES spectra of the peroxo and hydroperoxo states of CYP101. These results directly confirmed the changes in coordination geometry upon reduction of the ferrous oxygenated state (Makris et al., in press). The same approach is becoming more and more recognized in X-ray crystallography, where irradiation of the protein crystals also changes the state of redox centers, metals, flavins, etc.[69,174,256−259]

3.5. Peroxoferric Intermediates in Heme Enzymes: Role of Proton Transfer

In heme enzymes the porphyrin and proximal ligand provide the heme iron with five coordination sites. This prevents the side-on or η^2 coordination of peroxide, which is commonly observed in non-heme metalloenzymes but is very rare in model metalloporphyrin complexes.[39] The latter may strongly depend on the preparation methods and presence of the strong trans ligand, and these systems were recently reviewed by Valentine and co-workers.[187,188,260]

Another important role of the porphyrin in oxygen activation is the ability to donate the electron for the O−O bond cleavage.[261−264] Relatively easy oxidation of Fe−protoporphyrin IX, which serves as an additional buffer supply of electron to the dioxygen ligand, is a common feature in the mechanisms of several main families of heme enzymes, namely, peroxidases, heme catalases, cytochrome P450s, NOS, and peroxygenases. All of these are mononuclear heme enzymes which catalyze O−O bond cleavage in the peroxide ligated to the heme iron.[33,70] Donation of one electron from the porphyrin to peroxide ligand for the effective heterolytic scission of O−O bond results in formation of a cation radical on the porphyrin ring.[265] In contrast, homolysis of the iron-bound hydroperoxide would result in formation of a

hydroxyl radical, similar to the mechanism of the Fenton-type reaction, as observed for many transition-metal ion complexes.[266−268] Different porphyrin models for mechanistic studies of these reactions have been developed[269−282] and the results reviewed.[27,137,138,283−288] The application of metalloporphyrin models to mechanistic studies of P450 have been discussed recently.[139,289]

The oxygen activation in heme enzymes begins with binding dioxygen as an axial ligand to the Fe^{2+} heme iron or binding H_2O_2 to the Fe^{3+} heme iron. Although through different pathways, the common peroxo−ferric heme complex is thought to be a common precursor to the ferryl−oxo species (Figure 2). This stage of heme enzyme biochemistry is currently under detailed investigation with advances in instrumentation and biophysical methods useful in the isolation and stabilization of the reaction intermediates. In further discussion we will distinguish between the oxidase/oxygenase pathway, which uses dioxygen, and the peroxidase/peroxygenase pathway, which uses peroxide as an oxygen donor. A thorough analysis of these two pathways with respect to oxygen activation in P450 was provided by Ortiz de Montellano in ref 11. The obvious difference between these two mechanisms is the difference in the redox state of oxygen versus peroxide and the need for two additional reduction steps (one-electron reductions by exogenous electron donor) in the former pathway. The less obvious but equally crucial difference is the necessity to provide two protons delivered to the peroxide dianion heme ligand. The resultant equivalent of a rearranged hydrogen peroxide, iron-coordinated peroxo−water (Fe−O−OH_2) is the precursor to the heterolytic O−O bond cleavage to form the ferryl−oxo porphyrin complex and the H_2O product.

In most mechanistic studies the pivotal role of different protonation pathways for the bound oxygen derivatives is appreciated.[33,290] The same idea is common also in description of inorganic catalysis involving hydrogen peroxide in which the correlation between the ability to efficiently deliver the proton to the metal-bound peroxo or hydroperoxo anion, and the efficiency of catalytic decomposition of hydrogen peroxide is critical.[291] In oxygenase catalysis the ability to supply the necessary one or two protons required for the generation of the proper active compound from the iron-bound peroxide/hydroperoxide anion is now understood to be a crucial feature. Indeed, the fine-tuning and structurally provided spatial arrangements facilitate or suppress some or other microscopic pathways of all possible chemical reactions which may involve these activated oxygen compounds (Figure 2). In particular, the hydrogen-bond stabilized network of water molecules has been implied as an important part of the enzymatic mechanism, including the inherent activities, as well as the branching points between productive and nonproductive pathways in cytochrome P450.[63,69,208,232,292−294] The uncoupling of cytochrome P450 CYP101 mutants with the native substrate camphor and that of the wild-type CYP101 when metabolizing other substrates may have a thermodynamic or kinetic origin, but in any case it can be conceptualized via the operation of a distal pocket proton relay system. This relay is composed of several water molecules stabilized in the active center of the enzyme as well as two residues which are conserved in a large number of P450 systems—the famous "acid−alcohol pair", which in CYP101 is Asp251−Thr252.

The P450-type mechanism of oxygen activation was also suggested as an alternative cycle in peroxidase-type activity. Both enzymes utilize a high-valent ferryl−oxo porphyrin cation radical as an essential catalyst of oxidative transformation of substrates, or Compound I.[31,295] In P450 catalysis atmospheric dioxygen binds to the reduced heme iron of the enzyme, and this ferrous−oxygen or ferric−superoxide complex accepts one more electron from the protein redox partner to form the peroxo−ferric intermediate, which is quickly protonated to the hydroperoxo−ferric intermediate.[70,283,285] The formation of this intermediate from the ferrous−oxy complex and its subsequent transformations were recently investigated in detail using cryogenic radiolytic reduction.[208,213,232] The same intermediate is assumed to be a step toward formation of Compound I in peroxidases, and it is accepted as the main active species in the heme oxygenase catalysis.[23−25,209,296] However, contrary to P450 and HO, in peroxidases the natural formation of this active intermediate involves hydrogen peroxide as an oxygen donor. Hydrogen peroxide binds to the ferric heme bringing the two electrons and two protons necessary for Compound I generation. The key step in Compound I generation in peroxidases involves proton transfer from the proximal (closest to the iron center) to the distal oxygen atom of the bound hydrogen peroxide. This induces formation of oxo−water by the heterolytic splitting of the O−O bond and is thought to be promoted by simultaneous electron transfer from the porphyrin to the Fe−O bond.[297] As the net result of this sequence the water molecule and the ferryl−oxo porphyrin cation radical are formed from the ferric heme and hydrogen peroxide. A remarkable feature of this rearrangement is the fact that it proceeds with no other external source of electrons or protons with the imidazole side chain of the distal His being suggested as an intermediate catalyst, serving as an acceptor of a proton from the proximal oxygen on the first step and as the proton donor to the distal oxygen at the second step. The pK_a of the Fe-coordinated HOO(H) has been estimated to be 3.6−4.0 for HRP and CcP.[199,298] Thus, the iron-coordinated peroxide is in the form of an anion at neutral and alkaline pH. This complex is unstable, the hydroperoxide was described as a relatively weak ligand,[177] and the dissociation rate constant for the peroxide anion as the sixth ligand in Fe−microperoxidase-8 and Mn−microperoxidase-8 was estimated as 10−20 s^{-1} [178] and in superoxide dismutase as 50 s^{-1}.[299] Similar estimates can be made from other kinetic studies as reviewed,[32,300] which report both K_m and k_{cat} for the formation of Compound I in reaction of HRP with hydrogen peroxide. Binding constants or Michaelis constants in the range of 1−100 mM are reported for peroxidases with H_2O_2. Thus, taking into account the

fast binding rate, which cannot be slower than the experimentally observed k_{cat}, $\sim 10^7$ M^{-1} s^{-1}, the dissociation rate of HOO(H) may be as high as 10^3-10^4 s^{-1}. The latter number is near that typical for the dissociation kinetics of diatomic ligands in heme proteins[137,301] and still several orders of magnitude lower than the rates of water replacement as a ligand in octahedral porphyrin complexes, 10^6-10^7 s^{-1}, at room temperature.[302]

In contrast to the peroxidase pathway, in the P450 oxygenases where the dioxygen molecule has to be activated two protons per one O_2 molecule are consumed in addition to the two reducing equivalents to form Compound I. While reduction of the heme iron is provided by the protein redox partner, it is an essential part of the P450 enzyme to catalyze the transfer of two protons to the distal oxygen atom of the bound peroxide anion through the sophisticated structural arrangement and regulation of a proton relay system. From X-ray structural analysis of the oxygen complex this is proposed to involve several water molecules, stabilized in the active center of the enzyme through a hydrogen-bonded network interacting with the "conserved" acid−alcohol pair.[63,69,303] Through mutations of the residues involved in this network formation it was shown that the enzyme kinetics and coupling ratio are very sensitive to the hydrogen-bonding properties of these sites.[16,304,305] Similarly strong effects including the changes in ratios between different products formed have been found in other enzymes in the P450 family.[306−310] The kinetics of the productive and nonproductive turnover by human cytochrome P450s with various substrates were extensively studied by Guengerich and coauthors.[311−313] Their results directly show the existence of multiple steps, each potentially can be rate-limiting depending on specific reaction.

Homolytic and heterolytic scission of the O−O bond in alkyl peroxides and in hydrogen peroxide catalyzed by various heme enzymes has also been investigated in detail.[314−319] In P450 the ratio of homolytic to heterolytic cleavage of the O−O bond was repeatedly addressed.[315−317,320,321] The product distribution with cumene hydroperoxide as a substrate is used as a convenient test for the relative ability of a particular isozyme or mutant[315,322,323] to form Compound I. Attempts were also made to correlate the 'push' electron-donating effect[151] from the proximal thiolate with the spectroscopic markers of the porphyrin ring and the formation of Compound I (i.e., the heterolysis of O−O bond) and product.[322] Shaik and co-workers used DFT calculations to analyze the basis for the electronic 'push' from the proximal thiolate and its influence on the O−O bond heterolysis.[324] The general acid catalysis of heterolytic O−O bond scission via protonation from solvent water was also suggested.[317] Homolysis of the O−O bond in **5b** was identified[325] as an important reaction leading to inactivation of cytochrome P450s. The same authors later described how formation of hydroxyl radicals in the reconstituted CYP2B4 system results in covalent modification of the protein and degradation of the heme.[326]

In the absence of additional catalysis of heterolytic scission through the second protonation of the distal oxygen atom heterolysis or homolysis of the O−O bond is governed by the general thermodynamic stability of the reactant and product, as predicted by Lee and Bruice[327] for porphyrin models and recently analyzed by Que and Solomon.[328−334] If the iron−peroxide complex is predominantly in the high-spin state, as it is very often in non-heme metal enzymes with weak or moderate ligand field, homolysis of the O−O bond is favorable because the spin state of the transition state and product state also is high spin. For heterolytic O−O scission with second protonation of the leaving oxygen atom and formation of a water molecule, the second electron is supplied to the peroxide ligand by the porphyrin moiety and a porphyrin π-cation radical is formed. All these factors determine the behavior of the actual systems in the heterolysis/homolysis ratio as well as the overall product distribution. The parameters of the system, i.e., pH of the solvent, ligation of the metal, structure, and redox properties of porphyrin and peroxide, may also play important roles as they may favor the specific catalytic pathway with respect to others by shifting the HS−LS equilibrium, providing protonation of the coordinated peroxide and weakening of the O−O bond. Interestingly, detailed DFT calculations and Car−Parinello molecular dynamic studies of Fenton-like chemistry (in a mixture of Fe^{3+} and hydrogen peroxide in aqueous solution) showed that homolytic decomposition of H_2O_2 is favored for both HS and LS states.[335] This different conclusion reaffirms the importance of the porphyrin as an electron donor for the heterolytic scission of the O−O bond in coordinated hydroperoxide to form Compound I.[336]

The protonation/deprotonation events at the bound superoxo/peroxo/hydroperoxo states have been theoretically analyzed and claimed to account for the essential reactions in formation of the active oxo−ferryl porphyrin π-cation hydroxylating intermediate. This is Compound I in cytochrome P450 and peroxidases, the enzymes which formally have different functions and begin the active cycle with different heme ligand complexes ($Fe^{2+}-O_2$ in the case of cytochrome P450, $Fe^{3+}-H_2O_2$ for the peroxidases).[190−192,194,337−341] On the other hand, the variability of the different pathways of oxygen activation provides one with a tool to change the performance of the enzyme through different mutations or even to directly design the new types of activity into a well-known system.[342,343] Indeed, the proton delivery mechanisms operating in P450 catalysis were directly addressed with considerable success.[63,292,344−348]

3.6. Branch Points in P450 Catalysis: Reactivity of Peroxo States

While the formation of hydroperoxo−ferric heme is widely accepted as a common intermediate in the reactions of both oxygenases and peroxidases, it can undergo many potential transformations after its formation. In P450s, one can summarize these fates as follows: (i) direct participation (i.e., a direct catalyst) in substrate metabolism, (ii) an uncoupling reaction in which hydrogen peroxide is released from

the enzyme, and (iii) a second protonation of distal oxygen in which the high-valent Compound I species is formed through heterolytic cleavage and water release.

3.6.1. Catalytic Activity of Peroxo States

The potential reactivity of ferric−peroxo heme complexes as alternative oxidants in P450 catalytic transformations has been the focus of numerous articles and reviews,[349−353] including within this issue an excellent discussion by Shaik and co-authors. While generally accepted that the hydroxylation reaction proceeds by a high-valent iron−oxo intermediate, this reaction represents only one of the plethora of P450-catalyzed chemistries important in xenobiotic metabolism.[354] Despite the inability to directly monitor production of the Compound I intermediate by cryoradiolysis studies of CYP101, presumably owing to the intermediate's poor accumulation due to its high reactivity, ^{1}H ENDOR results of product-bound states intimate involvement of a high-valent iron−oxo in the monooxygenation of CYP101's native substrate.[208] The work of Coon and colleagues has suggested that the peroxo−ferric species can serve as both electrophilic and nucleophilic oxidants in microsomal P450 catalysis.[350] The use of multiple oxidants in P450 catalysis may account for the remarkable versatility of the enzyme family in a wide array of biotransformations. Therefore, assessment of peroxo, hydroperoxo, and Compound I species represents both an interesting question in terms of understanding the library of P450 oxygenations and a potentially crucial parameter in pharmaceutical design. As the chemical nature of the three oxidants varies significantly in terms of electrophilic character, for example, predictive strategies for drug oxidation products may rely on assessing the active oxidant of P450 that is operant in the given reaction.

As an electrophilic oxidant mutants at the conserved alcohol position (see next section) in CYP2B4 and CYP2E1 were shown to support the epoxidation of a number of substrates.[308,310] In particular, the T303A mutation of CYP2E1 resulted in enhanced epoxidation of a series of olefins accompanied by a decrease in allylic hydroxylation. An analogous mutation in CYP101 is known to selectively inhibit delivery of the second proton of dioxygen scission[60,61,355] and hence production of Compound I. Hence, these authors interpreted the result as evidence that the hydroperoxo species served as an alternative active oxidant in substrate epoxidation. The T252A mutant in CYP101, which is almost completely uncoupled from the hydroxylation reaction of camphor[60,61] and instead forms hydrogen peroxide according to the abortive process described in the subsequent section, has also been shown to catalyze epoxidation.[356]

Utilization of the ferric−hydroperoxo species as an electrophilic oxidant in P450 catalysis has similarly been cited as a means to explain the metabolism of other chemical moieties.[357] The threonine to alanine mutant (T268A) in CYP102 shows an enhancement in the ratio of sulfoxidation to N-dealkylation of probe substrates in comparison to the wild-type enzyme.[357] With the lack of a significant intrinsic isotope effect for the probe substrates, this is understood as hydroperoxo−iron serving as the sulfoxidation agent and Compound I as the N-dealkylation catalyst, the latter in agreement with earlier findings utilizing peroxide-mediated turnover.[358] Similar electrophilic activities of species **5b** in P450 catalysis have included the *ipso* substitution of phenol derivatives[359] and the metabolism of nitriles.[360] In addition, intermediates of cationic rather than radical character have been suggested by radical clock studies conducted by Newcomb and co-workers, also suggesting the possibility of peroxoferric complexes as direct catalysts in the oxidative transformation of electron-rich substrates.[361,362]

While suggestive, these experimental results cannot completely exclude the possibility that the reactivity changes observed upon mutation and substrate exchange are not due to redistribution of active-site waters that participate in the crucial hydrogen-bonding network and proton-delivery relay. For example, the results on CYP2E1 and CYP2B4 show enhancement and reduction of the rates of epoxidation upon threonine mutation, respectively.[310] Therefore, the phenotype of the conserved threonine mutant in this system is incomplete in the sense that it manifests an isozyme dependence on the probe substrates studied. In fact, it is known that many of the reducing equivalents in these P450s are wasted in uncoupling pathways,[293,363] including water production presumably by two-electron reduction of Compound I.[364] Studies on the branching of sulfoxidation and N-dealkylation in CYP102 can also be presented in an alternative fashion,[357] as mutation of the conserved alcohol in this isozyme is known to result in an incomplete and substrate-dependent uncoupling to hydrogen peroxide even with native substrates.[365]

Utilization of the P450 hydroperoxo−ferric species as an oxidant in epoxidation has also been addressed in numerous computational and model studies. Shaik and colleagues used DFT methods to compare the relative barriers for the epoxidation of ethane by a distally protonated ferric−hydroperoxo species versus Compound I.[195,366,367] They conclude that all monooxygenation pathways involving Compound I by the one-oxidant two-state reactivity (TSR) model are lower in energy than those involving a hydroperoxo oxidant by an order of 16−39 kcal/mol. In contrast, there is validation for the ability of peroxo adducts as epoxidation agents in a number of mimetic systems. Alkylperoxo (Fe−OOR) mimetics have shown epoxidation activity in a number of systems.[280,368,369] Furthermore, stable peracid adducts of porphyrin model systems, deficient in their conversion to a high-valent species, have been characterized to perform epoxidation but not hydroxylation chemistries.[370,371]

The protonated (**5b**) and unprotonated (**5a**) peroxo−ferric complexes have also been implicated as having nucleophilic character. One important physiological manifestation of this in a cytochrome P450 is evidenced by aromatase, which catalyzes the conversion of androstenedione to estrone.[372,373] A

number of studies have proposed that the last stage of this reaction, involving aromatization of the substrate and subsequent release of formate, involves a nucleophilic attack of the aldehyde by a peroxoanion or hydroperoxo species.[188,374,375] To address the mechanism of this process the deformylation of aldehydes has been examined for a number of P450 enzymes[309,321,376−379] and has further substantiated the potential nucleophilic character of ferric−peroxo species. Nucleophilic attack of the aldehyde group results in production of a peroxyhemiacetal intermediate, which upon homolytic cleavage forms an alkyl radical which can react with the heme to form a detectable heme adduct. In addition, the rate of aldehyde oxidation to the corresponding carboxylic acid is suppressed in P450 mutants which lack the stabilizing alcohol functionality in the active site as opposed to reactions involving heme alkylation reaction.[309] Therefore, in a manner analogous to the epoxidation via an electrophilic peroxoferric complex, these results suggest that the oxidation of aldehydes to carboxylic acids involves the Compound I species and that of the deformylation reaction a peroxoferric intermediate.

3.6.2. Peroxoferric Intermediates as a Branch Point for Monooxygenation and Peroxide-Forming Reactions

An alternative pathway for peroxoferric complexes involves the abortive release of hydrogen peroxide, presumably through the hydroperoxo intermediate. This pathway, which is often more prominent than the monooxygenation reaction in eukaryotic P450s, is intrinsically linked to a particular physiological role of cytochrome P450s. The production of hydrogen peroxide and other reactive oxygen species (ROS) from the autoxidation and peroxide shunt pathways is of important consideration as host defense responses which upregulate P450. ROS production can have deleterious physiological effects[380,381] as result of utilizing the majority of reducing equivalents toward the ultimate production of hydrogen peroxide.[382−384] Thus, higher levels of this 'uncoupling' reaction are seen in P450s which operate as a defense response to xenobiotic chemicals. In addition, the daunting range of size, shape, and electronic structures into the P450 active site renders it difficult to control access of waters, which can lead to prodigious uncoupling. This is in sharp contrast to P450s, which are fundamentally tied to specific anabolic or biosynthetic application, for example, the almost complete coupling in microbial P450s in which the prokaryote can grow on the P450 substrate as a sole carbon source (for example, *Pseudomonas putida* and camphor, and *Citrobacter braakii* and cineole).[72,385]

Chemically one can rationalize hydrogen peroxide production from peroxo−ferric heme enzymes simply involving the dissociation of the hydroperoxo anion ligand from the ferric enzyme. While the dissociation rate constants for these complexes are often difficult to measure, it has been estimated in microperoxidase as 10−20 s^{-1}.[178,386] In peroxidases, both protons from the hydrogen peroxide substrate are essential for heterolytic cleavage of the O−O bond to occur. The reaction stoichiometry (i.e., the necessity of two protons) is the same in the cytochrome P450s and other oxygenases, but in this case the enzyme has to provide the two protons necessary for heterolytic cleavage rather than rearrangement of protons from the peroxide cosubstrate. If the second proton is not available, O−O bond scission via a homolytic mechanism could still occur theoretically, resulting in a departing hydroxyl group and Compound II (Fe^{4+}=O), although the probability of simple dissociation of the hydroperoxo ligand is much higher given the high dissociation constant of the weak peroxide ligand. The effect of the electrostatics of the heme binding pocket is likely an additionally important consideration. For example, Mauk and co-workers have shown that the addition of a positively charged residue in the distal pocket can greatly increase the equilibrium constant for the binding of other anionic diatomic ligands, for example, cyanide.[387] In most P450s, in contrast, no positively charged residue is localized in proximity to the distal oxygen atom to stabilize the hydroperoxoanion ligand. As a result, P450s have a millimolar dissociation constant for cyanide, which is notably weakened in the presence of a bound hydrophobic substrate.[388]

The reaction of CYP101 with alternative substrates has likewise illustrated that efficient coupling of the enzyme is highly dependent on the identity of the substrate. Using mono-[389] and dihalogenated[390] analogues of the native substrate which block the normally oxygenated position results in a significant portion of uncoupling. In these examples there is uncoupling via peroxide release as well as the generation of a second metabolic water through reduction of the Compound I state. In the case of completely blocking the 5-site with fluorination, different regiospecificity for the monooxygenation reaction accompanies the introduced uncoupling reaction. Engineering the P450 CYP101 active site to metabolize a range of alternative substrates has also implicated the role of active-site water as a potential source of uncoupling.[293,304,391−397] By subsequently introducing mutations that introduce steric bulk to occlude this water, efficient coupling can be restored to the monooxygenation reaction of the enzyme and alternative substrate. Nonetheless, it is important to note that the coupling efficiency cannot simply be inferred by the measurement of binding parameters, neither the ability to convert to a high-spin state nor the absolute value of the binding free energy, associated with interaction of the substrate. This can easily be visualized through a comparison of the wild-type CYP101 enzyme in substrate-bound and -free states[398] as the substrate binding event is accompanied by displacement of a network of water molecules not simply the water which coordinates the heme iron.

Annealing studies of the CYP101 T252A hydroperoxo complex, in which the ferric−peroxo complex decays directly to the ferric-resting state with no intervening intermediates, strongly support the simple chemical model that uncoupling simply results from dissociation of the peroxide ligand from the enzyme.[208] Analogous results have been obtained with substrate-free P450, CYP119,[213] and CYP101 mutants complexed with a number of substrates. In all

cases studied the transition to the protonated hydroperoxo complex is always observed, presumably owing to the extremely favorable pK_a of the distal oxygen, and only following this proton transfer is uncoupling observed.

3.7. Heterolytic Cleavage of the O–O Bond

The final reactivity of the hydroperoxo complex involves protonation of the distal oxygen atom, resulting in a concerted cleavage of the O−O bond to afford Compound I and a departing water molecule.[297] While perhaps one of the most interesting aspects of the P450 catalytic scheme, the intrinsic high reactivity of this high-valent iron−oxo, through a very low barrier of a few kcal/mol[399] for substrate attack, has precluded its direct characterization in the case of the oxygen-dependent reaction of the cytochrome P450s. As a result, electronic assignment of this intermediate is largely based on analogies to peroxidase chemistry, as this 'green' intermediate has been characterized as an iron(IV)−oxo π-cation radical by optical, X-ray absorption, EPR, and Mössbauer spectroscopies of horseradish peroxidase and other heme enzymes.[122,400−407] Similar to cytochrome *c* peroxidase, a protein-based radical has been observed by Jung and co-workers in CYP101.[408]

While there has been no direct evidence for a stable high-valent intermediate in the dioxygen-driven reaction by the cytochrome P450s, recent spectroscopic data has allowed definitive assignment of the optical signature of the P450 Compound I intermediate when generated by exogenous oxidants.[409,410] As with other heme enzyme and model systems, mixing of the protein in the ferric resting state with peroxyacid results in heterolytic cleavage of the peroxide adduct to yield the high-valent iron−oxo species. While the instability of Compound I in most P450 systems has often precluded clear kinetic and spectral resolution in comparison with analogous studies on other heme enzymes,[411,412] the use of a thermostable P450 enzyme fortuitously offers a set of formation and decay rate constants which stabilize the intermediate over that of the mesophilic counterparts. This has allowed the direct observation and optical characterization of a P450 Compound I with absorption maxima at 370, 610, and 690 nm,[410] almost identical to those of the well-characterized Compound I in the thiolate-ligated chloroperoxidase[406,411] and observed in transient spectra of cytochrome P450 CYP101.[409] This optical spectrum also shows remarkable similarity to spectra calculated by DFT methods for a methylmercaptan-ligated model complex for the intermediate, demonstrating similar shifts from the spectrum of the ferric enzyme, particularly in the near-UV region.[413] It should be noted, however, that the percentage of Compound I intermediate generated in these studies is less than 20%, so the chance for precise resonance spectral measurements will need further work. Similar studies using peracids have yielded the isolation and spectroscopic assignment of other high-valent states, including the isolation of radical species on the protein,[179,408,414] although their relevance to the P450 catalytic scheme is not clear at present.

4. Role of the Distal Pocket in P450 Dioxygen Activation

Many aspects of the P450 monooxygenation chemistry can be explained and emulated by mimetics of the iron protoporphyrin IX macrocycle with appropriate ligands. Nonetheless, while many aspects of P450 reaction intermediate chemistry can be approximated by redox-linked changes in the heme−iron ligand set, moieties in the protein scaffold have a profound impact in modulating both the evolution and the reactivity of heme−oxygen intermediates. This has perhaps best manifested itself in the successful redesign of heme enzymes to catalyze the reaction chemistries of analogous bioinorganic catalysts, either through introduction of an accommodating active site or introduction of novel metal binding sites.[415−422,423−425]

With regard to the monooxygenation reaction catalyzed by the cytochrome P450s, the role of both the proximal ligand and distal pocket in controlling the formation and reactivity of active heme−oxygen adducts has proven vital. Rather than these reengineering perspectives, many efforts since the advent of facile molecular biology techniques have historically taken a microdissection approach. This is evidenced best in the work designed to probe the effect a single residue may have in controlling the critical catalytic proton-transfer steps. In the case of the P450 reaction coordinate, these follow the reduction of the oxyferrous intermediate and result ultimately in the heterolytic cleavage of the O−O bond to form a putative high-valent oxo−ferryl or Compound I species (Figure 2).

As noted, deviation from the P450 reaction coordinate from completion of the catalytic cycle represents an important theme in understanding 'normal' P450 function as many P450s are observed to stray from production of the high-valent ferryl−oxo intermediate. Therefore, development of a structural model of P450 dioxygen activation must attempt to account for this digression. While the CYP101 system presents somewhat of an anomaly in that pyridine nucleotide oxidation and dioxygen reduction are fully coupled with the native substrate, the structural studies of the wild-type enzyme with non-native substrates, mutant enzymes, and reaction intermediates or mimics adds to our understanding of common themes which can be extended to other enzymes in the superfamily. Thus, while the focus of our attention is largely derived from the growing database of CYP101 structures, which at present comprises a library of over 40 structures in the Protein Data Bank, it is possible to begin to ask which structural elements link the enzyme family mechanistically. With regard to discrete intermediates in P450 catalysis, this translates into the alteration of kinetic parameters describing the formation and reactivity of peroxo−ferric species upon mutagenesis or substrate exchange. As particular aspects of proton delivery are hard wired to accommodate the structural and mechanistic nuances of a particular P450 class, such as substrate recognition events, subsequent sections will discuss some important exceptions to the themes presented here.

The control of effective dioxygen activation, through the irreversible committed step of heterolytic O−O bond cleavage, is thus dictated by the protein in modulating effective proton-transfer events. Therefore, the first consideration of structural elements involved in proton donation to the peroxo anion or hydroperoxo species demands a detailed understanding of the mechanisms of proton transfer through proteins. In the examination of enzyme systems that catalyze the vectorial transfer of protons across membrane, several mechanisms of proton delivery have been described. Importantly, these include the participation of ionizable side chains in which the proton-transfer event is accompanied by a change in the charge state of the accompanying amino acid side chain. Assignment of these residues is usually derived from inference of their local pK_a value as well as via measurement of proton-transfer rates upon mutagenesis to a residue which cannot perform acid−base chemistry (i.e., a nonpolar residue) or one with a significantly altered side chain pK_a. In the evaluation of Grotthuss mechanism[426] of proton transfer, in which the proton-donor/acceptor pair maintain the same ionization state, all residues and solvent which can participate in effective hydrogen-bonding interactions must also be considered as potential conduits for proton transfer. Other mechanisms of proton transfer include quantum mechanical tunneling[427] as well as direct involvement of the peptide bond which has been implicated both in model peptides and enzyme systems[428 429]

The puzzle of distal pocket side chains and their implication in proton-coupled dioxygen activation by the cytochrome P450s occurred with the first P450 crystal structure of CYP101.[430,431] This structure not only made the structural assessment of early catalytic steps (those prior to dioxygen binding) possible but also illustrated the marked differences in distal pocket composition as compared to other dioxygen- and peroxide-activating enzymes. Notably, in contrast to the crystal structures of catalase and cytochrome *c* peroxidase, the active site of CYP101 and subsequent P450s since are found to be highly hydrophobic in nature with the lack of obvious acid−base catalytic residues in close proximity to the oxygen binding pocket. Specifically, CYP101 does not contain the His-Arg or His-Asp pairs thought to be operating in the 1−2 proton shift of peroxide activation by peroxidases.

The ubiquitous nature of cytochrome P450s and the development of high-throughput sequencing technologies have resulted in a database of over 3000 sequences to align and compare in a search for diagnostic features of the P450 proton-transfer mechanism. Such an analysis has shown an amazing degree of conservation of not only the hydrogen-donor/acceptor functionality of the CYP101 Thr252 (although sometimes a serine or asparagine) but also for an adjacent acidic side chain (Asp251 in CYP101). The alignment of the 58 human P450 sequences, as one example, shows that 80% of these sequences retain this acid−alcohol pair.

Mutation of the conserved threonine in CYP101 to a hydrophobic residue results in drastic phenotypic changes. While one observes near normal kinetic parameters of pyridine nucleotide oxidation, the mutant enzyme cannot effectively cleave the O−O bond and rather releases two-electron-reduced dioxygen in the form of hydrogen peroxide.[60,61] This phenotype is not exclusive to the CYP101 system, and similar mutants in other P450 isozymes show an increase amount of uncoupling upon mutation of this residue.[309,355,365,432] The potential role of this residue in mediating proton transfer to a peroxoanion species, either as a direct proton donor or in stabilizing hydrogen-bond interactions to solvent which served as the proton donor, has been explored through a series of mutagenesis experiments by Ishimura and colleagues.[10,62,346−348,433,434] As a result, the coupling activity is dramatically restored by mutants able to participate in hydrogen-bonding interactions, almost irrespective of the sterics of the mutant and pK_a of the mutant side chain. This mutagenesis data has resulted in a model in which the threonine hydroxyl participates in hydrogen-bond interactions with a secondary proton donor rather than serves this role itself. The structure of the T252A mutant of CYP101 shows subtle differences from that of the wild-type enzyme.[292] As a result of mutation, the Ala252 side chain C_β moves 1.4 Å away from the dioxygen binding pocket with respect to the wild-type enzyme. While the kink in the I helix is retained, the observation of new solvent molecules in the distal pocket has been inferred as a cause for the resulting destabilization of the ferric−hydroperoxo species in the T252A mutant. Likewise, the structures of substrate-free CYP101[435] and the enzyme bound to non-native substrates[363] all show an increased number of crystallographically resolved water in the distal pocket, and this apparent increase of solvation may lead to increased degrees of uncoupling. The destabilization of metal−peroxo complexes in protic solvents has been noted in a number of inorganic studies.[436,437]

Despite an overwhelming degree of conservation of the conserved threonine residue, a few notable exceptions have been structurally characterized recently. In the case of CYP107 (P450eryF), involved in the erythromycin biosynthetic pathway, an alanine residue takes the place of the conserved threonine. Again, the cleft of the I helix, like in the 252Ala mutant of CYP101, is retained, and a hydroxyl residue on the substrate forms a hydrogen bond with a water molecule that is almost superimposable with the typical placement of the threonine hydroxyl.[438−440] Both removal of the substrate hydroxyl[440] and reintroduction of the threonine residue[432] have effects consistent with the model of 'substrate assisted catalysis'.[439] Molecular dynamics experiments have also shown that effective proton transfer may be obtained by this mechanism[190,441]

In the case of CYP176A (P450cin), the homology of its native substrate (1,8-cineole) to that of CYP101's native substrate camphor does not appear to translate into a similar proton-transfer machinery.[385,442] Replacement of the conserved threonine with an asparagine in CYP176A results in a hydrogen bond with the substrate ether oxygen atom and the side chain adopts a rotameric configuration distinct from

Thr252 in CYP101. This results in a decreased size of the I-helix kink, although it is still apparent despite the typical helical hydrogen-bonding pattern of Asn242 with the (i-4) residue. Future structural studies on CYP176A and CYP165B1(OxyB),[65] which also has an Asn instead of a Thr, will elucidate an interesting alternative proton-transfer mechanism and shed light on the plasticity of this network in other P450 isozymes.

The conserved acid residue preceding the conserved threonine has similarly been implicated in mediating proton-transfer events that affect heterolysis to form Compound I. Mutagenesis of this residue in CYP101 results in a drastic decrease in the rate of pyridine nucleotide consumption while coupling is maintained,[344,345] linking the residue to a reduction in proton-transfer efficacy following oxyferrous reduction. Mutagenesis experiments at this position in both CYP101 and other P450s have generated a variety of various kinetic phenotypes.[347,202] In CYP101 an analysis of solvent isotope effects and proton inventory experiments in the wild type and D251N mutant[294,63] has shown an exquisite sensitivity to proton-transfer events following formation of the oxyferrous enzyme. While two protons are in flight during the transition state of the wild-type enzyme, a much greater value in the D251N mutant implied that proton transfer may rely on a chain of water molecules instead of the normal delivery mechanism.

Low-temperature radiolytic reduction of mutant P450s has provided additional insight into distal pocket proton-transfer mechanisms and complemented the interpretation that the conserved acid residue is intimately involved in mediating proton transfer to the peroxoanion species. As a result of oxyferrous reduction of the CYP101 D251N mutant, EPR and ^{1}H ENDOR results show an enhanced stabilization of the peroxoanion species in the mutant rather than immediate protonation to form the hydroperoxo species as shown in the wild-type enzyme upon reduction at 77 K.[208] Interestingly, a similar result has also been shown upon mutation of an aspartate residue (Asp140) in heme oxygenase,[209] which has also been shown to be intimately involved in mediating effective proton-transfer reactions in that heme enzyme.[25,443] Structurally, the D251N mutant in CYP101 shows most of the features of the distal pocket retained, although the side chain adopts altered salt-bridge contacts which result in greater access of the heme iron from the protein surface.[63] In light of the isotope effect experiments, this may imply a reliance of a greater number of solvent molecules in effective heterolytic cleavage.

5. Crystallographic Characterization of P450 Reaction Intermediates: Structural Basis for Proton Transfer

It was long appreciated that since the mechanism of P450 catalysis involves the transformation of one oxygen atom from molecular dioxygen into a water molecule, protons would be involved and a natural source of these protons would involve solvent waters. Hence, the high-resolution X-ray crystal structure of the stable ternary carbonmonoxy camphor complex of CYP101,[398] which serves as a rough analogue for the unstable O_2 complex, was examined in detail for structural water molecules that could play a central role in these proton-transfer reactions. Although the structure revealed several interstitial waters, particularly around the highly conserved Glu366, the water chain did not provide a continuous path from bulk solvent into the active site. It was therefore clear that crystal structures of critical intermediates along the reaction cycle were required to gain further insight into the proton-transfer pathway, particularly the structure of the oxy complex.

Crystal structure determinations of short-lived species are complicated by the long crystallization times which require crystallization under nonreactive conditions and means to generate the active complex fast and efficiently in the crystal, i.e., the ability to initiate the reaction effectively. Upon formation of a desired reaction intermediate, diffraction data can be collected either on the same time-scale as the reaction using fast data collection geometries such as the Laue method or by kinetically trapping the intermediate, thereby preventing its decay.[256]

The study of intermediates occurring along the reaction cycle of cytochrome P450 is facilitated by the requirement of the input of either electrons or oxygen before the reaction can proceed.[69] This need of reinitiating the reaction after an intermediate has been generated reduces the dephasing of the subsequent intermediates. The first reduction step can be realized chemically by diffusing dithionite into the crystals. The slowness of diffusion is not a concern in this case since the reduced ferrous form of P450 is stable under anaerobic conditions. The situation is slightly different for the next step of the reaction, introduction of oxygen, since the ferrous−oxy complex is unstable.[444,445] The use of a pressure cell allows rapid and complete oxygenation despite the low solubility of oxygen in water (0.2 mM) and the high concentration of protein in the crystal (higher than 10 mM). The second electron can be introduced by X-ray radiolysis, resulting in splitting of the O−O bond of the oxy complex, analogously to cryoradiolytic reduction using a radioactive source. However, in X-ray crystallographic investigations the situation is complicated by the fact that the radiolytic beam is not only a reductant but also the probe of the structure. Thus, unwanted X-ray radiolysis of a sample can only be prevented by not exposing it to X-rays, which ensues data collection schemes requiring limited exposure of many crystals resulting in "patched" datasets.[174,446]

The first unstable intermediate in the reaction cycle of CYP101 is the ferrous complex **3**. Its structure is very similar to the substrate-bound ferric resting state **2**.[69] In the oxy complex **4**[69] the iron moves into the heme plane and O_2 binds end-on (η^1) with an Fe−O−O angle of 142°. Camphor moves 0.3 Å toward Tyr96 due to the bulk of the oxy group. The backbone amide of the highly conserved Asp251 rotates and stabilizes a new water molecule (Wat901), which is in hydrogen-bonding distance to the bound oxygen molecule and the side chain hydroxyl group

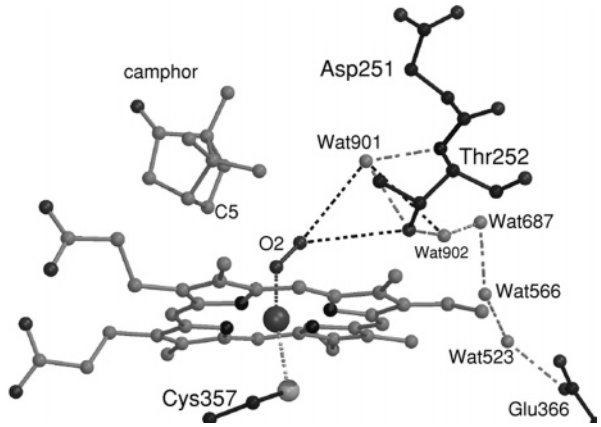

Figure 3. Crystal structure of the oxy complex of wild-type CYP101 (pdb code 1dz8) that catalyzes the 5-exo hydroxylation of camphor. Oxygen binding induces a rotation of the amide of Asp251, which ensues binding of two new water molecules, Wat901 and Wat902.

of Thr252, which rotated also compared to the ferrous complex. Another new water molecule, Wat902, also interacts with the hydroxyl of Thr252 and is part of a water chain that extends to the highly conserved Glu366 (see Figure 3). Mutation of Glu366 shows little influence on the catalytic activity,[347] thus implying that this proton-transfer link does not play a major role in catalysis. Given the technical difficulties in determining the structure of the short-lived, radiation-sensitive oxy complex, it is interesting to note that the structure of the stable cyanide complex of P450cam is virtually identical.[259] This suggests structure determination of cytochrome P450s cyanide complexes as models for oxy complexes. Upon irradiation of the P450−oxy crystals with long-wavelength X-rays the electron density corresponding to the distal O_2 oxygen atom disappears and a complex is formed that is consistent with Compound I (**6**).[69] The distance between the C5 atom of camphor and the oxo atom is 3.1 Å. It seems that immediate turnover is slowed by a crystal contact that restricts the mobility of Tyr96 and thus of the hydrogen-bonded camphor molecule, preventing hydrogen abstraction. This may be the reason a complex consistent with an oxo−ferryl species accumulates in crystals but not in frozen solutions. Raising the temperature of the crystals above the glass-transition temperature results in formation of the product, 5-*exo*-hydroxy-camphor.

The crystal structure of the oxy complex of wild-type CYP101 suggests a structural basis for the high uncoupling in the T252A mutant and the strongly reduced catalytic rate of the D251N mutant. The critical feature of the oxy complex is the O_2-induced rotation of the backbone amide of Asp251, which thereby creates a binding site for the new water molecule Wat901. The flipped conformation of Asp251 is stabilized by a hydrogen bond between the backbone carbonyl of Asp251 and the side chain of Asn255. This backbone rearrangement occurs already in the ferric complex of the T252A mutant (PDB code 2CP4[292]). However, due to the missing hydrogen bond with Ala252 the water molecule (Wat720) is displaced from the position of Wat901 by 1 Å. The oxygen-independent (and thus unregulated) binding and 'sloppy' positioning of Wat720 in the T252A mutant may be the cause for uncoupling. EPR studies on cryoradiolytically reduced ferrous−oxy complexes of T252A show that the first proton transfer occurs readily below the glass-transition temperature.[208] This is in line with the above proposal that the reacting species are already in place, making larger scale motions unnecessary.

In the structure of the D251N mutant of CYP101[63] the carbonyl oxygen of the Asn251 side chain forms a hydrogen bond with the amino group of the Asn255 side chain. This interaction prevents the formation of a hydrogen bond between the rotated conformation of the Asn251 amide and the Asn255 side chain in the oxy complex and thus the binding of Wat901 (Makris et al., unpublished results). The structural finding is consistent with the observation that proton transfer is rate limiting in this mutant.

The close proximity of Wat901 to the proximal oxygen atom of O_2 and the spectroscopic finding that the first proton transfer (**5a** → **5b**) occurs at cryogenic temperatures below the glass-transition temperature suggest that Wat901 is involved in the first proton-transfer step. Molecular dynamics simulations based on the crystal structure of the oxy complex[447] suggest a further water binding site and a side chain rotation of Asp251 into the active site analogous to the aforementioned proposal based on data from mutagenesis and proton inventory experiments.[294] Since the EPR spectra of cryoradiolytically reduced CYP101 indicate that the second proton transfer occurs only above the glass-transition temperature,[207,208] it is suggestive that the carboxylate switch is responsible for the second proton transfer. Thus, the structural mechanism for proton transfer in P450cam involves both a carbonyl (**5a** → **5b**)[69] and a carboxylate switch (**5b** → **6**).[294]

6. Alternative Proton-Transfer Pathways in Functionally Different Cytochrome P450s

As discussed, the concerted delivery of protons to heme-bound dioxygen is highly sensitive to the microenvironment of the distal pocket, evidenced by the drastic changes of peroxo formation and reactivity upon perturbation of the wild-type CYP101 system through substrate modification or mutagenesis. While common themes for effective proton delivery in the P450s exist, they are undoubtedly tuned by the protein structure. The study of CYP107[438−440] and CYP176A[448] has shed light on the alternative mechanisms for hydrogen-bonding networks to provide a proton-transfer pathway. We now turn our focus toward other enzymatic thiolate−iron porphyrin systems which catalyze chemistries quite distinct from the typical P450 system. To accommodate these drastic changes in function, P450s which perform nitric oxide reductase and peroxygenase chemistries must employ modifications from the paradigm presented above to compensate for the requirements of each ligand.

6.1. Nitric Oxide Reductases

CYP55 (P450nor) is involved in fungal denitrification and catalyzes reduction of NO to N_2O, a non-

standard P450 reaction: $2NO + NAD(P)H + H^+ \rightarrow N_2O + NAD(P)^+ + H_2O$. This reaction takes place without other protein redox partners. The role of proton transfer in CYP55-catalyzed NO reduction has been implicated in a number of computational[449,450] and experimental studies[451,452] even though a complete mechanistic assignment of particular nitrogen-ligated states remains elusive. Structural analysis of the CYP55 active site from *Fusarium oxysporum* by Shiro and colleagues reveals that, unlike CYP101, the active site comprises a number of hydrophilic residues and water molecules. A positive charge cluster of arginines and lysines has been shown to be crucial for interaction with NAD(P)H.[453] The X-ray crystal structure shows that Thr243[454] differs in the rotamer of the side chain from the corresponding Thr252 in CYP101 such that the hydroxyl points away from the oxygen binding site. Instead, it forms a hydrogen bond with Wat173 that mediates the connectivity with the I-helix main chain peptide carbonyl of the (i-4) residue Ala239. It is interesting to note that in CYP55 the residue corresponding to the highly conserved acidic residue is Ala242. Its backbone conformation and interactions are very similar to the "flipped" conformation of Asp251 observed in the oxy complex of CYP101. The carbonyl of Ala242 forms a hydrogen bond with the side chain of Asn246 (corresponding to Asn255 in CYP101), and the amide interacts with Wat175, which is positioned similar to Wat901 in CYP101. In CYP55 there are two water channels leading to the distal face and finally bulk solvent. The change in polarity of the distal pocket between CYP55 and CYP101 is transmitted even into the observed coordination geometry of bound NO and isocyanide complexes.[455,456]

Mutagenesis of the Thr243 residue, like the Thr252 series, results in a drastic reduction of activity, in this case the NADH-dependent reduction of NO to N_2O.[451] Nonetheless, structural analysis of several mutants at this position does not provide any rationale for the observed difference in function.[452] Thus, while the appearance of new water molecules in mutant forms is certainly correlated with a change in a possible proton-delivery network, it is difficult to eliminate the possibility that the residue could instead be crucial in facilitating electron transfer from NADH as the enzyme directly catalyzes electron transfer from pyridine dinucleotide to the heme iron.[457] In fact, this potential alteration in the role of a conserved threonine in proton delivery is likewise supported by the absence of a conserved acid residue (Ala242) in CYP55. This alteration of the distal pocket network to accommodate the unique mechanism of CYP55 is supported by the recently determined crystal structure of CYP55 in complex with the NADH analogue nicotinic acid adenine dinucleotide, NAAD.[454] Binding of NAAD induces global conformational changes of the B, F, and G helices accompanied by local changes at the active site. These comprise movement of the I helix away from the heme and a flip of the peptide bond between Ala239 and Gly240. This induces a slight bending of the I helix, resulting in a positioning of the amide of Gly240 and the hydroxyl of Thr243 for direct interaction with the carboxyl group of NAAD. Since the hydroxyl group of Thr243 is the only side chain interacting with the carboxyl group of NAAD and since Thr243 is rather distant from the catalytic site, the functional role of Thr243 seems to be stereoselective binding of the nicotinic ring but not proton delivery. The distance between the heme iron and the C4 atom of the nicotinic acid ring is 4.2 Å; NO would be located in between and face the pro-R side of C4. The structural data and a significant kinetic isotope effect of [4R-^{2}H]-NADH[458] on the reduction step strongly support the direct transfer of a hydride ion from the pro-R side of NADH to the Fe^{3+}−NO complex. The role of the conserved P450 hydroxyl may be fulfilled by Ser286.[459,460] Its side chain is close to the C4 atom of the nicotinic ring and the oxygen atom of heme bound NO, respectively, and NO reductase activity is abolished upon mutation (see Figure 4). Unlike for the Thr243 mutants, structural analysis of various Ser286 mutants shows drastic changes in hydrogen-bonding networks through the systematic destabilization of water networks.[459,460] The effects of these water networks involving Ser286 appear to be critical as even the minor S286T mutation results in a rotameric configuration which destabilizes the water network and is correlated with a loss of activity. Thus, the sequence A/G−G−X−D−T is highly conserved in the I helices in P450s, possibly endowing local flexibility for the peptide backbone. This motif is used for creation of an oxygen binding pocket in monooxygenase P450s but diverted in CYP55 for NAD(P)H binding.[454]

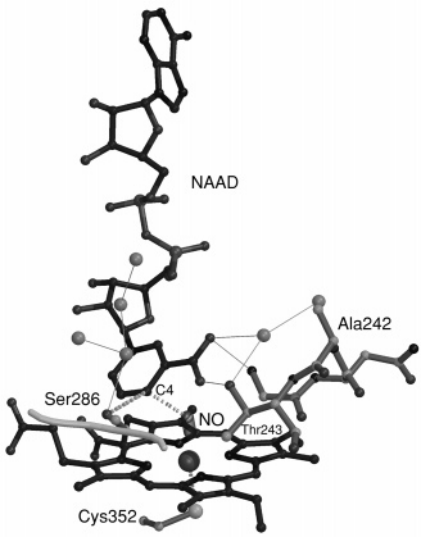

Figure 4. Active site of CYP55, a nitric oxide reductase, complexed with the NADH analogue NAAD. Thr243, the highly conserved Thr located in the I-helix, positions the nicotinic acid ring, whereas Ser286 seems to have taken up the Thr's traditional role in proton transfer. The R side of the C4 atom of the nicotinic acid ring is within 3.5 Å of the hydroxyl group of Ser286 and a modeled NO (based on pdb code 1cl6). There are two water chains extending into the active site. Hydrogen-bonding interactions are indicated by thin lines and water molecules by gray spheres.

6.2. P450 Peroxygenase Activity

While P450s are typically poor in their utilization of hydrogen peroxide as a dioxygen/electron/proton

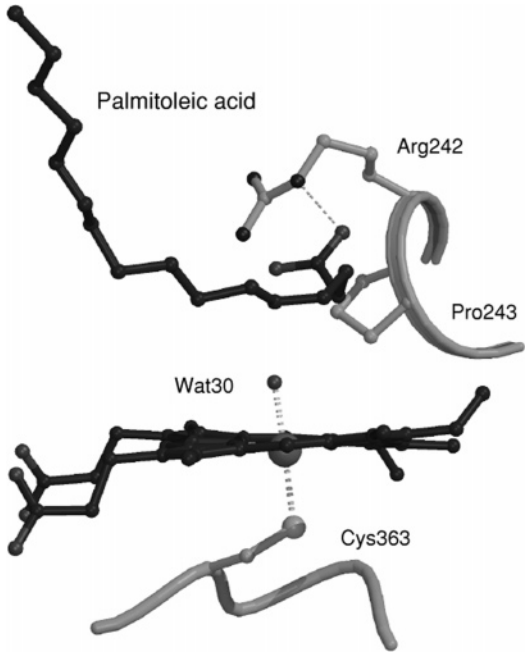

Figure 5. Active site of CYP152A1, a peroxygenase. Pro243 and Arg242 are at the positions of the acid−alcohol pair of the I helix. Arg242 forms a hydrogen bond with the carboxylate of the substrate, which is assisting in catalysis.

surrogate in monooxygenation chemistry, a number of P450s are shown to utilize the peroxygenase pathway in order to hydroxylate fatty acids.[461−463] Their evaluation is critical in order to assess the isozyme dependence of various P450s in the utilization of the peroxide shunt pathway in catalytic processes. To accommodate this fundamental difference in ligand chemistry, as now both protons and electrons are delivered to the ferric heme moiety, the enzyme is devoid of the conserved pair, which in turn is represented by Arg242 and Pro243 in CYP152A1 ($P450_{BS\beta}$) (see Figure 5). Mutagenesis results have succinctly demonstrated their critical role in both fatty acid binding and the hydrogen-peroxide-mediated hydroxylation reaction.[464] Likewise, both the presence and positioning of a substrate carboxylate is similarly crucial, presumably in an electrostatic interaction with this conserved arginine in peroxygenase.[388,465] Indeed, the recent crystal structure of $P450_{BS\beta}$ determined by Shiro and co-workers has suggested a very elegant mechanism of substrate-mediated peroxygenase catalysis.[466] The relative positions of the fatty acid carboxylate and Arg242 are similar to the positions of Glu183 and His105 in chloroperoxidase[467] and are likely to have similar roles in catalysis of the O−O bond scission of hydrogen peroxide.[466−468]

7. Summary and Future Directions

Since the excellent 1996 *Chemical Reviews* article entitled "Heme-containing Oxygenases" by Sono et al.,[13] an impressive number of new studies of cytochrome P450 chemistry, enzymology, and function have been published. These include several crystal structures of mammalian CYPs, complete step-by-step characterizations of reaction intermediates by spectroscopy or crystallography, and a variety of fundamental advances using computational analysis. These studies have yielded new insights into the influence of spin states on the reduction potential or chemical activity, the role of highly conserved residues such as the acid−alcohol pair in the I helix, and a possible structural framework for proton relay or electron transfer and the direct observation of new intermediate states in the catalytic cycle. New approaches such as spectroscopic analysis of cryoreduced samples, the realization and exploitation of the photoreductive powers of X-rays, and developments in quantum chemical analysis have contributed to the progress. In the past one was mostly limited to the bacterial CYP101 or readily available mammalian proteins purified from liver as tools. Therefore, one of the questions to be answered before publication of the next *Chemical Reviews* paper on this topic is how general are the findings obtained on CYP101, CYP102, or CYP2B4, e.g., do they apply for the larger class of membrane-bound P450 monooxygenases? New methods of solubilization of cytochrome P450s in homogeneous and monodisperse form offer the opportunity for testing the mechanistic hypothesis presented in this review.[94,469,470] With new tools in hand, more complicated systems may be tackled. While the basic findings to date will be found to be applicable to other CYPs, there will be many interesting variations on the theme. We started to see some of these, for example, the divergent use of the conserved Thr in P450nor to bind the nicotinic ring of the cofactor coupled with the hijacking of a structurally diverse threonine to fulfill the former's traditional function in proton or respective hydride transfer in P450nor. Given the observation of different backbone flips in the highly conserved sequence A/G−G−X−D−T in the I helices in P450s in different functional states, it is suggestive that it endows local flexibility for the peptide backbone. In light of this, direct measurement of dynamics will become critical. It is apparent that nature can also use various chemical features of the substrate to be metabolized to provide the critical hydrogen-bonding and active-site electrostatics necessary for efficient oxygenase catalysis.

An area for future effort is advancement of the understanding of molecular recognition processes in biological systems. Despite huge differences in substrate shape, size, and specificity, CYPs appear to use a common overall mechanism for substrate binding involving both open and closed conformations. It is now appreciated that such movements are important in excluding bulk water from the active site and thus controlling the detailed proton-transfer pathways necessary for catalysis. The open form appears to exist only transiently in CYP101 but is stable enough in other forms, such as CYP102, to be structurally characterized. The recent mammalian P450 structures appear often as the form of an "open" configuration due to specific ligands present or specific crystallization conditions. Common conformational states among different prokaryotic and mammalian[45,97] P450 enzymes indicate that the channel allowing access of the substrate to the active site is structurally conserved and provided by movement of the F/G helices and the B′ helix. Complicating the

picture of substrate-induced conformational changes in the P450 systems are substrates that are only recognized when coupled to other proteins or complex homo- and heterotropic cooperativity that are present in many mammalian metabolic P450s. The latter interactions are of critical importance in determining the metabolic profile and drug−drug interactions present in human P450 isozymes. Molecular recognition events in protein−protein complexes, involving oligomers of P450 and/or reductase, offer another dimension in the means by which Nature controls P450 metabolic reactivity.[94,471−476]

CYPs appear to have evolved separated key steps along their reaction pathways, possibly to allow for regulation. Ligand-induced movement of the I helix (SRS4) not only positions the catalytic residues located therein but also changes the water structure surrounding the heme iron. This regulates the heme reduction potential and oxygen binding by (de)-stabilizing the met-water molecule bound to the heme iron. At the simplest level of understanding this structural interplay couples oxygen binding to the presence of substrate, thereby reducing side reactions such as the production of superoxide, peroxide, and other toxic forms of reduced dioxygen.

The hunt for an unambiguous experimental identification of the ephemeral active oxygen species will most certainly continue. The plethora of new P450s at hand from various organisms will undoubtedly be helpful, as was the case for the thermophilic isolates such as CYP119. The availability of thousands of P450 genes allows the study of the influence of polymorphism on the sensitivity of certain drugs and their pharmacokinetics and bioavailability, ultimately resulting in a patient-optimized pharmaceutical intervention. Thus, again, one sees the cytochrome P450s providing a focus for the proteomics and individualized medical treatment that are exciting areas of current research. We look forward to another decade of exciting results from numerous laboratories throughout the world that will provide additional *Chemical Reviews* subject material.

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