Chapter 1

CYTOCHROME P450:
FROM PURSUIT OF REACTIVE INTERMEDIATES
TO
ENGINEERING NOVEL REACTIVITY

1.1. Cytochrome P450: a remarkable metabolic enzyme

Every living organism is composed of cells that are alive with chemical reactions, the processes that allow them to grow, reproduce, and respond to the environment. Beneficial nutrients are broken down to provide energy and synthetic building blocks, new molecules are built from scratch or modified for specific purposes, and unwanted or harmful chemicals are detoxified and excreted. Each of these metabolic processes must be accomplished in a very precise, controlled fashion; this often requires surmounting a significant reaction barrier. Thus, cells employ enzymatic machinery to catalyze these reactions.

Metabolic enzymes face a number of significant challenges. Splitting up large molecules into smaller or more useful components requires activating, breaking, and functionalizing some of the strongest and most inert chemical bonds found in organic molecules. Additionally, a series of reduction and oxidation reactions (redox) is often required, in which electrons are specifically transferred out of one chemical bond and into a new one. All of these transformations must occur in a tightly controlled fashion in order to synthesize complex molecules with specific information encoded in the molecular structure. Furthermore, they must be accomplished on a wide variety of substrates; exposure to toxins and xenobiotics in the environment provides a constant variety of sizes, geometries, and functionalities.

A remarkable class of enzymes called cytochrome P450 (often abbreviated as just "P450"), plays a critical role in these metabolic processes. These enzymes possess three characteristics that make them indispensible. First, the P450 center houses a thiolate-ligated heme active site capable of generating extremely reactive iron-oxo species. These reactive species can react with alkane C–H bonds – the most

common and inert bonds found in organic molecules. Secondly, the amino acids that directly surround the active site orient substrates in proximity to the reactive center, allowing the enzyme to form new bonds in a controlled fashion with high levels of regio- and stereoselectivity. Finally, flexible protein architecture around the active site allows P450s to envelop substrates with a wide variety of sizes, geometries, and functionalities, and thus, metabolize myriad small molecules.

Because of these three features, P450s are found in every living organism, from bacteria and archaea, to plants, fungi, and animals; there are even a number of viruses that carry the P450 genetic code. Since their initial discovery in rat liver microsomes in the 1940s, advances in genomics have led to the discovery of over 20,000 versions of P450,[1] and this number keeps rising. The remarkable reactivity displayed by P450s has made them a highly active field of research, involving many disciplines from medicine and toxicology to molecular biology, biochemistry, and biophysics. In addition, they are inspiration for the development of inorganic catalysts and synthetic methodologies. A fundamental question continues to fascinate scientists: how do these enzymes catalyze such difficult transformations? Specifically, what is the nature of the catalytically active species? How does the enzyme control the fundamental electron transfer (ET) events that are required to form the active species, and for it to then react with substrates? Furthermore, can we harness and modify P450s to drive catalysis in an entirely new way?

This Chapter begins by discussing the key features of P450s that contribute to their remarkable reactivity, including the nature of reactive intermediates along the catalytic pathway. Two soluble, bacterial enzymes are highlighted in particular: P450 BM3 from the soil bacterium *Bacillus megaterium*, and CYP119 from the thermophilic hot-springs archaeum *Sulfolobus acidocaldarius*. These two variants are the focus of the experimental work that will be presented and analyzed in

Chapters 2-6. This first Chapter next addresses a rapid, photochemical method for triggering ET within enzyme systems in order to generate and investigate reactive species. Implementation of this method is discussed in Chapters 2-5. Finally, this Chapter discusses a recent report of engineered P450-catalyzed cyclopropanations. By manipulating electron flow, these artificial P450s can be reprogrammed to catalyze cyclopropanation of styrene within the context of an *E. coli cell*. Electrochemical measurements that quantify this electronic manipulation are presented in Chapter 6.

1.2. P450: structural features that direct function

The first X-ray crystallographic characterization of a P450 was achieved in 1987 for the soluble, bacterial $P450_{CAM}$ from *Pseudomonas putida*.[2] Bacterial variants such as $P450_{CAM}$ often are stable, soluble, and easier to study. In contrast, mammalian variants are delicate and frequently are membrane bound. Due in large part to improvements to crystallization techniques, there are almost 600 P450 structures in the protein data base (PDB) to date, including 107 structures of human variants. This increasing wealth of data allows identification of common features and individual peculiarities. Entire volumes have been written on the relationship between P450 structure and function,[3] so this Chapter only briefly highlights the features that are pertinent to Chapters 2-6 of this Thesis.

Cytochrome P450s are largely α-helical proteins, with a tertiary structure that is highly conserved and unique to the superfamily; no non-P450 proteins share this fold.[4] The structures of P450 BM3 and CYP119 are shown in **Figure 1.1**. It is perhaps unsurprising that the most highly conserved regions are those adjacent to the active site, while those associated with substrate specificity may differ significantly among P450 variants.

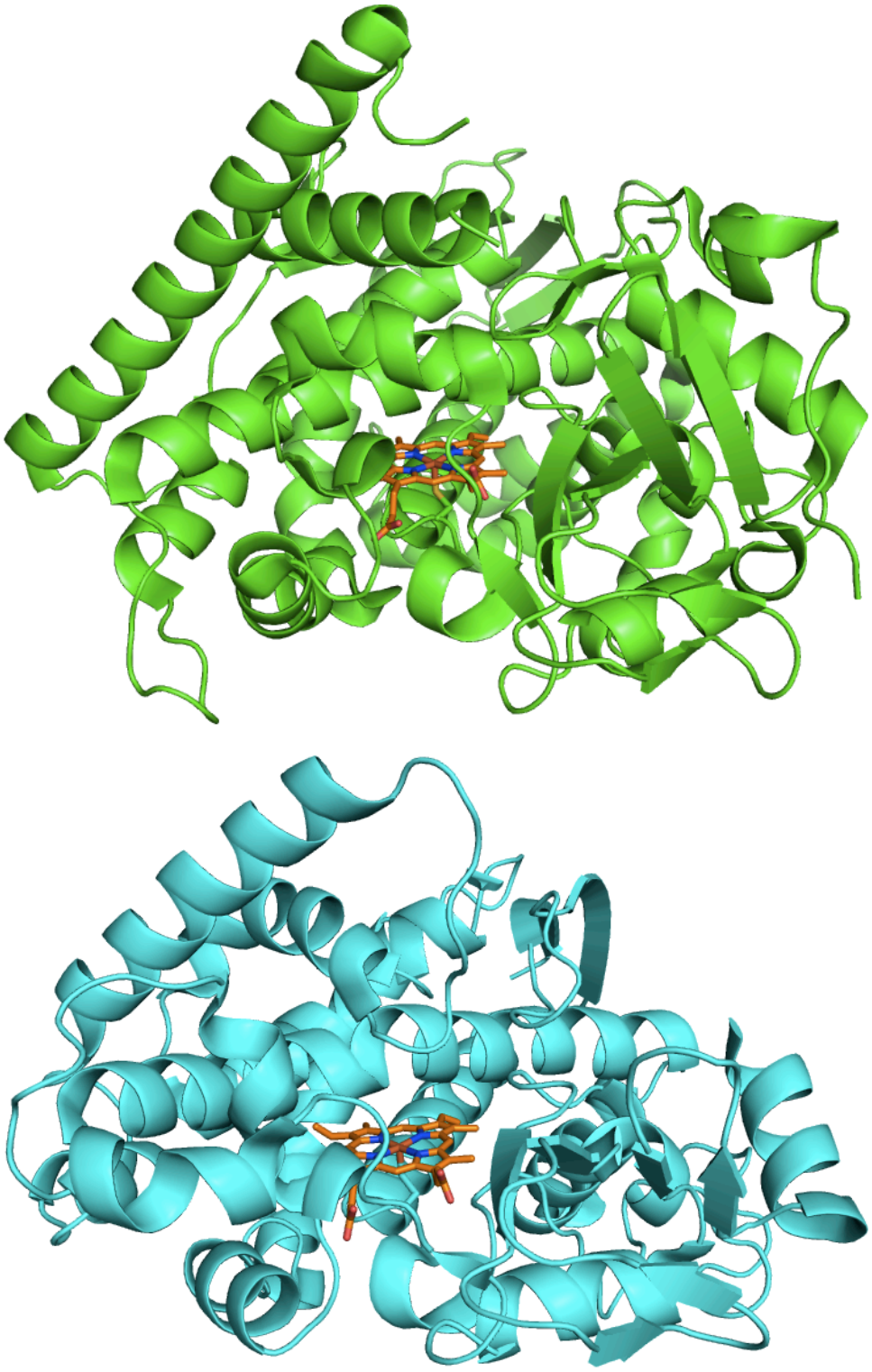

Figure 1.1. Structures of P450 BM3 heme domain from *Bacillus megaterium* and CYP119 from *Sulfolobus acidocaldarius*. BM3: top, green, PDB: 2IJ2. CYP119: bottom, cyan, PDB: 1IO7. Heme active sites are highlighted in orange. All figures in this Chapter were made using PyMol graphics software for Mac.

At the heart of cytochrome P450 lies the heme, iron protoporphyrin IX, that drives P450 chemistry. The iron center is ligated on the proximal face by an absolutely conserved cysteine (**Figure 1.2**, green); this ligation is absolutely critical for P450 function. This cysteine is part of a signature β-bulge containing the conserved sequence FxxGx(H/R)xCxG; this lies at the end of helix L, which runs beneath the heme (**Figure 1.2**, blue). This region of the protein fold is critical for holding the cysteine in place. Three hydrogen bonds from nearby backbone amide N-H donors also stabilize the thiolate ligand. This ligation and hydrogen bonding arrangement is shared with two other heme monooxygenases: chloroperoxidase and nitric oxide synthase.

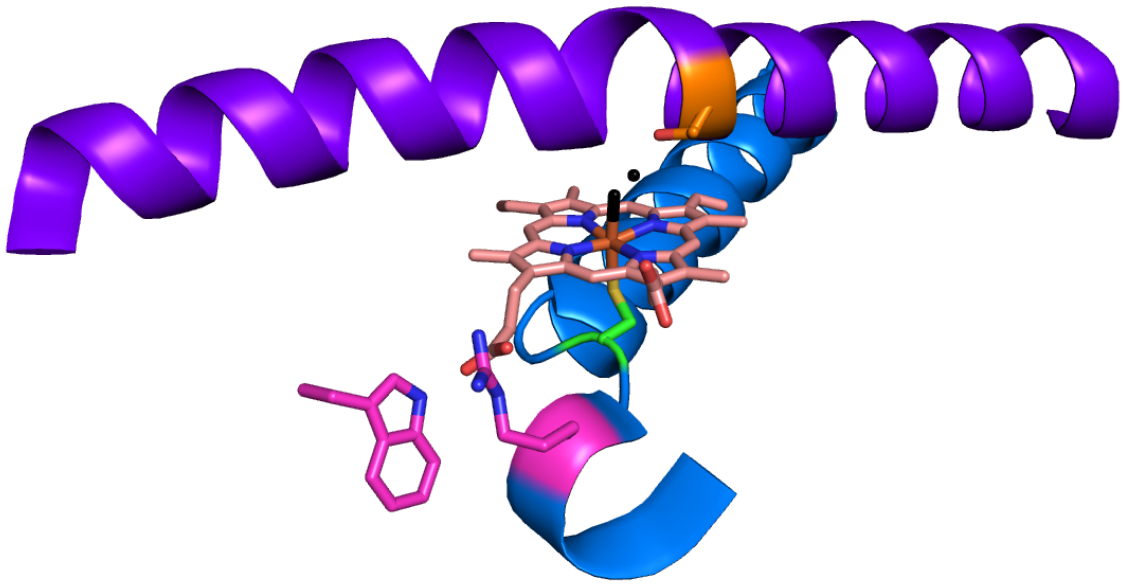

Figure 1.2. P450 BM3 active site. The heme is colored pale pink. The L helix (blue), and contains cysteine400 (green) which ligates the heme. In bright pink: arginine398 and tryptophan96 form hydrogen bonds with one of the heme propionates. The long I helix (purple) above the heme contains threonine268 (orange). The axial water molecule, and an additional, structured water molecule above the heme are shown as a black dot.

The P450 heme is not covalently bound to the protein structure, but rather is held in place by cysteine ligation, hydrophobic contacts, and hydrogen bonding

between the heme propionates and amino acid side chains. The precise nature of the hydrogen bonding partners differs among P450 variants. For P450 BM3, this includes a tryptophan96 and arginine398; site directed mutagenesis studies that replaced tryptophan96 with non-hydrogen bonding residues (e.g., alanine) saw greatly diminished heme incorporation in the expressed mutants. CYP119 contains a more extensive hydrogen bond network between the protein and the heme propionates (**Figure 1.3**). Histidine76, histidine315, and arginine80 form hydrogen bonds with one of the heme propionates, and arginine259 forms a hydrogen bond with the other.

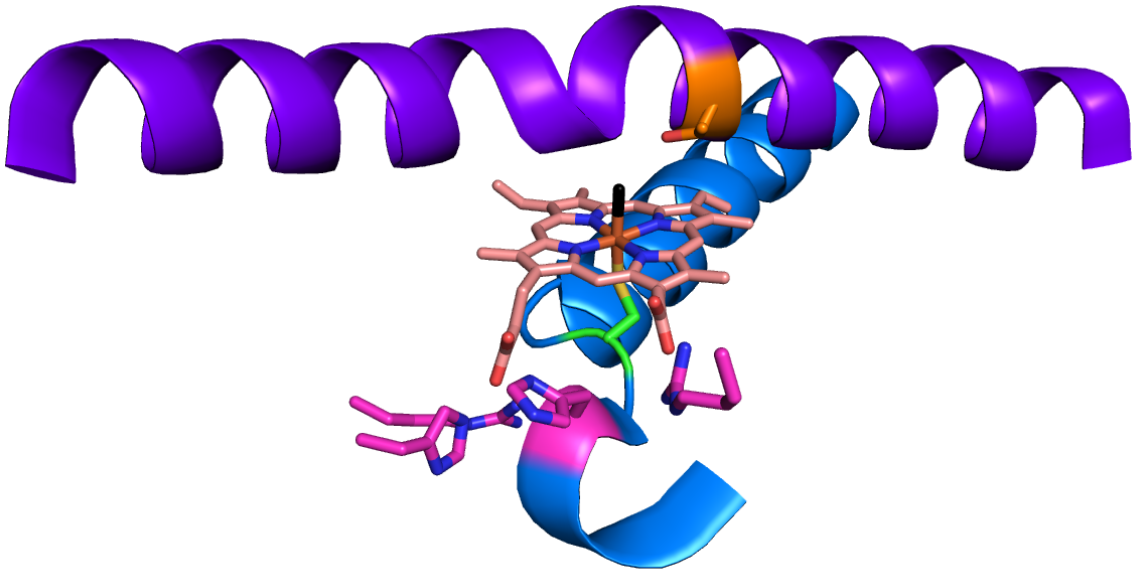

Figure 1.3. CYP119 active site. The heme is colored pale pink. The L helix (blue), and contains cysteine317 (green) which ligates the heme. In bright pink: histidine315, histidine76, and arginine80 form hydrogen bonds with one of the heme propionates, and arginine259 forms a hydrogen bond with the other. The long I helix (purple) above the heme contains threonine213 (orange). The axial water molecule is black.

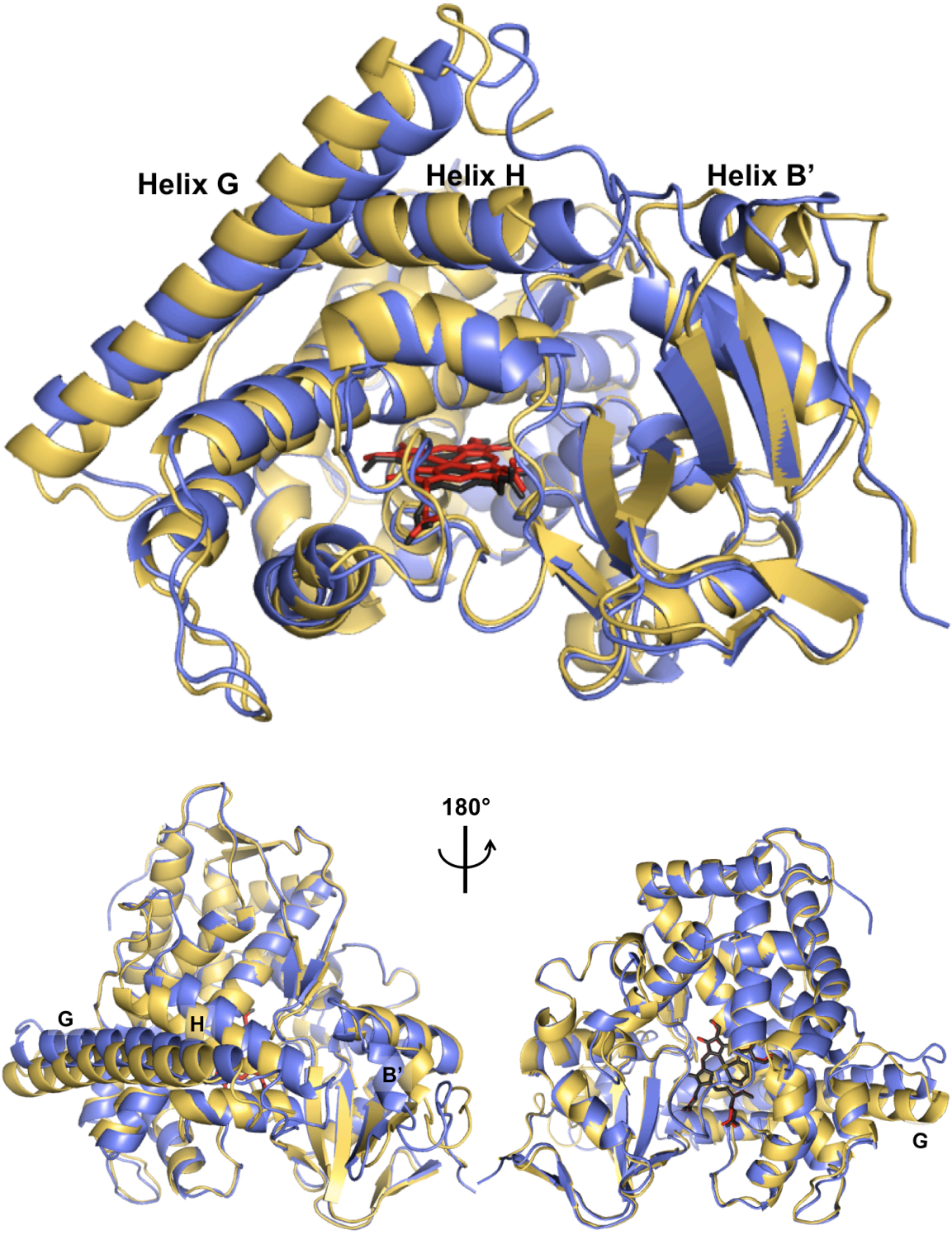

Figure 1.4. Overlay of substrate-free (open, gold) and substrate-bound (closed, blue) forms of P450 BM3. **Top**: view from the front. **Bottom**: views from distal (left) and proximal (right) faces.

The distal face of the P450 heme is in contact with the substrate binding pocket. In the absence of substrate, a water molecule acts as the distal axial ligand, making the iron center six coordinate and low spin. The long I helix (**Figure 1.2** and **Figure 1.3**, purple) runs along this distal face, and contains a conserved threonine side chain that is involved in oxygen activation, as discussed in Section 1.3. The substrate binding pocket of cytochrome P450 is largely hydrophobic, with key residues positioned to align substrate. While the heme active site is deeply buried, a long substrate-access channel provides a pathway for molecules to enter. Upon substrate binding, large conformation changes can be seen for many P450 variants. For example, in the presence of long chain fatty acids such as palmitic acid, the P450 BM3 G, H, and B´ helices tighten down on the pocket (**Figure 1.4**).

1.3. P450 activity: reactions and mechanisms

Broadly, P450s catalyze the metabolism of xenobiotics and the biosynthesis of signaling molecules. The most common and well-studied transformations are monooxygenase reactions, such as hydroxylation of aliphatic and aromatic carbons or heteroatoms, and epoxidations.[5] Dehydrations, ring formations and expansions, and reductive halogenations among many others have also been observed. As will be discussed in Section 1.6, the heme active site can also be engineered for non-native reactions including carbene transfer.

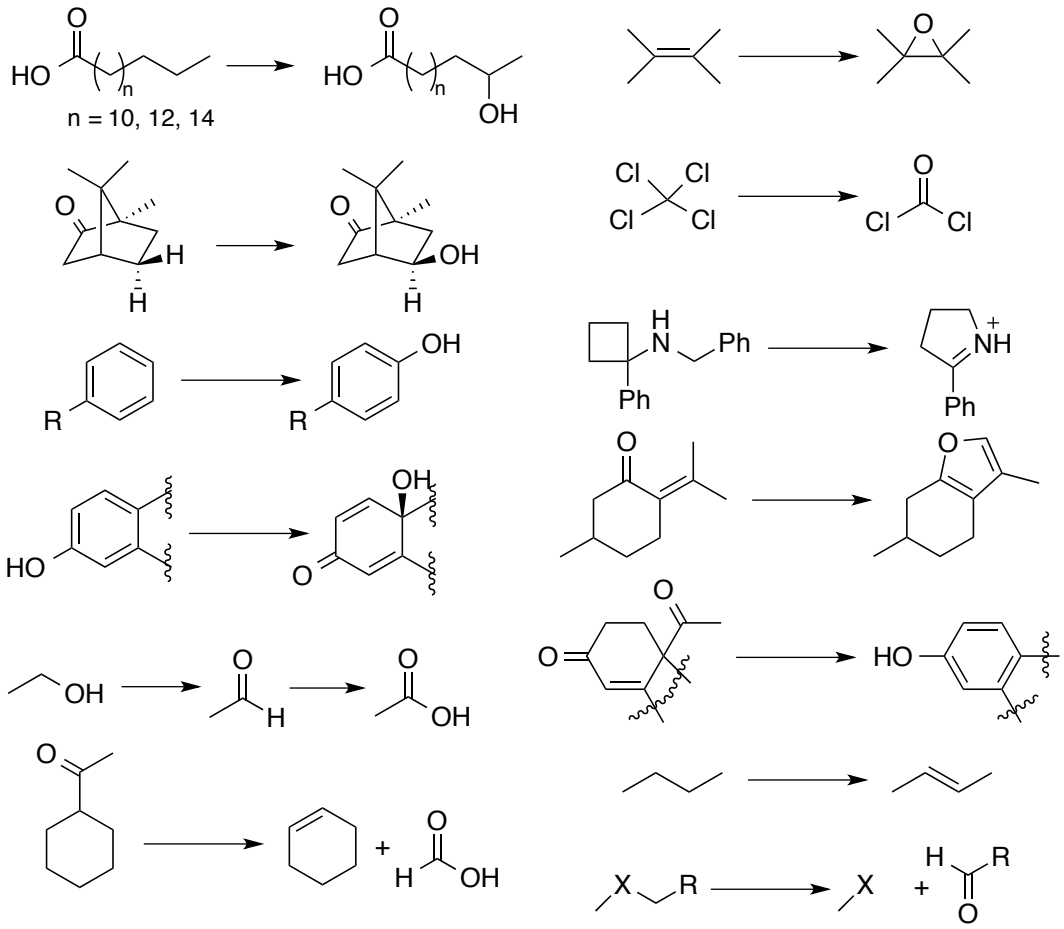

Figure 1.5. Various reactions catalyzed by cytochrome P450s.

Oxygen Activation

P450 monooxygenase activity requires binding and activation of molecular oxygen (O_2) with the input of high-energy electrons. Once the O–O bond is broken, a single oxygen atom is incorporated into an organic substrate molecule; the other is released as water. A canonical P450 catalytic cycle is shown in **Figure 1.6**; black rhombs represent the active site heme. Each step in this catalytic process relies on critical features in the P450 architecture.

The stepwise, reductive activation of dioxygen involves transient generation of reactive intermediates that include superoxide, peroxide, and high valent iron-

oxos. In order to prevent deleterious generation and release of these species, many P450s have an important gating mechanism prevents the flow of electrons, and thus, the activation of dioxygen, in the absence of substrate; both P450 BM3 and CYP119 fall into this class. In these enzymes, the resting state contains a six-coordinate, ferric (Fe^{III}) heme, which is ligated by the axial thiolate on the proximal face, and a loosely bound water molecule on the distal face (species 1 of **Figure 1.6**). Strong interactions of the iron 3*d* orbitals with these ligands cause the resting state to be low-spin.

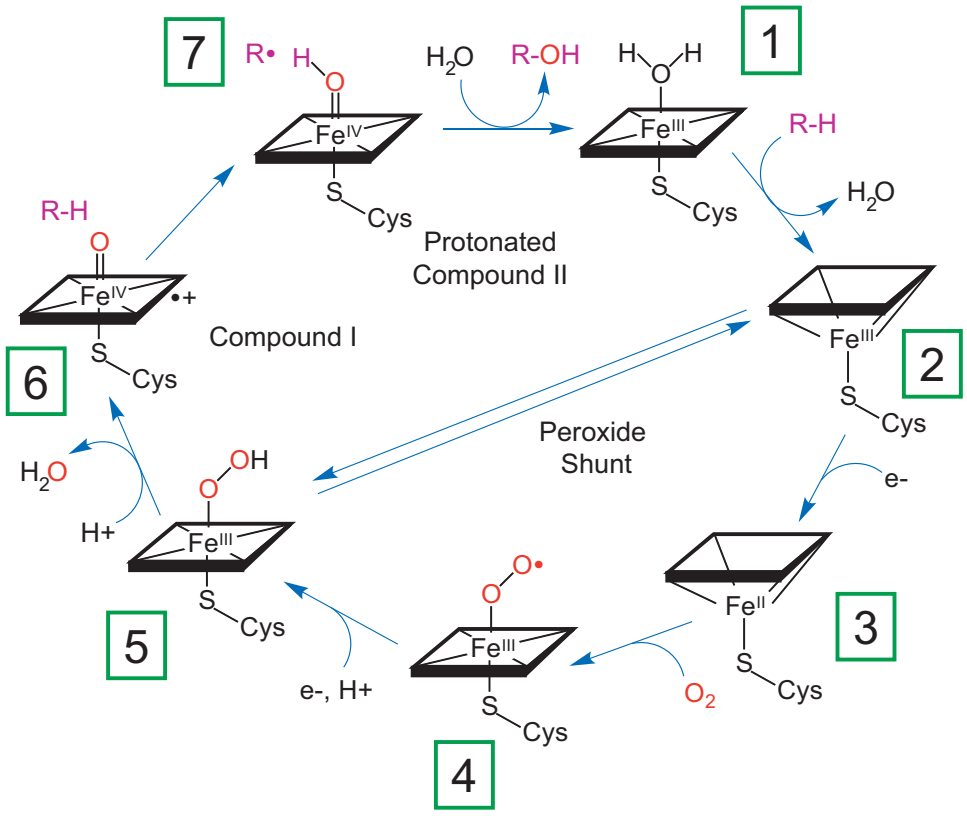

Figure 1.6. Canonical P450 catalytic cycle.

Due in large part to the strongly donating character of the axial thiolate, the resting state $Fe^{III/II}$ reduction potential is too negative to be reduced by native redox cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), and reduced flavin adenine dinucleotide ($FADH_2$) (**Table 1.1**).

Substrate binding causes displacement of the axial water molecule,[6] forming a five-coordinate, ferric heme in which the iron center is displaced below the ring of the porphyrin (species **2**).[7] The weaker ligand field in this geometry favors the high spin iron state, and shifts the $Fe^{III/II}$ reduction potential 50-150 mV positive,[8] facilitating one-electron reduction of the heme (species **3**).

Table 1.1. Relevant reduction potentials of wild type P450s and redox cofactors[8–10]

Enzyme/Cofactor	*E*°' (mV vs. NHE)
NAD^+/NADH ($2e^-$)	-320[a]
FAD/$FADH_2$ ($2e^-$)	-320[b]
FAD/FADH•	-264[b]
FADH•/$FADH_2$	-375[b]
WT BM3 $Fe^{III/II}$	-430[c,d]
WT BM3 $Fe^{III/II}$ (substrate bound)	-290[c]
WT CYP119 $Fe^{III/II}$	-420[d]

a: ref 9. b: ref 10. c: ref 8. d: Chapter 6 of this Thesis

The requisite electrons from NAD(P)H, and are transferred to the heme by various electron relays. For the subset of P450s known as "Class I" enzymes, a flavoprotein reductase acquires the two electrons from NADPH and dispatches them individually to an iron-sulfur protein, which in turn reduces the P450 oxygenase. Mammalian P450s associated with steroid synthesis and the majority of bacterial enzymes fall into this category. In Class II enzymes, a single flavoprotein reductase transfers the electrons from NADPH to the P450 oxygenase via the associated flavin mononucleotide cofactor (FMN).[11] The mammalian P450s involved in drug metabolism are an example of this class.[11] P450 BM3 belongs to a unique, "self-sufficient" class, in which the reductase and oxygenase domains are fused. This facilitates ET, and allows P450 BM3 to turnover product with some of the highest

rates: 930 min^{-1} for lauric acid, and 1470 min^{-1} for arachidonic acid.[12] For comparison, CYP119 oxidizes lauric acid at a rate of 11 min^{-1}.[13]

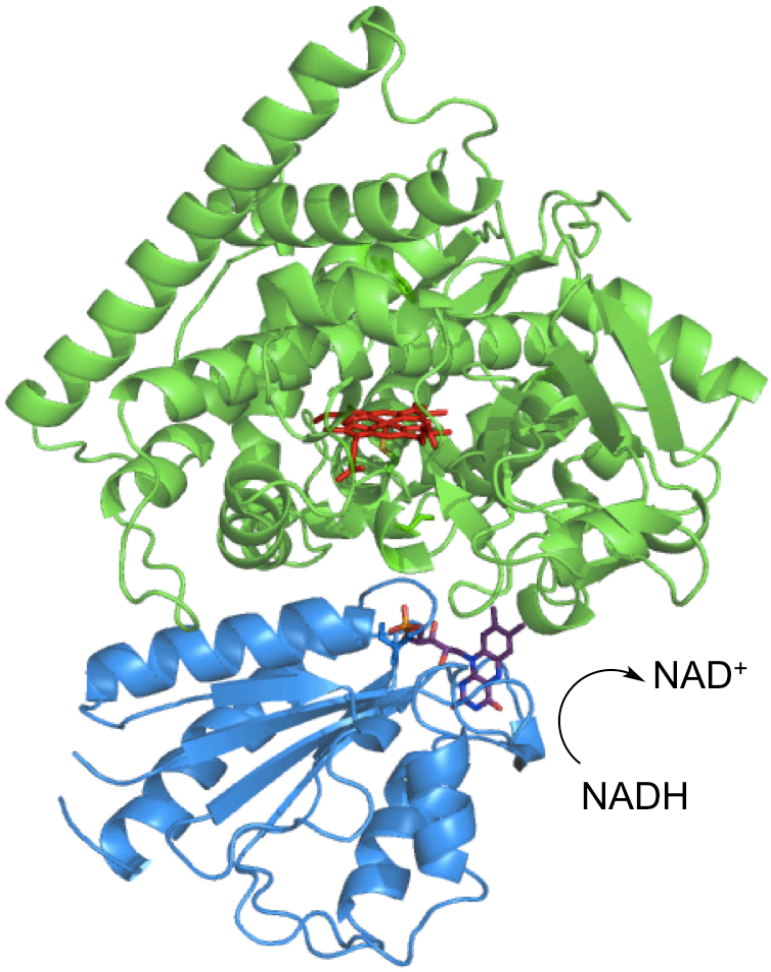

Figure 1.7. Structure of P450 BM3 oxygenase and reductase domains (PDB: 1BVY). The oxygenase domain is colored green (heme: red), and the reductase domain is colored blue (flavin: purple).

The ferrous heme readily binds oxygen, forming a ferric superoxide (species **4**). Further reduction by one electron and protonated forms a hydroperoxy species (species **5**). Two main factors affect heterolysis of the O–O bond at the heme active site. First, the "thiolate-push" from the strongly-donating thiolate ligand assists in heterolytic bond cleavage.[14] Second, a well-organized proton relay facilitates selective protonation of the distal oxygen. This relay includes the conserved

threonine side chain on helix I, as well as organized water molecules within the active site (**Figure 1.2**). The doubly-protonated distal oxygen departs as a water molecule, generating a ferryl, ligand-radical-cation species known as Compound I (CI, **Figure 1.6**, species **6**).

P450 active hydroxylation agents: ferryl compounds I and II

CI is an extremely reactive oxidant, which allows P450 to functionalize strong and unactivated C–H bonds. CI abstracts a hydrogen atom from substrate to form Compound II (CII, **Figure 1.6** 7), which then undergoes radical recombination with substrate to produce hydroxylated product. Release of product from the active site and re-ligation by a water molecule returns the enzyme to its resting state.

The native steps of substrate binding, ET, and O–O bond cleavage are slower than the reaction of CI with substrate. However, CI can be generated from the resting state by reaction with chemical oxygen-atom donors, including *meta*-chloroperoxybenzoic acid (*m*CPBA). This chemical oxidation was accomplished cleanly for highly purified samples of CYP119, and CI was spectroscopically characterized by UV-visible absorption, Mössbauer, and electron paramagnetic resonance (EPR) spectroscopies.[15,16] The apparent second order rate constant for reaction of CI with lauric acid (dodecanoic acid) is 1.1×10^7 $M^{-1}s^{-1}$.[15]

It is remarkable that a wide range of specific chemical transformations can be achieved by very similar reactive heme-oxygen intermediates. It is equally remarkable that an enzyme can house a reactive species capable of activating aliphatic C–H bonds, and emerge unscathed. How does the enzyme house such a

reactive species without reacting with the much weaker bonds that make up the protein itself?

One factor is the gating mechanism described earlier; many P450s cannot be reduced (and therefore cannot activate dioxygen to form CI) in the absence of substrate. Another factor is the thiolate ligation; this causes CII to be more basic than other heme enzymes which are ligated by histidine. Characterization of chloroperoxidase and P450 CII by Mössbauer spectroscopy indicates a protonated, Fe^{IV}-OH species.[17,18] CII in CYP158 from *Streptomyces coelicolor* has a reported pK_a of almost 12,[18] much higher than the pK_a of ~3.5 for histidine-ligated proteins such as horseradish peroxidase and myoglobin.[19] Abstraction of the hydrogen atom from substrate requires transfer of both the electron and the proton. By facilitating proton transfer through basicity of CII, a less-oxidizing CI is required.

Much progress has been made to understand the native P450 catalytic cycle. Chemical generation, trapping, and spectroscopic characterization of the CI and CII active species have been huge steps in the understanding of cytochrome P450 reactivity. However, the fundamental nuclear reorganization that drives O–O bond scission to form CI, and the events of hydrogen atom abstraction and radical rebound to form hydroxylated substrate, have yet to be observed directly. Formation of these intermediates requires rapid and precisely controlled ET.

1.4. Photo-triggered electron transfer in proteins

Photo-triggered processes provide a high degree of temporal precision for the gating of reactions. Absorption of a photon occurs nearly instantaneously, and light energy can be delivered in discrete packets using pulsed lasers. In these cases, the precision of temporal gating is limited by the length of a laser pulse; this can be a few nanoseconds (as in the case of experimental instrumentation for this Thesis)

or as little as a few femtoseconds. In order to translate the energy from photons into a desired reaction, such as ET, an additional component is needed: a photosensitizer.

When photosensitizers absorb a photon of the appropriate wavelength, a reactive electronic excited state is generated. Ruthenium tris(2,2′-bipyridine) ($[Ru(bpy)_3]^{2+}$) is a classic example.[20,21] The metal-to-ligand charge transfer (MLCT) absorption band (λ_{max} = 453 nm) is responsible for the orange-red color of this complex (**Figure 1.8**). Excitation into the MLCT band generates a reactive excited state that is both oxidizing at the metal center and reducing at the ligand (**Figure 1.9**, left). The excited state can relax by emission of a photon (λ_{max} = 626 nm), or can be "quenched" by ET to or from a redox partner.

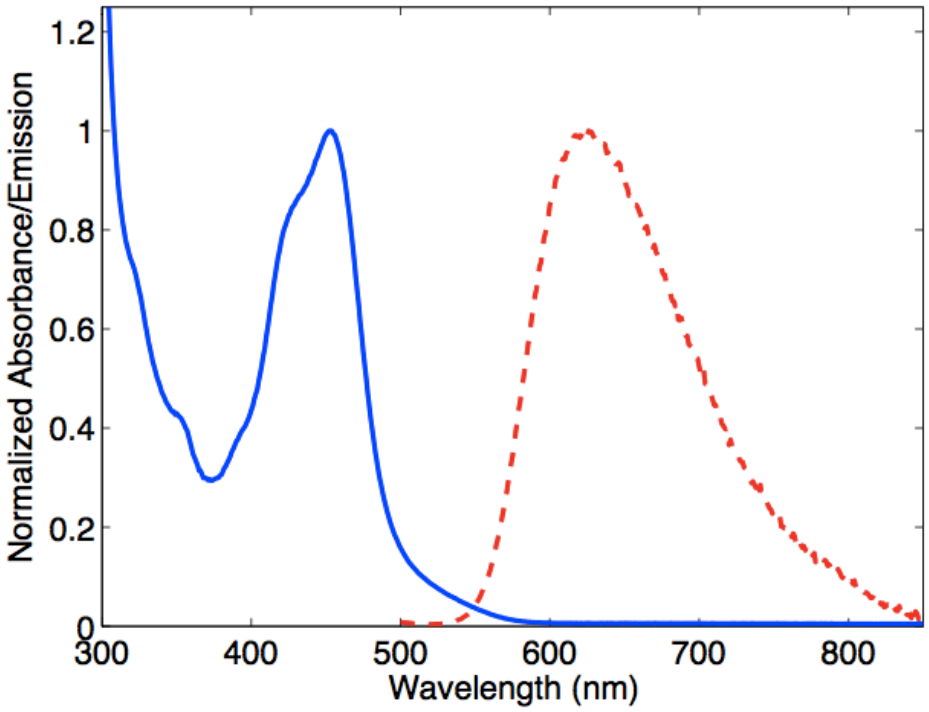

Figure 1.8. $[Ru(bpy)_3]^{2+}$ absorption (blue, solid) and emission (red, dashed) spectra in water, at room temperature. The emission spectrum was generated using 480 nm excitation.

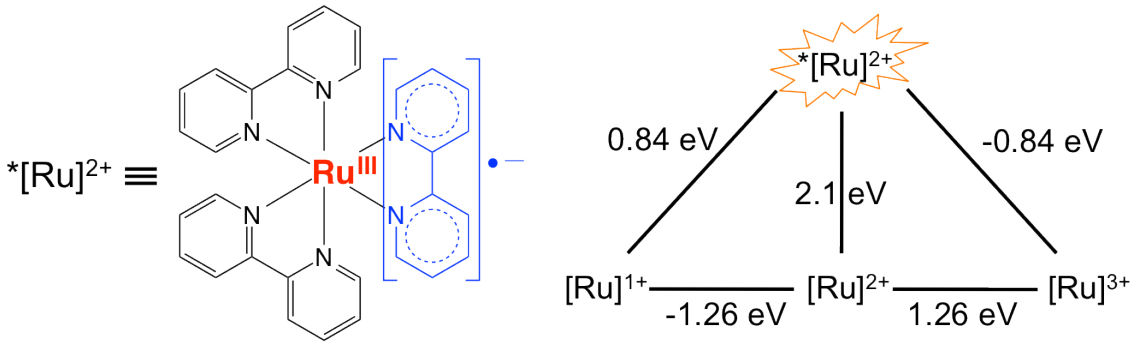

Figure 1.9. $[Ru(bpy)_3]^{2+}$ photochemistry. **Left**: MLCT excited state of $[Ru(bpy)_3]^{2+}$. **Right**: Modified Latimer diagram for $[Ru(bpy)_3]^{2+}$; potentials are vs. the normal hydrogen electrode (NHE), in water.[20,21]

In some cases, the photosensitizer's excited state reduction potential is sufficient to accomplish a desired ET reaction within the lifetime of the excited state (e.g., 600 ns for $[Ru(bpy)_3]^{2+}$ in deoxygenated water). However, in cases where additional driving force is required, or where the ET reaction is slower than luminescent relaxation of the photosensitizer, a method called "flash-quench" can be employed. In our example of $[Ru(bpy)_3]^{2+}$, a "flash" of light, such as a laser pulse, generates the photosensitizer excited state; this in turn is "quenched" by ET with exogenous small molecules. By selection of an appropriate quencher, either the more oxidizing $[Ru]^{3+}$ or more reducing $[Ru]^{1+}$ can be generated; this new species can be used to achieve ET with a system of interest. Flash-quench generates a photosensitizer state with additional driving force, and the separation of charges extends the lifetime of the reactive species.

By partnering photosensitizers with redox-active proteins, flash quench can be used to trigger biologically-relevant ET. In particular, the Gray lab has focused on systems in which the electron acceptor (or donor) is a metal center, such as copper in azurin, or heme iron in cytochromes, including cytochrome P450 (**Figure 1.10**). An advantage of these systems is that the metal center has strong absorbance in the

visible region. Furthermore, the absorption bands are sensitive to oxidation state of the metal center; ET reactions can be monitored using transient absorption spectroscopies. By varying the nature of the photosensitizer and the protein structure, the Gray lab and others have probed the mechanistic aspects of biological ET. Curious readers are directed toward a number of excellent reviews for further details.[22–24] An extension of this analysis, multistep ET ("hopping") is discussed in Chapter 4.

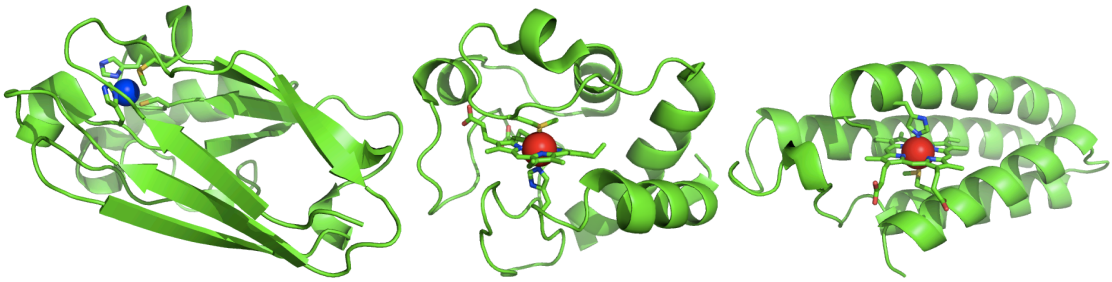

Figure 1.10. Redox active model proteins. Left to right: *P. aeruginosa* azurin (PDB: 1JZG, Cu in blue), horse heart cytochrome c (PDB: 1HRC, heme Fe in red), cytochrome b562 (PDB: 256B, heme Fe in red).

The bulk of this Thesis (Chapters 2-5) focus on the extension of these photo-triggered methods to generate redox intermediates and investigate ET mechanisms in cytochrome P450. To set the stage for the design of photosensitizer-P450 systems, we outline strategies for the selection of appropriate photochemical elements.

Photosensitizers

A wide variety of photosensitizers have be used for initiating ET within proteins. The Gray lab and others have employed ruthenium, rhenium, and osmium photosensitizers with a variety of ligand frameworks to study ET processes within metalloproteins, including heme proteins.[22,25] Selection of an appropriate

photosensitizer for each specific application requires assessment of several photochemical and photophysical aspects.

Electronic communication between the protein and photosensitizer is critically important. For systems in which the protein redox cofactor is solvent exposed, simple photosensitizers such as $[Ru(bpy)_3]^{2+}$ can be implemented. However, when the redox cofactor is buried inside the protein framework, more elaborate methods must be employed. Photosensitizer "wires" that bind noncovalently to the protein surface, or within the substrate channel, can be used (**Figure 1.11**).

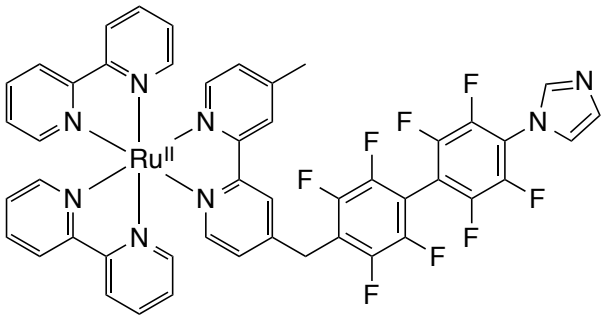

Figure 1.11. An example of a perfluorinated ruthenium wire.

A potential disadvantage of these wires is that noncovalent binding results in a distribution of free and bound photosensitizers. For enhanced control over binding location, the photosensitizer can be tethered to the protein surface (**Figure 1.12**).

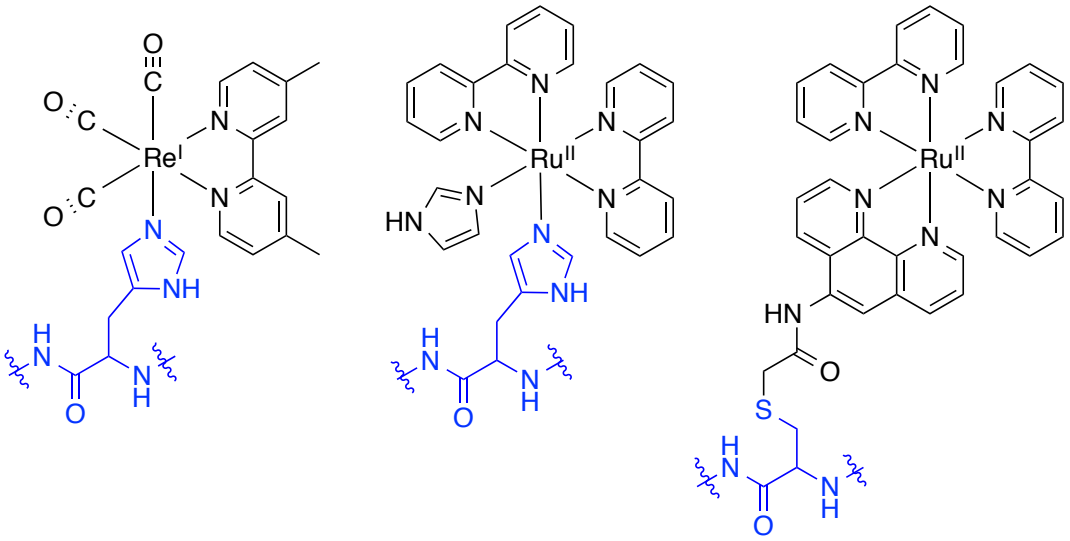

Figure 1.12. Photosensitizers for site-specific surface labeling at amino acids (blue). **Left**: $[Re(dimethyl\text{-}bpy)(CO)_3(imidazole)]^{1+}$ is ligated by hisitidine. **Center**: $[Ru(bpy)_2(imidazole)]^{2+}$ is ligated by histidine. **Right**: $[Ru(bpy)_2(iodoacetamidophenanthroline)]^{2+}$ is tethered to cysteine.

This can be achieved by direct coordination of the photosensitizer metal center by histidine amino acids (**Figure 1.12** left and center). Alternatively, nucleophilic residues such as cysteine can be modified with an iodoacetamido linker attached to the photosensitizer ligand, in order to generate a covalent thioether bond between protein and photosensitizer (**Figure 1.12,** right). Selective photosensitizer conjugation to a specific site on the protein surface is accomplished by removal of native histidine/cysteine residues, and installation of a single labeling site, using site directed mutagenesis.

Selection of the photosensitizer/protein conjugation method is also important. The cysteine tethering reaction is more rapid, and can be accomplished at low temperature (4 °C) within the span of a few hours.[26] By comparison, histidine labeling often takes days or weeks, and may require room- or elevated temperature (37 °C).[27,28] However, the more flexible iodoacetamido linker (cysteine-labeling) results in a photosensitizer-metalloprotein distance that is not fixed, as it is in

direct metal ligation by histidine. For experiments in which knowledge of discrete photosensitizer-protein distance is important, histidine labeling may be advantageous. Additionally, it is important to consider the native amino acid composition of the protein. For example, for large proteins that contain a high number of exposed histidine residues, cysteine labeling is more practical.

The photosensitizer must also provide sufficient driving force to accomplish the desired ET. Varying the metal center and the ligand framework provides a range of driving forces that span over 1 V.[29]

Absorption (of both photosensitizer and protein) is another key factor. It is desirable to excite the photosensitizer at a wavelength that is not also absorbed by the protein or its cofactors. Protein absorption reduces the number of photons available to excite the photosensitizer; it could cause undesired sample heating as excited protein molecules relax by thermal processes; or (particularly in the case of heme enzymes) it could cause protein photochemistry or luminescence that interfere with observation of the desired ET processes.

Small molecule quenchers

Flash-quench methods require an exogenous small molecule ET quencher (Q). For protein experiments, Q must be water soluble up to millimolar concentrations at mild pH (6-8), and must have an appropriate reduction potential to rapidly (ideally, diffusion limited) oxidize or reduce the photosensitizer.

In addition, the quencher must not have significant absorbance at wavelengths of interest for the photosensitizer-protein system; this is analogous to what was described for protein absorbance. Laser-triggered flash-quench ET process can be probed by transient absorption spectroscopies, therefore it is also desirable that the

quencher not absorb at the probe wavelengths (e.g., the Soret region for heme proteins: 390 – 440 nm).

A final consideration for quencher selection is redox reversibility. In a reversible cycle (such as depicted in **Figure 1.13**), the stable, reduced quencher serves as an electron source to complete the ET cycle and return the system back to its resting state. In an ideal reversible system, the flash-quench cycle can be repeated *ad infinitum*, with no degradation or persistent accumulation of intermediates. Reversibility is most desirable for rapid transient absorption laser studies, in which the averaging of many shots is required to achieve a sufficient signal-to-noise ratio. Alternatively, irreversible quenchers serve as a sacrificial oxidant; this supplies more time for slow reaction events, and allows for accumulation (and detection) of oxidized species. Irreversible quenchers are usually desirable for steady-state flash-quench processes, such as light-driven catalysis. Parameters for three water-soluble ET quenchers are given in **Table 1.2**.

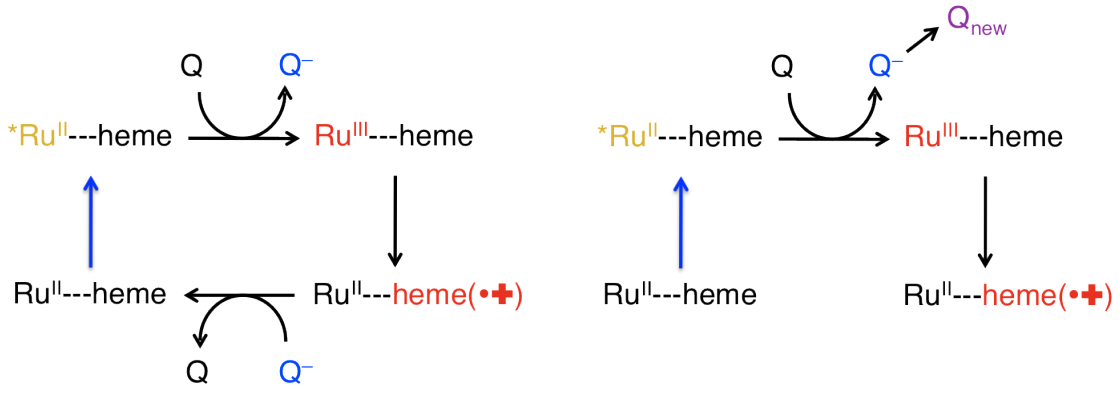

Figure 1.13. Flash-quench ET cycles. Oxidized species are highlighted in red, reduced species in blue. **Left**: Reversible flash-quench ET. **Right**: irreversible flash-quench.

Table 1.2. Parameters for a selection of oxidative quenchers.[30–33] Potentials are reported vs. NHE.

Q	$E^{\circ}(Q/Q^{-})$	ε (480 nm)	ε (400-440 nm)	solubility (pH 7)	reversibility
$[Ru(NH_3)_6]^{3+}$	0 V	*negligible*	*negligible*	> 50 mM	reversible
Methyl viologen	-0.45 V	*negligible*	*negligible*	> 50 mM	reversible
$[Co(NH_3)Cl]^{2+}$	0.29 V	30 $M^{-1}cm^{-1}$	15-25 $M^{-1}cm^{-1}$	< 20 mM	irreversible

1.5. Photo-triggered ET in P450: two pathways toward reactive heme species

Photosensitizers and flash-quench can be used in place of native ET partners to generate and investigate the active species CI and CII. Reductive flash-quench supplies the electrons necessary to activate dioxygen (**Figure 1.14**, blue arrows), much more rapidly than the ET with native redox partners. Alternatively, assuming microscopic reversibility of reaction in the catalytic cycle, one could envision a novel pathway in which direct oxidation of the ferric aquo heme (loss of one electron and one proton) generates CII, followed by a second one-electron one-proton oxidation to generate CI (**Figure 1.14**, red arrows). An attractive aspect of this "reverse," oxidative route is that the oxygen atom in CI, which would be incorporated into substrate, is derived not from dioxygen or reactive oxygen species, but from water. This method was previously implemented to generate CII and CI in histidine-ligated heme enzymes and model systems (vide infra). Inspired by these successes, we began the pursuit of P450 CI using the oxidative route; these experiments are discussed in Chapters 2-4. Reductive ET and associated reactions are discussed in Chapter 5.

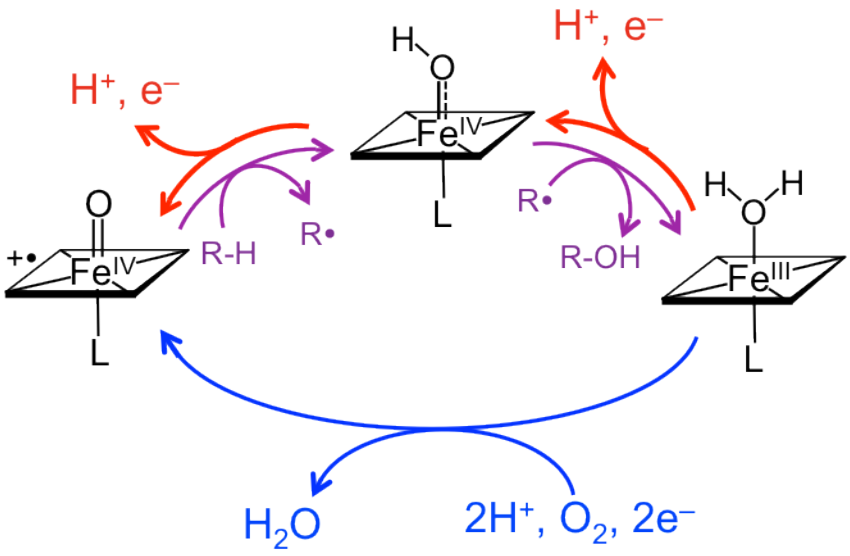

Figure 1.14. Pathways for formation of high valent CI and CII. Blue arrows: reductive activation of dioxygen (native catalytic cycle). Red arrows: oxidative activation of water. Purple arrows: hydroxylation of substrate (RH).

Precedence for photochemical heme oxidation

Photochemical heme oxidation was first achieved for the model system microperoxidase-8 (MP8), the heme-containing 8-amino acid fragment of horseheart cytochrome *c* (**Figure 1.16**, right). Laser flash-photolysis of a sample composed of $[Ru(bpy)_3]^{2+}$, an excess of $[Ru(NH_3)_6]^{3+}$ as an oxidative quencher, and MP8 resulted in one-electron oxidation of the exposed heme. The position, shape, and intensity of the heme Soret and Q-bands are sensitive to oxidation state and heme environment. CII formation was observed by a shift to higher energy of the Soret band at pH values above 7. In more acidic solutions, CII was not produced, but rather an Fe^{III} porphyrin radical cation species was characterized by loss of the Soret absorbance (a "bleach") (**Figure 1.15**). Analogous flash-quench oxidation was also achieved for the histidine-ligated enzyme horseradish peroxidase (HRP) (**Figure 1.16**, left).

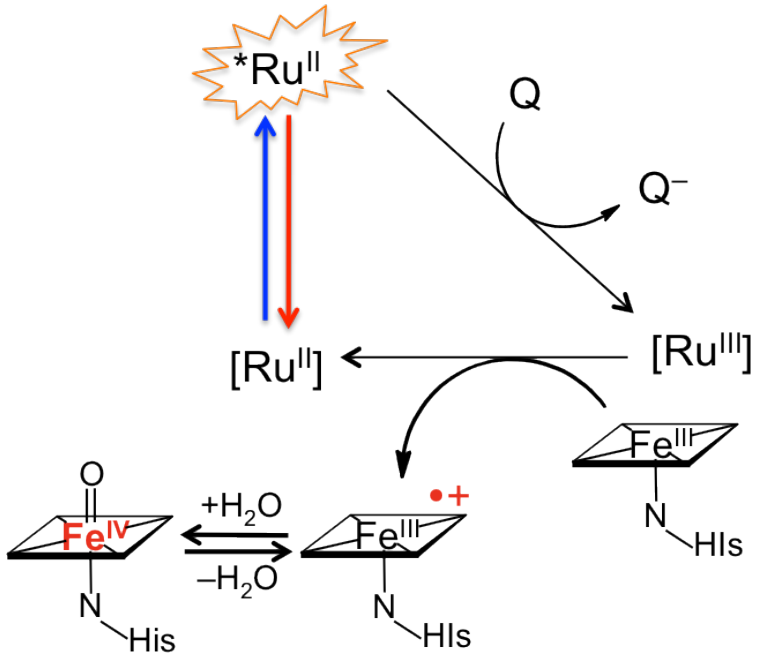

Figure 1.15. Flash-quench oxidation of a heme protein active site. Blue arrow indicates excitation with blue light (e.g., 480 nm), the red arrow indicates emission of red light (e.g., 630 nm).

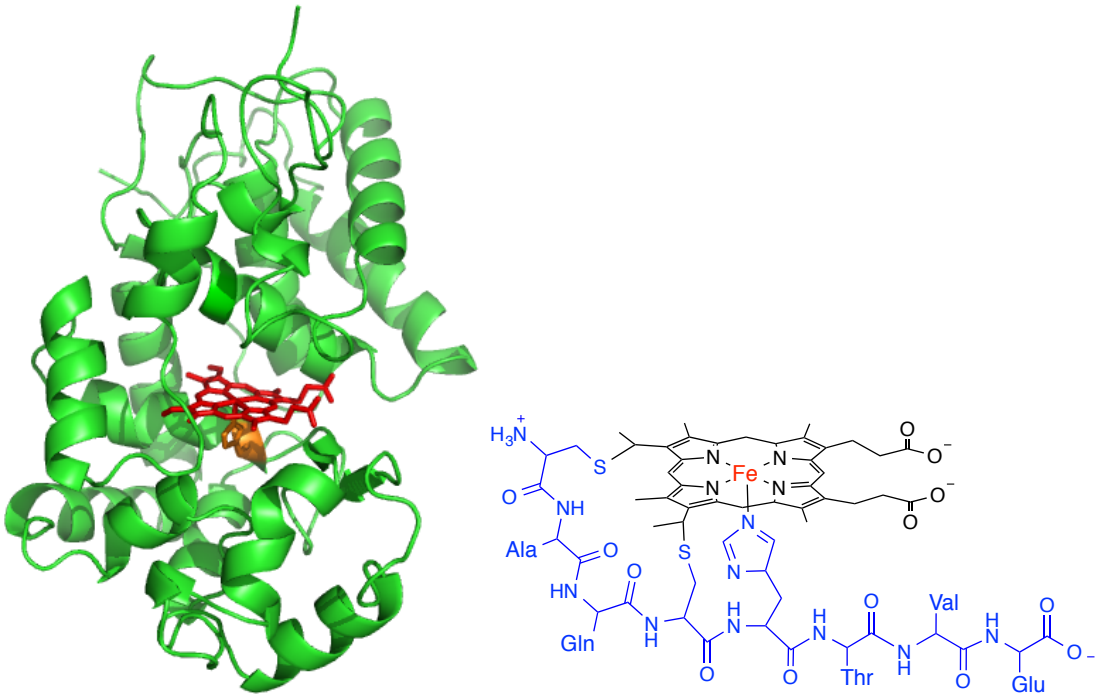

Figure 1.16. Histidine-ligated hemes. **Left**: Horseradish Peroxidase (HRP, PDB: 1HCH). **Right**: Microperoxidase-8 from horseheart cytochrome *c*.

P450 considerations: the buried heme

The analogous use of $[Ru(bpy)_3]^{2+}$ was unsuccessful for oxidation of the P450 heme, likely due to deep burial of the active site within the protein scaffold. Ruthenium "wires" were synthesized as substrate mimics; rapid flash-quench heme reduction was accomplished using an imidazole-terminated perfluorinated ruthenium wire (**Figure 1.11**).[34] However, this method both blocks the substrate channel and displaces the axial water molecule that is necessary for oxidative formation of CII. Covalent tethering of the photosensitizer to the P450 surface has finally enabled the desired oxidative ET (Chapter 2).

1.6. New Frontiers: Novel P450 Active Species for Non-Native Catalysis

Cytochrome P450s offer a seemingly boundless platform in which to design and examine redox catalysis. As described earlier, the catalytic scope of native P450 catalysis is already vast (**Figure 1.5**). With small adjustments from directed evolution and rational design, P450 catalysis can be extended further to include the biodegradation of halocarbons,[35] and oxidation of gaseous hydrocarbons.[36–38] By modifying the surface with photosensitizers, P450 catalysis can even be driven with light.[39,40] As described in the previous section, the development of photochemical methods may be used to accomplish P450 catalysis by activating water, instead of oxygen or other reactive oxygen species.

However, the extent of catalysis is restricted by the nature of the active species; atom-transfer reactions catalyzed by CI are limited to oxygenation reactions. Development of alternative atom transfer methods would greatly expand utility of P450 catalysts for industrial transformations.

One particularly attractive new reaction target is enzymatic cyclopropanation. The controlled formation of new carbon-carbon bonds is one of the most fundamental challenges in synthetic chemistry. Specifically, generation of the strained cyclopropane motif often requires the formation of two new C–C bonds in a regioselective fashion, as well as control of the stereochemistry at all three carbon centers. Cyclopropanes are found in myriad organic compounds including natural products, pyrethroid insecticides, fragrances, and therapeutics,[41] and the development of cheap, efficient, selective, and sustainable cyclopropanation methods is an active area of research.[42] The development of enzymatic cyclopropanation routes can take advantage of selectivity imparted by the protein scaffold under mild temperatures and pressures. However, this reactivity requires formation of a novel active species.

Inspiration for P450-catalyzed carbene transfer came from small molecule late transition metal porphyrins (primarily Rh, Fe, Ru, Os), which catalyze the cyclopropanation of alkenes in the presence of diazo carbene precursors.[43–47] The reaction is thought to proceed via initial formation of a metallo-carbenoid, followed by carbene transfer to the alkene.

Early in 2013, Coelho *et al.* reported carbene transfer from ethyldiazoacetate (EDA) to styrene to form cyclopropanated products catalyzed by cytochrome P450 BM3.[48] Both wild-type and engineered P450 mutants were capable of catalyzing the reaction, but specific mutants achieved high turnover numbers. In particular, the single T268A mutation was sufficient to raise the turnover number from 5 (wild-type) to 323, with high selectivity for trans 1:99 *cis*:*trans* ratio and 96% enantioselectivity for the *trans S*,*S* diastereomer. Additional mutations to the active site and periphery were used to reverse this *cis*:*trans* selectivity; the highest

selectivity was reported as 92:8 *cis*:*trans*, with 97% *S*,*S* enantioselectivity for the *cis* form.

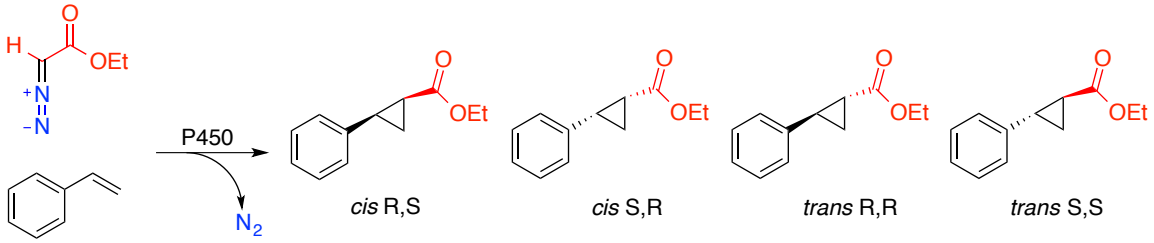

Figure 1.17. P450-catalyzed cyclopropanation of styrene.

In analogy to small molecule metalloporphyrin studies,[47] the authors proposed a mechanistic scheme that employs a high-valent carbenoid active species (**Figure 1.18,** right).

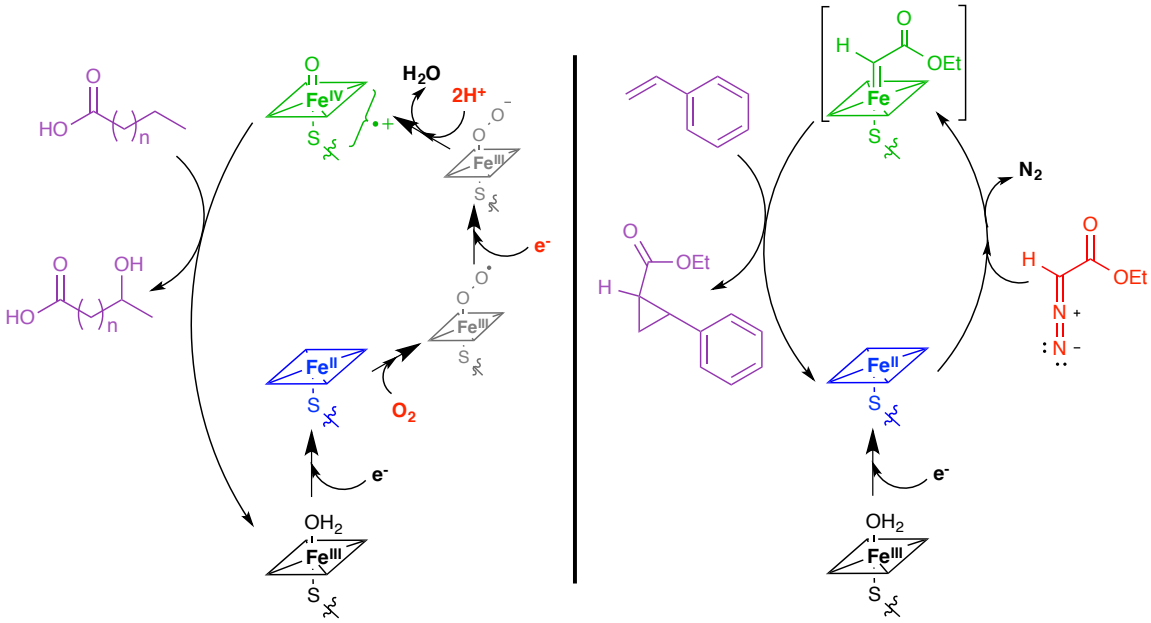

Figure 1.18. Native and engineered P450 catalytic schemes. **Left**: Consensus P450 catalytic cycle for oxygenations, showing hydroxylation of a fatty acid. **Right**: Proposed catalytic scheme for the cyclopropanation of styrene.

Many details of the proposed mechanism have yet to be experimentally verified, including the identity of the active cyclopropanating agent. However, there is

significant evidence that ferrous (not ferric) P450 is required to activate ethyldiazoacetate (EDA). Cyclopropanation activity required the presence of strong reductant, such as dithionite, and the net reaction is inhibited by carbon monoxide, which readily binds to ferrous hemes.[49]

While this in method offers an exquisite proof-of-concept for the development of enzymatic cyclopropanations, the extension of this reactivity to *in vivo* systems would avoid time consuming isolation and purification steps. P450 BM3 is an ideal candidate for such development, as this "self-sufficient" enzyme contains both heme oxygenase and flavin reductase domains fused in a single polypeptide chain. No additional redox partner proteins are required. However, the biological electron source for heme reduction (a critical step for activation of EDA) is NADH. The aforementioned mutants show little to no cyclopropanation activity in the presence of this cofactor.

This observed inactivity is due to a substrate gating mechanism described previously (Section 1.3); styrene binds with low affinity (K_M ~ 5 mM)[48] and is inefficient at effecting the low-to-high spin state change. Enzyme engineering to increase the binding affinity might facilitate gating; however, this would need to be achieved for every individual substrate. Incorporation of inert perfluorinated substrate mimics has been implemented to artificially prop the gate open and facilitate oxidation of tiny, ill-binding substrates such as ethane and methane.[38] However, use of these decoy molecules *in vitro* is expensive, and implementation for *in vivo* systems has not been established.

Alternatively, the enzyme could be reprogrammed to bypass this substrate gating mechanism by altering the resting state reduction potential. In Chapter 6, we

describe a series of electrochemical experiments that probe electron flow in a new generation of P450 mutants engineered for cyclopropanation.

1.7. Conclusions

Rapid and efficient ET is critical for the formation of the reactive P450 intermediates responsible for catalysis, as well as for their selective functionalization of organic substrates and biological molecules. The remaining Chapters in this Thesis explore the details of photochemical generation of high-valent heme intermediates in ruthenium-P450 conjugates, photochemical heme reduction and gas binding as ways to monitor the native P450 cycle, and electrochemical characterization of P450 axial mutants in relation to cyclopropanation activity.

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