5.6: Monooxygenases

Metal-containing monooxygenase enzymes are known that contain heme iron, nonheme iron, or copper at their active sites.[2] For most of these enzymes, there is only limited information about the nature of the active site and the mode of interaction with dioxygen or substrates. But there are three monooxygenase enzymes that strongly resemble well-characterized reversible dioxygen-carrying proteins (see preceding chapter), suggesting that dioxygen binding to the metalloenzyme in its reduced state is an essential first step in the enzymatic mechanisms, presumably followed by other steps that result in oxygenation of substrates. The enzymes are:

1. cytochrome P-450,[73] a heme-containing protein whose active site resembles the dioxygen-binding sites of myoglobin or hemoglobin in many respects, except that the axial ligand to iron is a thiolate side chain from cysteine rather than an imidazole side chain from histidine;
2. tyrosinase,[74] which contains two copper ions in close proximity in its active site and which has deoxy, oxy, and met states that closely resemble comparable states of hemocyanin in their spectroscopic properties; and
3. methane monooxygenase,[75,76] which contains two nonheme iron ions in close proximity and which resembles hemerythrin in many of its spectroscopic properties.

In addition to these three, there are also monooxygenase enzymes containing single nonheme iron[77] or copper ions,[78] or nonheme iron plus an organic cofactor such as a reduced pterin at their active sites.[79] Just as with the dioxygenase enzymes, we do not know how similar the mechanisms of the different metal-containing monooxygenase enzymes are to one another. The enzyme for which we have the most information is cytochrome P-450, and we will therefore focus our discussion on that system. Speculations about the mechanisms for the other systems are discussed at the end of this section.

1. Cytochrome P-450

Cytochrome P-450 enzymes are a group of monooxygenase enzymes that oxygenate a wide variety of substrates.[73] Examples of such reactions are:

1. hydroxylation of aliphatic compounds (Reaction 5.59);
2. hydroxylation of aromatic rings (Reaction 5.60);
3. epoxidation of olefins (Reaction 5.61);
4. amine oxidation to amine oxides (Reaction 5.62);
5. sulfide oxidation to sulfoxides (Reaction 5.63); and
6. oxidative dealkylation of heteroatoms (for example, Reaction 5.64).

$$(5.59)$$

$$(5.60)$$

$$(5.61)$$

$$(5.62)$$

$$(5.63)$$

$$Ph-O-CH_3 \longrightarrow Ph-OH + HCHO \qquad (5.64)$$

Some of these reactions have great physiological significance, because they represent key transformations in metabolism, as in lipid metabolism and biosynthesis of corticosteroids, for example.[73] Cytochrome P-450 is also known to catalyze the transformation of certain precarcinogens such as benzpyrene into their carcinogenic forms.[73]

Many of the P-450 enzymes have been difficult to characterize, because they are membrane-bound and consequently relatively insoluble in aqueous solution. However, cytochrome P-450$_{cam}$, which is a component of the camphor 5-monooxygenase system isolated from the bacterium *Pseudomonas putida*, is soluble and has been particularly useful as the subject of numerous spectroscopic and mechanistic studies, as well as several x-ray crystallographic structure determinations.[80] This enzyme consists of a single polypeptide chain, mainly α-helical, with a heme b group (Fe-protoporphyrin IX) sandwiched in between two helices, with no covalent attachments between the porphyrin ring and the protein. One axial ligand complexed to iron is a cysteinyl thiolate. In the resting state, the iron is predominantly low-spin Fe^{III}, probably with a water as the other axial ligand. When substrate binds to the resting enzyme, the spin state changes to high-spin, and the non-cysteine axial ligand is displaced. The enzyme can be reduced to an Fe^{II} state, which is high-spin, and resembles deoxyhemoglobin or myoglobin in many of its spectroscopic properties. This ferrous form binds dioxygen to make an oxy form or carbon monoxide to make a carbonyl form. The CO derivative has a Soret band (high-energy π-π* transition of the porphyrin ring) at 450 nm, unusually low energy for a carbonyl derivative of a heme protein because of the presence of the axial thiolate ligand. This spectroscopic feature aids in the isolation of the enzyme and is responsible for its name.

a. "Active Oxygen"

Camphor 5-monooxygenase is a three-component system, consisting of cytochrome P-450$_{cam}$ and two electron-transfer proteins, a flavoprotein, and an iron-sulfur protein (see Chapters 6 and 7). The role of the electron-transfer proteins is to deliver electrons to the P-450 enzyme, but these may be replaced *in vitro* by other reducing agents. The reaction sequence is in Figure 5.10.
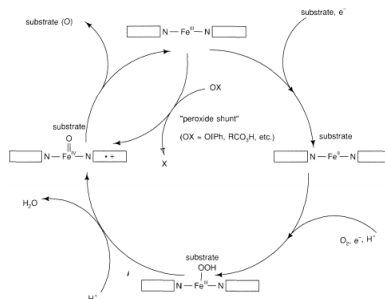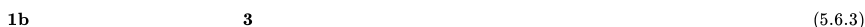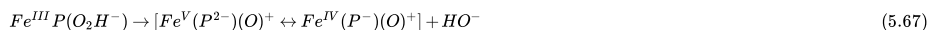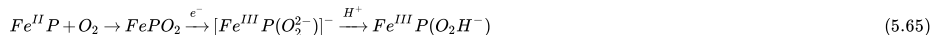

Figure 5.10 - Proposed mechanism for cytochrome P-450. The resting enzyme is shown at the top of the cycle. Substrate binds to the enzyme at a position close to the iron center, but it is not directly coordinated to the metal ion. The enzyme-substrate complex is then reduced to the ferrous form. Dioxygen then binds to form an oxy complex (not shown). The oxy complex is then reduced by another electron and protonated, giving a ferric-hydroperoxy complex shown at the bottom of the cycle. The ligand bound here to the Fe^{III} center is HO$_2^-$, i.e. deprotonated hydrogen peroxide. The ferric hydroperoxy form of the enzyme-substrate complex then undergoes heterolytic O—O bond cleavage, giving a high-valent Fe^{IV} oxo center, with the porphyrin ligand oxidized by one equivalent (see text). This species then transfers a neutral oxygen atom to the bound substrate, which is then released, giving the oxygenated product and regenerating the resting form of the enzyme. The "peroxide shunt" refers to the mechanism proposed for the cytochrome P450-catalyzed oxygenation of substrates by single-oxygen-atom donors (see text). It is believed that the same high-valent iron-oxo intermediate is generated in these types of reactions as well.

For cytochrome P-450, the question that is possibly of greatest current interest to the bioinorganic chemist is just what mechanism enables activation of dioxygen and its reaction with substrate. It seems clear that dioxygen binds to the ferrous state of the enzyme-substrate complex, and that the resulting oxy ligand, which presumably is similar to the oxy ligand in oxyhemoglobin and oxymyoglobin, is not sufficiently reactive to attack the bound substrate. The oxy form is then reduced and the active oxidant is generated, but the nature of the active oxidant has not been deduced from studies of the enzyme itself, nor has it been possible to observe and characterize intermediates that occur between the time of the reduction and the release of product. Three species are potential candidates for "active oxygen," the oxygen-containing species that attacks the substrate, in cytochrome P-450. They are:

1. a ferric peroxo, **1a**, or hydroperoxo complex, **1b**, formed from one-electron reduction of the oxy complex (Reaction 5.65);
2. an iron(IV) oxo complex, **2**, formed by homolytic O—O bond cleavage of a ferric hydroperoxo complex (Reaction 5.66); and
3. a complex at the oxidation level of an iron(V) oxo complex, **3**, formed by heterolytic O—O bond cleavage of a ferric hydroperoxo complex (Reaction 5.67).

The hydroxyl radical, HO•, although highly reactive and capable of attacking P-450 substrates, is considered to be an unlikely candidate for "active oxygen" because of the indiscriminate character of its reactivity.

$$Fe^{II}P + O_2 \rightarrow FePO_2 \xrightarrow{e^-} [Fe^{III}P(O_2^{2-})]^- \xrightarrow{H^+} Fe^{III}P(O_2H^-) \tag{5.65}$$
$$\mathbf{1a} \qquad\qquad \mathbf{1b} \tag{5.6.1}$$

$$Fe^{III}P(O_2H^-) \rightarrow Fe^{IV}P(O) + HO\cdot \tag{5.66}$$
$$\mathbf{1b} \qquad\qquad \mathbf{2} \tag{5.6.2}$$

$$Fe^{III}P(O_2H^-) \rightarrow [Fe^V(P^{2-})(O)^+ \leftrightarrow Fe^{IV}(P^-)(O)^+] + HO^- \tag{5.67}$$
$$\mathbf{1b} \qquad\qquad \mathbf{3} \tag{5.6.3}$$

$(P^{2-}$ = porphyrin ligand; P^- = one-electron oxidized porphyrin ligand)

An iron(V) oxo complex (or a related species at the same oxidation level), **3**, formed via Reaction (5.67), is the favored candidate for "active oxygen" in cytochrome P-450.[81] This conclusion was initially drawn from studies of reactions of the enzyme with alkylhydroperoxides and single-oxygen-atom donors. Single-oxygen-atom donors are reagents such as iodosylbenzene, OIPh, and periodate, IO_4^-,capable of donating a neutral oxygen atom to an acceptor, forming a stable product in the process (here, iodobenzene, IPh, and iodate, IO_3^-). It was discovered that ferric cytochrome P-450 could catalyze oxygenation reactions using organic peroxides or single-oxygen-atom donors in place of dioxygen and reducing agents. Usually the same substrates would give the identical oxygenated product. This reaction pathway was referred to as the "peroxide shunt" (see Figure 5.10). The implication of this discovery was that the same form of "active oxygen" was generated in each reaction, and the fact that single-oxygen-atom donors could drive this reaction implied that this species contained only one oxygen atom, i.e., was generated subsequent to O—O bond cleavage. The mechanism suggested for this reaction was Reactions (5.68) and (5.69).

$$Fe(III)P^+ + OX \rightarrow \mathbf{3} + X \tag{5.68}$$
$$\mathbf{3} + substrate \rightarrow Fe(III)P^+ + substrate(O) \tag{5.69}$$

b. Metalloporphyrin Model Systems

Studies of the reactivities of synthetic metalloporphyrin complexes in oxygen-transfer reactions and characterization of intermediate species observed during the course of such reactions have been invaluable in evaluating potential intermediates and reaction pathways for cytochrome P-450. Logically, it would be most desirable if one could mimic the enzymatic oxygenation reactions of substrates using iron porphyrins, dioxygen, and reducing agents. However, studies of such iron-porphyrin-catalyzed reactions have failed to produce meaningful results that could be related back to the P-450 mechanism. This is perhaps not surprising, since the enzyme system is designed to funnel electrons into the iron-dioxygen-substrate complex, and thus to generate the active oxidant within the confines of the enzyme active site in the immediate proximity of the bound substrate. Without the constraints imposed by the enzyme, however, iron porphyrins generally will either (1) catalyze the oxidation of the reducing agent by dioxygen, leaving the substrate untouched, or (2) initiate free-radical autoxidation reactions (see Section II.C). A different approach was suggested by the observation of the peroxide shunt reaction (Reactions 5.68 and 5.69) using organic peroxides or single-oxygen-atom donors, and the earliest successful studies demonstrated that Fe(TPP)Cl (TPP = tetraphenylporphyrin) would catalyze the epoxidation of olefins and the hydroxylation of aliphatic hydrocarbons by iodosylbenzene[81](Reactions 5.70 and 5.71).

$$(5.70)$$

$$(5.71)$$

Reactions (5.70) and (5.71) were postulated to occur via an iron-bound oxidant such as **3** in Reaction (5.67). This hypothesis was tested by studying the reaction of dioctyl Fe(PPIX)CI with iodosylbenzene, which resulted in 60 percent hydroxylation at positions 4 and 5 on the hydrocarbon tail (see 5.72), positions for which there is no reason to expect increased reactivity except for the fact that those particular locations are predicted from molecular models to come closest to the iron center when the tail wraps around the porphyrin molecule.[82]
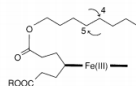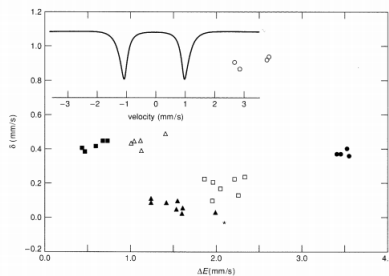

Figure 5.11).[83]

Figure 5.11 - Comparative Mössbauer data for iron-porphyrin complexes: ■ *high-spin Fe[III]* , ☐ *low-spin Fe[III]* , ● *intermediate-spin (admixed) Fe[III]* , ○ *high-spin Fe[II]* , △ *low-spin Fe[II]* , ▲ *oxo- and imido-Fe[IV]*, ✱ *dimethoxyiron(IV)TMP. Insert shows the zero-field Mössbauer spectrum of ^{57}Fe-dimethoxyiron(IV)TMP at 4.2 K. (From Reference 83.)*

Visible absorption spectra of porphyrin complexes are due largely to π-π* transitions of the porphyrin ligand. The bright green color is unusual for iron-porphyrin complexes, which are usually red or purple. (However, this green color has been seen for compound I of catalase and peroxidases; see Section VI below.) The unusually long-wavelength visible absorption bands that account for the

green color result from the fact that the porphyrin ring has been oxidized by one electron. Similar visible absorption bands can be seen, for example, in other oxidized porphyrin complexes, such as $Co^{III}(P^{\bullet-})^+$, formed by two-electron oxidation of $Co^{II}(P^{2-})$(see 5.73).[84]
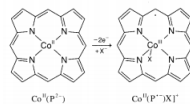
$$(5.73)$$

Oxidized porphyrin ligands also give characteristic proton NMR spectra, which are seen for the green porphyrin complex as well.[81,83]

Magnetic measurements indicate that the green porphyrin complex contains three unpaired electrons. Detailed analysis of the Mössbauer spectra has indicated that the two unpaired electrons on the Fe^{IV} ion are strongly ferromagnetically coupled to the unpaired electron on the porphyrin, accounting for the resulting $S = \frac{5}{3}$ state.[81,83]
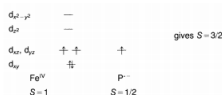
$$(5.74)$$

Studies of the reactions of this species with P-450-type substrates demonstrate that this species is reactive enough to make it an attractive candidate for" active oxygen" in the enzymatic mechanism.[81,83]

Synthetic analogues for two of the other candidates for "active oxygen" have also been synthesized and their reactivities assessed. For example, Fe^{III} and Mn^{III}-porphyrin peroxo complexes analogous to **1a** in Reaction (5.65) have been synthesized. The x-ray crystal structure of the Mn complex shows that the peroxo ligand is bound to the metal in a triangular, side-on fashion (see 5.75). The Fe complex is believed to have a similar structure.[85,86]


$$(5.75)$$

Studies of this species indicate that **1a** in Reaction (5.65) would not have the requisite reactivity to be a candidate for "active oxygen" in the cytochrome P-450 mechanism, since it will not even oxidize triphenylphosphine, PPh_3, to triphenylphosphine oxide, $OPPh_3$, one of the more facile oxygenation reactions known.[87] Attempts to examine the protonated form, **1b** in Reaction (5.65), however, indicate that it is highly unstable, and its reactivity has not yet been thoroughly examined.[87] Fe^{IV}-oxo-porphyrin complexes analogous to **2** in Reaction (5.66) have also been prepared in solution and characterized by NMR.[60,61] Such complexes will react with PPh_3 to give $OPPh_3$, but are relatively unreactive with olefins and totally unreactive with saturated hydrocarbons. Thus **2** is also ruled out as a candidate for "active oxygen" in P-450 mechanisms.

These reactivity studies, and the observation of the peroxide shunt described above, indicate that $Fe^V(P^{2-})(O)^+$ or $Fe^{IV}(P^{\bullet-})(O)^+$ is the most likely candidate for "active oxygen." These two formulations are, of course, isoelectronic, and it is tempting to conclude that the latter is the more likely formulation of the enzymatic intermediate. However, it is important to remember that the model systems lack the axial cysteinylligand present in cytochrome P-450. The effect of the relatively easily oxidized sulfur ligand on the electron distribution within that intermediate is not known, since model systems for high-valent iron-oxo complexes containing axial thiolate ligands have not been synthesized.

The mechanism of reactions of the high-valent oxo complex **3** in Reaction (5.67) with a variety of substrates is an area of active interest.[81,88] Such studies are generally carried out by generation of the species *in situ* from the reaction of a ferric porphyrin with a single-oxygen-atom donor, such as a peracid or iodosylbenzene.[89] In hydroxylation reactions of aliphatic hydrocarbons, the initial step appears to be abstraction of a hydrogen atom from the substrate to form a substrate radical and an Fe^{IV} hydroxide complex held together in a cage created by the enzyme active site so that they cannot diffuse away from each other (Reaction 5.76). This step is then followed by recombination of the OH fragment with the substrate radical to make the hydroxylated product (Reaction 5.77). This mechanism is referred to as the "oxygen rebound mechanism."[83]
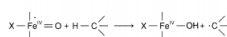

$$(5.76)$$
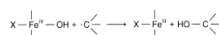
$$(5.77)$$

The radical character of the intermediates formed in this reaction is supported by the observation that such reactions carried out using synthetic porphyrins and single-oxygen-atom donors in the presence of $BrCCl_3$ give substantial amounts of alkyl bromides as products, a result that is consistent with radical intermediates and inconsistent with either carbanion or carbonium-ion intermediates.[83]

In the enzymatic reactions themselves, there is also strong evidence to support a stepwise mechanism involving free-radical intermediates. For example, cytochrome P-450$_{cam}$ gives hydroxylation of d-camphor only in the 5-*exo* position, but deuterium-labeling studies show that either the 5-*exo* or the 5-*endo* hydrogen is lost (Reaction 5.78).[88]
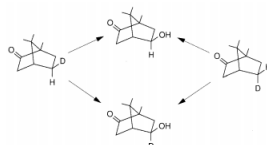
$$(5.78)$$

Such results are obviously inconsistent with a concerted mechanism in which the oxygen atom would be inserted into the 5-*exo* C—H bond in one step; so there would be no chance for the hydrogens in the two positions to exchange. (Remember that alcohol protons exchange rapidly with water and therefore are not expected to remain deuterated when the reaction is carried out in H_2O.)

The crystal structure of reduced cytochrome P-450$_{cam}$ with CO bound to the iron and the substrate camphor bound[90] adjacent to it has been examined and compared with the crystal structure of the oxidized enzyme with camphor bound. The former is expected to be similar in structure to the less-stable oxy complex. The comparison shows that the substrate camphor is closer to the iron center in the oxidized enzyme. It is therefore possible that a similar movement of the substrate occurs during the catalytic reaction after either a 5-*exo* or a 5-*endo* hydrogen is abstracted, and that the new position of the camphor molecule then restricts the hydroxylation step to the 5-*exo* position. It is interesting to note that the 5-*exo* position on the camphor that is hydroxylated is held in very close proximity to the Fe^{III} center, and therefore to the presumed location of the oxo ligand in the high-valent oxo intermediate in the structure of the ferric enzyme plus camphor derivative (Figure 5.12). Crystal structures of the ferric form of cytochrome P-450$_{cam}$ with norcamphor and adamantanone bound in place of camphor have also been determined.[90] These alternative substrates are smaller than camphor, and appear to fit more loosely than camphor. It is therefore reasonable to assume that they "rattle around" to a certain extent in the substrate binding site, which probably accounts for the less-specific pattern of hydroxylation observed for these alternative substrates.
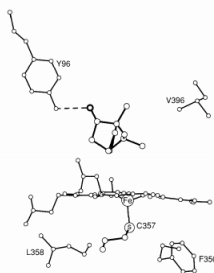
Figure 5.12 - Edge-on view of the P-450 active-site region with the substrate camphor molecule bound.[80] The substrate camphor is located in a hydrophobic pocket directly above the heme and is oriented by a hydrogen bond between the carbonyl oxygen of the camphor and Tyr-96. The position that is hydroxylated in the oxygenation reaction, i.e., the 5-exo position, is the closest point of approach of the substrate to the expected position of the oxygen atom bound to iron in the highvalent iron-oxo intermediate.

Mechanisms for olefin epoxidations catalyzed either by the enzyme or by model porphyrin complexes are not as well understood as those for hydroxylation of aliphatic hydrocarbons. Some of the possibilities that have been proposed[88,91] are represented schematically in Figure 5.13.
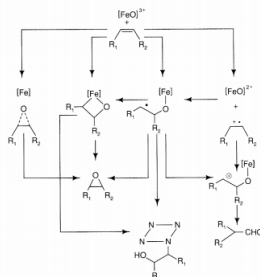
Figure 5.13 - Schematic representation of possible mechanistic pathways for olefin epoxidation by **3**. The mechanisms described are, from right to left, concerted addition of oxygen to the double bond, reaction via a metallocylic intermediate, reaction via a ring-opened radical intermediate, and reaction proceeding via an initial electron-transfer step.[91]

c. O—O Bond Cleavage

The evidence is persuasive that the "active oxygen" species that attacks substrate in cytochrome P-450 is a high-valent iron-oxo complex. However, the mechanism of formation of that species in the catalytic reaction with dioxygen is less well-understood. Heterolytic O—O bond cleavage of a ferric porphyrin hydroperoxide complex, **1b** (Reaction 5.67), is the logical and anticipated route, but it has not yet been unequivocally demonstrated in a model complex.[92,93] The catalase and peroxidase enzymes catalyze heterolytic O—O bond cleavage in reactions of hydrogen peroxide, but in them the active sites contain amino-acid side chains situated to facilitate the developing charge separation that occurs in heterolytic cleavage (see Section VI). The crystal structure of cytochrome P-450$_{cam}$ shows no such groups in the activesite cavity, nor does it give any clue to the source of a proton to protonate the peroxide ligand when it is produced.[80] Also, we have little experimental evidence concerning possible roles that the cysteinyl sulfur axial ligand might play in facilitating O—O bond cleavage. These issues remain areas of active interest for researchers interested in cytochrome P-450 mechanisms.

Other Metal-containing Monooxygenase Enzymes

As mentioned above, much less is known about the structural characteristics and mechanisms of the nonheme metal-containing monooxygenase enzymes. From the similarities of the overall stoichiometries of the reactions and the resemblance of some of the enzymes to dioxygen-binding proteins, it is likely that the initial steps are the same as those for cytochrome P-450, i.e., dioxygen binding followed by reduction to form metal-peroxide or hydroperoxide complexes. It is not obvious that the next step is the same, however (i.e., O—O bond cleavage to form a high-valent metal-oxo complex prior to attack on substrate). The problem is that such a mechanism would generate metal-oxo complexes that appear to contain metal ions in chemically unreasonable high-oxidation states, e.g., Fe^V, Cu^{III}, or Cu^{IV} (Reactions 5.79-5.81).

$$(Fe^{III} - OOH)^{2+} \rightarrow (Fe^V O)^{3+} + OH^- \tag{5.79}$$

$$(Cu^{II} - OOH)^+ \rightarrow (Cu^{IV} O)^{2+} + OH^- \tag{5.80}$$

$$(Cu^{II} - OO - Cu^{II})^{2+} \rightarrow 2(Cu^{III} O)^+ \tag{5.81}$$

An alternative mechanism is for the peroxide or hydroperoxide ligand to attack the substrate directly; i.e., O—O bond cleavage could be concerted with attack on substrate. Another possibility is that the oxygen atom is inserted in a metalligand bond prior to transfer to the substrate. Neither of these alternative mechanisms has been demonstrated experimentally. These various possibilities remain to be considered as more information about the monooxygenase enzymes becomes available.

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