2. Conversion to nitroso compounds: Under favorable conditions (protic media) the σ-adducts of some nucleophiles are converted into nitroso compounds. The latter are very active and undergo a variety of interesting transformations.[44]

3. Vicarious nucleophilic substitution: This occurs when nucleophiles, particularly carbanions, contain leaving groups X that can be eliminated as HX. This reaction is the main subject of this Account. Amination of some nitroarenes with hydroxyloamine and aminotriazole in basic medium also belongs to the category.[45]

4. Annulation: This process occurs when, in σ-adducts of carbanions containing leaving groups, the negative charge is less delocalized.[32,33]

5. ANRORC reaction: In many heterocycles containing leaving groups X, σ-adducts undergo ring opening, HX elimination, and ring closure. The final results are often identical with conventional nucleophilic replacement of X, but in fact H is replaced.[46]

6. There are many variants of "cine" and "tele" substitutions in which σ-adducts are further converted via elimination of leaving groups from different positions.[47]

Only when the structures of the arenes and the nucleophiles and also the conditions are such that none of these (or similar) reactions can proceed with a sufficient rate, then, due to reversibility of the σ^H-adducts formation, slower formation of the σ^X-adducts leads to the conventional nucleophilic replacement of halogen via the S_NAr mechanism. We can therefore claim that this well-known and important reaction is in fact a secondary process.

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Control of the Catalytic Activity of Prosthetic Heme by the Structure of Hemoproteins

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The three general biological functions of hemoproteins are the transport of electrons (e.g., cytochrome b_5), the transport of oxygen (e.g., hemoglobin), and the catalysis of redox reactions (e.g., cytochrome P450, horseradish peroxidase). Despite the differences in the chemistry they support, all of these proteins have iron protoporphyrin IX (heme) as their prosthetic group. Their different functions therefore stem from differences in the way the protein interacts with the heme and with potential substrates. The mechanisms by which the protein controls the intrinsic reactivity of the heme are of both theoretical and practical interest. The heme in the electron carrier hemoproteins is exceptional in that it has two strong axial ligands and generally does not bind molecular oxygen or peroxides. The heme in all the other hemoproteins has one accessible coordination site which allows it to bind and react with peroxides and other ligands (Figure 1). The reaction of ferric hemoproteins with peroxides results in cleavage of the oxygen–oxygen bond and oxidation of the iron to the ferryl [Fe^{IV}=O] state. If the peroxide bond is cleaved heterolytically, as it usually is, the one-electron change in iron oxidation state is paralleled by oxidation of the porphyrin to a radical cation (e.g., horseradish peroxidase) or a protein residue to a radical species (e.g., cytochrome *c* peroxidase).[1-3] Cytochrome P450 is unique among the hemoproteins in that it can react with peroxides but usually reduces molecular oxygen to generate its own peroxide equivalent. It is not known whether the Fe^{IV}=O species presumed to be the active oxidant of cytochrome P450 is matched by a porphyrin or protein radical. These mechanistic alternatives are illustrated schematically in Figure 1. Once activated, the oxygen of the ferryl complex ($[FeO]^{3+}$) can be transferred to a substrate (RH) or can simply abstract one or more electrons from the substrate (P$^\bullet$ stands for a porphyrin or protein radical):

$$PFe^{IV}{=}O + R^\bullet + H^+ \leftarrow P^\bullet Fe^{IV}{=}O + RH \rightarrow PFe^{III} + ROH$$

Electron abstraction is characteristic of the peroxidases and oxygen transfer of the cytochrome P450 monooxygenases, but it is not unusual for hemoproteins to function, at least in the test tube, in more than one of these catalytic modes.

The Active Site of Cytochrome $P450_{cam}$. Cytochrome $P450_{cam}$, in contrast to eukaryotic cytochrome

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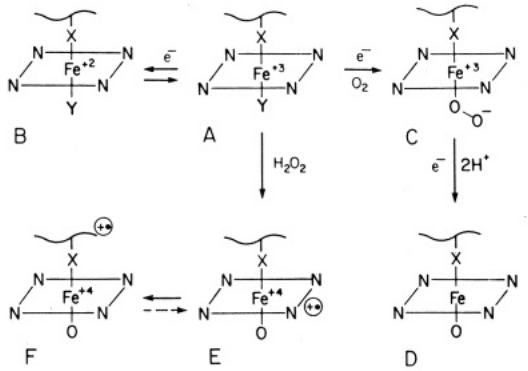

Figure 1. Interrelation of hemoprotein states: (A) resting ferric hemoprotein with ligands X and Y; (B) electron transport, X = histidine, Y not readily exchanged; (C) oxygen transport, X = histidine, Y exchanged for O_2 in Fe^{2+} state; (D) cytochrome P450, X = cysteine thiolate, Y readily exchanged; (E) peroxidase, X = histidine, Y readily exchanged. The radical may move from the porphyrin (as in E) to the protein (as in F). The distribution of electrons in D, which is at the same oxidation state as E and F, is not known.

P450 enzymes, is a soluble, crystallizable protein that is amenable to structural and mechanistic studies. $P450_{cam}$ catalyzes the 5-exo-hydroxylation of camphor in *Pseudomonas putida*, a strain of bacteria that degrades camphor to obtain carbon atoms and energy. Spectroscopic and physicochemical studies,[4,5] capped by the crystallographic work of Poulos,[6-8] have provided great insight into the structure of cytochrome $P450_{cam}$. Four aspects of this structure are particularly relevant to the present discussion of the relationship between active site architecture and catalytic outcome: (a) the identification of a cysteine thiolate as the fifth heme ligand (four ligands are provided by the porphyrin),[4-8] (b) the demonstration that the region of the active site in which the substrate and molecular oxygen are bound is composed entirely of lipophilic residues,[6-8] (c) the demonstration that the heme is held by the pincer action of two peptides, one of which physically covers pyrrole ring B (Figure 2),[6] and (d) the finding that the heme is buried deep inside the protein and that access to the active site requires transient thermal or allosteric broadening of a protein channel.[6-8]

The Active Sites of Membrane-Bound P450 Enzymes. Eukaryotic cytochrome P450 enzymes are invariably attached to cell membranes. Some P450 enzymes are essential for the biosynthesis and catabolism of endogenous sterols and lipids, whereas others are primarily involved in the oxidation of lipophilic xenobiotics to polar, excretable metabolites. The very properties that make cytochrome $P450_{cam}$ so accessible to structural studies differentiate it from the membrane-bound eukaryotic enzymes. It is consequently important to determine the extent to which conclusions based on $P450_{cam}$ hold for the membrane-bound enzymes. The spectroscopic properties[4,5] and the conservation of a specific cysteine peptide in all the P450 enzymes sequenced to date[9] confirm that the fifth heme ligand in the membrane-bound enzymes is also a thiolate anion. Much less is known about the residues in the substrate- and oxygen-binding regions of the active site beyond the fact that nonpolar residues are favored by a clear relationship between substrate lipophilicity and substrate affinity.[10-12] A strong indication that the active sites of the membrane-bound enzymes resemble that of cytochrome $P450_{cam}$ is provided, however, by evidence that the heme is also held by the pressure of a peptide on pyrrole ring B.[13,14] This conclusion derives from the regiochemistry and stereochemistry of the prosthetic heme alkylation that accompanies the oxidation of terminally unsaturated hydrocarbons by cytochrome $P450_b$. Cytochrome $P450_b$ is one of the rat liver enzymes that is primarily involved in the metabolism of foreign compounds. $P450_b$ generally oxidizes terminal olefins and acetylenes to respectively epoxides and ketenes. Periodically, however, transfer of the catalytically activated oxygen to the internal carbon of

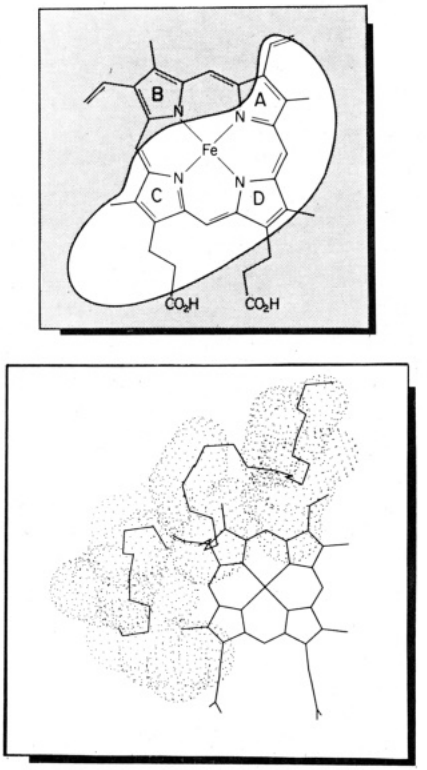

Figure 2. Comparison of the active site of $P450_b$ (top) predicted by heme alkylation experiments with that of $P450_{cam}$ (bottom) taken from the crystallographic coordinates of Poulos et al. (ref 6; personal communication). The dots in the $P450_{cam}$ representation correspond to the volume defined by the van der Waals radii of the protein residues in the immediate vicinity of the prosthetic heme group (residues 243–254).

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the π-bond results in simultaneous addition of one of the heme nitrogens to the terminal carbon.[13,15,16] The critical finding, in terms of the structure of the active site, is that linear olefins alkylate pyrrole ring D whereas linear acetylenes alkylate pyrrole ring A (see Figure 2).[13] The internal carbon of the π-bond must be located close to the ferryl oxygen in these reactions because the ferryl oxygen is transferred to it. Reaction of the terminal carbon of the linear (sp-hybridized) acetylenes with the nitrogen of pyrrole ring A therefore places the hydrocarbon chain in the region above pyrrole ring C. If the hydrocarbon chain and the internal carbon of a linear olefin are similarly positioned, the terminal carbon of the olefin will point toward the nitrogen of pyrrole ring B or D due to the sp^2-hybridization state of the internal carbon. However, since only ring D is alkylated, pyrrole ring B must not be accessible and therefore must be protected by the protein. Independent support for the resulting active site model (Figure 2) is provided by the absolute stereochemistry of the heme adduct.[14] Both enantiomers of octene oxide are produced from octene, so that the oxygen can be delivered to both faces of the olefin, but heme alkylation only occurs when the oxygen is delivered to the *re* face. This is precisely the absolute stereochemistry predicted by the active site model.

The Active Site of Cytochrome *c* Peroxidase. The only peroxidase for which a crystal structure is available is yeast cytochrome *c* peroxidase.[17-21] This enzyme differs from horseradish peroxidase and other peroxidases in that it catalyzes the one-electron oxidation of a protein (cytochrome *c*) rather than of a small molecule. The active site structures of both peroxidases nevertheless appear to be related (ref 22; next section). A comparison of the crystal structures of cytochrome *c* peroxidase and cytochrome $P450_{cam}$ shows that their active sites differ markedly. First, the fifth heme ligand in cytochrome *c* peroxidase is an imidazole rather than a thiolate,[17,23] although the imidazole is hydrogen bonded to a vicinal aspartate and thus has some anionic character.[21] Second, the heme is sandwiched between two protein helices but, in contrast to $P450_{cam}$, is completely covered by them except for the outer perimeter of pyrrole rings A and D. Third, the oxygen activation site of cytochrome *c* peroxidase has several polar residues, salient among which are an imidazole, an arginine,[18] and several water molecules.[21] It has been proposed that the imidazole facilitates peroxide heterolysis by transferring the proton from the oxygen that binds to the iron to the oxygen that is eliminated. Polar interactions of the departing oxygen with the arginine are also thought to facilitate the peroxide cleavage.[19,24]

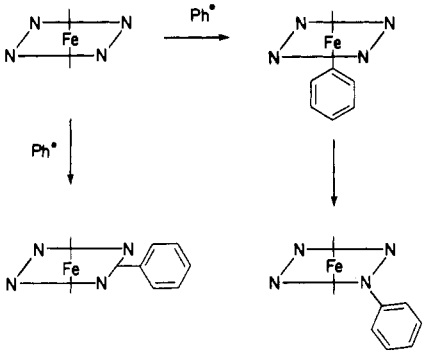

Figure 3. Schematic representation of the addition of the catalytically generated phenyl radical to the prosthetic heme group to give the Fe–phenyl and N–phenyl adducts obtained with myoglobin and cytochrome P450 or the *meso*-phenyl adducts obtained with horseradish peroxidase.

The activation of oxygen by cytochrome *c* peroxidase thus occurs, in contrast to that by cytochrome $P450_{cam}$,[6-8] in a highly polar environment. Finally, the heme in cytochrome *c* peroxidase is located within the protein but, unlike $P450_{cam}$, is reached via a well-defined channel that terminates close to the exposed edge of pyrrole ring D.[21]

The Active Site of Horseradish Peroxidase. The primary sequence of horseradish peroxidase is known,[25] but efforts to obtain crystals suitable for X-ray analysis have not been successful.[26] Protein modification experiments, however, have identified a histidine-containing peptide that is thought to be the heme coordination site[27,28] and an aspartate that is possibly located in the active site.[29] Alignment of the protein sequences of horseradish peroxidase and cytochrome *c* peroxidase led Welinder[22] to propose that both structures are made up of two similarly assembled domains. NMR studies suggest that the imidazole ligand is hydrogen bonded when the heme is pentacoordinated, as in cytochrome *c* peroxidase, but loses the proton and acquires a negative charge when the heme is hexacoordinated.[30] Substantial information on the structure of the active site of horseradish peroxidase is provided by recent heme alkylation experiments using aryl- and alkylhydrazines. The reaction of phenylhydrazine with myoglobin,[31-33] hemoglobin,[33-35] catalase,[36] cytochrome P450,[37] and metalloporphyrins[35] results in alkylation of the iron or nitrogen atoms of the heme. In contrast, the reaction of phenylhydrazine with horseradish peroxidase results partially in attachment of the phenyl

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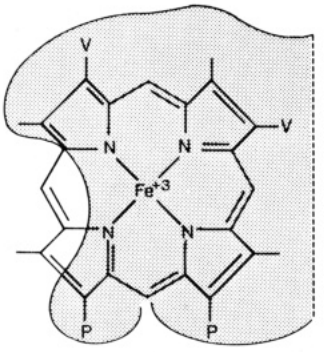

Figure 4. Model of the active site of horseradish peroxidase predicted by heme alkylation experiments.

group to the δ meso carbon (Figure 3)[38] and partially in conversion of the heme to the 8-hydroxylmethyl derivative. The same meso carbon is alkylated by the alkylhydrazines, but the hydroxymethyl derivative is not simultaneously formed.[39] Two structural conclusions can be drawn from these results. First, the iron and pyrrole nitrogens of the heme are not accessible to the radical released from the hydrazines because their reactions with other hemoproteins indicate that nitrogen-alkylated products are favored if these sites are exposed. Second, only that sector of the heme perimeter defined by the edge of pyrrole ring D and the δ meso carbon is physically accessible to substrates. Specific involvement of only one of four meso carbons and one of four methyl groups in the reaction is otherwise inexplicable. The resulting active site model (Figure 4) bears a striking resemblance to the active site of cytochrome *c* peroxidase in that very nearly the same region of the heme is exposed in both enzymes. Further support for the proposed active site structure is provided by NMR nuclear Overhauser experiments, which suggest that substrates bind to the enzyme in the vicinity of the 8-methyl group.[40] The model is also consistent with the conclusion, based on NMR studies, that the heme is buried in the protein.[41] Finally, it readily rationalizes the earlier finding that the reaction of horseradish peroxidase with cyclopropanone hydrate results exclusively in alkylation of the δ meso carbon.[42]

The active site model for horseradish peroxidase predicts that the ferryl oxygen should not be transferred to substrates by horseradish peroxidase. A few examples exist of the incorporation of oxygen into substrates by peroxidative turnover of the enzyme, but in most cases the origin of the oxygen is unknown. The oxidation of 2,4,6-trimethylphenol to 4-(hydroxymethyl)-2,6-dimethylphenol by horseradish peroxidase[43] is an exception in that the hydroxyl has been shown to derive from water and molecular oxygen.[44] The hydroxyl is thus introduced by addition of water to the quinonemethide or oxygen to the carbon radical rather than by ferryl oxygen transfer. The hydroxylation of

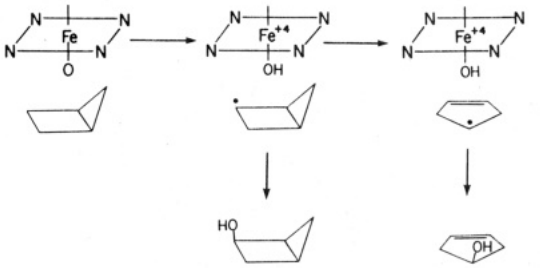

Figure 5. Competition of ring opening with radical recombination in the cytochrome P450 catalyzed hydroxylation of bicyclo-[2.1.0]pentane.

N-methylcarbazole by horseradish peroxidase has also been shown to result in incorporation of oxygen from molecular oxygen and the medium.[45] Incorporation of oxygen from H_2O_2 into the product has only been reported for the sulfoxidation of thioanisoles.[46] The significance of this observation for the normal function of the enzyme, however, is obscured by the fact that it is based on prolonged (4–12-h) incubations of approximately equimolar mixtures of horseradish peroxidase, H_2O_2, and the thioanisoles. These prolonged incubation conditions may allow abnormal reactions to surface. The reactions of horseradish peroxidase are otherwise consistent with physical separation of the ferryl oxygen from the substrate-derived radical.

P450 and Free-Radical Intermediates. The cumulative evidence strongly supports the conclusion that cytochrome P450 catalyzed hydrocarbon and nitrogen oxidations involve sequential one-electron steps rather than concerted insertion of the oxygen into the substrate.[47] The strongest evidence for stepwise oxidation of saturated hydrocarbons is provided by the loss of stereochemistry associated with the hydroxylation of norbornane,[48] camphor,[49] and ethylbenzene[50] and by the regiochemical scrambling that occurs during the allylic hydroxylation of cyclohexene and other olefins.[51] The hydroxylation of these substrates passes through an intermediate that can isomerize before the carbon–oxygen bond is formed. The general validity of a nonconcerted mechanism, however, and the radical (vs. ionic) nature of the intermediates have been obscured by the absence of rearrangements during the hydroxylation of substrates containing the cyclopropylmethyl group.[52–55] This ambiguity reflects the belief that the rate of ring opening of the cyclopropylmethyl radical (approximately 1×10^8 s^{-1})[56] should compete with re-

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combination of the diradical intermediate in the hydroxylation reaction. Recent work shows, however, that the rearrangement is observed if a substrate is used that rearranges considerably faster ($k > 10^9$ s^{-1}) than the cyclopropylmethyl radical itself.[55] Bicyclo[2.1.0]pentane is thus oxidized by cytochrome P450 to a 7:1 mixture of *endo*-bicyclo[2.1.0]pentan-2-ol *and* 3-cyclopentenol (Figure 5). It appears, therefore, that the radical recombination step is so rapid that only substrates with rearrangement rates in the 10^9-s^{-1} rather than 10^8-s^{-1} range compete with it. The enzymatic hydroxylation consequently masquerades as a concerted process because the carbon radical intermediate is trapped by the iron-coordinated hydroxyl "radical" before it can rearrange or break out of the solvent cage.

The oxidation of nitrogen compounds is thought to occur by peroxidase-like removal of one electron from the nitrogen atom or, more precisely, from the highest occupied (usually nitrogen-containing) molecular orbital.[47] Loss of a proton from the carbon adjacent to the nitrogen of the radical cation thus obtained yields a delocalized carbon radical that is trapped by the iron-coordinated hydroxyl "radical". The relatively high pK_a value[57] for a proton adjacent to a nitrogen radical cation[47] suggests, in fact, that the ferryl oxygen first accepts the electron and then, acting as a base, pulls off the required proton. The complexity of nitrogen oxidation reactions, which increases the chances of a diversion of the catalytic process, has made it possible to detect radical products in the oxidation of substrates in which the proton and nitrogen are separated by conjugated double bonds. Specifically, the cytochrome P450 catalyzed oxidation of 3,5-bis(carbethoxy)-2,6-dimethyl-4-alkyl-1,4-dihydropyridines results in extrusion of the 4-alkyl substituent as a spin-trappable (EPR-detectable) radical.[58]

The oxidation of quadricyclane by cytochrome P450 provides further evidence that cytochrome P450 catalyzes electron-transfer reactions comparable to those of the peroxidases. Quadricyclane, which has a very low oxidation potential (approximately 0.9 V vs. SCE), is oxidized exclusively by cytochrome P450 to *endo*-bicyclo[3.1.0]hexane-2-carboxaldehyde (Figure 6).[59] The rearrangement required for the formation of this product is readily explained by electron transfer to the ferryl complex followed by combination of the ferryl oxygen with the carbon radical. This strengthens the argument that cytochrome P450 is a monooxygenase rather than a peroxidase largely because it traps the radical intermediate generated in the first catalytic step before it diffuses out of the active site. Conversely, classical peroxidases do not function as monooxygenases because they fail to similarly capture the radical intermediate.

Horseradish Peroxidase and Free-Radical Intermediates. Horseradish peroxidase, except in very special circumstances,[60] catalytically removes one electron from its substrates. The formation of detectable free radicals and radical coupling products is therefore the rule rather than, as in the case of P450, the exception. This reactivity difference is illustrated by the oxidation of phenols. Phenols are oxidized by cytochrome P450 to catechols and hydroquinones[61,62] but by horseradish peroxidase to phenoxy radicals and radical condensation products (e.g., ref 43). The difference in the reactivities of the two enzymes is readily explained by the differences in their active site structures. The abstraction of a hydrogen or an electron by cytochrome P450 produces a radical pair that collapses so rapidly as to place its very existence in doubt. In contrast, the active site of horseradish peroxidase *is so constructed that the substrate radical is barred from contact with the ferryl oxygen by the protein structure.* Electrons are thus delivered to the heme edge in the vicinity of pyrrole ring D (Figures 4 and 7). The first electron so delivered neutralizes the porphyrin radical cation of compound I. The second electron, which is relayed to the iron by the π-system of the neutral porphyrin, reduces the hypervalent iron to the ferric state. This catalytic stratagem precludes interception of the substrate radical by the ferryl oxygen and thus virtually ensures its escape from the active site.

The Active Sites of Hemoglobin and Myoglobin. Hemoglobin and myoglobin, which normally transport oxygen, also catalyze peroxidative[63,64] and monooxygenative reactions[65] when provided with either H_2O_2 or oxygen plus reducing equivalents. The reaction of these hemoproteins with H_2O_2 produces the usual ferryl species and a protein radical (Figure 1).[2,3] The fifth ligand in these proteins is an imidazole that is not strongly hydrogen bonded and is therefore a poorer electron donor than the imidazole ligand of the peroxidases. The oxygen-binding site contains the imidazole but not the arginine employed by the peroxidases to cleave the peroxide bond.[66] Dioxygen bond cleavage is consequently less facile and probably occurs concurrently by homolytic and heterolytic mechanisms, both of which ultimately yield Fe^{IV}=O and a protein radical. The protein radical is probably located on a tyrosine residue.[2,3] Relatively large substrates reach the iron coordination site despite the fact that a well-defined entry channel is not visible in the crystal structures. This is clearly demonstrated by the reaction of phenylhydrazine with myoglobin, which produces the phenyl radical and an adduct in which the phenyl group is σ-bonded to the heme iron (Figure 3).[31,32,34,35] The route taken by phenylhydrazine into the heme site is discernible in the crystal structure of the resulting heme adduct because the entry channel fails to reclose completely behind the phenyl group.[31] In addition to the oxidation of substrates in the active site, it is possible that electron transfer to the heme edge also occurs because the periphery of pyrrole rings C and D is exposed to the medium.[66] In addition, a third oxidative

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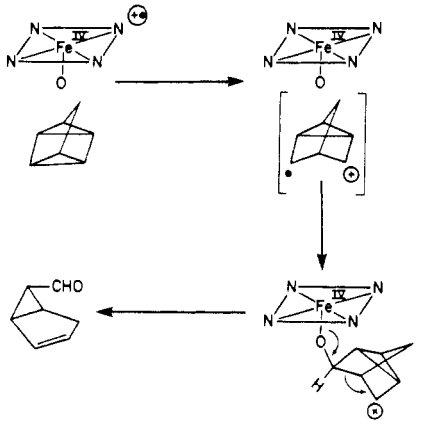

Figure 6. Radical cation pathway proposed for the oxidation of quadricyclane by cytochrome P450.

Figure 7. Schematic representation of the edge–electron transfer mechanism postulated for the oxidation of phenols and other substrates by horseradish peroxidase.

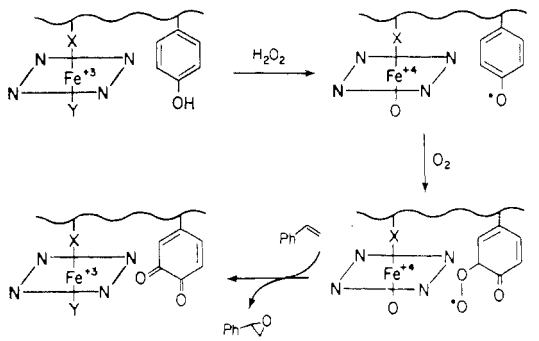

Figure 8. Schematic representation of the oxidation of myoglobin or hemoglobin by H_2O_2 to a protein (probably tyrosine-centered) radical that binds oxygen and cooxidizes styrene.

mechanism was recently unearthed by studies of the epoxidation of styrene by hemoglobin and myoglobin (Figure 8).[67] The relevant findings are that up to 70% of the oxygen incorporated into the styrene oxide derives from O_2 rather than H_2O_2 and that the epoxidation is accompanied by a substantial loss of the olefin stereochemistry. Neither of these findings is compatible with a ferryl oxygen transfer reaction. The results are readily explained, however, by addition of molecular oxygen to the protein radical to give a peroxy radical that epoxidizes the olefin. It is likely that this cooxidation mechanism cooexists with conventional ferryl oxygen transfer because a significant fraction of the epoxide oxygen still derives from the peroxide, and the stereochemistry of the olefin is only partially scrambled.[67] Mechanistic heterogeneity is not unexpected because hemoglobin and myoglobin have presumably been optimized by evolution to transport oxygen rather than to catalyze redox reactions. This mechanistic polymorphism is probably also responsible for the fact that the reaction of phenylhydrazine with hemoglobin yields both the *N*-phenyl adducts characteristic of cytochrome P450[33,34] and a *meso*-phenyl biliverdin. The latter is presumably obtained by further oxidation of a peroxidase-like *meso*-phenyl adduct (Figure 3).[33]

Summary. Hemoprotein catalysis occurs in two distinct phases: (a) cleavage of the dioxygen bond to give the catalytic ferryl species and (b) oxidation of the substrate by electron abstraction or oxygen transfer. The first of these two steps is assisted, in the classical peroxidases, by hydrogen bonding and polar interactions between the protein and the departing oxygen atom as well as by electron donation by the fifth ligand. The fifth ligand effect is dominant in the case of cytochrome P450 enzymes, which have an electron-rich thiolate ligand but apparently little in the way of hydrogen bonding or ionic groups to facilitate dioxygen bond cleavage. The absence of polar active site residues is consistent with the preferential turnover of lipophilic substrates by cytochrome P450 because the substrates must be bound close to the ferryl oxygen. It is interesting, in this context, that the other hemoproteins that apparently have a thiolate fifth ligand (prostacyclin synthase,[68] thromboxane synthase,[69,70] and chloroperoxidase)[71,72] also transfer oxygen to lipophilic substrates. Chloroperoxidase, like cytochrome P450, epoxidizes styrene[73] with exclusive incorporation of oxygen from the peroxide and with complete retention of the olefin stereochemistry.[44] Transfer of electrons to the heme edge of horseradish peroxidase is consistent, on the other hand, with exclusion of substrates from the active site by the polar machinery that facilitates dioxygen bond cleavage and stabilizes the ferryl oxygen. This stabilization is responsible for the remarkable stability of the ferryl species of horseradish peroxidase. In contrast, the oxidative fragility of chloroperoxidase and cytochrome P450, both of which are readily destroyed by catalytic turnover in the absence of substrates, argues for the presence of an unstabilized ferryl species. In the absence of ferryl stabilization or of protection by readily oxidized substrates, the protein and the heme are themselves oxidized. This is readily seen in the reaction of hemoglobin and myoglobin with H_2O_2, which results in heme loss and oxidation of the protein.[2,3] The resulting protein radical(s) react with oxygen, mediate cooxidation reactions, and initiate protein cross-linking reactions. The active sites of hemoproteins thus reflect a highly functional architecture in which protein structure controls the intrinsic reactivity of the common prosthetic heme group. As described here, the topology and function of this architecture can be profitably explored by prosthetic heme alkylation reactions that exploit the catalytic actions of the hemoproteins themselves.

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