soon be possible to perform kinetic measurements of MCD with a subnanosecond time resolution.[43] MCD could then be used to follow dynamic changes in porphyrin electronic structure induced by fast perturbations, such as a laser photolysis pulse. Examples of such applications might include the photolysis of axial ligands from metalloporphyrins[44] or from hemeproteins,[45] or the photochemical reduction of cytochrome proteins.[46] Recent advances in superconducting materials may lead to magnets for future MCD studies which could offer higher field strengths and lower operating costs than present superconducting magnets.[47]

A systematic understanding of porphyrin MCD spectroscopy in terms of porphyrin electronic structure and its variation with structural perturbations is emerging from studies of porphyrins at equilibrium. This understanding, while interesting in itself, will also be important to the application of MCD as a kinetic–spectroscopic probe of the role played by porphyrin structure in the chemical reactions of life.[48]

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Mechanism of Oxygen Activation by Pteridine-Dependent Monooxygenases

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The mammalian aromatic amino acid hydroxylases (phenylalanine, tyrosine, and tryptophan hydroxylase; PAH, TH, and TPH, respectively) are a unique class of monooxygenases in their use of tetrahydropterins (Figure 1) as obligatory cofactors. These enzymes play important roles in mammalian metabolism: PAH initiates the detoxification of high levels of phenylalanine[1,2] while TH and TPH catalyze the committed steps in the biosynthesis of the neurotransmitters dihydroxyphenylalanine[3,4] and serotonin,[5,6] respectively; hence the latter are targets for therapeutic intervention. In addition, two other types of tetrahydropterin-dependent monooxygenases are known, a mammalian glyceryl ether cleavage enzyme[7,8] and a group of bacterial PAHs.[9,10] While the enzymes operate on different substrates, a commonality of substrate structure is apparent, and recent data have provided important sequence[11,12] and mechanistic[10,13] links between the different mammalian and bacterial hydroxylases. These enzymes are almost certainly descended from the same protein family,[14,15] with evolutionary divergences likely

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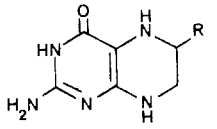
Table I
Hydroxylations of Unactivated Aromatic Rings by Chemical Oxidizing Systems

chemical oxidizing system	solvent	reaction performed
A.1. Fe^{2+}, H_2O_2, EDTA	kPhos, pH 7.2	benzene → phenol
A.2. Fe^{2+}, O_2, ascorbate, EDTA	kPhos, pH 7.2	benzene → phenol
A.3. Fe^{3+}, O_2, catechol	kPhos, pH 7.2	benzene → phenol
A.4. Cu^{+}, O_2	acetone	naphthalene → 1-naphthol
B.1. Fe^{2+}, H_2O_2	MeCN	chlorobenzene → chlorophenol
B.2. $(C_6H_5)_2C{=}N_2 + O_2$	MeCN	*N*-acetyl-L-phenylalanine ethyl ester → *N*-acetyl-L-tyrosine ethyl ester
B.3. $F_3CCOOOH$	$CHCl_3$	chlorobenzene → chlorophenol



BPH_4, R = $(CHOH)_2CH_3$

6-MPH_4, R = CH_3

Figure 1. Tetrahydropterin structures.

occurring in response to more stringent catalytic requirements and changes in their mechanisms of regulation. As such, these enzymes provide fascinating and significant systems to study for chemists, biochemists, molecular biologists, and evolutionary biologists alike.

While the metabolic conversion of phenylalanine to tyrosine was demonstrated as early as 1913,[16] the seminal work on the purification and characterization of the phenylalanine hydroxylation system was performed by Kaufman in the late 1950s (reviewed in ref 17). Kaufman demonstrated the following stoichiometry for PAH catalysis:

$$\text{phenylalanine} + O_2 + BPH_4 \rightarrow \text{tyrosine} + BPH_2 + H_2O \quad (1)$$

in which the four-electron reduction of oxygen thermodynamically drives the hydroxylation of phenylalanine (a formal two-electron oxidation) in tandem with the two-electron oxidation of tetrahydrobiopterin (BPH_4) to its quinonoid dihydropterin (BPH_2). The mixed-function oxygenase nature of the enzyme was demonstrated by $^{18}O_2$ incorporation into [^{18}O]tyrosine and $H_2{}^{18}O$.[18] The physiological cofactor for PAH is BPH_4[19] although synthetic tetrahydropterins can be substituted and in practice are typically used for assays due to their greater ease of handling versus BPH_4 (e.g., 6-methyltetrahydropterin (6-MPH_4), Figure 1). Groundwork provided by Kaufman's PAH characterization aided in the discovery and isolation of TH[20,21] and TPH[22] in the mid-1960s. However, due to the relatively large quantity of PAH available from rat liver as well as its soluble nature and ease of purification by substrate-induced hydrophobic binding,[23] much more work has been done on this enzyme than the other hydroxylases. Thus, PAH serves as the prototype aromatic amino acid hydroxylase, a fact reflected in the discussion below.

The purpose of this Account is to focus on the chemistry by which PAH (and, by extension, the other aromatic amino acid hydroxylases) generates an intermediate from O_2 capable of performing the energetically difficult task of hydroxylating an unactivated aromatic ring. We find this process especially interesting for at least two reasons. First, it will be seen that the tetrahydropterin cofactor directly participates in the process of O_2 activation: it does not simply serve as a source of reducing equivalents. Second, all of the mammalian hydroxylases contain one nonheme iron per subunit[13,24,25] while at least one example of bacterial PAH (from *Chromobacterium violaceum*) contains copper.[10] Since each enzyme requires the presence of its metal center for activity, this strongly suggests a role for the metal in oxygen activation. Thus, our goal is to summarize experimental results germane to the question of oxygen activation by PAH and correlate these results into a rational mechanistic and structural proposal ultimately to serve as a framework for discussion and further experimental design.

Chemical Models for PAH

Mechanistic elucidation of complex enzymatic reactions is often facilitated by an understanding of chemical systems that perform the same reaction. There exist a plethora of chemical systems that utilize Fe^{2+} (or Cu^{+}), a reducing cofactor, and O_2 (or reduced species) to oxygenate unactivated aromatic rings. Table I selectively summarizes the types of systems that potentially could serve as models for oxygen activation by PAH. The table is arbitrarily divided into two groups: (A) systems that oxygenate via free radical mechanisms and (B) systems that oxygenate via electrophilic oxygen transfers. Reactions A.1, A.2, and A.3 (the "Fenton",[26] "Udenfriend",[27] and "Hamilton"[28] systems, respectively) as well as reaction A.4[29] result in the generation of either hydroxyl radicals (•OH) or other poorly characterized radical oxidizing species. These species indiscriminately add to the aromatic substrate, resulting in formation of multiple products. Reaction B.1 is a modified Fenton system under conditions in which the redox potential

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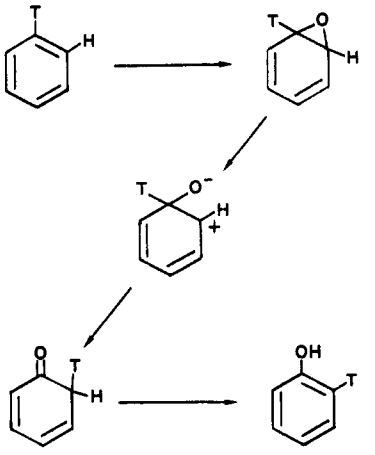

Figure 2. The NIH shift.

of the ferrous iron is increased enough to enable formation of a ferryl $(Fe{=}O)^{2+}$ species, the putative electrophilic oxygen source.[30] Reaction B.2 chemistry results in the generation of a carbonyl oxide, also an electrophilic oxygen donor.[31] Finally, peracids (reaction B.3) are capable of donating oxygen in either an electrophilic or nucleophilic manner.[32] Table I provides only an overview of the types of systems that potentially could be applied to PAH chemistry of catalysis, as all reactions have been performed with a variety of substrates, reducing cofactors, and/or solvents.

Mechanism with Respect to Substrate

As introduced above, one can conceptually distinguish two general modes of oxygen addition to the aromatic ring of phenylalanine, either through delivery of electrophilic (atomic) oxygen or via a radical pathway. Put another way, this issue revolves around whether oxidation is occurring in one two-electron or two one-electron steps. Current evidence implicates a concerted transfer of electrophilic oxygen. The key result was provided by an initially surprising observation made at the NIH in the mid-1960s.[33,34] When *p*-tritiophenylalanine was hydroxylated by either bacterial or liver PAH,[33] 90–95% of the product was *m*-tritiotyrosine; i.e., the tritium had migrated instead of being lost to solution. This migration, now known as the "NIH shift", can most easily be explained by the intermediacy of an arene oxide (Figure 2). Tritium is retained after arene oxide opening followed by hydride migration and rearomatization with preferred loss of H^+ (vs T^+). In contrast, hydroxylation of aromatic rings by hydroxyl radicals (a two one-electron process) results in complete loss of tritium from the product.[35] The higher than predicted degree of tritium retention during PAH catalysis implies that either a kinetic isotope effect or a stereospecific base selection for loss of H^+ (vs T^+) is occurring in the rearomatization step. However, when 3,5-ditritiophenylalanine was used as a PAH substrate, no loss of tritium was observed in the product.[36] This important result demonstrates that there is no stereoselectivity in proton abstraction with PAH such as might be the case if an enzyme base was involved. In addition, NIH shifts of F^-, Cl^-, and Br^- have also been demonstrated with PAH hydroxylating the appropriate para-substituted phenylalanines.[34,37] For example the major (>90%) product of *p*-chlorophenylalanine hydroxylation by PAH is *m*-chlorotyrosine.[37]

Table II
Magnitudes of NIH Shifts for Chemical and Enzymic Aromatic Hydroxylating Systems

system	substrate	solvent	% NIH shift[a]
Fe^{2+}, H_2O_2	[4-^{2}H]chlorobenzene	MeCN	32
$(C_6H_5)_2C{=}N_2$	1-chloronaphthalene	MeCN	≈50
F_3COOOH	[4-^{2}H]chlorobenzene	$CHCl_3$	75
PAH	[4-^{3}H]Phe	kPhos, pH 7.2, 25 °C	>90
TH	[4-^{3}H]Phe	kPhos, pH 7.2, 25 °C	84[b]
TPH	[5-^{3}H]tryptophan	kPhos, pH 7.2, 25 °C	85

[a] Percent retention of tritium resulting from its migration to the adjacent carbon during the hydroxylation event (cf. Figure 2). [b] Implied for conversion of phenylalanine to tyrosine. Product of reactions is 3,4-dihydroxyphenylalanine, formed with an overall 42% tritium retention.

Although the intermediacy of an arene oxide in PAH catalysis was questioned,[38] we have now provided the first direct evidence that epoxidations by PAH are chemically feasible.[39] 2,5-Dihydrophenylalanine, prepared by Birch reduction of phenylalanine, is a very good substrate for PAH in that its K_M and V_{max} are comparable to those of the normal substrate. The major product of dihydrophenylalanine oxidation by PAH is the 4,5-oxide of dihydrophenylalanine, with other products arising from solvolysis of this intermediate. PAH must be activated allosterically both by substrate binding[23] and by reduction of its Fe^{3+} center to Fe^{2+} [40,41] for hydroxylation of 2,5-dihydrophenylalanine to occur, in parallel to the requirements for conversion of phenylalanine to tyrosine.

The magnitude of the NIH shift observed with PAH hydroxylation also deserves comment. Table II compares the extent of NIH shift observed with PAH and a number of chemical electrophilic oxygen hydroxylating systems. It is noted that the extent of NIH shift observed is dependent on the types of hydroxylating systems utilized, however, all are purported to proceed through the intermediacy of the epoxide-opened species in Figure 2.[30–34] This implies that the extent of NIH shift depends on the stability of the cation in the solvent and the availability of scavengers. The active site of PAH may provide a stabilizing environment for the cation as well as shielding it from general solvent scavenging. This obviously provides an advantage over solution chemistry as ultimately the only species formed from phenylalanine will be tyrosine, in contrast to the model systems that typically exhibit formation of multiple products.[26–32]

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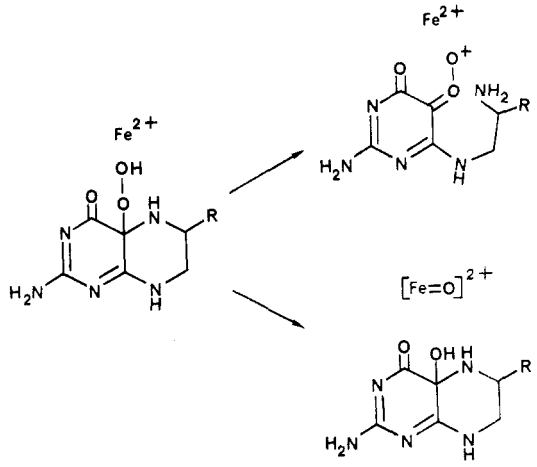

Figure 3. Possible structures for the PAH hydroxylating species.

Mechanism with Respect to Cofactor and Metal Center

The discovery and characterization of the NIH shift was especially significant in that an enzyme-associated reaction served as a mechanistic guide toward the further understanding of solution chemistry. The range of chemical species capable of performing the hydroxylation of an unactivated aromatic ring (see above) provides a foundation for proposing potential intermediates in oxygen activation by PAH. However, to serve as a potential PAH model, the chemical systems must also perform the NIH shift concomitant with substrate hydroxylation. Three activated oxygen species fulfill these requirements (Table II): peracids,[32] carbonyl oxides,[31] and certain metal–oxygen complexes in nonaqueous solution.[30] One can therefore propose, with foresight provided by experiments to be discussed below, three equivalent structures for the hydroxylating intermediate of PAH utilizing the chemical components required for enzyme turnover (Figure 3): a 4a-hydroperoxytetrahydropterin (4a-OOH adduct), a carbonyl oxide, and an iron–oxygen species (structure unspecified). The carbonyl oxide and various iron–oxygen species are potentially derivable from the 4a-OOH adduct (Figure 3), strongly suggesting the latter's intermediacy in PAH turnover. In addition, the chemical feasibility of the 4a-OOH adduct has strong precedents from flavin chemistry and biochemistry. The 4a-hydroperoxyflavins blocked at N-5 have been synthesized,[42] and the nonblocked species are strongly implicated through spectroscopic and other techniques as intermediates in turnover of certain bacterial hydroxy aromatic acid hydroxylases[43] and firefly luciferases.[44] The 4a position is the preferred site of electrophilic addition in tetrahydropterins as shown by a variety of chemical studies.[45–47]

Strong evidence exists implicating the intermediacy of a 4a-oxygen cofactor adduct in PAH turnover. Our

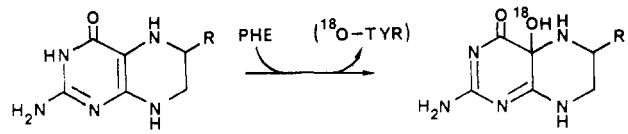

Figure 4. Generation of a 4a-OH adduct during PAH turnover.

laboratory demonstrated that, during PAH turnover, an intermediate is generated concurrent with substrate hydroxylation that decays to quinonoid dihydropterin and can be monitored by UV spectroscopy. Utilizing the synthetic cofactor 6-MPH_4 (Figure 1) 90% selectively enriched with ^{13}C in the C-4a position,[48] NMR evidence was acquired demonstrating that the intermediate was the 4a-carbinolamine (4a-OH) of 6-MPH_4 (Figure 4),[47] thereby confirming a hypothesis of Kaufman, who first noted the UV spectrum of the intermediate.[49] A key piece of evidence was the observation of a ^{13}C NMR signal for the carbinolamine carbon at 94.4 ppm, which decayed in a first-order fashion at the rate appropriate for the carbinolamine dehydration under the experimental conditions. In addition, we showed that 4a-OH-6-MPH_4 must be derived from an intermediate in PAH oxygen activation by demonstrating an ^{18}O shift in the ^{13}C NMR signal of 4a-OH-[^{13}C-4a]-6-MPH_4 when generated during PAH turnover in an atmosphere 50% enriched in $^{18}O_2$.[50] The observed ^{18}O-induced splitting (0.027 ppm) is again diagnostic of a carbinolamine carbon. In addition the ratio of the ^{18}O to ^{16}O signal intensities (51:49) matched that of both the $^{18}O_2$ atmosphere and the resultant oxygen incorporated as the tyrosine hydroxyl (52:48). Thus, the products of PAH turnover of phenylalanine, 6-MPH_4, and O_2 are tyrosine and 4a-OH-6-MPH_4, the latter forming without exchange of its hydroxyl group with the solvent (Figure 4). These experiments define the 4a-OH adduct as a residue of the hydroxylating intermediate generated during PAH turnover and conclusively argue against the cofactor as simply a source of reducing equivalents for the enzyme.

The identity of the 4a-OOH adduct as the initially formed tetrahydropterin–O_2 species in PAH turnover can be inferred from studies of unnatural substrates and cofactors that "uncouple" tetrahydropterin oxidation from substrate hydroxylation when substituted for the normal reaction components.[51–53] In the absence of hydroxylation, it is reasonable to invoke a two-electron reduction of O_2 to H_2O_2 at the expense of the tetrahydropterin reducing equivalents.[51,52] Although we demonstrated that H_2O_2 is not stoichiometrically released in uncoupled turnover,[53] it does form as an intermediate and small amounts can be detected.[53] This result implies that the dioxygen oxygen–oxygen bond is intact in the tetrahydropterin–oxygen adduct that forms on the catalytic pathway prior to hydroxylation. Thus, H_2O_2 most likely forms from breakdown of the 4a-OOH adduct if hydroxylation does not occur, re-

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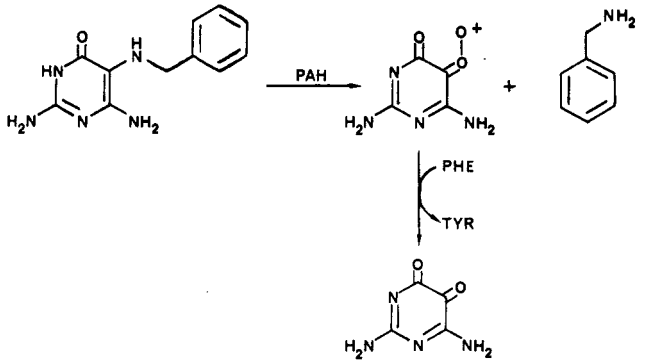

Figure 5. Possible mechanism of PAH oxygen activation with triaminopyrimidine cofactor.

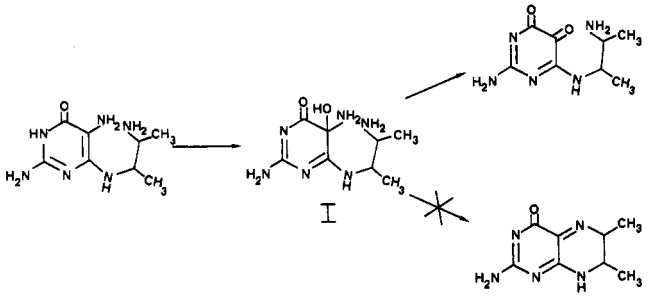

Figure 6. Molecular assay for cofactor ring closure during PAH turnover.

sulting in the direct formation of quinonoid dihydropterin and an Fe^{2+}–H_2O_2 complex capable of further reaction[53] (vide infra). While the 4a-OOH adduct almost certainly forms during PAH turnover, carbonyl oxide or iron–oxygen species remain alternative candidates for the identity of the hydroxylating intermediate (Figure 3).

The initial proposal of a ring-opened carbonyl oxide as the PAH hydroxylating intermediate was made by Hamilton, who questioned the ability of an unactivated hydroperoxide to perform the hydroxylation of an aromatic ring.[54] A number of experiments to detect a carbonyl oxide in PAH turnover have appeared in the literature but, on balance, are inconclusive. The most compelling evidence for the carbonyl oxide is from Ayling,[55–57] who studied the ability of PAH to utilize 5-amino-substituted pyrimidines as cofactors. Both 5-(benzylamino)-2,6-diamino-4-oxypyrimidine (Figure 5) and 2,5,6-triamino-4-oxopyrimidine are poor (less than 10% of the maximal activity) cofactors for PAH. During turnover, the bond analogous to the C_{4a}–N_5 bond in BPH_4 is cleaved in each compound, resulting in the liberation of either benzylamine or ammonia, respectively, with the resultant ketopyrimidine possessing the expected oxygen incorporated from O_2.[57] This is consistent but not demanding of a carbonyl oxide (Figure 5) and could reflect a different mode of decomposition for the hydroxy adducts derived from the pterin and pyrimidine cofactors. Other experiments with a butylamino-substituted pyrimidine (Figure 6), also a poor cofactor for PAH,[58] did not lead to measurable recovery of the cyclic quinonoid dihydropterin anticipated by analogy to the Ayling experiments described above. Thus the intermediate cyclization reaction required of pterin cofactors proceeding through a carbonyl oxide intermediate could not be demonstrated. That this cyclization should be predominantly enzyme catalyzed is mandated by our finding that 4a-OH-6-MPH_4 forms with a high degree of stereoselectivity during PAH turnover; i.e., the major enantiomer formed from 6-MPH_4 is R-4a-OH-6-MPH_4,[50] in contrast to the racemate expected from a solution process. Third, recent studies from Ayling's laboratory utilizing strained pyrimidodiazepine derivatives as PAH cofactors whose carbinolamine adducts prefer to ring cleave failed to provide evidence for a ring-opened product species as a consequence of a carbonyl oxide mechanism.[59] Thus this mechanistic suggestion remains speculative.

The second alternative to the 4a-OOH adduct as the hydroxylating intermediate of PAH is a ferryl complex $(Fe{=}O)^{2+}$, generated as diagrammed in Figure 3. While heme-associated iron–monooxygen complexes capable of performing aromatic hydroxylations are well established for cytochrome P_{450},[60] less evidence exists regarding the ability of nonheme ferryl complexes to exist in aqueous solution and serve as oxygen-transfer agents. (In this discussion, we presume that the iron ligand environment in PAH is nonheme in nature, as evidenced by the enzyme's complete lack of a visible absorption spectrum.) However, it has recently been demonstrated that ferrous EDTA forms a ferryl complex when it reacts with H_2O_2 in aqueous solution[16] as does "free" ferrous iron in acetonitrile,[62,63] but the latter does not apparently hydroxylate unactivated aromatic rings. We approached this question with PAH by evaluating whether the enzyme cleaves peroxides homolytically (one-electron reduction) or heterolytically (two-electron reduction):

$$PAH{\cdot}Fe^{2+} + ROOH \rightarrow PAH{\cdot}Fe^{3+} + RO^{\cdot} + {}^{-}OH \quad (2)$$

$$PAH{\cdot}Fe^{2+} + ROOH \rightarrow PAH{\cdot}(Fe{=}O)^{2+} + RO^{-} \quad (3)$$

Evidence for a heterolytic cleavage would imply the chemical competence of the enzyme to form and stabilize a ferryl complex by reductive cleavage of the putative 4a-OOH adduct to the 4a-OH adduct. The four peroxides employed in these studies ranged in oxygen-transferring ability in the order of H_2O_2 < *tert*-butyl peroxide < phenylperacetic acid < *m*-chloroperoxybenzoic acid. We have four lines of evidence suggesting that PAH cleaves peroxides primarily homolytically.[64] First, a tetrahydropterin peroxidase activity for PAH was demonstrated[53] in that incubation of peroxides with the enzyme results in the stoichiometric oxidation of 6-MPH_4 to its quinonoid dihydropterin (4a-OH-6-MPH_4 is not an intermediate in this reaction). When this assay was used, a linear relationship was shown to correlate the rate of peroxide

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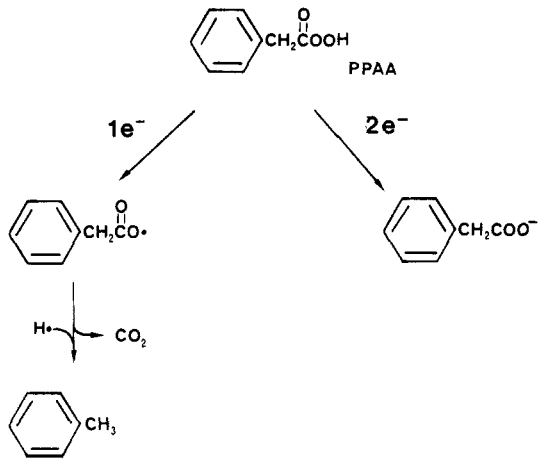

Figure 7. Phenylperacetic acid (PPAA) processing by PAH.

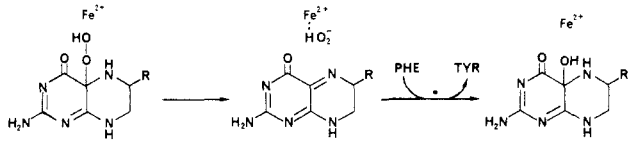

Figure 8. Alternative hydroxylating species for PAH.

oxygen–oxygen bond cleavage and the enthalpy required to cleave the bond in a homolytic (but not heterolytic) fashion. Second, catechols, which preferentially bind to ferric but not ferrous iron, were employed to trap the ferric form of PAH, indicating that the enzyme was converted to its ferric state per peroxide turned over during the tetrahydropterin oxidase activity. As shown in eq 2 and 3, the formation of ferric PAH implies a homolytic bond cleavage. Third, phenylperacetic acid (PPAA) when utilized as a probe for the mechanism of bond cleavage (ref 65, Figure 7) generated stoichiometrically the decarboxylated product, toluene. When the peroxide bond of PPAA cleaves homolytically, decarboxylation of the resultant phenylacetoxyl radical is observed.[65] Finally, the water-soluble hydroxyl radical trap *p*-nitroso-*N*,*N*-dimethylaniline was employed to detect ·OH release from PAH during H_2O_2 metabolism. Although the scavenging of ·OH was not stoichiometric with respect to the enzyme, the extremely reactive nature of this species makes it likely that the radical oxidizes amino acid residues on the enzyme before release into solution can occur. Collectively, the above experiments strongly imply that PAH cleaves peroxides primarily homolytically and suggest that the enzyme does not have the ability to stabilize a ferryl complex.

On the other hand, the observation of homolytic cleavage of peroxides by PAH raises the question of whether aromatic hydroxylation may result from Fenton chemistry at the active site (Figure 8). For that to be true the quinonoid form must be rehydrated stereospecifically by water derived exclusively from O_2 and not the medium. One can imagine active-site restrictions that would sequester the various intermediate species so that this process with its chemical products also would be feasible.

There are additional likely roles for the ferrous center in turnover that may include providing an initial oxygen binding site and overcoming, through orbital interactions, the spin-forbidden barrier of reacting triplet O_2 with the singlet organic molecule BPH_4. The alternative mechanism of 4a-OOH adduct generation is through a hydrogen abstraction/recombination involving an intermediate pterin semiquinone, analogous to the mechanism proposed for oxygen activation by dihydroflavin-dependent hydroxylases.[66] However, dihydroflavins react with O_2 by this mechanistic pathway quite rapidly in aqueous solution (10^7 M^{-1} s^{-1}) in the absence of metals,[66] whereas tetrahydropterins react quite slowly (10^{-3} M^{-1} s^{-1}).[67] Consequently, at saturating oxygen the first-order rate constant is ca. 10^{-6} s^{-1} wheras the turnover of PAH is ca. 10 s^{-1}.

In summary, the actual identity of the hydroxylating species in the PAH reaction remains open. Three components in addition to the enzyme are required for turnover: O_2, ferrous iron, and a tetrahydropterin. The intermediacy of the 4a-OH adduct is firmly established whereas the presence of the 4a-OOH is strongly inferred. We have calculated that a putative 4a-OOH tetrahydropterin adduct based on its pK_a will be almost identical with an N_5-blocked 4a-hydroperoxyflavin[60] in oxygen-transferring ability, placing it closer to a peracid than a hydroperoxide. Whether this species then requires further activation by the various means described above requires additional experiments, particularly to clarify the nature of the iron and active-site ligands and the iron redox potential.

Oxygen Activation by Other Pteridine-Dependent Monooxygenases

As mentioned in the introduction, each of the mammalian and bacterial aromatic amino acid hydroxylases shares chemical components required for generating a hydroxylating species. Each possesses a metal center and catalyzes the overall conversion of BPH_4 to BPH_2 concomitant with substrate hydroxylation.[10,13,24,25] In addition, a number of mechanistic observations imply that the enzymes may all proceed by the same pathway of oxygen activation as does mammalian PAH.

For example, NIH shifts have also been demonstrated with TPH and TH. TPH converts 5-tritiotryptophan to 4-tritio-5-hydroxytryptophan with about 85% retention of tritium[34] while TH converts *p*-tritiophenylalanine to 3,4-dihydroxyphenylalanine with about 42% tritium retention, implying an 84% retention in the intermediate tyrosine, assuming constant fidelity of hydroxylations.[68] An NIH shift cannot be established for hydroxylation of tyrosine by TH due to the facilitation of the epoxide-opening reaction by the *p*-hydroxyl of tyrosine, which precludes the requisite hydride shift (cf. Figure 2). (The *m*-hydroxyl of dihydroxyphenylalanine formed by TH is derived from O_2.[69]) Another key to evaluating whether the same hydroxylating species is forming in each enzyme is to demonstrate the intermediacy of the 4a-OH adduct during turnover. We have done this with two additional enzymes: TH purified from rat adrenal derived pheochromocytoma cells[13] and a copper-containing bacterial PAH from *Chromobacterium violaceum*.[10] This demonstration is not a trivial process for either enzyme due

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to the transient nature of the 4a-OH adduct and the slower rates of their turnovers versus rat liver PAH. For each enzyme, every hydroxylation event involves the formation of the 4a-OH adduct, a situation entirely analogous to PAH.

It is especially interesting to note that the copper-containing PAH appears to follow the same mechanism of oxygen activation as do the iron enzymes. The greater ease of obtaining resolved ESR spectra with the Cu^{2+} form of the bacterial enzyme prompted us to examine the proximity of the metal ion center to the pterin cofactor. ESR spectra of the enzyme recorded in the presence of [5-^{14}N]6,7-dimethyltetrahydropterin or [5-^{15}N]6,7-dimethyltetrahydropterin were computer simulated and found to be consistent with the pterin serving as a direct donor ligand to the copper center through the N-5 position. Presuming that the pyrazine ring–metal interaction is maintained upon reduction of the cupric ion to the active cuprous form, this study provides support for the underlying assumption of close proximity between the metal center and the pterin implicit in the mechanism depicted in Figure 3.[70]

The mechanistic links between PAH and the other aromatic amino acid hydroxylases provide a rationale for the enzymes sharing the common pathway of oxygen activation outlined above. Some differences do exist, as demonstrated by comparing TH with PAH. Although phenylalanine is the more difficult substrate to hydroxylate, PAH has a V_{max} about 100 times greater than that of TH. Conversely, PAH autoinactivates during turnover whereas TH does not. These facts do not obviously correlate with a commonality of oxygen activation. One possible explanation of the above differences may be the degree to which each enzyme must use its ferrous center to activate oxygen. As mentioned above, structural and energy considerations predict the 4a-OOH adduct to be close to a peracid in oxygen-transferring ability. Peracids are capable of hydroxylating tyrosine but not phenylalanine in aqueous media. Thus TH may hydroxylate simply by providing a passive environment for the generation of 4a-OOH adduct in the proximity of its substrate. With PAH, additional oxygen activation is needed, which is provided by the interaction of the 4a-OOH adduct with the ferrous center. The consequence of this interaction with PAH is the possibility of ferrous-induced homolytic cleavage of the 4a-OOH adduct's oxygen–oxygen bond, resulting in the generation of hydroxyl radicals, which play a part in enzyme inactivation. Thus PAH, which may simply be an "evolved" TH, may be less stable due to its requirement for generating a more potent hydroxylating intermediate.

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Registry No. PAH, 9029-73-6; O_2, 7782-44-7; Fe, 7439-89-6; monooxygenase, 9038-14-6; tetrahydropterin, 1008-35-1.

EPR Studies of Long-Range Intramolecular Electron–Electron Exchange Interaction

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If asked to name long-range interaction phenomena in chemical systems, many chemists would probably cite electron transfer, fluorescence resonance energy transfer, or perhaps NMR coupling. Electron–electron exchange interactions, in contrast, would likely be identified as short range. The fact that exchange interaction between two paramagnetic centers is often not recognized as a long-range phenomenon is largely a function of the techniques that have typically been used to study it. Electron–electron exchange has been studied by magnetic susceptibility measurements in which interactions weaker than kT are too small to detect. Since interaction that is strong enough to be observed by magnetic susceptibility typically occurs only between unpaired electrons that are separated by a few angstroms, the impression has evolved that electron–electron exchange interaction is a short-range phenomenon. When collisions between paramagnetic molecules in solution give rise to exchange, the strongest interaction is during the time of closest approach, which again emphasizes the short-range terms. In the EPR spectra of solids with high concentrations of unpaired electrons, exchange-narrowing also requires close proximity of the paramagnetic centers.

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