Two Distinct Electrophilic Oxidants Effect Hydroxylation in Cytochrome P-450-Catalyzed Reactions

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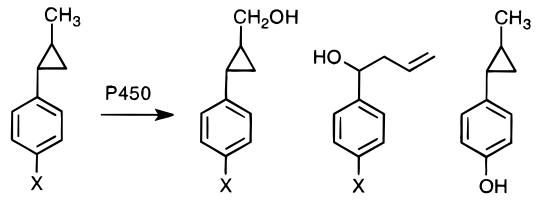
The cytochrome P-450 (P-450) enzymes, ubiquitous in nature, contain an iron−protoporphyrin IX complex (heme) with a thiolate from protein cysteine as the fifth ligand to the heme iron. Among the many oxidations catalyzed by P-450 enzymes are the remarkable hydroxylations of unactivated C−H bonds in hydrocarbons and other compounds. For the past two decades, the mechanism of the hydroxylation reaction has been cast in terms of initial production of an "iron−oxo" species, similar to the known Compound I of peroxidase chemistry (Figure 1).[1] The iron−oxo abstracts a hydrogen atom from hydrocarbon to give an alkyl radical intermediate, and the radical subsequently displaces "OH" from the iron atom in a homolytic substitution reaction termed "oxygen rebound".[2]

Attempts to determine the lifetime of the intermediate via the use of highly reactive "radical clocks" indicated that the mechanistic paradigm for P-450-catalyzed hydroxylations is not complete. Specifically, hydroxylations of hypersensitive probes suggest that the "radical" in P-450 hydroxylation is not an intermediate but a component of the transition structure of an insertion reaction with a lifetime of <100 fs[3,4] and that a competing process is involved.[4,5] These results led to new mechanistic proposals for P-450-catalyzed hydroxylations that invoke some type of competition in the hydroxylation reaction or immediately after the hydroxylation event.[4,6,7] Recent studies with two P-450 enzymes and their mutants lacking threonine in the active site indicated that an alternative explanation exists, that more than one electrophilic oxidizing species is produced in the natural course of oxidation by P-450 enzymes.[8] We report here results of hypersensitive radical probe studies with P-450 isozymes and their mutants that not only confirm the existence of two reactive electrophilic oxidants in P-450 reactions but also demonstrate that both oxidants can effect hydroxylation.

The probe substrates **1** and **2** were employed in oxidations by



purified P-450 isozyme 2B4, two truncated P-450 isozymes

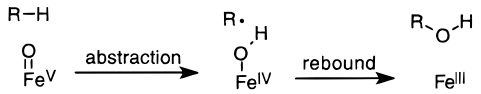


Figure 1. The hydrogen abstraction−oxygen rebound mechanism for P-450-catalyzed hydroxylation, where Fe represents heme-bound iron.

(Δ2B4 and Δ2E1), and mutants of the truncated isozymes in which a threonine in the active site was replaced with alanine (Δ2B4 T302A and Δ2E1 T303A).[8−10] Both of these probes were previously used in studies of hydroxylations by the P-450 isozyme 2B1,[5,11,12] and probe **1** has been studied with 2B4[11] and P-450$_{cam}$.[13] Hydroxylation reactions[14] at the methyl positions of the probes give either unrearranged alcohols (**C**) or rearranged alcohols (**A**); the rearranged alcohols can be produced by ring opening of either a radical or a cationic intermediate. The aromatic ring of probe **1** also is oxidized to phenol **1P** by P-450 enzymes, but no phenolic products are observed with probe **2**.

Most of the oxidations were relatively clean in that known oxidation products were produced. The exception was oxidation of probe **1** by the Δ2E1 T303A mutant. In this reaction, two new products with GC retention times similar to those of other oxidation products were found in significant amounts. Both of these new products had mass spectral fragmentation patterns similar to that of phenol **1P** including a strong molecular ion at $m/z = 148$. We tentatively assigned these compounds as the ortho and meta phenols from **1**. A series of control studies was performed to determine alcohol product stabilities with completely competent enzyme systems and product recoveries from reactions in which a component of the competent system was excluded; good recoveries were found in all cases (Supporting Information). Table 1 contains a summary of product distributions obtained in oxidations of probes **1** and **2** with various P-450 enzymes; detailed results are in the Supporting Information; major changes were found with the mutants.

Competing pathways apparently exist for oxidation of probe **1** at the methyl position and the phenyl group. Replacement of threonine in the active site resulted in an increase in the amount of phenyl oxidation from a minor (15−20%) process to a major (ca. 60%) process. The pattern of reactivity is the same as that observed previously with 2B4 and 2E1 and their T → A mutants in reactions with alkenes.[8] Importantly, although the regioselectivity of oxidation of **1** changed, the ratio of cyclic to acyclic

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(9) P450 2B4 is purified from liver microsomes of rabbits treated with phenobarbital. Δ2B4 is the recombinant isozyme with N-terminal amino acids 2−27 deleted, and Δ2B4 T302A is the Thr-302 to Ala mutant of this isozyme.[10] Δ2E1 is the recombinant rabbit liver ethanol-inducible P450 isozyme with N-terminal amino acids 3−29 deleted, and Δ2E1 T303A is the Thr-303 to Ala mutant of this isozyme.[8]

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(14) Oxidations were performed with 0.6 nmol of P450 and 1.2 nmol of reductase by the method previously described (volumes, phospholipid, buffer, etc.)[8] with the exception that the mixtures were incubated for 2 min rather than 5 min prior to addition of substrate and NADPH.

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Table 1. Products Formed from P-450 Oxidations of Probes **1** and **2**[a]

isozyme	probe **1** M/P[b]	probe **1** C/A[c]	probe **2** C/A[d]
2B1[e]	2	4	4
P-450$_{cam}$[f]	5	7	
2B4	5.7	6.5	6.1
Δ2B4	5.7	6.0	5.3
Δ2B4 T302A	0.8	4.6	2.5
Δ2E1	4.4	9.3	9.6
Δ2E1 T303A	0.5 (1.3)[g]	8.4	1.6

[a] Summary of results; complete results are in the Supporting Information. [b] Ratio of (**1C** + **1A**) to **1P**. [c] Ratio of **1C** to **1A**. [d] Ratio of **2C** to **2A**. [e] References 5 and 12. [f] Reference 13. [g] The ratio of (**1C** + **1A**) to all phenolic products is given; the ratio of (**1C** + **1A**) to **1P** is in parentheses.

products from methyl oxidation was essentially unaltered for the isozymes and their respective mutants. These observations appear to be consistent only with the existence of two types of electrophilic oxidants in the P-450 reactions as previously described.[8] One of the oxidants gives mainly methyl hydroxylation products, while the other gives mainly phenols from oxidation of the aromatic ring. Either the lifetime of the phenyl group oxidizing species is increased by the removal of threonine from the active sites of the enzymes, or the proportions of the two oxidants are altered.[15]

In oxidations of probe **2**, the electron-withdrawing CF_3 group prevents the arene oxidation reaction, and only products from methyl oxidation are observed.[5] The ratios of unrearranged to rearranged alcohol products from **2** were significantly changed with the respective mutants, and the results for the 2E1 system were especially dramatic. In the context of the two active electrophilic oxidants implicated from reactions of probe **1** in this work and reactions of alkenes previously studied,[8] the results with probe **2** indicate that both oxidizing species can effect methyl group hydroxylation in probe **2**.

The events in P-450-catalyzed oxidations involve substrate binding, P-450 reduction by reductase, oxygen binding, a second reduction, two protonations, and substrate oxidation.[1] The important points here are the details of the actual oxidation reactions and the timing of the protonation reactions. Threonine is highly conserved in the active sites of the P-450 enzymes,[16] and the few crystal structures available for bacterial P-450 enzymes show that threonine is within hydrogen bonding distance of a putative hydroperoxy−iron complex.[17] Active-site threonine is proposed to serve as a proton source, possibly via relay with water, following the second reduction step in the sequence.[18]

The probe results are consistent with the mechanistic description in Figure 2.[8] Protonation of a peroxo−iron species, a nucleophilic oxidant important in P-450-catalyzed deformylation reactions,[10] gives a hydroperoxo−iron complex. Proximal protonation of this species would give hydrogen peroxide−iron,

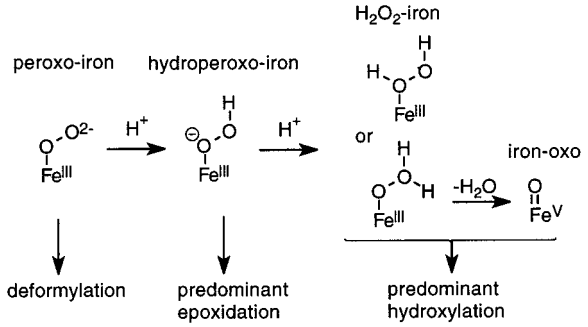

Figure 2. Multiple oxidants generated by cytochrome P-450 enzymes, where Fe represents heme-bound iron.

whereas distal protonation would give water-complexed iron−oxo. In the mutants lacking threonine in the active site, the lifetime (or population if an equilibrium is involved) of the hydroperoxo−iron species is increased. The hydroperoxo−iron species is an effective electrophilic epoxidizing agent, but the important point of this work is that both agents can bring about hydroxylation. One can reconcile the minor variations in the ratios of cyclic to acyclic alcohols from probe **1** observed with the mutants as arising from a small amount of hydroxylation effected by the hydroperoxo−iron species even when phenyl group oxidation was possible. The ultimate oxidant, formed by protonation of the hydroperoxo−iron complex, preferentially hydroxylates. The possibility that the ultimate oxidant can also effect epoxidations or phenyl group oxidations cannot be determined from this work, but it is noteworthy that the P-450-like enzyme chloroperoxidase from *Caldariomyces fumago* activated by H_2O_2, which must bypass a hydroperoxo−iron stage, hydroxylates the methyl group in probe **1** but does not produce appreciable amounts of phenol **1P**.[19]

The implications of two active electrophilic oxidants that can effect hydroxylation in P-450 reactions are profound. With respect to the specificity of P-450 oxidation reactions, this family of enzymes as a whole generally exhibits low selectivity. Substrate positioning by the protein is a possible mode of achieving selectivity, but differences in the preferred course of reactions of two electrophilic oxidants provide an alternative method while maintaining the requisite high reactivity necessary for hydroxylation reactions.

Proposals for the mechanisms of P-450-catalyzed hydroxylation reactions which start with an assumption that one oxidizing species is involved, usually taken to be an iron−oxo, should now take into account the demonstration of two competent hydroxylating species. The important questions become (1) Which previous results can be accommodated by the competition of two oxidants rather than competitions in the oxidations by one oxidant? and (2) What are the details of the reactions of each oxidant? Probes such as **1**, in which the major reaction of each oxidant apparently differs, might provide information in regard to the latter question, but it seems clear that conceptually new experimental approaches that can differentiate between reactions of the two oxidants or produce one or both of the oxidants independently are desired.

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Supporting Information Available: Complete tables of enzyme oxidation studies and control reactions (5 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(15) An alternative explanation is that methyl versus phenyl oxidation is controlled by substrate orientation and mutation results in a redistribution of the binding orientations of the substrate. We cannot rule out this possibility unequivocally, but we consider it to be unlikely on the basis of the similarities in alcohol versus phenol product distributions for the various P450 isozymes and the fact that metabolic switching, requiring free tumbling of the substrate in the active site, was observed in 2B1 oxidations of isotopically substituted probe **1**.[12.]

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