Hypersensitive Radical Probes and the Mechanisms of Cytochrome P450-Catalyzed Hydroxylation Reactions

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ABSTRACT
The title probes are precursors to kinetically calibrated, aryl-substituted cyclopropylcarbinyl radicals that rearrange with picosecond lifetimes. Applications in studies of cytochrome P450-catalyzed hydroxylation reactions are reviewed. Initially confusing results regarding lifetimes of radicals in the hydroxylation reactions were resolved when second-generation probes that distinguish between radicals and cations were employed. The results indicate that two electrophilic oxidizing species are involved in P450-catalyzed hydroxylations, an iron-oxo species that inserts oxygen and a hydroperoxo-iron species that inserts OH^+. The cationic rearrangement products are ascribed to reactions of the protonated alcohol products formed from the latter.

I. Introduction

The cytochrome P450 (P450) enzymes are among the most widely distributed enzymes in nature; they are found in bacteria, plants, and mammals.[1] Their active sites are characterized by an iron−protoporphyrin IX (heme) complex in which the fifth ligand to iron is a protein cysteine, as seen in crystal structures[2,3] of these enzymes (Figure 1). The broad occurrence of P450s is paralleled by the vast array of oxidation reactions that they catalyze. Through the use of reducing equivalents from NADPH or NADH and molecular oxygen, they produce water and iron−oxygen complexes that oxidize unactivated alkanes, alkenes, arenes, and heteroatoms.[4] The hydroxylation of unactivated alkanes is one of the most remarkable reactions effected by Nature because of the thermodynamics of the process; the C−H bond dissociation energies of simple hydrocarbons are 96, 99, and 101 kcal/mol for 3°, 2°, and 1° centers, respectively.[5] The net reaction for alkane hydroxylation is represented by eq 1.

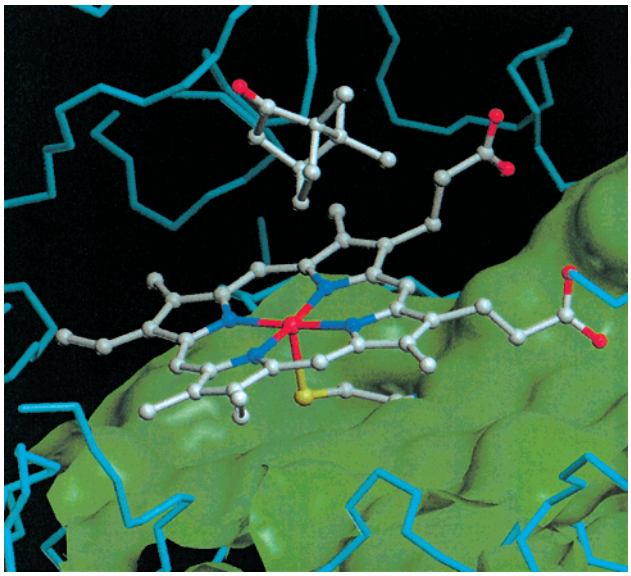

FIGURE 1. Portion of the crystal structure of $P450_{cam}$ with bound substrate showing the heme and protein cysteine ligation of iron; from ref 3, PDB file 1AKD.

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O \qquad (1)$$

Early views of the mechanism by which P450 enzymes catalyze alkane hydroxylation focused on an insertion pathway in which a high-valent iron-oxo species, equivalent to compound I of peroxidase enzymes, transferred oxygen via an insertion reaction. This was later supplanted by an "oxygen rebound" mechanism (Figure 2) that was based largely on seminal mechanistic probe studies by Groves and co-workers.[6−8] In these works, radical intermediates were implicated by observations such as epimerization in the hydroxylation of norbornane-d_4 (**1**)[7] and allylic rearrangement in hydroxylation of deuterated cyclohexene **2**.[8] Strong support for the production of a short-lived radical intermediate was provided in the finding by Ortiz de Montellano and Stearns that some ring opening occurred during the P450-catalyzed hydroxylation of bicyclo[2.1.0]pentane (**3**) at the C2 position, whereas no ring opening occurred during hydroxylation of the methyl group in methylcyclopropane (**4**);[9] the ring opening of the bicyclo[2.1.0]pent-2-yl radical, although not calibrated fully, was known to be faster than ring opening of the cyclopropylcarbinyl radical. Other experimental results indicated that any radical intermediate must be short-lived. For example, a very high level of stereospecificity (>90% retention) was found in the terminal methyl group hydroxylations of both enantiomers of geraniol, chiral by virtue of isotopic substitution on that methyl group (**5**).[10] It is noteworthy that hydroxylations at cyclopropylcarbinyl positions as in **4** without rearrangement excluded an obligate cationic intermediate.

II. Hypersensitive Probe Calibration

Probe studies of P450-catalyzed hydroxylations were in qualitative agreement that a radical intermediate was

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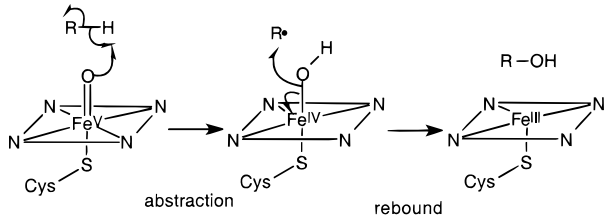
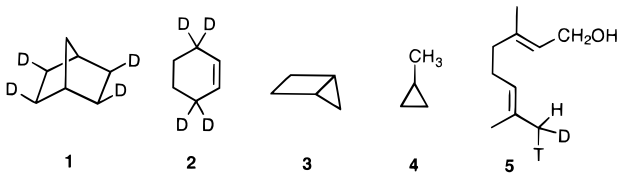


FIGURE 2. Hydrogen abstraction−oxygen rebound mechanism for hydroxylation.



formed, but the rate constants of the radical rearrangement reactions generally were not available. If they were known, then the product distributions observed in, for example, oxidation of probe **3** could be used to determine the rate constants of the "oxygen rebound" step. Such calibrated radical rearrangements are often referred to as "radical clocks" that are used to "time" a competing radical process.[11−13]

Our interest in radical reaction kinetics and the results of the Ortiz de Montellano and Stearns study with bicyclo[2.1.0]pentane[9] led us to develop an indirect method that could be used for the calibration of fast radical reactions. Eventually, this evolved into a technique applicable for radicals with lifetimes as short as 1 ps. The method is based on the use of Barton's PTOC (pyridine-2-thione-*N*-oxycarbonyl) esters as radical precursors and radical trapping by the highly reactive group 16 hydrides thiophenol and benzeneselenol.[14−17] The simplicity and breadth of the method has permitted the calibration of a variety of fast-reacting alkyl radicals, bicyclo[2.1.0]pent-2-yl,[18] cubylcarbinyl,[19] 2-(alkoxycarbonyl)cyclopropylcarbinyl,[20,21] 2-methylenecyclopropylcarbinyl,[22] and 2-arylcyclopropylcarbinyl[16,23−27] radicals (Figure 3). The array of calibrated fast radical clocks permits timing of events that occur on the subpicosecond time scale. This account describes experiments using calibrated hypersensitive probe substrates in P450-catalyzed hydroxylation reactions, initially with the aim of "timing" the putative oxygen rebound step.

III. Mechanistic Studies of P450-Catalyzed Hydroxylation Reactions

Calibrations of the bicyclo[2.1.0]pent-2-yl radical rearrangement kinetics by our group[18] and Ingold's group, via competitive nitroxyl radical coupling,[28] gave similar results. Using the rate constant for ring opening of this radical and the product distribution found in P450-catalyzed oxidation of the hydrocarbon **3**,[9] one computes a rate constant for the oxygen rebound step of 1.4×10^{10} s^{-1}. A quantitative value for the rebound step was now available, but, unfortunately, this value was never found again in studies with other calibrated clocks, which gave increasingly confusing results. In work conducted after

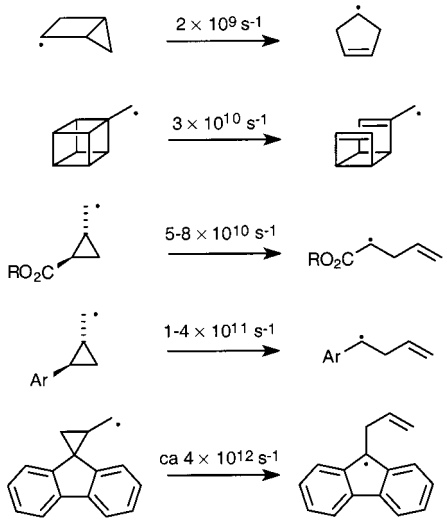
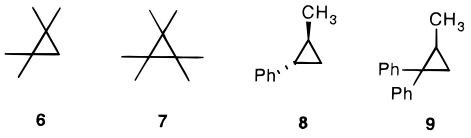


FIGURE 3. Some ultrafast radical rearrangements. Rate constants shown above the arrows are for reactions at ambient temperature.

calibrations of fast and very fast radical rearrangements became available, Atkinson and Ingold[29] found rearranged alcohol products from 1,1,2,2-tetramethylcyclopropane (**6**), hexamethylcyclopropane (**7**), *trans*-2-phenylmethylcyclopropane (**8**), and 2,2-diphenylmethylcyclopropane (**9**) in addition to **3**. From the known rate constants for rearrangement, oxygen rebound rate constants ranging from 1.4×10^{10} to 7×10^{12} s^{-1} were computed. The results were ascribed to a special reactivity of the radical from **3** and a special steric effect of the enzyme that slowed the rearrangements of the cyclopropylcarbinyl radicals derived from **8** and **9** by constraining the aryl groups; that is, the rebound kinetics found with three of the five cases available were considered nonrepresentative.



Our group first studied P450 hydroxylations in a collaboration with the groups of Ingold (at the NRC in Canada) and Hollenberg (at Wayne State University and later the University of Michigan). In an attempt to characterize the hydroxylation of probe **8** more completely, kinetic isotope effects (KIEs) were determined for the enantiomers of the probe.[30] If the postulate that enzyme steric effects slowed the ring opening of the radical was correct, then the two enantiomers should give different results due to their different diastereomeric complexes with the enzyme. In fact, the two enantiomers gave quite similar results in terms of their overall reactivity, the observed KIEs, and, most importantly, the product distributions.[30]

The postulate that the enzyme constrained the phenyl group in the radical from probe **8** and affected the kinetics of ring opening was further vitiated when a rigid bicyclic analogue was studied. P450-catalyzed hydroxylation of **10** gave several products, including those from oxidation of the methyl group. From the ratio of rearranged to un-

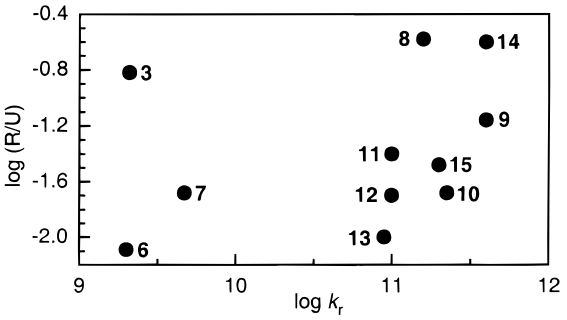
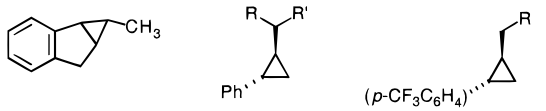


FIGURE 4. Log−log plot of the ratio of rearranged to unrearranged products from oxidations of various probes against the rate constants for rearrangement. The numbers identify the probes. The regression line for these data (not shown) has a slope of 0.2 ± 0.4 at the 95% confidence interval, and the linear correlation coefficient is $r = 0.28$.

rearranged products and the rate constant for radical ring opening, the computed rebound rate constant at 37 °C was (1−2) × 10^{13} s^{-1}.[31] This rate constant exceeds that calculated for decomposition of a transition state at this temperature, an impossible result if a discrete radical intermediate was involved in the oxidation.



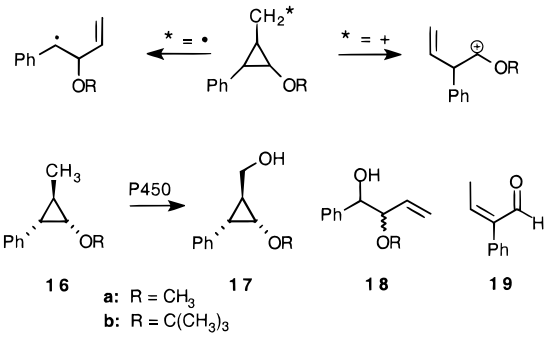
10

11: R = CH_3, R' = H
12: R = C_2H_5, R' = H
13: R = R' = CH_3

14: R = H
15: R = CH_3

With six sets of "quantitative" results available, the inconsistency of either the oxygen rebound mechanism or the kinetic calibrations became apparent. A log−log plot of the ratio of rearranged to unrearranged alcohol products against the rate constants for radical rearrangements should have shown a linear correlation with a slope of 1, but the data were more likely not correlated.[31] Increasing the number of probe substrates only results in more scattered data. For example, Figure 4 shows a log−log plot of the ratio of rearranged to unrearranged alcohol products found in oxidations of probes **3**, **6**−**10**, and the substituted systems **11**−**15**[32,33] against the rate constants for rearrangements of the corresponding radicals. All oxidations were conducted with microsomes from livers of rats treated with phenobarbital and/or the purified isozyme CYP2B1, which is the major rat liver isozyme induced by phenobarbital treatment.

The data in Figure 4 seem almost random. Possible explanations are (1) the abstraction−rebound mechanism for P450 hydroxylation is incomplete, (2) the kinetic calibrations of the radical reactions are grossly in error, or (3) the enzyme exerts profound effects on the kinetics of radical reactions. We reject point 3 because alkyl radical reactions are generally insensitive to the polarity of the medium and steric effects in the enzyme active site appear to be minor. For example, CYP2B1 oxidizes a broad spectrum of substrates, suggesting a large active site, and the KIE study with probe **8** indicated that the substrate freely tumbled in the enzyme, thus permitting metabolic



16 **17** **18** **19**

a: R = CH_3
b: R = $C(CH_3)_3$

FIGURE 5. (Top) Concept of second-generation hypersensitive probes; production of a cyclopropylcarbinyl radical results in ring opening to a benzylic radical product, whereas formation of a cyclopropylcarbinyl cation leads to fragmentation favoring an oxonium ion. (Bottom) Products produced from cytochrome P450 oxidations of second-generation hypersensitive probes.

switching.[30] A recent study indicated that even the highly specific isozyme $P450_{cam}$ permits free tumbling of adamantane in the active site.[34] Point 2 seems equally unlikely because the absolute errors in radical kinetics would have to amount to up to 3 orders of magnitude. Moreover, some of the "corrected" kinetic values would be absurd; for example, it would be necessary for some of the aryl-substituted cyclopropylcarbinyl radicals to ring open *less rapidly* than other radicals that do not have aryl groups. One appears to be left with explanation 1; the mechanistic picture is incomplete.

We reasoned that a possible explanation for the scattered data in Figure 4 was that some rearrangement of the probes resulted from cationic species which, for all probes listed above, would rearrange with the same skeletal reorganization as the corresponding radicals. Requisite cationic intermediates produced in the hydroxylation reaction were excluded, but a competing pathway involving cations remained possible. To test for cations and still maintain the hypersensitivity of the aryl-substituted cyclopropane systems, we designed a new type of probe based on a trisubstituted cyclopropyl architecture in probes **16** (see Figure 5).[25,35] Production of a radical at the methyl group in these probes gives highly regioselective cleavage, favoring the benzylic radical product (>50:1), and production of a cation at the methyl position results in ring opening toward the alkoxy group to the limit of detection (>1000:1). The cyclopropylcarbinyl radicals derived from probes **16** were calibrated by the PTOC-selenol method.[25]

Oxidation of probe **16b** by the P450 isozyme CYP2B1 gave a mixture of products, including four from oxidation of the methyl group (Figure 5, Table 1).[36] The most abundant of these by far was the unrearranged alcohol **17b**, but both diastereomers of ring-opened alcohol **18b** were present, as was aldehyde **19**. Aldehyde **19** is the product formed from methyl group oxidation with ring opening toward the alkoxy group, to give a hemiacetal, followed by hydrolysis to give an unsaturated aldehyde product and then isomerization in the buffer solution of the enzyme reaction mixture. Alcohols **18b** are the products expected from a radical ring-opening reaction, but

Table 1. Products from Cytochrome P450-Catalyzed Oxidations of Probes 16[a]

probe	isozyme	non-rad/rad[b]	% cation[c]
16b	microsomes	27	2
	2B1	24	1
16a	2B1	8	3
	2B4	13	3
	Δ2B4	20	2
	Δ2B4 T302A	19	5
	Δ2E1	10	2
	Δ2E1 T303A	10	13

[a] Results from refs 36 and 37. [b] Ratio of (**17** + **19**) to radical derived products **18**. [c] Percentage of cationic product **19**.

the observed amounts were too small to accommodate a true radical intermediate. In a more recent study, probe **16a** was hydroxylated with several P450 isozymes including mutants discussed below, and similar results were obtained.[37] Specifically, the major product from oxidation of the methyl group in **16a** was unrearranged alcohol **17a**, and minor amounts of radical-derived products **18a** and cation-derived product **19** were found. Once again, the amounts of radical-derived product **18a** were too small to accommodate a true intermediate. Using the rate constants for ring opening of the radicals derived from probes **16**,[25] corrected according to the recent recalibration of PhSeH trapping kinetics,[38] and the product ratios in Table 1, one computes lifetimes for the "radical" in oxidations of probes **16** in the range of 80−200 fs. The apparent rate constants for radical trapping are on the order of vibrational rate constants, not chemical rate constants, and the lifetimes are similar to those of a transition state, about 170 fs at ambient temperature. These values preclude formation of a discrete radical intermediate, and the hydroxylation reactions apparently involve asynchronous insertions with a small amount of ring opening occurring in competition with collapse of the transition structure.

IV. The Cationic Species in P450 Hydroxylations

Cationic rearrangements were found with methylcubane as well as with **16**.[37] The production of cations explains why the results with the other hypersensitive probes were so scattered. The rearranged products from the other probes were likely derived from both the fast ring openings that compete with collapse of the transition structure and any cationic intermediates formed. This conclusion resolves one puzzle, the scattered results in Figure 4, but also presents another. How are the cationic products formed?

The results of the P450-catalyzed hydroxylation of probe **16b**, reported late in 1995, led to several possible explanations for production of cationic products. We reasoned that oxidation of the "radical" in the transition state might occur to give a cation. Alternatively, the first-formed product of the hydroxylation reaction might be a protonated alcohol, produced by insertion of the elements of OH^+, and a solvolysis-type reaction competed with deprotonation to give the neutral alcohol product.[36] Another explanation was that an agostic complex between the probe and the heme-iron rearranged in competition with consummation of the hydroxylation reaction.[39] An especially perceptive alternative delineated the state crossing effects expected in reaction of a high-spin ensemble of the activated iron-oxo and substrate; state crossing to a low-spin ensemble would permit insertion, whereas reaction on the high-spin surface would result in a radical pair in which oxidation of the radical to a cation was possible.[40] All of these explanations were cast in terms of reaction of a single oxidizing species for P450, either the iron-oxo (which "everyone" believed was formed despite its elusive character) or an iron-complexed hydrogen peroxide (proposed by us for the protonated alcohol route).

Contemporary results mainly from the laboratory of M. J. Coon at the University of Michigan provided an important new twist for P450 oxidation reactions that seems to have resolved the question about the origin of cationic species. In studies aimed at better characterizing the active oxidant(s) of P450, Coon and Vaz worked with expressed wild-type P450 isozymes and mutants in which a highly conserved threonine in the active site was replaced with alanine.[41,42] The expressed enzymes, which contain a small deletion at the N-terminal end of the protein, were the rabbit hepatic phenobarbital-induced isozyme (Δ2B4) and the rabbit hepatic ethanol-inducible isozyme (Δ2E1) and their respective mutants, Δ2B4 T302A and Δ2E1 T303A. In brief, these researchers found that the ratios of epoxides to allylic alcohols formed in oxidations of alkenes varied considerably for the wild-type enzymes and their respective mutants. The conserved active site threonine in P450 is thought to be involved in proton-transfer reactions that form the P450 oxidant(s), and the results of Vaz and Coon suggest that the mutations disrupted the protonation sequence. The bottom line with respect to mechanisms is that their results suggested that two electrophilic oxidant forms were involved in P450 oxidations, one a preferential epoxidizing agent and the other a preferential hydroxylating agent.

Our group teamed up with Coon's and Hollenberg's groups to study hypersensitive probe oxidations by the wild-type and mutant P450 enzymes.[37,43] Oxidations of probes **8** with a series of enzymes gave results in excellent agreement with those of the earlier studies; specifically, the amount of methyl group oxidation (hydroxylation) relative to phenyl group oxidation (epoxidation) increased dramatically with the mutants, indicating that two oxidants were at play. Importantly, the ratio of rearranged to unrearranged products from methyl oxidation in **8** was relatively constant despite the alteration in regioselectivity, whereas oxidation of the substituted probe **14**, where phenyl group oxidation is suppressed by the CF_3 group, resulted in a large change in the ratio of rearranged to unrearranged alcohols for both wild-type and mutant pairs. These results indicate that both electrophilic oxidant forms must be capable of effecting hydroxylation when the epoxidation pathway is not available.

The oxidation of the second-generation probes **16** by several P450 enzymes, including the wild-type mutant

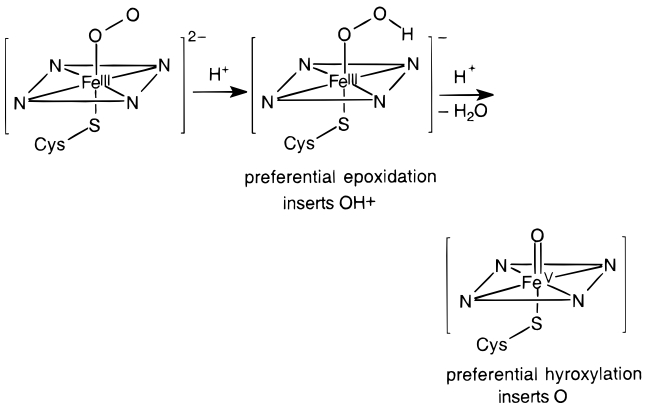
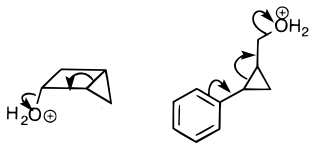


FIGURE 6. Possible identities and modes of action of the electrophilic oxidants in P450.

pairs, was noted earlier.[36,37] The alkoxy groups divert the cationic species, thus allowing one to determine the extent of radical-derived rearranged products. If one now focuses on the cation-derived products in oxidations of probe **16a** (Table 1), one sees that the amount of cationic species was greater with the mutants.

Combining these observations with the known sequence of events in P450 oxidations gives the mechanistic conclusions in Figure 6. Substrate binding, reduction of the ferric form of the P450 enzyme, oxygen binding, and a second reduction gives the peroxo-iron species that starts this scheme. Protonation of the peroxo-iron on the distal oxygen atom gives a hydroperoxo-iron. A second protonation on the distal oxygen and loss of water gives the iron-oxo species similar to compound I of the peroxidases. One of the two electrophilic oxidant forms apparently is the iron-oxo, which is generally believed to be the oxidizing species. The second electrophilic oxidant, the preferential epoxidizing agent, apparently is the hydroperoxo-iron complex, although iron-complexed hydrogen peroxide formed by protonation of this complex is also a possibility.[44] The preferential epoxidizing species is the major (or only) source of cationic species in the P450 hydroxylations, and this species apparently effects hydroxylation by insertion of OH^+. A similar OH^+ insertion was implicated in reaction of heme oxygenase.[45]

It appears, therefore, that the origin of the cationic species in P450 hydroxylations is likely to be first-formed protonated alcohols produced by insertion of OH^+ into the C−H bond by the preferential epoxidizing species. Deprotonation of these strong acids undoubtedly will be fast, but it must compete with solvolytic-type rearrangement reactions. This picture is supported by the nature of the probes in which rearranged alcohol products have been found; in all cases, the strained structures of the probes and the cation-stabilizing substituents should result in fast solvolytic fragmentation reactions as indicated in **20** and **21**.



20 **21**

V. Mechanistic Studies of Other Oxidation Systems

Hypersensitive probes described here have been used in studies of hydroxylation by chloroperoxidase (CPO) from *Caldariomyces fumago*. This CPO is unique among the peroxidases in that its active site is structurally equivalent to those of the P450 enzymes, containing an iron-heme complex with a protein cysteine as the fifth ligand to iron. Zaks and Dodds reported hydroxylation of **8** with no rearrangement.[46] In our studies with CPO,[47] hydroxylation of **8**, **11**, and **13** gave only unrearranged alcohol products, but we also found that the rearranged products were not stable in the presence of the enzyme and H_2O_2. Nonetheless, the estimated lifetime of a putative radical intermediate in CPO was <3 ps, in general agreement with the results in P450 studies.

Several of the probes also have been used in studies of oxidations by the soluble methane monooxygenase (sMMO) system from *Methylococcus capsulatus* (Bath).[48−50] Although the active site of the MMO hydroxylase enzyme is dramatically different than those of the P450s, containing a diiron moiety and no porphyrin, recent results suggest that the mechanisms might be quite similar. Exceedingly short (≤250 fs) "radical" lifetimes (i.e., insertions) and cationic rearrangement products from methylcubane and probe **16b** highlight the similarities.

Other enzyme applications of hypersensitive probes include a study of an alkane monooxygenase from *Pseudomonus oleovorans*[51] and the use of a hydroxy-substituted version in a study of galactose oxidase.[52] Nonenzymatic hydroxylations studied with hypersensitive probes include those effected by dimethyldioxirane[53] and those effected by a variety of Gif-type oxidants.[54] Related vinyl-substituted hypersensitive probes have been used in mechanistic studies of epoxidation by $P450_{cam}$,[55] by an MMO mimic,[56] and by dimethyldioxirane.[57]

VI. Conclusion.

The cytochrome P450 enzymes are among the more important proteins found in nature, and the complex mechanisms by which they catalyze oxidation reactions have been intensely studied. In our work, a total of 11 hypersensitive radical probes have been employed, and seven different P450 enzymes have been used. The key factors of the probes that provided useful information are the ultrafast rates of rearrangement and the fact that quantitative information is provided from the known rate constants. The results are consistent with insertion pathways for hydroxylation and the production of a nonrequisite cationic species that can lead to rearranged products. The premise that the latter is produced by insertion of OH^+ from the hydroperoxo-iron species to give protonated alcohol products that can rearrange in a solvolytic-type reactions nicely explains the mechanistic confusion from early work using probes that could not differentiate between radical and cationic intermediates.

There is a caveat, however. Whereas we believe the mechanistic picture for P450-catalyzed hydroxylation is reasonably well explained, the view of some is that it is complicated and not yet resolved. In addition to studies of a broader range of P450s, experimentalists might attempt to design studies to test the multistate scenario for hydroxylation currently being addressed computationally.[40,58]

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