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Cytochrome P450-Catalyzed Hydroxylation of Mechanistic Probes that Distinguish between Radicals and Cations. Evidence for Cationic but Not for Radical Intermediates

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Abstract: Oxidation of the mechanistic probes *trans,trans*-2-methoxy-3-phenylmethylcyclopropane and methylcubane by six cytochrome P450 isozymes has been studied. The probes differentiate between radical and cationic species in that different structural rearrangements occur for the two types of intermediates. The P450 isozymes are the phenobarbital-inducible hepatic isozymes P450 2B1 (from rat) and P450 2B4 (from rabbit), the expressed truncated isozymes P450 Δ2B4 and P450 Δ2E1 (ethanol-inducible, from rabbit), and mutants of the latter two in which an active site threonine was replaced with alanine, Δ2B4 T302A, and Δ2E1 T303A. Cationic rearrangement products were found from both probes. Oxidations of *trans,trans*-2-methoxy-3-phenylmethylcyclopropane gave small amounts of radical-derived rearrangement products indicating that hydroxylation occurs via insertion reactions with transition state lifetimes in the 80−200 fs range. A mechanistic description of cytochrome P450-catalyzed hydroxylations that is in accord with the present and previous radical probe results is presented. This description incorporates the recent demonstrations that two electrophilic oxidants are produced in the natural course of P450 oxidation reactions and that both electrophilic oxidant forms can effect hydroxylation reactions. Following production of a peroxo-iron species, protonation gives a hydroperoxo-iron species. Protonation of the hydroperoxo-iron species gives an iron-oxo species and water. Hydroxylations by both the hydroperoxo-iron and iron-oxo species occur by insertion reactions. The hydroperoxo-iron species inserts the elements of OH^+ producing protonated alcohol products that can react in solvolysis-type reactions to give cationic rearrangement products. The iron-oxo species reacts by insertion of an oxygen atom.

The cytochrome P450 enzymes (P450s) are ubiquitous in nature and effect numerous oxidations of physiologically important natural and foreign substrates including the remarkably difficult hydroxylation of unactivated C−H bonds in hydrocarbons and other compounds.[2] The engine for oxidation is an iron-protoporphyrin IX complex (heme) with a thiolate from protein cysteine serving as a fifth ligand to iron. Many of the steps in the sequence of the oxidation reactions are well characterized, but the identity of the final oxidant(s) and the reaction mechanisms are still poorly understood. The well-characterized portion of the sequence involves substrate binding, reduction of the resting ferric form of the enzyme to a ferrous state, binding of oxygen to give a superoxide complex, and a second reduction step. Although subsequent oxidation of substrate is fast, attempts at spectral identification of the ultimate

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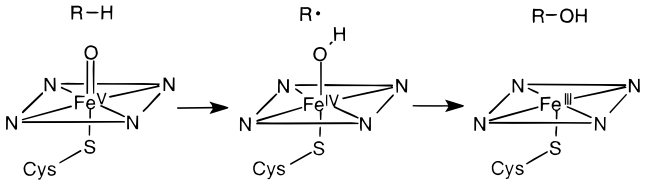

Figure 1. The hydrogen abstraction, oxygen-rebound mechanism for P450-catalyzed hydroxylation. Abstraction of hydrogen atom by the iron-oxo species is followed by a homolytic displacement of OH that returns the resting enzyme.

oxidant in P450[3,4] and the related nitric oxide synthase[5] have met with partial success, and recent developments in transient state crystallographic techniques appear to have identified such a species in the reaction cycle of $P450_{cam}$.[6] The formal addition of two electrons and two protons to oxygen produces the equivalent of hydrogen peroxide, and these monooxygenases produce water in addition to the oxidized substrate.

The active electrophilic oxidant in P450 usually has been assumed to be a high-valent iron-oxo species, structurally similar to the intermediate Compound I that is known to form in reactions of the heme-containing peroxidase enzymes with hydrogen peroxide.[7] A consensus view of the mechanism of P450-catalyzed hydroxylations by an iron-oxo species evolved over the past two decades, primarily based on the results of mechanistic probe studies and kinetic isotope effects.[2,7] In that mechanism, the iron-oxo species abstracts a hydrogen atom from substrate to give an iron-hydroxy species and an alkyl radical intermediate; the alkyl radical then displaces hydroxy from iron in a process termed "oxygen-rebound"[8] (Figure 1).

Results of the past few years indicate that the hydroxylation reaction is more complex than previously thought. The mechanistic picture began to cloud when ultrafast "radical clocks"[9] were used in attempts to "time" the oxygen rebound step; the amounts of rearranged products did not correlate with the radical rearrangement rate constants. Moreover, hydroxylation of a probe that could distinguish between radical and cationic species by one P450 isozyme indicated that cationic rearrangements were complicating studies with probes/clocks, and the results suggested that hydroxylation occurred by an insertion reaction instead of abstraction and recombination.[10] The latter study presented a new paradox in that it provided evidence that cations could be formed in hydroxylation reactions whereas it is well established that carbocations cannot be requisite intermediates. As discussed later, possible explanations for these results were presented recently.[10−12]

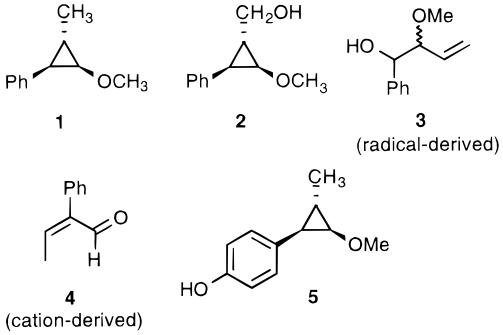

Figure 2. Substrate **1** and its putative products from P450-catalyzed oxidations.

As the mechanistic picture became more complicated, so also did the view of the active oxidant(s) in P450. Studies with expressed P450 isozymes and their mutants lacking a conserved threonine in the active site indicated that multiple oxidizing species are produced in the natural course of P450 oxidations.[13,14] That work implicated two electrophilic oxidant species, one a preferential epoxidizing agent and the other a preferential hydroxylating agent, and a subsequent study indicated that both of these could effect hydroxylation reactions when epoxidation was not possible.[15]

In this work, we report oxidations of two hypersensitive radical probe/clock substrates with wild-type and mutant P450 isozymes. In their capacity as mechanistic probes, both substrates have the potential to differentiate between radical and cationic intermediates, and cationic rearrangement products were found from both. One substrate also serves as a radical clock, and in this mode we find "radical" lifetimes that are too short for a true radical intermediate. We present a mechanistic description of P450-catalyzed hydroxylation reactions that is consistent with the present and previous results. The new features of this description are (1) two electrophilic oxidants, a hydroperoxo-iron complex and an iron-oxo complex, effect hydroxylations via insertion processes and (2) cationic rearrangements occur from solvolytic-type reactions of protonated alcohols, the first-formed products from hydroxylation by the hydroperoxo-iron species.

Results

Substrates and Oxidation Products. One of the probe substrates used in this work was *trans*,*trans*-2-methoxy-3-phenylmethylcyclopropane (**1**) which is shown in Figure 2 with its oxidation products. The mechanisms of the rearrangement reactions are discussed later. Oxidation of the cyclopropyl methyl group in **1** can give the unrearranged alcohol **2**, two diastereomers of benzylic alcohol **3** derived from rearrangement of a cyclopropylcarbinyl radical, and aldehyde **4** derived from rearrangement of a cyclopropylcarbinyl cation. Oxidation of the phenyl group in probe **1** would give phenol **5** or other regioisomeric phenols. Oxidation of the methoxy methyl group in probe **1** would produce a formaldehyde hemiacetal that hydrolyzes to give the demethylated cyclopropanol product, but we did not attempt to identify this product.

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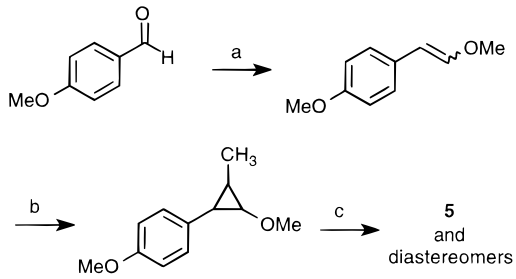
Scheme 1[a]

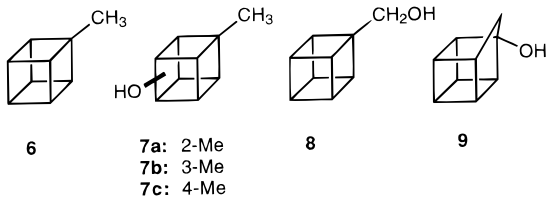


[a] Conditions: (a) $CH_3OCH(Li)P(Ph)_3$; (b) Et_2Zn, CH_3CHI_2; (c) NaSEt.

Products **2**, **3**, and **4** were identified in product mixtures from P450 oxidations of probe **1** by comparison of the GC retention times and mass spectral fragmentation patterns to those of authentic samples. Compounds **2**[16] and **4**[10] were previously known. A mixture of diastereomers of **3** was prepared by reaction of a methoxyallyl-tin reagent with benzaldehyde by the general procedure reported by Koreeda and Tanaka.[17]

An authentic sample of phenol **5** was not isolated as a pure compound, but it was possible to demonstrate that phenol **5** was not a significant product in the P450-catalyzed oxidations of substrate **1**. A mixture of **5** and its three diastereomeric isomers was prepared by the method shown in Scheme 1. Thus, reaction of *p*-anisaldehyde with the methoxymethyl Wittig reagent gave a ca. 1:1 mixture of *cis*- and *trans*-1-methoxy-2-(*p*-methoxyphenyl)ethene that was treated with an ethyl carbenoid reagent to give a mixture of four diastereomeric products. Demethylation of these compounds with sodium ethylthiolate gave **5** and its diastereomers which were isolated as a mixture. GC analysis of the mixture showed four components with baseline resolution, all with similar mass spectral fragmentation patterns and strong molecular ions. GC analyses of the P450 oxidation products of **1** demonstrated that none of the components of the mixture containing **5** was present in significant amounts, and separation of **5** from its diastereomers was deemed pointless.

The second probe substrate used in this work was methylcubane (**6**) which is shown below with its possible oxidation products. Again, the mechanisms of the rearrangement reactions



are discussed later. Oxidation of the cubyl positions in **6** gives three regioisomeric methylcubanol products (**7**). Oxidation of the methyl group in **6** could give unrearranged cubylmethanol (**8**) or the cube-expanded product 1-homocubanol (**9**) from a cationic rearrangement process. Several radical-derived rearrangement products from **6** are possible in principle, all in which the cube skeleton has been destroyed;[18] these products are expected to be highly unstable (see below).

Compounds **8** and **9** were identified in the product mixtures from P450-catalyzed oxidation of probe **6** by comparison of the GC retention times and mass spectral fragmentation patterns to those of authentic samples. Compound **8** is an intermediate in the preparation of **6**,[19] and compound **9** is produced by acid-catalyzed rearrangement of **8**.[20] The regioisomeric methylcubanols **7** were detected as products from oxidation of **6** by a methane monooxygenase (MMO) system,[21] but they were not isolated. In the P450-catalyzed oxidations of **6** conducted in this work, we observed by GC three products that had mass spectra the same as those reported for compounds **7** in the MMO study[21] in addition to products **8** and **9**.

The identities of the products **7**, as well as those of products **8** and **9**, were verified by treatment of the P450 product mixtures with acetic anhydride and pyridine to give the corresponding acetates followed by GC and mass spectral analyses. Oxidation of probe **6** by one P450 isozyme, specifically P450 2B1, followed by derivatization to the acetates was previously shown to give the acetates **7a**-OAc, **7c**-OAc and **8**-OAc, as determined by comparison to authentic samples, and another acetate product.[19] The fourth acetate product found in that work was identified as **7b**-OAc on the basis of the similarity of its GC retention time and mass spectral fragmentation pattern to those of acetates **7a**-OAc and **7c**-OAc.[19] The same acetates were obtained from derivatization of the product mixtures obtained with all P450 isozymes used in this work. In addition, acetate **9**-OAc was present after derivatization of product mixtures from reactions with the Δ2B4 and Δ2E1 isozymes and their mutants; an authentic sample of **9**-OAc was prepared from **9**.

From analyses of the alcohol products from **6** and their acetate derivatives, we conclude that cubanols **7** were partially decomposed during the GC analysis. In several cases, the GC peak shapes for **7** were not symmetrical, but more importantly, the ratios of products **7** to product **8** obtained from the analyses of the alcohols and the acetates were not the same. Furthermore, product **9** appeared to be partially decomposed in the acetate derivatization procedure; again, this was deduced from the ratios of products observed before and after derivatization. Because the ratios of the products from oxidation of the methyl group in substrate **6** were most important for this work, we quantitated the alcohol mixtures with the result that the yields of cubanols **7** discussed below are variable. We emphasize that the ratios of products from methyl group oxidation of substrate **6** (i.e. products **8** and **9**) are not affected by partial loss of products **7**.

P450-Catalyzed Oxidation Reactions. Probes **1** and **6** were oxidized by various hepatic P450 isozymes and mutants. P450 2B1 and P450 2B4 are the phenobarbital-inducible isozymes from rat and rabbit, respectively. The truncated isozyme P450 Δ2B4 is the expressed rabbit liver enzyme with N-terminal amino acids 2−27 deleted, and P450 Δ2B4 T302A is the Thr-302 to Ala mutant of this isozyme.[14] P450 Δ2E1 is the expressed rabbit liver ethanol-inducible isozyme with N-terminal amino acids 3−29 deleted, and P450 Δ2E1 T303A is the Thr-303 to Ala mutant of this isozyme.[13]

The enzyme-catalyzed oxidations were performed under conditions similar to those employed in previous studies.[15] Following the oxidation reactions, the products were extracted into CH_2Cl_2. Product quantitation was accomplished by GC analysis on a Carbowax column with FID detection. Product identifications were achieved as noted above. For some oxidation reactions, the turnovers were so small that the amounts of products were not sufficient for high-quality GC-mass spectral analyses, and the identities of the products in those cases rely

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

isozyme	**2**	**3**	**4**	turnover[b]
2B1	85	11	4	24
2B4	90	7	3	166
Δ2B4	93	5	2	336
Δ2B4 T302A	90	5	5	20
Δ2E1	89	9	2	44
Δ2E1 T303A	79	9	12	22

[a] Average relative percentage yields of **2**, **3**, and **4** from duplicate or triplicate runs. [b] Average turnover values.

on the GC retention times and the expectation that similar product mixtures were formed with all enzymes. Small peaks with GC retention times consistent with phenol **5** and its diastereomers were observed in most reactions of **1**, but we could not confirm the identity of **5** in the enzyme product mixtures by GC-mass spectral analysis due to the small amounts of materials.

Table 1 summarizes the results from P450-catalyzed oxidations of probe **1**; complete results are given in the Supporting Information. Unrearranged alcohol **2**, radical-derived rearrangement products **3**, and cation-derived rearrangement product **4** were produced in all cases. As noted, phenol product **5** was not produced in significant amounts in these oxidations, but small peaks with appropriate retention times for product **5** were present in the GC traces. If we assume that the largest peaks observed in this region of the GC trace were due to **5**, then the amount of **5** formed in various P450 oxidations was in the range of tenths of nanomoles.

Products **2**−**4** were stable to the GC conditions used for analysis. Control reactions were performed to determine whether these products were stable to the enzyme reaction conditions and could be recovered in good yield. The detailed results of the control reactions are given in the Supporting Information. Samples of the authentic oxidation products in amounts similar to those found in the oxidations of substrate **1** were employed with (A) reaction mixtures containing fully competent enzyme oxidation systems, (B) mixtures lacking the enzymes, and (C) mixtures lacking NADPH, and the amounts of material remaining after incubation, reaction, and workup were determined. In the case of cyclic alcohol **2** and the acyclic alcohols **3**, the test substrates were returned in high yields. Acyclic alcohols **3** and aldehyde **4** were not produced in the control reactions for product **2** which indicates that **3** and **4** were not formed in secondary reactions.

Aldehyde **4** was unstable when tested in the absence of a cosubstrate. A reaction with the fully competent P450 2B1 enzyme system containing 29 nmol of **4** resulted in complete loss of the aldehyde. However, when aldehyde **4** (25 nmol) was admixed with probe **1** in a reaction with the 2B1 isozyme, no loss of product was observed. Given that **4** was obtained in a yield of only 0.5 nmol from the oxidation of **1** by the 2B1 isozyme, this result suggests that substrate **1** served as a competitive inhibitor for enzyme-catalyzed consumption of **4**, although it is possible in principle that the amounts of **4** produced and destroyed in the control reaction were the same. To test the latter possibility, a reaction was conducted with the 2B1 isozyme in which *trans*-2-(*p*-methoxyphenyl)methylcyclopropane (**10**)[22] was used as a surrogate for substrate **1**, and aldehyde **4** was present in a small amount (1.6 nmol). Aldehyde **4** was stable in this test reaction indicating that substrate **10** was a competitive inhibitor for consumption of **4** and reinforcing

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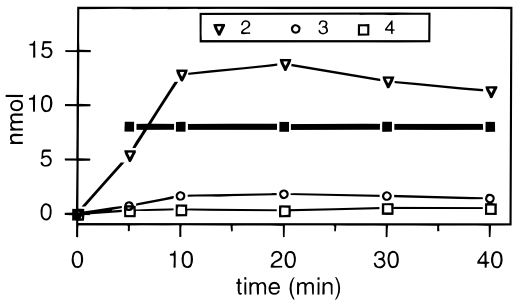


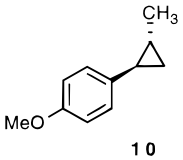
Figure 3. Time course study for oxidation of **1** with P450 2B1. The yields of each product (in nmol) are given; the thick line is the ratio of (**2** + **4**) to **3**.

Table 2. Products from Cytochrome P450-Catalyzed Oxidations of Probe **6**[a]

isozyme	(**7a** + **7b** + **7c**)	**8**	**9**	turnover[b]
2B1 (batch 1)[c]	31	68	0[d]	nd
2B1 (batch 2)[c]	36	66	0[d]	24
2B4	11	89	0[d]	98
Δ2B4	19	80	1	61
Δ2B4 T302A	31	48	21	12
Δ2E1 (batch 1)[c]	42	48	10	21
Δ2E1 (batch 2)[c]	24	70	6	105
Δ2E1 T303A	29	56	15	14

[a] Average percentage yields of **7**−**9** from duplicate or triplicate runs. [b] Average turnover value; nd = not determined. [c] Results from two samples of enzymes. [d] Trace of product **9** (<1%) detected in GC-MS analysis.

the conclusion that substrate **1** also protects product **4** by competitive inhibition.



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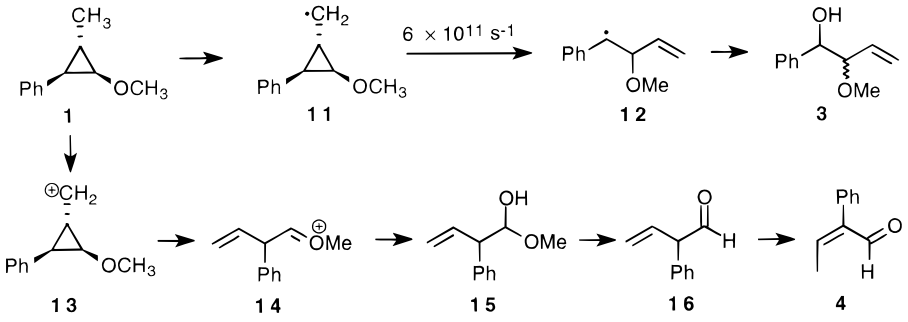
As a further test of stabilities of the products from substrate **1**, a series of reactions with the 2B1 isozyme was conducted over varying times. Figure 3 shows the yields in nanomoles of products **2**, **3**, and **4** for a series of reactions conducted between 5 and 40 min. Apparently, an enzyme was destroyed or NADPH was consumed within 10 min of initiating the reactions. It is possible that small amounts of products were lost on extended standing, but most importantly, the ratio of ([**2**] + [**4**])/[**3**], indicated by the heavy line in Figure 3, was invariant in the time course study. This ratio provides the lifetime of the putative radical formed by hydrogen atom abstraction as discussed later.

Table 2 contains a summary of results from P450-catalyzed hydroxylations of methylcubane (**6**); complete results are given in the Supporting Information. Because methylcubane is quite volatile, we were unable to determine percent recoveries of unreacted substrate. The three methylcubanol products (**7**) and cubylmethanol (**8**) were observed in all oxidations. 1-Homocubanol (**9**) was observed by GC using FID detection in reactions with four of the six isozymes. For the two cases where product **9** was not observed with FID detection, the 2B1 and 2B4 isozymes, GC-MS analyses of the reaction mixtures showed that traces of **9** (<1% relative yield) were present.[23]

The GC and GC-mass spectral analyses of the product mixtures before and after acetate derivatization provided firm identifications of the products. The results from oxidations of probe **6** are qualitatively meaningful. They are, however, less reliable in a quantitative sense than those from oxidations of

Scheme 2



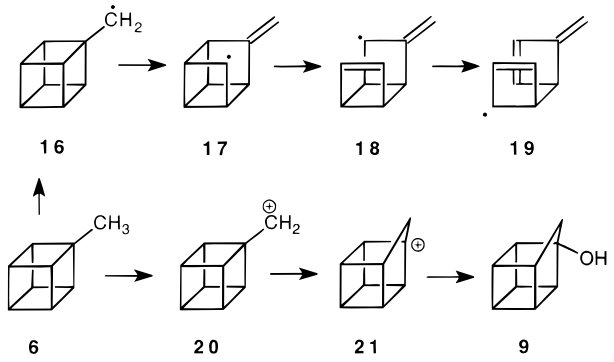
probe **1**. Synthetic routes for production of authentic samples of the methylcubanols (**7**) are not available, so GC response factors could not be determined. We made the reasonable assumption that the flame ionization response factors for compounds **7** were equal to that for the isomeric compound **8**. A further complication in quantitation resulted from the apparent partial decomposition of compounds **7** on the GC column as noted above.

Control reactions with products **8** and **9** were conducted in a manner similar to those conducted with the products from substrate **1**. Results are given in the Supporting Information. Good consistency in product recoveries was observed, but the yields were somewhat reduced from those found with products **2**−**4**. The slight reductions in recoveries were independent of the presence of enzymes, and we conclude that these compounds were not appreciably oxidized. The loss apparently is due to either the distribution of products **8** and **9** between the buffer and CH_2Cl_2 used for extraction or, more likely, some evaporation of the products during the solvent removal step preceding analysis. The recovery−stability results present yet another caution regarding the precision of the quantitative data for studies with substrate **6**. Most importantly, however, the control experiments demonstrate that product **9** was not formed from **8** in reactions of fully competent enzyme systems.

Discussion

How the Probes Work. The hydrogen abstraction−oxygen rebound mechanism for P450-catalyzed hydroxylations shown in Figure 1 was generally accepted at the beginning of the 1990s. It is qualitatively consistent with the observations of rearranged products from oxidations of substrates that are precursors to highly reactive radicals, but it is not quantitatively consistent. For example, oxidations of kinetically calibrated hypersensitive radical probes gave apparent rebound barriers ranging from 4 kcal/mol found with bicyclo[2.2.0]pentane[24−26] to 0 kcal/mol found with a constrained aryl-substituted cyclopropane probe.[27] We proposed that the inconsistencies might be the result of multiple oxidation pathways, with both radical and cationic rearrangements possible, and a shortcoming of the probe designs; that shortcoming was that all probes from which rearranged products had been obtained would suffer the same skeletal reorganization from reactions of radicals or cations.[28] Both of the substrate probes used in the present work display distinctly different rearrangement patterns for radicals and cations.[29]

Scheme 3



The reaction manifolds for the cyclopropane-based probe **1** are shown in Scheme 2. The cyclopropylcarbinyl radical **11** rearranges with high regioselectivity, 160:1 at ambient temperature,[16,28] to the benzylic radical **12**; in the context of enzyme-catalyzed hydroxylations, benzyl alcohols **3** are produced from this pathway. An incipient cyclopropylcarbinyl cation (**13**) opens with even higher regioselectivity, >1000:1, to oxonium ion **14**.[28] The hydroxylation product from the cationic route is an unstable hemiacetal (**15**) that hydrolyzes to the β,γ-unsaturated aldehyde **16** that, in turn, was found to isomerize to aldehyde **4** in the enzyme buffer medium.[10] In addition to the high fidelity of the two rearrangement pathways, relative rate constants for the radical ring opening of **11** in competition with PhSeH trapping were determined.[16] Using the product ratios from that work and the recently recalibrated rate constants for reactions of PhSeH with alkyl radicals,[30] one computes a rate constant for ring opening of **11** at 37 °C of 6×10^{11} s^{-1}.

The reaction manifolds for oxidation of the methyl group methylcubane are shown in Scheme 3. The cubylcarbinyl radical (**16**) experiences a cascade of bond-cleavage reactions.[18,31] The first rearranged radical (**17**) has not been trapped, and it is

(23) Substrate **6** was previously studied with microsomes from livers of phenobarbital-treated rats, which contain mainly P450 2B1, and the purified 2B1 isozyme (ref 19). In that study, the alcohol products were converted to acetates that were analyzed, and the amounts of products **7**-OAc and **8**-OAc found were comparable. Given the apparent decomposition of alcohols **7** in GC analyses, the yields of **7** relative to **8** found here are in reasonable agreement with those previously reported.

(24) Ortiz de Montellano, P. R.; Stearns, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 3415−3420.

(25) Newcomb, M.; Manek, M. B.; Glenn, A. G. *J. Am. Chem. Soc.* **1991**, *113*, 949−958.

(26) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5687−5698.

(27) Newcomb, M.; Le Tadic, M. H.; Putt, D. A.; Hollenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 3312−3313.

(28) Newcomb, M.; Chestney, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 9753−9754.

(29) It is noteworthy that the concept of studying P450 hydroxylations with a probe that can differentiate between radicals and cations was put forth by Groves. Specifically, bicyclo[4.1.0]heptane was used as a probe because radicals and cations at the cyclopropylcarbinyl position of this species give different rearrangement products. P450-catalyzed hydroxylation of this probe, however, resulted in no rearrangement products. See: White, R. E.; Groves, J. T.; McClusky, G. A. *Acta Biol. Med. Ger.* **1979**, *38*, 475−489.

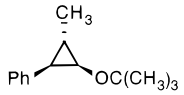
(30) Newcomb, M.; Choi, S.-Y.; Horner, J. H. *J. Org. Chem.* **1999**, *64*, 1225−1231.

(31) Choi, S.-Y.; Eaton, P. E.; Newcomb, M.; Yip, Y.-C. *J. Am. Chem. Soc.* **1992**, *114*, 6326−6329.

possible that simultaneous bond cleavages occur taking **16** directly to **18**. Radical **18** can be trapped, but in the absence of reactive trapping agents, it further reacts to give **19**.[18] An incipient cubylcarbinyl cation (**20**) suffers a bond migration to give the 1-homocubyl cation (**21**).[20,32,33] The regioselectivities of the two pathways apparently are high, none of the "wrong" rearrangement product has been reported for either pathway, but the relatively high reactivities of the observed and putative products have precluded precise determinations of regioselectivity. Rate constants for rearrangement of radical **16** to radical **18** are known (e.g., 3×10^{10} s^{-1} at ambient temperature),[31] but it is unlikely that radical-derived rearrangement products from hydroxylation of **6** would be stable. The strained tricyclooctadiene products from hydrogen atom trapping of **18** are not stable upon standing at ambient temperature,[18] and the corresponding alcohols should also be subject to solvolytic reactions in the enzyme medium. A recent report[21] that a radical-derived rearrangement product was obtained by methane monooxygenase (MMO) oxidation of **6** appears to be in error as the published[21] mass spectrum of the putative radical rearrangement product is the same as that of authentic 1-homocubanol (**9**),[34] and **9** was shown to be a product from MMO oxidations of **6** in a more recent work.[34]

Probe **6** was also oxidized at the cube positions giving products **7**. Formation of cubanols **7** requires an electrophilic oxidant,[35] and alkoxyl radical abstraction from **6** occurs on the cube.[19,36] Whereas the formation of products **7** speaks to the character of the oxidizing species, it does not provide readily interpreted mechanistic information.

Multiple Reaction Pathways in P450-Catalyzed Hydroxylations. That radical and cationic rearrangement pathways occur in P450 hydroxylation reactions[28] is clearly demonstrated by the results with probe **1** listed in Table 1. The discrimination factor for this probe, the product of the regioselectivities for the two types of intermediates, exceeds 100 000, and there is little doubt about the radical origin of product **3** and the cationic origin of product **4**. A similar demonstration of two distinct pathways for rearrangement was previously reported for the 2B1 isozyme with a related probe.[10] Specifically, oxidations of probe **22** by microsomes from livers of phenobarbital-treated rats, which contain mainly P450 2B1, and by the purified P450 2B1 isozyme gave mixtures of unrearranged product, radical-derived rearrangement product, and cation-derived rearrangement product in a product distribution similar to those found here with probe **1**.



22

The observations of cationic rearrangement products in the P450 hydroxylations are the unexpected results because there is no pathway for formation of these species in what is, or was, clearly the consensus view of P450-catalyzed hydroxylation (i.e., Figure 1). Thus, the results from oxidations of methylcubane (**6**) are important. The cationic rearrangement product 1-homocubanol (**9**) was formed in appreciable amounts with several of the P450 isozymes. Given the differences in the structural features of probes **1** and **6** and their different modes of rearrangements, it is difficult to formulate any rationalization wherein the rearrangement products **4** and **9** are produced other than from cationic species. The demonstrations that authentic samples of unrearranged alcohols **2** and **8** gave no detectable **4** and **9**, respectively, when tested with the enzymes indicates that the rearrangement products were produced in the hydroxylation reactions. One appears to be left only with the explanation that cationic species were produced in part in the enzyme-catalyzed reactions. Multiple reaction pathways are strongly suggested.

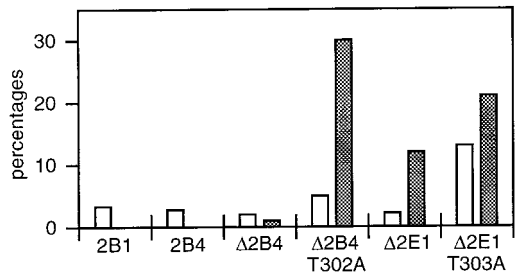


Figure 4. Percentages of cationic rearrangement products relative to total methyl group oxidation found in P450-catalyzed hydroxylations of substrates **1** (unfilled) and **6** (filled).

Moreover, the patterns in the product distributions for the two probes are roughly correlated in a manner consistent with multiple reaction pathways. Figure 4 shows the percentages of cationic rearrangement products from **1** and from oxidation of the methyl group in **6** for the various enzymes studied. Increased amounts of cationic rearrangement products were found with the mutants, which were designed to disrupt the normal sequence of events in P450 oxidations. Later in discussion, we will attribute the cationic rearrangement products to reaction of a specific P450 oxidant species.

Radical Lifetimes in P450-Catalyzed Hydroxylations. That both radical- and cationic-type rearrangements can occur in P450-catalyzed hydroxylations is important for understanding the results of previous mechanistic probe studies. Both pathways occurred for the probes studied here, and there is little reason to believe that they could not also occur when other, "non-distinguishing" probes were employed. Because both radical and cationic rearrangements of those other probes ultimately would give the same products, one has no method for determining how much of any observed rearranged product should be ascribed to a radical pathway. Therefore, all "radical lifetimes" calculated from the product distributions found in P450-catalyzed hydroxylations of those probes can only be upper limits.

From the design of probe **1**, one is confident that benzylic alcohol products **3** are not from cations; they must be produced in a radical process. Table 3 lists the ratios of non-radical-derived hydroxylation products (**2** and **4**) to radical-derived products **3** for each isozyme studied. These ratios can be used with the rate constant for rearrangement of the cyclopropylcarbinyl radical **11** to compute the apparent "rebound" rate constants[37] and the lifetimes ($1/k$), both of which are also listed in Table 3. The consistency of the values for both of the wild-type and mutant pairs is satisfying. When compared to results from other probes, these results clearly demonstrate the importance of separating radical-derived and cation-derived rear-

(32) Eaton, P. E. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1421−1436.
(33) Smith, B. J.; Tsanaktsidis, J. *J. Org. Chem.* **1997**, *62*, 5709−5712.
(34) Choi, S.-Y.; Eaton, P. E.; Kopp, D. A.; Lippard, S. J.; Newcomb, M.; Shen, R. *J. Am. Chem. Soc.* **1999**, *121*, 12198−12199.
(35) Hrovat, D. A.; Borden, W. T. *J. Am. Chem. Soc.* **1994**, *116*, 6459−6460.
(36) Della, E. W.; Head, N. J.; Mallon, P.; Walton, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 10730−10738.

(37) The radical lifetimes were calculated by a conventional competition kinetics. The apparent rebound rate constant is equal to the ratio of nonradical-derived to radical-derived products, ([**2**] + [**4**])/[**3**], times the rate constant for ring opening of radical **11**, 6×10^{11} s^{-1}. See: Newcomb, M. *Tetrahedron* **1993**, *49*, 1151−1176.

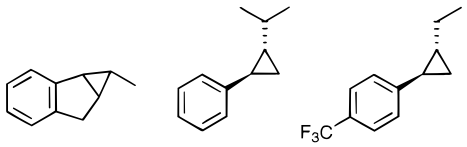
Table 3. Radical Lifetimes in Cytochrome P450-Catalyzed Oxidations of Probe **1**[a]

isozyme	non-rad/rad[b]	k_{ox}[c]	lifetime (fs)[d]
2B1[e]	8.1 ± 0.7	5×10^{12}	200
2B4	13.0 ± 0.6	8×10^{12}	130
Δ2B4	20.0 ± 0.1	1.2×10^{13}	80
Δ2B4 T302A	19.2 ± 0.4	1.2×10^{13}	80
Δ2E1	9.6 ± 1.7	6×10^{12}	170
Δ2E1 T303A	9.8 ± 3.3	6×10^{12}	170

[a] Average values from duplicate runs unless noted. [b] Ratio of (**2** + **4**) to **3**; stated error is one standard deviation. [c] Apparent oxygen rebound rate constant. [d] Lifetime of radical in femtoseconds. [e] Average value from eight runs.

rangement products. For example, hydroxylations of a hypersensitive probe in which no epoxidation pathway was available with the wild-type and mutant pairs used here resulted in ratios of rearranged to unrearranged products that varied dramatically.[15]

The computed radical lifetimes of 80−200 fs are in good agreement with the value of 100 fs found in oxidation of probe **22** with the P450 2B1 isozyme.[38] Previous results with other probes are also seen to be consistent with these lifetimes when one understands that the values computed from those studies can only be upper limits. Some of those upper limits are quite short, for example, lifetimes of <200 fs were found in P450-catalyzed hydroxylations probes of **23**−**25**.[27,39] Lifetimes of 80−200 fs are too short for a true intermediate, and the corresponding rate constants listed in Table 3 are in the range expected for vibrational rate constants, not for chemical rate constants. That is, the lifetimes are those of transition states.[40] *True radical intermediates are not formed.*



23 **24** **25**

If no radical intermediates are formed, then the hydroxylation reactions must be insertion processes. The small amounts of radical rearrangement products found reflect a bifurcation in the transition state between collapse without ring opening and ring opening followed by collapse. Although dynamicists might have no problem with the concept of a bifurcation from a transition structure, many chemists and biochemists find this notion unusual. Another way to perceive the process is that collapse of the "radical" formed by abstraction occurs with no barrier but still requires an exquisitely short period of time (a vibrational period) during which some ring opening occurs.

Multiple Cytochrome P450 Oxidants and the Origins of the Cation Species. The implication of a cationic species resulting in rearranged products is clear from the results with the substrates studied in this work and those found previously with probe **22**.[10] That a cationic species can be formed in the hydroxylation reaction might explain why other probes gave conflicting results in regard to the apparent lifetimes of radicals, but it also presents a new mechanistic puzzle. One must formulate a mechanism wherein cationic rearrangements are possible, but carbocations are not required. The latter condition is absolute because requisite carbocations would result in ring-opened products from all methylcyclopropane probes.

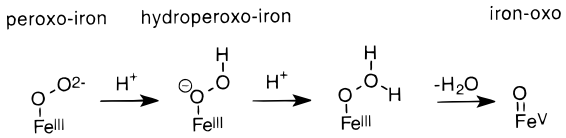


Figure 5. The consensus pathway for production of the iron-oxo species from the peroxo-iron species where Fe represents heme-bound iron.

Some recent mechanistic descriptions of P450 hydroxylations have attempted to address the origin of cationic products. Those explanations include rearrangement of the probe while bound to iron in an agostic complex,[11] competing production of a spin-paired complex (insertion) and non-spin-paired complex (radical intermediate) with electron transfer from the radical,[12] and insertion of the elements of OH^+ followed by solvolysis of the resulting protonated alcohol in competition with deprotonation,[10,39] the latter implying that the ultimate oxidant was iron-complexed hydrogen peroxide instead of iron-oxo. Elements of these mechanistic proposals might explain cationic rearrangements, but they contained shortcomings that have been discussed.[39] We believe that a problem with these descriptions is that they have been cast in terms of a single oxidizing species, whereas a unified mechanistic view must incorporate recent evidence that two distinct electrophilic oxidants are active in P450-catalyzed reactions.

The sequence of events in P450-catalyzed oxidation reactions involves substrate binding, reduction of the ferric form of the enzyme, oxygen binding to give a ferrous-dioxygen species, and a second reduction step to give a peroxo-iron species that ultimately gives oxidizing species. Neither the active oxidant(s) in P450 nor any intermediate following the ferrous-dioxygen species has been characterized unambiguously, but the common belief has been that the oxidant is an iron-oxo species related to the spectroscopically observed Compound I of peroxidase chemistry.[2] A commonly accepted route to the iron-oxo species, supported by recent computational work by Harris and Loew,[41] is shown in Figure 5. Initial protonation of the distal oxygen in the peroxo-iron complex gives a hydroperoxo-iron species, and a subsequent protonation of the distal oxygen gives a species that loses water to give an iron-oxo complex similar to Compound I of the peroxidases. The computational results further indicated that the loss of water from the penultimate complex shown in Figure 5 to give the iron-oxo species has no barrier, and that initial protonation of the proximal oxygen in the peroxo-iron species would give a considerably higher energy species than that formed by distal oxygen protonation.[41]

Despite the widespread belief that an iron-oxo species is the active oxidant in P450 reactions, the diverse oxidations effected by these enzymes suggest that different types of oxidants are involved in some specific functions. For example, the iron-oxo species is an electrophilic oxidant that would seem to be the appropriate character for epoxidation and hydroxylation reactions. Alternatively, the deformylation reaction effected by P450 aromatase and similar deformylations of various xenobiotic aldehydes by microsomal P450s[42,43] are consistent with nucleo-

(38) On the basis of the recalibration of PhSeH trapping kinetics (ref 30), the rate constant for rearrangement of the radical derived from probe **22** (ref 16) must be adjusted. This, in turn, results in an adjustment of the radical lifetime in the 2B1 oxidation of **22** (ref 10) from 70 to 100 fs.

(39) Toy, P. H.; Newcomb, M.; Hollenberg, P. F. *J. Am. Chem. Soc.* **1998**, *120*, 7719−7729.

(40) The lifetime of a transition state at ambient temperature from classical transition state theory is 170 fs.

(41) Harris, D. L.; Loew, G. H. *J. Am. Chem. Soc.* **1998**, *120*, 8941−8948.

(42) Vaz, A. D. N.; Roberts, E. S.; Coon, M. J. *J. Am. Chem. Soc.* **1991**, *113*, 5886−5887.

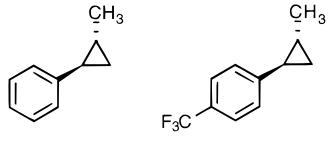
(43) Coon, M. J.; Vaz, A. D. N.; Bestervelt, L. L. *FASEB J.* **1996**, *10*, 428−434.

philic oxidants that react in Baeyer−Villiger-type processes. In fact, a nucleophilic peroxo-iron porphyrin was recently shown by Valentine and co-workers to mimic aromatase behavior.[44] The nucleophilic oxidizing species produced in reactions of P450 is not responsible for oxidations studied here, however, because the oxidant must have electrophilic character.

The mutants used in this work were designed to study the potential disruption of the sequence of electrophilic oxidant formation in Figure 5. Threonine is highly conserved in the active sites of P450 enzymes[45] indicating a specific role for the hydroxy group, and in crystal structures of bacterial P450 enzymes, threonine is located within hydrogen-bonding distance of a putative hydroperoxo-iron species.[46] One proposed role for the conserved threonine is protonation of the hydroperoxo-iron species, possibly via a relay.[47]

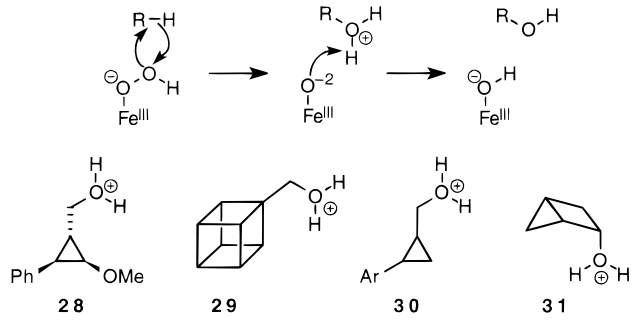
Recent studies with both Δ2B4 and Δ2E1 and their respective mutants indicated that two *electrophilic* oxidants are produced in the natural course of P450 oxidations.[13] In that work, the ratios of epoxidation to allylic oxidation products for a series of alkenes was found to differ for the isozymes and their respective mutants. The conclusions were that both the hydroperoxo-iron species and the ultimate oxidant were electrophilic oxidizing entities and that the epoxidation was the preferred reaction of the former whereas hydroxylation was the preferred reaction of the latter. The proposed effect of substitution of alanine for threonine in the mutants was either to slow the overall rate of formation of the ultimate oxidant or to affect an equilibrium reaction involving protonation;[13] on the basis of the computational finding of a barrierless loss of water following the second protonation,[41] one would now favor the former possibility.

More recent studies with the same wild-type and mutant enzymes supported the conclusion that two electrophilic oxidant forms exist and, moreover, indicated that both species were capable of effecting hydroxylation.[15] Those studies involved the hypersensitive probes **26** and **27**. Consistent ratios for the rearranged and unrearranged products from methyl group oxidation in probe **26** (where an arene epoxidation pathway exists) were found for the wild-type and mutant pairs, but the ratios of rearranged to unrearranged products varied dramatically for probe **27** (where arene epoxidation is not possible). The deduction from those observations was that each electrophilic oxidant was displaying a characteristic product ratio for methyl group oxidation, that most of the methyl group oxidation in probe **26** was attributable to one oxidizing species, and that both oxidizing species effected methyl group hydroxylation in probe **27**.[15]



26 **27**

The demonstration of two active electrophilic oxidant forms for P450, the hydroperoxo-iron species and the iron-oxo, provides a straightforward explanation for the production of cation-derived rearrangement products. Hydroxylation by the iron-oxo species would involve insertion of an oxygen atom,



28 **29** **30** **31**

Figure 6. Likely mechanism for hydroxylation by the P450 hydroperoxo-iron species where Fe represents heme-bound iron and first-formed protonated alcohols from various probes.

but hydroxylation by the hydroperoxo-iron species would involve insertion of the elements of OH^+ (Figure 6). The latter reaction gives protonated alcohol products such as **28** and **29** for the two probes studied in this work or **30** and **31** for probes used in previous studies (see Figure 6). Intermediates **28**−**30** would be expected to be highly reactive with respect to solvolytic reactions with anchimeric assistance, and the bicyclo[2.1.0]pentyl system is also known to be quite reactive in cationic processes that cleave the central C−C bond.[48] It would appear that all probes that have been found to give rearranged hydroxylation products in P450 oxidations are reasonable candidates for cationic rearrangements from first-formed protonated alcohols.

We suggest, therefore, that cationic rearrangement products derive from hydroxylation reactions effected by the hydroperoxo-iron species. If the formulation of the effects of the Thr to Ala replacement in the mutant enzymes proposed here is correct, then larger percentages of hydroxylation by the hydroperoxo-iron species should have occurred with the mutants, resulting in larger amounts of cationic rearrangement products. In general, this is the case as shown in Figure 4. Supporting evidence also is available in the results from oxidations of probe **27** which cannot react by arene epoxidation; specifically, substantially more rearranged products were produced with the mutants than with the wild-type isozymes suggesting a larger percentage of cationic rearrangement products.[15]

A Unified Mechanistic View of P450-Catalyzed Hydroxylations. A unified description of P450-catalyzed hydroxylation reactions thus involves the following features. (1) Both the hydroperoxo-iron complex and the iron-oxo species can effect hydroxylation reactions. (2) Hydroxylations by both species involve insertion processes, that is, no radical intermediates are produced in the reactions. (3) Hydroxylation by the iron-oxo species involves insertion of an oxygen atom giving a neutral alcohol product directly. (4) Hydroxylation by the hydroperoxo-iron species involves insertion of the elements of OH^+, and the first formed product is a protonated alcohol. (5) Protonated alcohols formed in the latter process can rearrange by cationic routes. Figure 7 summarizes the properties of the oxidants in P450 implicated by the present work and previous studies.[13−15]

(44) Wertz, D. L.; Sisemore, M. F.; Selke, M.; Driscoll, J.; Valentine, J. S. *J. Am. Chem. Soc.* **1998**, *120*, 5331−5332.

(45) Nelson, D. R.; Strobel, H. W. *Biochemistry* **1989**, *28*, 656−660. Gotoh, O.; Fujii-Kuriyama, Y. In *Frontiers in Biotransformation*; Ruckpaul, K., Rein, H., Eds.; Akademie: Berlin, 1989; Vol. 1, pp 195−243.

(46) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687−700. Ravichandran, K. G.; Boddupalli, S. S.; Hasemann, C. A.; Peterson, J. A.; Deisenhofer, J. *Science* **1993**, *261*, 731−736. Hasemann, C. A.; Ravichandran, K. G.; Peterson, J. A.; Deisenhofer, J. *J. Mol. Biol.* **1994**, *236*, 1169−1185. Cupp-Vickery, J. R.; Poulos, T. L. *Nat. Struct. Biol.* **1995**, *2*, 144−153.

(47) Imai, M.; Shimada, H.; Watanabe, Y.; Matsushima-Hibiya, Y.; Makino, R.; Koga, H.; Horiuchi, T.; Ishimura, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7823−7827. Martinis, S. A.; Atkins, W. M.; Stayton, P. S.; Sligar, S. G. *J. Am. Chem. Soc.* **1989**, *111*, 9252−9253.

(48) Wiberg, K. B.; Williams, V. Z., Jr.; Friedrich, L. E. *J. Am. Chem. Soc.* **1970**, *92*, 564−567.

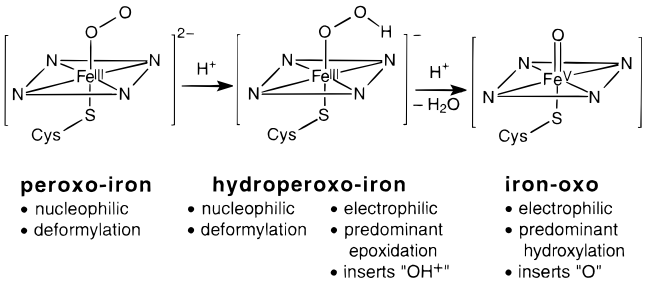


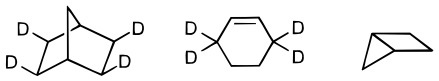
Figure 7. Multiple oxidants generated by cytochrome P450 enzymes; the character and typical reactions of each species are listed.

A seemingly unusual feature of this mechanistic picture is the insertion of OH^+ in the reaction of the hydroperoxo-iron species, but there is good evidence for such reactions. Acid-catalyzed hydroxylation of hydrocarbons by hydrogen peroxide has long been thought to involve OH^+ insertion,[49] and such an insertion is supported by computational results.[50] Perhaps a more meaningful analogy is found in the mechanism of reaction of heme oxygenase, the enzyme that oxidizes heme to biliverdin. On the basis of successful H_2O_2 shunting of the reaction and the failure of other oxidants such as *m*-CPBA to effect reactions, Wilks and Ortiz de Montellano concluded that the heme oxygenase "ferric peroxide" species (structurally the same as the hydroperoxo-iron in Figures 5 or 7) reacted by insertion of OH^+ into a heme C−H bond.[51] The formulation of this aspect of the heme oxygenase reaction sequence[52] is the same as that shown in Figure 6 for reaction of the hydroperoxo-iron species in P450.

It is possible that OH^+ insertion involves reaction of an iron-hydrogen peroxide complex instead of a hydroperoxo-iron complex, and we cannot exclude this possibility. Such a reaction is more akin to acid-catalyzed insertion reactions of hydrogen peroxide noted above. Reaction of iron-complexed hydrogen peroxide was a speculation offered previously to account for production of cationic species in P450 hydroxylations,[10] and Pratt et al. reported evidence suggesting that such a species was involved in at least some P450-catalyzed oxidations.[53] If our formulation of the effects of the Thr to Ala replacement in the mutants is correct, however, the hydroperoxo-iron species is the "other" oxidant, and some evidence supports this view. Specifically, the mutant enzymes reacted with probe **26** to give predominantly phenol products,[15] but phenols were not formed when the same probe was oxidized with the P450-like enzyme chloroperoxidase activated with H_2O_2.[54]

The unified view appears to be consistent with most of the recent high-level computational studies of P450-catalyzed and related hydroxylations, the only real difference being the incorporation of two active oxidants. We noted earlier the computational study of the oxidants in P450 (Figure 5) that provided barriers for the various processes.[41] In a quite recent work, the Shaik group found that reaction of an alkyl radical with a porphyrin radical cation/hydroxy-iron(III) complex on a low spin surface gives an alcohol and prophyrin/iron(III) in a barrier-free process.[55] A ramification of this result is that hydroxylation of an alkane by an iron-oxo species in a low-spin ensemble is predicted to occur effectively via a "concerted insertion" process. High-level computations of hydroxylations by methane monooxygenase enzymes also appear to be converging on the conclusion that collapse of the "radical" with an Fe−OH complex is barrier-free[56] or that the hydroxylation reaction occurs by concerted insertion.[57]

The unified view also is consistent with the results that originally led to the conclusion that hydroxylation occurred by hydrogen abstraction and oxygen rebound (i.e., Figure 1). The most important early evidence came from mechanistic probe studies.[7] Examples include deuterium epimerization in oxidation of **32**,[58] allylic rearrangement in oxidation of **33**,[59] and some ring opening in the hydroxylation of bicyclo[2.1.0]pentane (**34**).[24] The epimerizations and allylic shifts do not require discrete intermediates as these rearrangements should be fast enough to compete with collapse of transition structures. The rearrangement of **34** does require an intermediate, but as noted above, the species that rearranges could be the protonated alcohol **31**.



32 **33** **34**

The other evidence often cited for the abstraction/rebound mechanism is the observation of primary kinetic isotope effects (KIEs) in P450-catalyzed hydroxylations that are similar to those observed in hydrogen atom abstractions by alkoxyl radicals. For example, a recent such study by Dinnocenzo, Jones, and co-workers demonstrated a high correlation between KIEs in P450-catalyzed hydroxylations with those found in reactions of the same substrates with the *tert*-butoxyl radical.[60] Whereas the observed correlations are undoubtedly accurate, the inference that such similarities in KIEs result from the same mechanisms can be questioned. One important lesson from modern computational studies of KIEs is that the assumption that a single vibrational mode is isolated in the transition state of a hydrogen atom transfer reaction is a gross oversimplification.[61] Moreover, Shaik and co-workers point out that the KIE for oxygen atom insertion from an iron-oxo species reacting with an alkane on a low spin surface is expected to mirror that of a hydrogen atom abstraction reaction.[55] That is, the insertion reaction does resemble a hydrogen abstraction to a point and should have a similar KIE, even though no intermediate radical is produced.

In summary, the results from oxidations of probes **1** and **6** by the various P450 isozymes used in this work suggest that production of cationic intermediates apparently is a common feature of P450-catalyzed hydroxylations. We believe these species are most likely protonated alcohols produced by insertion of OH^+ in reactions of the hydroperoxo-iron species formed in the natural course of P450 oxidation reactions. The results from

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oxidations of probe **1** also indicate that hydroxylations by both the iron-oxo and hydroperoxo-iron species occur by insertions, not by initial hydrogen abstractions that give radical intermediates. The unified mechanistic picture of P450-catalyzed hydroxylation, two electrophilic oxidants reacting by insertions, appears to be consistent with virtually all previous results.

Experimental Section

Materials. *trans,trans*-2-Methoxy-3-phenylmethylcyclopropane (**1**), alcohol **2**, and aldehyde **4** were prepared as previously reported.[16] 2-Methoxy-1-phenyl-3-buten-1-ol (**3**) was prepared following the procedure of Koreeda and Tanaka,[17] and the preparation of a 17:1 mixture of *erythro* and *threo* diastereomers of **3** is reported in the Supporting Information. *trans,trans*-2-(*p*-Hydroxyphenyl)-3-methoxy-1-methylcyclopropane (**5**) was obtained as a mixture with its three diastereomers from the reaction sequence shown in Scheme 1; details of the preparation are given in the Supporting Information. Methylcubane (**6**) and cubylmethanol (**8**) were prepared by reported methods.[18,19] 1-Homocubanol (**9**) was prepared by acid-catalyzed reaction of **8** in a slight modification of a reported method.[20]

Enzyme-Catalyzed Oxidations. The preparations of the truncated and mutant P450 isozymes have been reported.[13,14] Oxidations with 2B4, Δ2B4, Δ2B4 T302A, Δ2E1, and Δ2E1 T303A were conducted by the method previously described[13] with the exceptions that the amounts of P450 isozymes and reductase were 0.2 and 0.4 nmol, respectively, and the incubation period prior to addition of substrate and NADPH was reduced from 5 to 2 min. For the oxidations by P450 2B1, 0.6 nmol of P450 and 0.6 nmol of reductase were used in procedures that were otherwise the same as previously reported.[39] Approximately 1.3 μmol of substrates **1** and **6** was used in each reaction.

Enzyme-catalyzed oxidation reactions were conducted at 37 °C. With the exception of the time course study of **1** with P450 2B1, all reactions were allowed to proceed for 30 min. The reactions were quenched by placing the reaction mixtures in an ice bath. The reaction mixtures were extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over $MgSO_4$, filtered, and mixed with a solution containing an internal standard. The mixtures were concentrated under a nitrogen stream to a volume of ca. 0.2 mL and analyzed by GC on Carbowax 20M bonded-phase columns. Quantitation was achieved on an FID-equipped GC using a 15 m × 0.5 mm column. Product identification was achieved by the use of a GC equipped with a mass selective detector using a 25 m × 0.25 mm column. The results are summarized in Tables 1 and 2, and detailed listings of product yields are provided in the Supporting Information.

Control reactions were designed to evaluate the stability of the products and the amounts of products that could be recovered. Typically, the product under evaluation was added to reaction mixtures in approximately the amount produced by enzyme-catalyzed oxidation of the parent probe, and the reactions were worked-up and analyzed in the same method as described above. Detailed results from these studies are given in the Supporting Information.

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Supporting Information Available: Synthetic details for the preparation of **3** and **5**, NMR and mass spectra for **5**, and tables of results from enzyme-catalyzed oxidation studies and control studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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