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Hydroxylation by the Hydroperoxy-Iron Species in Cytochrome P450 Enzymes

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Abstract: Intramolecular and intermolecular kinetic isotope effects (KIEs) were determined for hydroxylation of the enantiomers of *trans*-2-(*p*-trifluoromethylphenyl)cyclopropylmethane (**1**) by hepatic cytochrome P450 enzymes, P450s 2B1, Δ2B4, Δ2B4 T302A, Δ2E1, and Δ2E1 T303A. Two products from oxidation of the methyl group were obtained, unrearranged *trans*-2-(*p*-trifluoromethylphenyl)cyclopropylmethanol (**2**) and rearranged 1-(*p*-trifluoromethylphenyl)but-3-en-1-ol (**3**). In intramolecular KIE studies with dideuteriomethyl substrates (**1**-d_2) and in intermolecular KIE studies with mixtures of undeuterated (**1**-d_0) and trideuteriomethyl (**1**-d_3) substrates, the apparent KIE for product **2** was consistently larger than the apparent KIE for product **3** by a factor of ca. 1.2. Large intramolecular KIEs found with **1**-d_2 ($k_H/k_D = 9-11$ at 10 °C) were shown not to be complicated by tunneling effects by variable temperature studies with two P450 enzymes. The results require two independent isotope-sensitive processes in the overall hydroxylation reactions that are either competitive or sequential. Intermolecular KIEs were partially masked in all cases and largely masked for some P450s. The intra- and intermolecular KIE results were combined to determine the relative rate constants for the unmasking and hydroxylation reactions, and a qualitative correlation was found for the unmasking reaction and release of hydrogen peroxide from four of the P450 enzymes in the absence of substrate. The results are consistent with the two-oxidants model for P450 (Vaz, A. D. N.; McGinnity, D. F.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3555), which postulates that a hydroperoxy-iron species (or a protonated analogue of this species) is a viable electrophilic oxidant in addition to the consensus oxidant, iron-oxo.

The ubiquitous cytochrome P450 enzymes (P450s) catalyze a wide range of oxidations in nature including the energetically difficult hydroxylations of unactivated C−H positions. As the catalysts for oxidations of drugs, pro-drugs, and xenobiotics, as well as numerous natural substrates, P450s have attracted considerable interest from the pharmacological community. Their mechanisms of action are also of interest to the chemical community because of the high-energy intermediates that must be formed to hydroxylate a C−H bond at ambient temperature. The result is that P450s, their models, and related oxidants are the topics of thousands of publications each year.

The active sites of P450 enzymes contain iron in heme with thiolate from protein cysteine as the fifth ligand to iron, which is in the Fe(III) oxidation state in the resting enzyme.[1] The well-characterized portion of the catalytic cycle[1,2] of P450s involves complexation of substrate, reduction of Fe(III) to Fe(II), and reversible binding of molecular oxygen. Subsequent rapid steps in the cycle involve a second reduction, two protonation reactions, creation of a molecule of water, and oxidation of substrate. The iron−oxygen complexes produced in the latter reaction steps are shown in Figure 1. The second reduction step gives a peroxo-iron species. Protonation of this intermediate on the distal oxygen gives a hydroperoxy-iron species. A second protonation on the distal oxygen with subsequent or concomitant loss of water gives the iron-oxo species. If the second protonation occurs on the proximal oxygen atom, iron-complexed hydrogen peroxide is formed, and hydrogen peroxide can be released as product in an "uncoupled" process. Recent advances in "cryo-reduction" methodology (radiolytic reductions at low

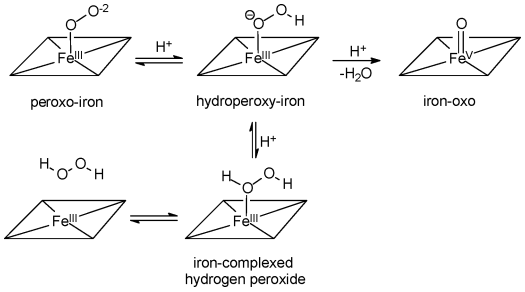

Figure 1. The iron−oxygen intermediates formed in cytochromes P450. The parallelogram represents heme.

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temperatures) permitted ESR characterization of the peroxo-iron and hydroperoxy-iron species.[3,4] Iron-oxo, although widely believed to be an oxidant, could not be detected even at ultralow temperatures,[3,4] but evidence for transient iron-oxo production in reactions of P450 enzymes with *m*-chloroperoxybenzoic acid in the absence of substrates has been reported.[5,6] Iron-complexed hydrogen peroxide has not been detected in P450 studies, but this species is formed on the pathway to the release of hydrogen peroxide in an uncoupled reaction.[1] It is also a requisite intermediate when P450s are "shunted" with hydrogen peroxide;[7] in shunt reactions, oxidations are effected by P450 and hydrogen peroxide in the absence of the reductase enzymes that transfer electrons to the P450s.

Iron-oxo has long been thought to be the active electrophilic oxidant in P450,[1] but results from our laboratories[8−12] and others[13,14] suggested that two electrophilic oxidants are formed in P450 reactions. One oxidant would be iron-oxo, and the other would be hydroperoxy-iron, possibly hydrogen bonded, or the protonated analogue, iron-complexed hydrogen peroxide. Much of the evidence for two oxidants in P450 involved studies where multiple oxidation pathways were implicated,[15] but the two-oxidants model is not the only possible explanation for multiple reaction pathways. Shaik, Schwartz, and co-workers reported computational results that suggested that iron-oxo might have two accessible spin states that, in principle, could react differently, providing a "two-states" alternative for multiple reaction pathways,[16] although that conclusion was recently questioned.[17,18]

Both the two-oxidants and the two-states models can rationalize selected experimental results, but recent studies designed specifically to test for the two-oxidants model provided evidence that hydroperoxy-iron (or a hydrogen-bonded or protonated version of this species) is an active oxidant in P450-catalyzed reactions. In one approach, Jin et al. showed that mutant cytochrome $P450_{cam}$ T252A was capable of epoxidizing alkenes even though it does not hydroxylate camphor, the natural substrate of the enzyme; the conclusion was that the hydroperoxy-iron intermediate effects epoxidation reactions.[14] In another study, highly variable kinetic isotope effects (KIEs) were obtained for two products formed by P450-catalyzed oxidations of the methyl group in a hypersensitive probe substrate, and the conclusion was that both the iron-oxo and the hydroperoxy-iron were effecting hydroxylations with different KIEs.[19] We report here KIE studies related to the latter work involving hydroxylation reactions of both enantiomers of a hypersensitive cyclopropane probe with five P450 enzymes that provide further support for the two-oxidants model.

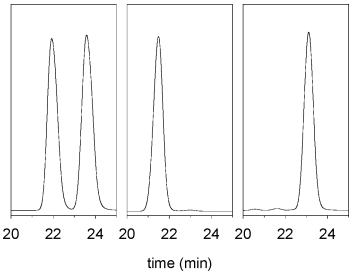
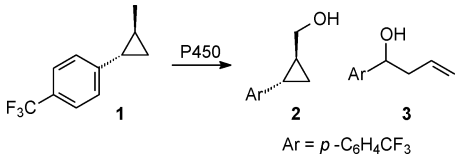


Figure 2. Portions of HPLC traces of amides from *trans*-2-(*p*-trifluoromethylphenyl)cyclopropanecarboxylic acid and (*S*)-1-phenylethylamine: racemic acid (left), (+)-acid (center), (−)-acid (right).

Results

Substrates and Products. The reactions studied were P450-catalyzed oxidations of both enantiomers of *trans*-2-(*p*-trifluoromethylphenyl)cyclopropylmethane (**1**), which was previously shown to be a substrate for hepatic P450s.[9] Oxidations occur at the methyl position to give unrearranged alcohol **2** and rearranged alcohol **3**.[9] The trifluoromethyl group is electron withdrawing, and it suppresses aromatic oxidation reactions that ultimately give phenol products; phenol products are obtained in high relative yield with the analogous substrate lacking the trifluoromethyl group.[20]



The enantiomers of **1** were obtained by resolution of *trans*-2-(*p*-trifluoromethylphenyl)cyclopropanecarboxylic acid followed by reduction reactions.[9] The analysis of the % ee of the enantiomers of purified acid was accomplished by conversion of the acid to an amide with (*S*)-1-phenylethylamine and HPLC analysis of the mixture. Figure 2 shows the results for a diastereomeric mixture of amides from racemic acid and amides from the enantiomerically enriched acids. Samples used for preparation of the substrates were 99.3% ee for the (+)-enantiomer of the acid and 98.4% ee for the (−)-enantiomer.

Enantiomers of substrate **1** had been prepared previously,[9] but the absolute configuration of the enantiomers was not determined. The structure of the amide from the (−)-enantiomer

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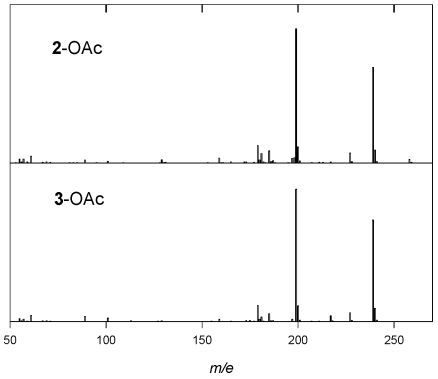

Figure 3. Mass spectral fragmentation patterns (CI, methane) for the acetate derivatives of alcohols **2** and **3**.

of the acid and (*S*)-1-phenylethylamine was determined by X-ray crystallography.[21] The absolute configuration of the (−)-acid is (*R*,*R*).

The resolved carboxylic acids were converted to substrates **1** by the method previously reported.[9] Thus, $LiAlH_4$ reduction of the resolved acids gave alcohols **2**. Conversion of alcohols **2** to the corresponding mesylates at low temperature followed by $LiBHEt_3$ reduction afforded substrates **1**-d_0. The dideuteriomethyl substrates, **1**-d_2, were prepared in the same manner but with $LiAlD_4$ in the initial reduction. For the preparation of trideuteriomethyl substrates, **1**-d_3, $LiAlD_4$ and $LiBDEt_3$ were employed. The ^{1}H NMR spectra of both enantiomers of **1**-d_3 showed <1% hydrogen content at the methyl positions, confirming the high deuterium incorporation.

Analytical Methods. The products from P450-catalyzed oxidations were analyzed by GC to determine yields, and GC-MS was employed to determine isotopic composition. With electron impact ionization, the ion produced from ring-opened alcohol **3** efficiently cleaves the allyl group that contains the isotopic label, and a molecular ion is not observed. Thus, the alcohol products were converted to acetate derivatives and analyzed with chemical ionization (CI) techniques. In the CI mass spectra of the acetates from both **2** and **3**, a significant peak at $(M - 19)^+$ ($m/e = 239$) from loss of one fluorine atom is observed (Figure 3). This convenient result ensures that the isotopic compositions of the fragments we analyzed were the same as those of the alcohol products.

In GC-MS analyses of the acetate derivatives of **2** and **3**, we continuously monitored selected ion channels and integrated the peaks. This method avoids problems in determining isotope ratios when the isotopomers are partially resolved by GC separation. For studies with **1**-d_2, the mono- and dideuterio products were formed, and we monitored the mass 240 and 241 ion channels. For studies with mixtures of **1**-d_0 and **1**-d_3, we monitored the mass 239 and 241 ion channels. Typical results are shown in Figure 4.

The precision of the method over a range of concentrations was tested with one of the product mixtures from P450 2B1 oxidation of a mixture of **1**-d_0 and **1**-d_3. The yields of oxidized products were largest with this enzyme and were up to 10 times greater than the amounts found with other P450 enzymes. Following the standard GC-MS analysis, samples of the product mixture were diluted by factors up to 1000, and the resulting

(21) Chandrasena, R. E. P.; Newcomb, M.; Surve, B. C.; Wink, D. J., unpublished results.

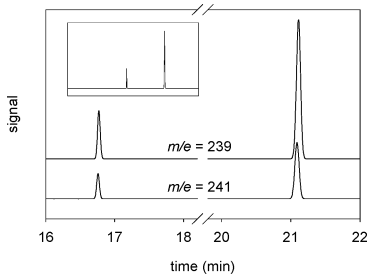

Figure 4. Representative GC-MS results from oxidation of a mixture of **1**-d_0 and **1**-d_3 where the two ion channels were monitored simultaneously. The acetate derivative of **3** elutes at 16.8 min, and that of **2** elutes at 21.1 min. The inset shows the $m/e = 239$ trace from 10 to 25 min.

diluted mixtures were analyzed by GC-MS (Table S1 in Supporting Information). The ratios of d_0 to d_2 products found were the same in these samples. With a sample diluted by a factor of 10 000, the signal-to-noise ratio was so poor that accurate product ratios were not obtained. The dilution study shows that GC-MS measurements were made within the linear range of the instrument.

P450-Catalyzed Oxidations. Oxidations were conducted with P450s 2B1, Δ2B4, Δ2B4 T302A, Δ2E1, and Δ2E1 T303A.[10,22,23] These are mammalian hepatic enzymes that were expressed in *E. coli* and purified. P450 reductase also was expressed in *E. coli* and purified.[22] P450 Δ2B4 and P450 Δ2E1 have short deletions at the N-terminal ends of the proteins that have no apparent effect on the catalytic activity. In the mutants, a highly conserved active-site threonine was replaced with alanine.[10,23] Active-site threonine is thought to be involved in the protonation reactions of the iron−oxygen intermediates, and the two wild-type−mutant pairs oxidize multiple reactive site substrates with different regioselectivities.[10,24]

Oxidation reactions were conducted at 10 °C for 30 min. Under the conditions we employed, the P450 enzymes remained active over the course of the oxidations (Figure S1 in Supporting Information). A series of trial runs wherein (*R*,*R*)-**1**-d_0 at varying concentrations was oxidized by P450 2B1 in a crude Michaelis−Menten study indicated that saturation conditions were obtained when the substrate was ca. 100 μM (Figure S2 in Supporting Information). To ensure that, for intermolecular KIE studies, the KIEs were in the k_{cat} terms and not in K_M, reactions were conducted with substrate concentrations of ca. 500 μM. Control reactions wherein substrate **1** in reaction mixtures containing buffer but lacking enzymes was treated with 2 and 4 μmol of H_2O_2 gave no oxidized products, ensuring that H_2O_2 released by the enzymes (see below) did not complicate the results.

Two types of oxidation studies were performed. In one set of studies, **1**-d_2 was oxidized to obtain intramolecular KIEs. Duplicate experiments were conducted for each enzyme−substrate combination (Supporting Information). The weighted averages were determined to give the results in Table 1 where we have multiplied the measured ratios of d_2 to d_1 products by 2 to obtain the apparent KIE values. Also listed in Table 1 are the ratios of the apparent KIEs for products **2** and **3**. The

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Table 1. Apparent Kinetic Isotope Effects for Oxidations of Substrates **1**-d_2 at 10 °C[a]

enzyme[b]	substrate	KIE_{app} **2**	KIE_{app} **3**	KIE ratio[c]
2B1	(*R*,*R*)-**1**	10.72 ± 0.02	8.64 ± 0.04	1.24
	(*S*,*S*)-**1**	11.16 ± 0.03	9.54 ± 0.01	1.17
Δ2B4	(*R*,*R*)-**1**	9.19 ± 0.01	7.53 ± 0.04	1.22
	(*S*,*S*)-**1**	9.52 ± 0.01	8.15 ± 0.04	1.17
Δ2B4 T302A	(*R*,*R*)-**1**	9.83 ± 0.02	7.67 ± 0.02	1.28
	(*S*,*S*)-**1**	9.76 ± 0.02	8.24 ± 0.05	1.18
Δ2E1	(*R*,*R*)-**1**	9.96 ± 0.02	8.72 ± 0.05	1.14
	(*S*,*S*)-**1**	8.48 ± 0.01	7.0 ± 0.2	1.21
Δ2E1 T303A	(*R*,*R*)-**1**	9.85 ± 0.03	8.08 ± 0.02	1.22
	(*S*,*S*)-**1**	9.71 ± 0.04	8.14 ± 0.06	1.19

[a] Apparent KIEs are the ratios of d_2 to d_1 products multiplied by 2. [b] P450 enzymes. [c] Ratio of KIE for product **2** to that for product **3**.

Table 2. Apparent Kinetic Isotope Effects in Competitive Oxidations of **1**-d_0 and **1**-d_3 at 10 °C

enzyme[a]	substrate	KIE_{app} **2**	KIE_{app} **3**	KIE ratio[b]
2B1	(*R*,*R*)-**1**	2.29 ± 0.04	1.80 ± 0.04	1.3
	(*S*,*S*)-**1**	1.71 ± 0.01	1.28 ± 0.01	1.3
Δ2B4	(*R*,*R*)-**1**	1.99 ± 0.04	1.68 ± 0.01	1.2
	(*S*,*S*)-**1**	2.32 ± 0.01	1.63 ± 0.01	1.4
Δ2B4 T302A	(*R*,*R*)-**1**	3.70 ± 0.01	3.18 ± 0.01	1.2
	(*S*,*S*)-**1**	2.62 ± 0.01	2.31 ± 0.01	1.1
Δ2E1[c]	(*R*,*R*)-**1**	8.5	8.2	
	(*S*,*S*)-**1**	8.3	6.2	
Δ2E1 T303A	(*R*,*R*)-**1**	8.03 ± 0.16	7.32 ± 0.01	1.1
	(*S*,*S*)-**1**	8.18 ± 0.01	7.69 ± 0.01	1.1

[a] P450 enzymes. [b] Ratio of KIE for product **2** divided by that for product **3**. [c] Low yields of d_2 products were obtained in these reactions, and the values should be considered approximate.

consistency of the ratios of KIEs is an important point that will be discussed later.

The second type of experiment was conducted to determine intermolecular KIEs in the hydroxylation reactions, which are not necessarily the same as intramolecular KIEs due to masking effects. Intermolecular KIEs could be determined by comparing the results of independent experiments with **1**-d_0 and **1**-d_3, but we used a design where oxidations of the two substrates were conducted in one experiment. In this manner, there can be no differences in the activity of the P450 enzymes, and (unlikely) differences in product release rates do not affect the results. Again, the results (Table 2) are weighted averages. Reactions were conducted with all P450s. The yields of product **3** from oxidations of **1**-d_3 catalyzed by P450 Δ2E1 were too small to permit accurate quantitation, however, and approximate values for the KIEs with this enzyme are listed.

The intermolecular KIE results appear to be more random than the intramolecular KIE results because extensive masking[25,26] occurred with P450 2B1, Δ2B4, and Δ2B4 T302A. Nonetheless, the ratio of KIEs for products **2** and **3** converged at 1.2, reflecting an underlying uniformity in the results that was present in the intramolecular KIE data.

The intramolecular KIE values in Table 1 are large, suggesting the possibility that H-atom tunneling effects were complicating the results. We investigated the temperature dependence of the KIEs in oxidations of (*S*,*S*)-**1**-d_2 by two P450 enzymes to evaluate whether changes in tunneling effects were important.

(25) Masking and unmasking are briefly discussed in the Appendix in the Supporting Information.
(26) For an overview of masking effects in P450-catalyzed reactions, see: Higgins, L.; Bennett, G. A.; Shimoji, M.; Jones, J. P. *Biochemistry* **1998**, *37*, 7039−7046.

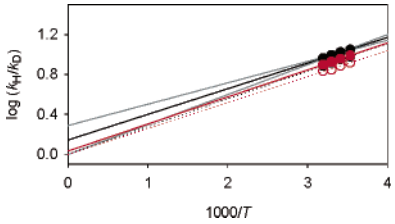

Figure 5. Arrhenius plots for variable temperature KIE studies. Product **2** results are solid circles and solid lines, product **3** results are open circles and dotted lines, P450 2B1 results are black, P450 Δ2B4 T302A results are red, and the 95% confidence interval for product **2** oxidations by P450 2B1 is gray.

Table 3. Hydrogen Peroxide Release from P450 Enzymes at 30 °C[a]

enzyme[b]	pH 6.8	pH 7.4
Δ2B4	17.9	10.6[c]
Δ2B4 T302A	34.6	19.4[c]
Δ2E1	76.1	28.3
Δ2E1 T303A	115	58.5

[a] Turnover in nmol of H_2O_2/nmol of P450/min. Average of six determinations from two separate experiments. [b] P450 enzymes. [c] The results for P450s Δ2B4 and Δ2B4 T302A at pH 7.4 were previously reported; see ref 28.

Reactions were conducted between 10 and 40 °C, and the results are given in Table S2 (Supporting Information). The (A_H/A_D) values for these data were in the range expected for cases where tunneling effects are not biasing the results.[27] Values of log(A_H/A_D) were within the 95% confidence interval of zero (Figure 5), and the largest deviation from zero was positive; in the region where tunneling is found for H and not D, log(A_H/A_D) has a negative value.[27]

In discussion, we will highlight the uncoupling reactions of P450 enzymes where NADPH is consumed and H_2O_2 is released; such uncoupling reactions can "unmask" a masked intermolecular KIE. Hydrogen peroxide release was determined for reactions of P450s Δ2B4, Δ2B4 T302A, Δ2E1, and Δ2E1 T303A in the absence of substrate, and the results are given in Table 3.[28] In qualitative terms, Δ2E1 released more H_2O_2 than Δ2B4, and both mutants were more active than their wild-type parents. In addition, the Δ2B4 T302A mutant has been found to generate more H_2O_2 than wild-type Δ2B4 in the presence of typical substrates,[28] and preliminary results with *p*-substituted phenols also reveal this trend with the Δ2E1 pair of cytochromes.[29] Hydrogen peroxide release from P450 2B1 in the absence of substrate at pH 7.5 was reported to be 33 nmol/nmol of enzyme/min,[30] but we caution it is not appropriate to compare the results from different laboratories without standardization experiments.

Discussion

A key objective of the present study was to attempt to distinguish between the "two-oxidants" model and the "two-states" model for P450-catalyzed oxidations. Evidence that multiple reaction pathways are available for P450-catalyzed oxidations has been accumulating for years,[15] and these two

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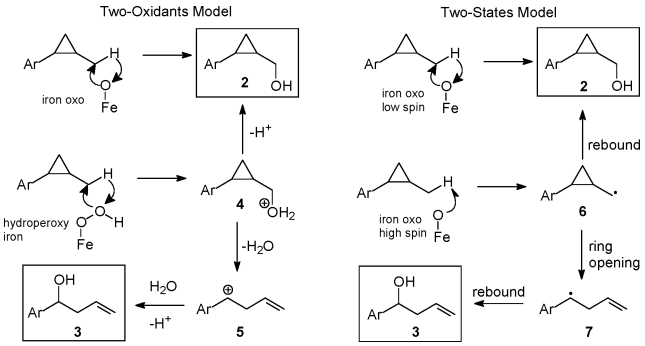
Scheme 1



models evolved to rationalize the results. In the two-oxidants model, both iron-oxo and another electrophilic oxidant effect oxidation reactions. We refer to the other oxidant as hydroperoxy-iron (FeOOH), but there is no evidence to distinguish between hydroperoxy-iron, a hydrogen-bonded analogue of this species, or iron-complexed hydrogen peroxide. In the two-states model, low-spin and high-spin states of iron-oxo are proposed to be energetically accessible.[16−18]

The two-oxidants and two-states models are compared in Scheme 1 in the context of substrate **1** studied in this work. In the two-oxidants model, iron-oxo reacts by an insertion reaction that produces cyclic alcohol **2** directly. The hydroperoxy-iron species effects oxidations by insertion of the elements of OH^+ to give, initially, the protonated alcohol intermediate **4**, that can be deprotonated, producing **2**, or can react by a solvolytic-type heterolysis to give the ring-opened product **3** via the cationic intermediate **5**. In the two-states model,[16] a low-spin state of iron-oxo reacts by an insertion reaction to produce **2**. The high-spin state of iron-oxo reacts by a hydrogen atom abstraction reaction that gives the radical intermediate **6**, which can be trapped in a rebound step (displacement of OH from iron) or ring open to give **3** via radical **7**. Both models accommodate iron-oxo insertion reactions, and the difference involves the identity of the "other" oxidant; is it hydroperoxy-iron or a second spin state of iron-oxo?

Figures 6 and 7 show the ratios of products **2** to **3** obtained in the intra- and intermolecular KIE studies, respectively. In experiments with **1**-d_2, the ratio of [**2**]/[**3**] was larger for the d_2 products than for the d_1 products, and, in experiments with the **1**-d_0 and **1**-d_3 substrates, the [**2**]/[**3**] ratio was larger for the d_0 products than for the d_2 products. The changes in product ratios are graphic representations of the larger apparent KIEs for product **2** than for product **3**. The figures also show the absolute values of the product ratios for the various enzymes. In general, the results in the two figures show high correspondence. Especially noteworthy is the large change in product ratios for the mutant enzymes in comparison to their wild-type parents.

Both the intramolecular and the intermolecular studies indicate that two KIEs are involved in the formation of products **2** and **3**. They could be two relatively large KIEs, one for formation of each product, or one large KIE for formation of both products with a small KIE superimposed. If the latter holds, then the small KIE is approximately 1.2, which is on the order of a secondary KIE, but it is important to note at the outset that the ratio of 1.2 for the apparent KIEs for the two products cannot possibly result from a secondary KIE. Secondary KIEs would be expected to be quite small for reactions of aryl-substituted

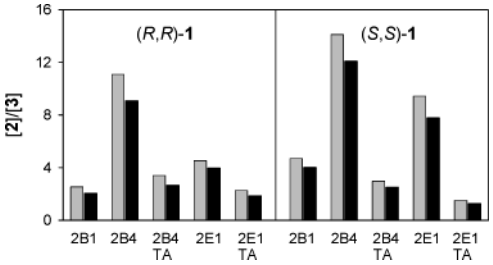


Figure 6. Ratios of product **2** to product **3** obtained in oxidations of substrates **1**-d_2. The d_2 products are gray bars, and the d_1 products are black bars. TA in the enzyme name indicates the active-site Thr to Ala mutant.

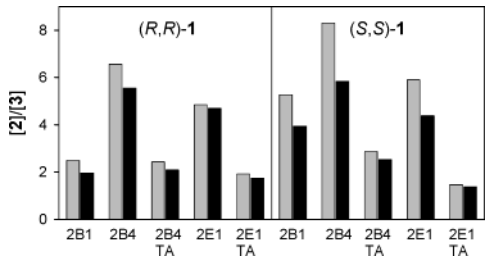
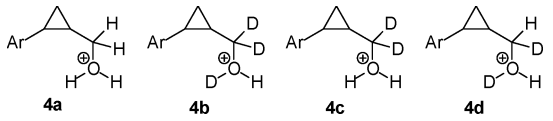


Figure 7. Ratios of cyclic to acyclic products from reactions of d_0 substrates (gray bars) and d_3 substrates (black bars). TA in the enzyme name indicates the active-site Thr to Ala mutant.

cyclopropylcarbinyl radicals or cations,[31−33] but, irrespective of that, they can be excluded from first principles. In either model that explains P450-catalyzed oxidations, a secondary KIE in the corresponding putative intermediate would result in the correct prediction of apparent KIEs for products **2** and **3** in one set of experiments (intra- or intermolecular studies) and an incorrect prediction of apparent KIEs for these products in the other set of experiments. Thus, the small apparent KIE of ca. 1.2 must be due either to a small primary KIE in addition to a large KIE or to a constant ratio of KIEs for the processes leading to the two products.

Two-Oxidants Model. The results of the present study support the "two-oxidants" model. This model contains three possible isotope-sensitive reactions. In addition to KIEs for reactions of the two oxidants, a small primary KIE will exist in the deprotonation reactions of protonated alcohol intermediate **4**. In the intermolecular KIE studies, **4a**, produced by insertion of OH^+ into a C−H bond in **1**-d_0, will be deprotonated somewhat faster than **4b**, produced by insertion into a C−D bond in **1**-d_3. Because the deprotonation reaction competes with the heterolytic fragmentation reaction, intermediate **4a** will give more of the cyclic product **2** than intermediate **4b**. Therefore, one expects that the ratio of cyclic product **2** to rearranged product **3** will decrease in the oxidation of the d_3 substrate, as found experimentally. In the intramolecular KIE studies, the intermediates formed are **4c** from insertion of OH^+ into the C−H bond and **4d** from insertion into a C−D bond. Intermediate **4c** will be deprotonated faster than intermediate **4d** due to a KIE in the deprotonation reaction, and this will result in greater yields of the cyclic product containing two deuterium atoms, again as observed.

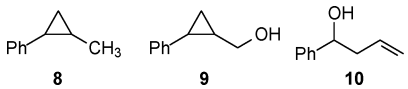
(31) Newcomb, M.; Johnson, C. C.; Manek, M. B.; Varick, T. R. *J. Am. Chem. Soc.* **1992**, *114*, 10915−10921.
(32) Newcomb, M.; Choi, S. Y.; Toy, P. H. *Can. J. Chem.* **1999**, *77*, 1123−1135.
(33) Wiberg, K. B.; Shobe, D.; Nelson, G. L. *J. Am. Chem. Soc.* **1993**, *115*, 10645−10652.



The qualitative results, that is, the product ratios in Figures 6 and 7, implicate formation of intermediates **4** and hydroxylation by the hydroperoxy-iron species in P450. A quantitative evaluation of the results indicates that hydroxylation of substrate **1** involved predominantly oxidation by hydroperoxy-iron and that hydroxylation by iron-oxo was a minor process that resulted in "noise" in the KIE values. This conclusion is based on the consistent ratios of the apparent KIEs for products **2** and **3**, the consistent values for the total KIEs for each enzyme found in the intramolecular studies, and the high correspondence for deprotonation KIEs and fragmentation rate constants in reactions of intermediate **4** obtained from the intra- and intermolecular data.

The KIEs for product **2** were ca. 1.2 times as large as the KIEs for product **3** for any enzyme−substrate combination, irrespective of the absolute values. If two oxidants (iron-oxo and hydroperoxy-iron) with the same inherent KIEs in the oxidation steps were involved in formation of the products, the difference in KIEs for **2** and **3** should be attenuated depending on the amounts of products arising from each oxidant. Moreover, if the KIEs for iron-oxo and hydroperoxy-iron differed considerably, then the KIE for product **2**, formed in reactions of both oxidants, would be randomly different from that for **3**, formed only in the hydroperoxy-iron reaction. The consistent ratio of KIEs for **2** and **3** indicates either (1) that most of the oxidation of substrate **1** was effected by hydroperoxy-iron or (2) that approximately equal relative percentages of intermediate **4** were produced in all 10 enzyme−substrate combinations we studied and the inherent KIEs for reactions of iron-oxo and hydroperoxy-iron are similar. The former explanation is simpler; the latter requires an extraordinary number of coincidences.

A comparison of the present results for substrate **1** and previous KIE studies[19] of the related substrate **8** reinforces the above conclusion. Unlike the situation with substrate **1**, the ratios of KIEs for products **9** and **10** from substrate **8** were not constant with various P450s. In fact, the ratios of KIEs for the two products were not the same in intramolecular and intermolecular KIE studies with one enzyme. Three isotope-sensitive reactions were needed to model the results, oxidation by iron-oxo, oxidation by hydroperoxy-iron, and deprotonation of the protonated alcohol intermediates.[19]



The intramolecular KIE results are analyzed for the model of predominant oxidation by hydroperoxy-iron according to Scheme 2. The important rate constants are those for insertion of OH^+ into a C−H bond (k_H) or a C−D bond (k_D), those for deprotonation of the isotopomers of **4** by loss of a proton (k_h) or deuteron (k_d), and that for heterolytic fragmentation of **4** (k_F). We make the reasonable assumption that heterolytic fragmentations of the isotopomers of **4** would have negligible secondary KIEs.

Table 4 contains kinetic values evaluated according to Scheme 2. The intramolecular KIE is given by twice the ratio of d_2 to

Scheme 2

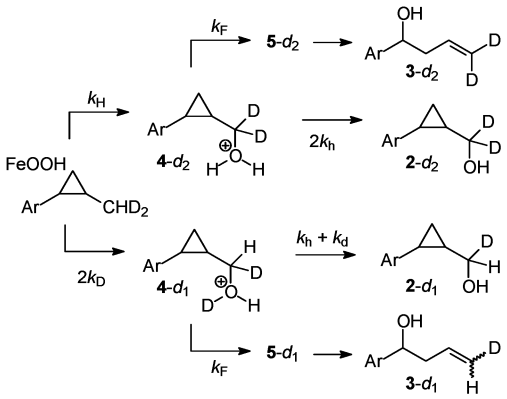


Table 4. Kinetic Values from Intramolecular KIE Studies Analyzed According to Scheme 2[a]

enzyme	substrate	k_H/k_D [b]	$2k_h/(k_h + k_d)$ [c]	$2k_h/k_F$ [d]
2B1	(*R*,*R*)-**1**	10.0	1.24	2.5
	(*S*,*S*)-**1**	10.8	1.17	4.7
Δ2B4	(*R*,*R*)-**1**	9.0	1.22	11.2
	(*S*,*S*)-**1**	9.4	1.17	14.1
Δ2B4 T302A	(*R*,*R*)-**1**	9.2	1.28	3.4
	(*S*,*S*)-**1**	9.3	1.18	3.0
Δ2E1	(*R*,*R*)-**1**	9.7	1.14	4.5
	(*S*,*S*)-**1**	8.3	1.21	9.4
Δ2E1 T303A	(*R*,*R*)-**1**	9.2	1.22	2.3
	(*S*,*S*)-**1**	9.0	1.19	1.5

[a] For reactions at 10 °C. [b] Intramolecular KIE from eq 1. [c] From eq 3. [d] From eq 2.

d_1 products (eq 1). The ratio of the deprotonation to heterolytic fragmentation rate constants is given by the ratio of the two d_2 products (eq 2). The ratio of deprotonation rate constants for **4**-d_2 and **4**-d_1, given by eq 3, is also equal to the observed ratios of apparent KIEs for **2** and **3** listed in Table 1.

$$k_H/k_D = 2(\mathbf{2}\text{-}d_2 + \mathbf{3}\text{-}d_2)/(\mathbf{2}\text{-}d_1 + \mathbf{3}\text{-}d_1) \quad (1)$$

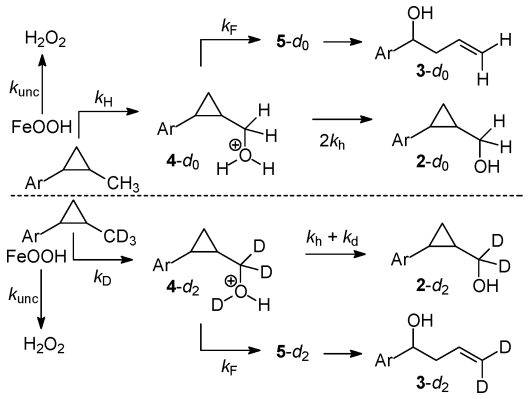
$$2k_h/k_F = \mathbf{2}\text{-}d_2/\mathbf{3}\text{-}d_2 \quad (2)$$

$$2k_h/(k_h + k_d) = (\mathbf{2}\text{-}d_2/\mathbf{3}\text{-}d_2)/(\mathbf{2}\text{-}d_1/\mathbf{3}\text{-}d_1) \quad (3)$$

The results in Table 4 show quite good agreement. The KIEs for hydroxylation of **1** by the hydroperoxy-iron species in each enzyme differ only slightly. The agreement in the KIEs for the hydroxylation reactions of each enantiomer of **1** catalyzed by the P450 Δ2B4 and Δ2B4 T302A wild-type/mutant pair is excellent, with an average difference from the mean for these four reactions of 1%. One intramolecular KIE for hydroxylation, that for oxidation of (*S*,*S*)-**1** by P450 Δ2E1, appears out of line given the good agreement for the other reactions; it is possible that this is a case where hydroxylation by iron-oxo was substantial enough to perturb the overall apparent KIE noticeably. If the (*S*,*S*)-**1** results for Δ2E1 are excluded, then the average difference from the mean for the remaining three KIEs for Δ2E1 and its mutant is less than 3%. It is important to note that the uniformity in the actual KIEs for oxidation by a P450 and its mutant is a consequence of consecutive isotope-sensitive reactions and cannot be obtained in a model with competing isotope-sensitive reactions.

The KIE for the deprotonation reactions of intermediates **4**, that is, $2k_h/(k_h + k_d)$, is quite consistent, as we have noted earlier

Scheme 3



in the context of the ratios of KIEs. This value translates into a rate constant for loss of D^+ from **4**-d_1 that is about 70% as fast as loss of H^+ from **4**-d_1 or from **4**-d_2, indicating a highly exothermic proton transfer reaction that competes with the undoubtedly fast solvolytic ring opening of **4**. The KIE in the deprotonation reaction of the protonated alcohol is specific to the substrate, and it is predicted to be a constant in our studies when different enzymes are employed because the only substrates were the enantiomers of **1**. This consistency for different enzymes provides strong support for the two-oxidants model.

The rate constants for deprotonation of **4** (k_h) are expected to be enzyme specific, and they should display small differences for the two enantiomers of **1** due to diastereomeric interactions in the enzymes' active sites. The heterolytic fragmentation reaction should be substrate specific and constant in our studies. Thus, changes in the $2k_h/k_F$ ratios reflect mainly differences in the deprotonation rate constants. In general, the trends in $2k_h/k_F$ are in reasonable agreement in that, for example, deprotonations in P450 Δ2B4 are much faster than in its mutant for both enantiomers of substrate, and both enantiomers of substrate are deprotonated slowly in P450 Δ2E1 T303A in comparison to the other enzymes.

It is noteworthy that the deprotonation reactions of intermediates **4** are considerably retarded for both mutants in comparison to their wild-type analogues. A modeling study of P450 2E1 indicates that Thr303 is located adjacent to bound substrates,[34] and this observation offers an attractive explanation of the results for P450 Δ2E1 and its T303A mutant. Specifically, the conversion of Thr303 to Ala removed a basic residue from a position that might be adjacent to the protonated alcohol moiety in **4**. This speculation could be tested by modeling **1** and **4** in the active site of P450 2E1.

For the intermolecular KIE studies, the model is modified as shown in Scheme 3, where the dotted line separates reactions occurring in different enzyme−substrate complexes. The second reduction step in the P450 reaction sequence is irreversible,[1] and, assuming that binding of substrate is not isotope sensitive, the intermolecular KIEs would be $k_H/k_D = 1$ (a masked KIE) unless some other reaction of the oxidant is possible that can "unmask" the masked KIE.[25,26] In Scheme 3, the "unmasking" reaction is the uncoupling reaction, release of H_2O_2 in competition with the oxidation of substrate, which has the rate constant k_{unc}. Because the rate of the uncoupling reaction relative to the rate of hydroxylation determines the extent of unmasking, one

(34) Park, J. Y.; Harris, D. *J. Med. Chem.* **2003**, *46*, 1645−1660.

Table 5. Kinetic Values from Intermolecular KIE Studies Analyzed According to Scheme 3[a]

enzyme	substrate	KIE_{obs} [b]	k_{unc}/k_H [c]	$2k_h/(k_h + k_d)$ [d]	$2k_h/k_F$ [e]
2B1	(*R*,*R*)-**1**	2.1	0.09	1.27	2.5
	(*S*,*S*)-**1**	1.6	0.04	1.34	5.3
Δ2B4	(*R*,*R*)-**1**	1.9	0.08	1.18	6.6
	(*S*,*S*)-**1**	2.2	0.10	1.42	8.3
Δ2B4 T302A	(*R*,*R*)-**1**	3.5	0.24	1.16	2.4
	(*S*,*S*)-**1**	2.5	0.13	1.13	2.9
Δ2E1[f]	(*R*,*R*)-**1**	8	1.2	1.0	5
	(*S*,*S*)-**1**	8	1.5	1.3	6
Δ2E1 T303A	(*R*,*R*)-**1**	7.8	1.12	1.10	1.9
	(*S*,*S*)-**1**	8.0	1.26	1.06	1.5

[a] For reactions at 10 °C. [b] Masked intermolecular KIE from eq 4. [c] From eq 7. [d] From eq 6. [e] From eq 5. [f] The values for P450 Δ2E1 are low precision.

can calculate the relative rates of the two processes from the observed KIE in the intermolecular studies and the intrinsic KIE determined from the intramolecular studies (see below). KIE masking of the oxidation reaction will not have an effect on the deprotonation and heterolytic fragmentation rate constants, and the values of k_h, k_d, and k_F obtained from the intermolecular KIE results should match those from the intramolecular studies.

Table 5 contains rate constants from the intramolecular KIE studies derived from the model in Scheme 3 using eqs 4−7. The observed KIE (KIE_{obs}), given by eq 4, is partially masked. The rate constants for deprotonation and heterolytic fragmentation of intermediates **4** are unaltered by the intermolecular design of the experiments, and eqs 5 and 6 are analogues of eqs 2 and 3. The ratio of rate constants for uncoupling and hydroxylation (k_{unc}/k_H) can be calculated with eq 7 if the intrinsic KIE value (KIE_{int}) is known. To calculate KIE_{int} for each enzyme−substrate combination, we used the intramolecular KIEs from Table 4 (k_H/k_D) and multiplied those values by $(1.15)^3 = 1.5$. The intramolecular experiment gives a primary KIE divided by a secondary KIE, whereas the intermolecular experiment gives a primary KIE times two secondary KIEs,[35] and we assume that the secondary KIE is normal and approximately equal to 1.15 as found for P450-catalyzed hydroxylation of octane.[36]

$$KIE_{obs} = (\mathbf{2}\text{-}d_0 + \mathbf{3}\text{-}d_0)/(\mathbf{2}\text{-}d_2 + \mathbf{3}\text{-}d_2) \quad (4)$$

$$2k_h/k_F = \mathbf{2}\text{-}d_0/\mathbf{3}\text{-}d_0 \quad (5)$$

$$2k_h/(k_h + k_d) = (\mathbf{2}\text{-}d_0/\mathbf{3}\text{-}d_0)/(\mathbf{2}\text{-}d_2/\mathbf{3}\text{-}d_2) \quad (6)$$

$$k_{unc}/k_H = (1 - KIE_{obs})/(KIE_{obs} - KIE_{int}) \quad (7)$$

For a given enzyme−substrate combination, the values in Table 5 show excellent agreement with the corresponding values in Table 4. The average deviation from the means for the $2k_h/(k_h + k_d)$ values is 5%, and the average deviation from the means for the $2k_h/k_F$ values is 11%. Importantly, the qualitative ranking of $2k_h/k_F$ for each enzyme−substrate combination is essentially unchanged in the two data sets, and the larger $2k_h/k_F$ values for P450 Δ2B4 and P450 Δ2E1 as compared to their mutants found

(35) Hanzlik, R. P.; Hogberg, K.; Moon, J. B.; Judson, C. M. *J. Am. Chem. Soc.* **1985**, *107*, 7164−7167.
(36) Jones, J. P.; Rettie, A. E.; Trager, W. F. *J. Med. Chem.* **1990**, *33*, 1242−1246.

from analysis of the intramolecular results are reproduced in the analysis of the intermolecular results, albeit somewhat attenuated.

For P450s 2B1, Δ2B4, and Δ2B4 T302A, the large degree of KIE masking[25,26] reflects the fact that the hydroxylation reactions of **1**-d_0 are 4−25 times faster than the uncoupling reactions for these enzymes. For P450s Δ2E1 and Δ2E1 T303A, however, release of hydrogen peroxide is faster than the hydroxylation reaction, which results in largely unmasked values for KIE_{obs} that approach KIE_{int}. It is reasonable to assume that, to a first approximation, the absolute values of the hydroxylation rate constants k_H are similar for the various enzymes, in which case the major changes in k_{unc}/k_H are due to changes in k_{unc}. Therefore, the lifetimes of the FeOOH species ($\tau = 1/k_{unc}$) in P450 2B1 are up to 40 times longer than those in Δ2E1. Knowledge of these lifetimes is important for studies that follow reactions of the hydroperoxy-iron species directly, such as cryo-reduction experiments.[3,4]

The hydrogen peroxide turnover values in the absence of substrate correlate with the values of k_{unc}/k_H at the qualitative level. For the enzymes studied in this work, P450 Δ2B4 released the smallest amount of H_2O_2 and had the smallest value of k_{unc}/k_H, and P450 Δ2B4 and its mutant had smaller values of k_{unc}/k_H and smaller amounts of H_2O_2 turnover than P450 Δ2E1 and its mutant. Hydrogen peroxide turnover/release values in the absence and presence of substrate are not necessarily identical, but the trend is clear that P450 Δ2E1 and its mutant appear to be "leaky" in comparison to P450 Δ2B4 and its mutant in regard to both the H_2O_2 release rates and the extent of unmasking. This correlation provides support for oxidation by hydroperoxy-iron and, thus, the two-oxidants model, because hydrogen peroxide release would not unmask a KIE for iron-oxo (see below).

Two-States Model. The two-states model has two distinct reactions (Scheme 1), and it is conceivable at the superficial level that all of product **2** was formed in reaction of the low-spin state with one KIE and all of product **3** was produced in the high-spin state reaction with a different KIE. Although this model can fit the ratio of apparent product KIEs, it is not in accord with other aspects of the results as detailed below.

The KIEs expected for reactions in the two-states model were computed by Shaik and co-workers who reported that the KIE in reactions of the high-spin state will be greater than the KIE in reactions of the low-spin state.[37] Irrespective of the extent of reaction by the two spin states or details of the partitioning of putative radical **6**, the prediction using those results[37] is that the KIE in product **3** will be greater than the KIE in product **2**. Therefore, the ratio of cyclic to acyclic product would be predicted to increase in the oxidation of the d_3 substrate, which is the opposite of the experimental results in Figure 6. In a similar manner, the ratio of products is predicted incorrectly for the intramolecular results shown in Figure 7.

The predicted[37] relative KIEs also were in the wrong direction for the products formed in P450-catalyzed oxidations of substrate **8**.[19] However, after the appearance of the communication reporting results for **8**, Shaik and co-workers published a new study for this substrate in which the relative order of KIEs for the low- and high-spin states was reversed from the original report.[38] Computed absolute KIE values for intramolecular competitions were similar to the experimental values when the computations included tunneling using the Bell correction. From the Bell-corrected results, the KIE for product **9** is now predicted to be 1.22 times greater than that for product **10** in intramolecular studies and 1.12 times greater in intermolecular studies.[38] For comparisons to experimental results, Shaik and co-workers averaged four of the six sets of results from the intramolecular KIE studies of **8** and did not consider the values from the intermolecular studies. They stated that the new computational results are in "perfect accord with experiment" and provide a "proof" that a two-state mechanism is operative in the P450-catalyzed oxidations of **8**.[38]

The criteria applied for these conclusions are elusive. The ratios of intramolecular KIEs for products **9** and **10** for the six combinations of enzymes and substrate ranged from (0.93 ± 0.01) to (1.24 ± 0.02) (deviations at 2σ), and the ratios of the intermolecular KIEs for **9** and **10** (four combinations of enzymes and substrate) ranged from 1.3 to 2.8 with an average of 1.9.[19,39] The demonstrably significant variations in the experimental KIE ratios were not addressed in the recent computational report, and the remarkably large errors in the two-states model predictions for the intramolecular KIE ratios were not noted.[38] In our view, the new KIE predictions do not provide convincing evidence that the two-state model is operative in oxidations of **8**, and, in fact, they indicate that it is not. As noted above, we concluded that the results with substrate **8** could not be modeled with only two isotope-sensitive reactions; a third KIE was required.[19]

Unlike the case with **8**, the results with substrate **1** can be modeled with two isotope-sensitive reactions. Thus, any "two-reactions" model can fit the consistent ratio of product KIEs because the only necessary conditions are that the products are formed in different reactions and the KIEs for these reactions are not the same. The two-states model is inadequate in explaining other results for substrate **1**, however, that include (1) the variable apparent KIEs for the products from reactions of each P450 with the enantiomeric substrates, (2) the unmasking effects found in the intermolecular studies, and (3) the changes in product ratios observed for the wild-type and mutant enzyme pairs. The same shortcomings exist if one attempts to rationalize the results for **8**.

In the two-states model, the only route to product **3** is ring opening of radical **6** produced in the high-spin reaction. As a consequence of the constant ratio of KIEs for the two products, the model then requires that the low-spin reaction gave only product **2**. One would expect that a particular P450 enzyme would display the same KIE for the products formed from the two enantiomeric substrates in the intramolecular studies, but the apparent KIEs for **2** and for **3** formed from a given enzyme vary by up to 25%.

The variations in apparent KIEs for products **2** and **3** from oxidation of the two enantiomers by a given enzyme cannot be explained adequately by any model that postulates two competing reactions with different KIEs. The substrates differ only in

(37) Ogliaro, F.; Filatov, M.; Shaik, S. *Eur. J. Inorg. Chem.* **2000**, 2455−2458.

(38) Kumar, D.; de Visser, S. P.; Shaik, S. *J. Am. Chem. Soc.* **2003**, *125*, 13024−13025.

(39) The intramolecular KIE results in ref 19 were from comparisons of independent reactions of **8**-d_0 and **8**-d_3. In more recent studies, we used the same experimental design as in the present study (i.e., competition between **8**-d_0 and **8**-d_3) and found a range of KIE ratios from 1.4 to 2.3 with an average of 1.75 for 10 examples of enzymes and substrate (Newcomb, M.; Chandrasena, R. E. P., unpublished results).

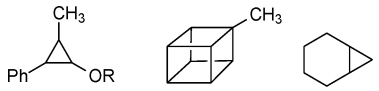
their configurations, and the basic energetics for the competing reactions in a given P450 are expected to be the same. As we demonstrated above, however, a model with consecutive isotope-sensitive steps not only explains the variability in apparent KIEs for the products but also gives consistent actual KIEs for the initial oxidation step by a given P450. The question thus arises: can the two-states model be salvaged by reduction to a "one-state" model, wherein all reactions occur via the high-spin iron-oxo (with one KIE), and the radical intermediate **6** is distributed to products **2** and **3** in a second isotope-sensitive step? It cannot. An isotope-sensitive trapping reaction for **6** would involve secondary KIEs from the remaining H/D atoms on the radical, and, as noted earlier, any secondary KIE effects will have opposite results in the intra- and intermolecular studies. That is, any secondary KIE effect will be larger for product **2** than for product **3** in one set of studies and smaller in the other.

The unmasking effects found in the intermolecular KIE studies are difficult to explain in the context of the two-states model. Because the formation of the peroxo-iron intermediate is irreversible,[1] an intramolecular KIE of $k_H/k_D = 1$ will be found unless some other pathway for reaction of the oxidant exists that "unmasks" the masked KIE.[25,26] If the hydroperoxy-iron intermediate is an oxidant, then release of hydrogen peroxide (the uncoupling reaction) serves as a KIE unmasking reaction. The uncoupling reaction cannot unmask a KIE if the iron-oxo species is the oxidant, however, because formation of iron-oxo by loss of water from the ($FeOOH_2$) species is computed to be highly exothermic and irreversible.[40] Thus, a different competing reaction is needed to unmask KIEs for iron-oxo. In principle, another unmasking reaction is possible, an oxidase-type reaction wherein iron-oxo is further reduced to give water, but the extent of oxidase activity (if any) in the reactions studied here is not known.

In regard to the changes in ratios of products **2** and **3** for the wild-type and mutant enzyme pairs, the two-states model offers no satisfactory explanation for why the mutation should have a profound effect. This problem for the model also surfaces in wild type−mutant pair studies in the form of variable regioselectivities in oxidations of substrates with multiple reactive sites (see below). We noted that the two-states model requires that essentially all of product **2** was formed by the low-spin reaction and all of product **3** was formed in the high-spin reaction. Therefore, the ratios of product **2** to product **3** would directly reflect the amounts of reaction through each channel. For the results found here, the difference in activation energies for oxidations by the two states must change by as much as 1.0 kcal/mol in the case of oxidations of (*S*,*S*)-**1** by P450 Δ2E1 and its T303A mutant. How the Thr to Ala mutation could have such a large differential effect on the activation barriers for the two similar processes postulated in the two-states model is unknown.

Although computational in origin,[16] the two-states model is largely qualitative. It successfully rationalizes selected P450 experimental results but is inconsistent with other results, including some important trends. For example, it predicts that only radical-derived rearrangement products will be formed in mechanistic probe studies[16,38] and does not explain the cationic rearrangement products consistently found in studies with probes that distinguish between cationic and radical intermediates (see below). This deficiency cannot be addressed with an ad hoc rationalization that radicals were initially produced and then oxidized to cations because cationic products are found for cyclopropylmethyl probes that are precursors to radicals with lifetimes as short as 2 ps[12,41] when no rearranged products are found from related probes that are precursors to radicals with lifetimes up to 10 ns.[42] A recent challenge to the model arose from computational studies that indicated that only one spin state of P450 iron-oxo was energetically accessible,[17,18] calling into question the premise of the two-states model for P450.

Comparison with Other Mechanistic Studies. The results of the present work and the related study with substrate **8** provide support for the two-oxidants model and indicate that the species we have called hydroperoxy-iron is the predominant oxidant of substrate **1**. Formation of protonated alcohol **4** by insertion of OH^+ into a C−H bond from the hydroperoxy-iron species (or iron-complexed hydrogen peroxide) leads to rearrangement product **3** by a cationic process, not a radical pathway. Both conclusions, that hydroperoxy-iron is an oxidant form in P450 and that rearrangements involve cationic intermediates, are consistent with previous mechanistic studies.



11a: R = CH_3 **12** **13**
11b: R = Bu^t

The formation of cation-derived rearrangement products in P450-catalyzed oxidations previously was observed when hypersensitive probe substrates **11** were used with P450 2B1 (**11a**)[8] and with the same P450 enzymes used in the present study (**11b**).[12] Probe substrates **11** were designed such that a radical intermediate will open by breaking the cyclopropyl bond to the benzylic carbon, whereas a cationic intermediate will open by breaking the bond to the ether-substituted carbon.[41] A cation-derived product also was found from methyl group oxidation of methylcubane (**12**), which gives a distinct cationic rearrangement product.[12] In recent works, a cation-derived rearrangement product was found in oxidations of norcarane (**13**) by a variety of P450 enzymes.[43,44] Most of the mechanistic probes that have been applied in P450 studies give the same products from radical and cationic rearrangements, and no conclusions about the pathway for rearrangements of these substrates can be made. The result is that cationic rearrangement products were found in all cases where rearranged products were obtained from a substrate that provided a means for differentiation between radical and cationic intermediates.[42]

Oxidations by the hydroperoxy-iron intermediate in P450 were implicated in the mechanistic probe studies discussed above. One pathway to the cationic rearrangement products involves solvolysis reactions of protonated alcohols such as that shown in Scheme 1 for intermediate **4**, and insertion of OH^+ from hydroperoxy-iron or iron-complexed hydrogen peroxide was postulated.[8] In early work, Pratt et al. concluded that iron-

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complexed hydrogen peroxide was an important oxidant in P450 on the basis of product differences found when H_2O_2 and iodosobenzene were used in shunt reactions.[13]

A compelling case for hydroperoxy-iron as an oxidant is provided by several studies of P450 wild-type enzymes and their mutants in which the highly conserved active-site Thr was replaced with Ala, including the P450s used in this work.[15,45] From changes in the regioselectivity of oxidations of simple alkenes (epoxide versus allyl alcohol products), Vaz et al. concluded that P450s Δ2B4 and Δ2E1 and their threonine-to-alanine mutants had two active electrophilic oxidants.[10] In that work, the authors suggested that the hydroperoxy-iron species was a preferential epoxidizing agent, and iron-oxo was a preferential hydroxylating agent. The regioselectivity changes found in oxidations of probe **8**, coupled with product ratio changes in oxidations of probe substrate **1** by the same wild-type and mutant pairs, further supported the two-oxidants model with the added condition that the hydroperoxy-iron species must be able to effect hydroxylation to some extent.[11]

Similar results were found with another wild-type and mutant P450 enzyme pair.[46] The relative amounts of N-dealkylation and sulfoxidation in oxidations of *p*-(*N*,*N*-dimethylamino)-thioanisole varied by a factor of 4 for P450 BM3 and its T268A mutant (equivalent to the mutants used in the present study). The absence of a KIE in competitive oxidations of undeuterated substrate and its perdeuteriomethyl analogue, coupled with the demonstration of an intrinsic KIE for N-demethylation and fast tumbling of substrate in the active site, resulted in the conclusion that two distinct oxidants existed that did not interchange.[46] The authors noted that, in principle, the two oxidants might be two spin states of iron-oxo, but that can only be the case if interconversion between the two spin states is slower than the oxidation reaction, which is not expected.

Studies with $P450_{cam}$, the best characterized P450 enzyme, were recently reported that also indicate that the hydroperoxy-iron species is an electrophilic oxidant.[14] The $P450_{cam}$ T252A mutant, which apparently does not produce iron-oxo, does not oxidize camphor, the natural substrate of the enzyme. Jin et al. reported that the T252A mutant was capable of epoxidizing alkenes, however, and they concluded that epoxidation reactions were effected by the hydroperoxy-iron intermediate produced in the mutant.[14]

Multiple electrophilic oxidant forms also were implicated in a recent study of the effect of anions on oxidations catalyzed by P450 2D6 and other P450 enzymes.[47] For P450 2D6, carbonate was found to slow O-demethylation reactions but have no effect on the kinetics of N-demethylation reactions. Most interestingly, when the P450 was shunted with cumyl hydroperoxide, carbonate had no obvious effect on the rate of an O-demethylation reaction. Because the shunt reaction is thought to produce iron-oxo directly, the authors concluded that carbonate differentially affected the production of iron-oxo in the normal reaction sequence in comparison to production of hydroperoxy-iron.[47]

Support for the two-oxidants model also is found in an evaluation of the magnitude of the KIEs obtained in the present work. The intramolecular KIE values indicate a relatively low reactivity for the hydroperoxy-iron species. The intramolecular KIEs we obtained are equal to a primary KIE divided by a secondary KIE.[35] Invariably, the secondary KIEs for P450-catalyzed hydroxylations are normal, and the average value for the secondary KIE in hydroxylation of the methyl group in octane was $k_H/k_D \approx 1.15$.[36] If that value applies in the functionalization of the methyl group in **1**, then the primary KIE at 40 °C for reaction of substrate (*S*,*S*)-**1** with P450 2B1 would be $k_H/k_D = 10$, which is larger than the primary KIE found for oxidation of the methyl group in octane by the same P450 enzyme at 37 °C (e.g., 9.1−9.2).[36,48] Because the C−H bond energy of the methyl group in substrate **1** is about 3 kcal/mol smaller than that of a methyl group in an alkane,[49] however, hydroxylation of **1** should have a smaller KIE than hydroxylation of octane. It appears possible, therefore, that the large KIEs found for reaction of substrate **1** are those for the less reactive hydroperoxy-iron species, whereas the smaller KIE found for octane might be for reaction of a more highly reactive iron-oxo species or for a mixture of reactions from hydroperoxy-iron and iron-oxo. Consistent with this conclusion, the primary KIEs determined for authentic iron-oxo intermediates produced from porphyrin−iron complexes and sacrificial oxidants were found to be $k_H/k_D = 7.5-8.7$ for oxidation of the tertiary C−H positions of adamantane at 20 °C,[50] substantially smaller than the primary KIEs found here for oxidation of substrate **1** at 10 °C ($k_H/k_D = 10.3-12.4$).

Alternative electrophilic oxidants to iron-oxo have been implicated in systems related to P450 enzymes, providing additional support for the two-oxidants model. In heme oxygenase, the enzyme that degrades heme, a hydroperoxy-iron species is thought to be the active species that oxidizes the heme macrocycle.[51] In several studies with iron−porphyrin complexes that model P450 and other heme-containing enzymes, multiple oxidants have been implicated from changes in product yields as a function of sacrificial oxidant or experimental conditions.[52−57] The conclusions in the model studies are that both iron-oxo and a complex between iron and the sacrificial oxidant are active oxidants, in direct analogy to the two electrophilic oxidants postulated in the two-oxidants model.

Conclusion

The present study adds to the growing evidence that hydroperoxy-iron is a second electrophilic oxidant species in cytochromes P450, effecting oxidations by insertion of OH^+ into a C−H bond. The intermediate protonated alcohol thus formed is rapidly deprotonated to give neutral alcohol products. Because

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hydroperoxy-iron is the predominant oxidant of substrate **1**, we were able to calculate the k_H/k_D values in both the oxidation reaction and the deprotonation reactions of each P450 and relative rate constants for oxidation and hydrogen peroxide release (uncoupling reaction) in each enzyme. The relative rate constants for fragmentation and deprotonation of intermediate protonated alcohol **4** also were obtained, and these will be useful for comparison to results with other P450 substrates.

We consider the complete characterization of the hydroperoxy-iron oxidant to be an important goal both for understanding the biological oxidations and for chemical modeling of the oxidants. One important question involves the detailed identity of the oxidant: is it hydroperoxy-iron, hydrogen-bonded hydroperoxy-iron, or iron-complexed hydrogen peroxide? Another question involves the reactive character of the species.

The present results implicate hydroxylation by hydroperoxy-iron, and this is supported by previous results from our laboratories.[11,19] Other properties have been ascribed to this species, however. Thus, Vaz et al.[10] concluded that hydroperoxy-iron was a preferential epoxidizing agent in comparison to hydroxylation, Jin et al.[14] concluded that it could epoxide alkenes but could not hydroxylate the high-energy C−H bond in camphor, Volz et al.[46] concluded that it was a preferential sulfoxidizing agent in comparison to oxidation of an *N*-methyl group, and Hutzler et al.[47] concluded that it preferentially oxidizes an *N*-methyl group in comparison to an *O*-methyl group. None of these conclusions are necessarily in conflict with one another, and the collective message apparently is that hydroperoxy-iron is a less reactive oxidant than iron-oxo.

Our results also present a caution regarding characterizations of the reactivity of the iron-oxo species. Previous P450 reactivity studies have been conducted with the assumption that iron-oxo was the only oxidant, but it is quite possible that the results reflect a mixture of the reactivities of the two electrophilic oxidants. Methods for unambiguous production of one oxidant and the exclusion of the other will undoubtedly be important for fully understanding the complex oxidizing system in cytochrome P450.

Experimental Section

Substrates and Products. The preparation of enantiomerically enriched substrates **1** and alcohols **2** and **3** followed the reported methods.[9] For dideuteriomethyl substrates **1**-d_2, $LiAlD_4$ was used in the initial reduction step. For tridueteriomethyl substrates, $LiAlD_4$ and $LiBDEt_3$ were used for the reduction steps. The ^{1}H NMR spectra of samples of **1**-d_3 showed <1% protium content in the region for methyl group absorbance. The enantiomeric purities of the samples of resolved acids used for the preparation of **1** were determined by conversion of the acid to an amide by reaction with (*S*)-(+)-2-phenylethylamine and analysis by HPLC (C-18 column, water−acetonitrile, 1:1, v:v); Figure 2 shows the results.

***N*-(1-Phenylethyl)-*trans*-2-(*p*-trifluoromethylphenyl)cyclopropanecarboxamide.** The procedure of Zhang et al. was used.[58] The above acid (0.41 g, 1.78 mmol) was added to 2 mL of $SOCl_2$, and the mixture was heated at reflux for 1 h. Excess $SOCl_2$ was removed by rotary evaporation, the residue was dissolved in 50 mL of CH_2Cl_2, and the CH_2Cl_2 was removed by rotary evaporation. The crude acid chloride was added to a solution of (*S*)-(+)-1-phenylethylamine (0.3 g, 2.5 mmol) in 10 mL of CH_2Cl_2 containing 300 μL of Et_3N at 0 °C. The mixture was stirred at room temperature for 3 h and quenched by addition of HCl solution. The mixture was washed with a 2 N HCl solution (3 × 20 mL), dried over Na_2SO_4, and filtered. Solvent was removed in vacuo to give crude product that was analyzed by HPLC. Recrystallization of the sample prepared from the (−)-acid from ethyl acetate gave a sample that was suitable for X-ray structure analysis.[21]

P450-Catalyzed Oxidations. Cytochrome P450 enzymes and P450 reductase were expressed in *E. coli* and purified as previously described.[10,22,23] A stock solution of substrate was prepared from substrate **1** (2 mg in 100 μL of methanol) and DLPC (2 mg) in 1.0 mL of 50 mM phosphate buffer, pH 7.4; the mixture was sonicated and stored at 0 °C. A stock solution of NADPH was prepared by addition of 15 mg of NADPH to 375 μL of the same buffer, and this solution also was stored at 0 °C. Reaction mixtures were prepared by mixing 1 nmol of P450 enzyme, 2 nmol of P450 reductase, 60 μL of DLPC, and the above buffer solution to bring the total volume to 1.9 mL at 0 °C. After standing at 0 °C for 10 min, 10 μL of the stock solution of substrate was added. The mixture was stored for 15 min at 0 °C and then placed in a 10 °C water bath. After 5 min at 10 °C, the reaction was initiated by addition of 100 μL of the NADPH stock solution. Reactions were maintained at 10 °C for 30 min and then quenched by the addition of 2 mL of CH_2Cl_2. The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (2 × 2 mL). The combined organic phase was dried ($MgSO_4$) and filtered. For quantitation studies, an internal standard (1-phenylpropan-1-ol) was added, the mixture was concentrated under a stream of nitrogen to ca. 0.2 mL, and the resulting mixture was analyzed by GC (Carbowax) using flame ionization detection.

Isotopic Compositions of 2 and 3. These were determined by GC-MS analysis of the corresponding acetates. Following the P450-catalyzed oxidation reactions, the combined organic phases were concentrated to ca. 0.5 mL under a nitrogen stream. Pyridine (50 μL) and acetic anhydride (100 μL) were added, and the mixtures were allowed to stand at room temperature overnight. The reaction mixtures were diluted to 1.5 mL (CH_2Cl_2) and washed with a 1 N HCl solution (3 × 1 mL) and a 1 N NaOH solution (3 × 1 mL). The solutions were dried ($MgSO_4$), filtered, and concentrated to ca. 0.1 mL under a nitrogen stream. The mixtures were analyzed by GC-MS (Carbowax) with methane gas chemical ionization. The mass spectra and representative results are shown in Figures 3 and 4.

Intermolecular KIE Studies. Stock solutions of substrates containing **1**-d_0 and **1**-d_3 were prepared as described above. The ratio of substrates in each was determined by GC-MS using chemical ionization by monitoring the $(M - 19)^+$ (loss of F) channels at $m/e = 181$ and 184. For studies with (*S*,*S*)-**1**, the d_0/d_3 ratio was 1.25. For studies with (*R*,*R*)-**1**, the d_0/d_3 ratio was 1.04. The ratios of d_0 to d_2 products were corrected by the ratios of the substrates to give the apparent KIE values.

Determination of H_2O_2 Production. Reconstitution of the P450 enzymes with P450 reductase and DLPC was previously reported.[28,59] Reaction mixtures (total volume 1.2 mL) were prepared in triplicate and consisted of P450 (0.08 μM Δ 2B4 or Δ2B4 T302A, 0.04 μM Δ2E1 or Δ2E1 T303A), reductase in an amount equal to that of the P450, and DLPC in a 75:1 molar ratio with total protein (reductase + P450). The protein−phospholipid mixtures were allowed to stand at room temperature for 30 min, diluted with potassium phosphate buffer (pH 6.8 or 7.4, final concentration 0.1 M), and equilibrated for 5 min at 30 °C before initiation of the reactions with excess NADPH (0.7 M). Reactions were carried out at the same temperature with shaking under air for 5 min with the Δ2E1 pair of proteins at pH 6.8 and for 8−15 min in all other cases. After termination of the reactions by addition of perchloric acid (final concentration, 2%) and precipitation of the denatured protein, H_2O_2 was quantified spectrophotometrically as the ferrithiocyanate complex in 1.0-mL aliquots of the supernatant

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fraction.[28] Zero-time controls contained all of the components of the reconstituted enzyme system but were treated with perchloric acid before addition of NADPH. Standard curves were constructed with known amounts of H_2O_2 added to perchlorate-denatured, zero-time mixtures. The turnover numbers listed in Table 3 are the averages of values obtained in two separate experiments. No H_2O_2 was formed when the flavoprotein or NADPH was omitted from the reaction mixtures.

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Supporting Information Available: Appendix discussing masking effects, tables of results from the dilution study, variable temperature studies, intra- and intermolecular KIE studies, and the results of the time-course and Michaelis−Menten studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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