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Kinetic Isotope Effects Implicate Two Electrophilic Oxidants in Cytochrome P450-Catalyzed Hydroxylations

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The cytochromes P450 are a superfamily of heme-containing enzymes that catalyze a wide range of oxidations in nature.[1] The reaction cycle for P450-catalyzed oxidations involves substrate binding, reduction of iron, oxygen complexation, and a second reduction step to give the peroxo-iron species (Figure 1). Subsequent protonation on the distal oxygen gives a hydroperoxy-iron intermediate, and a second protonation on the distal oxygen with loss of water gives an iron-oxo species. The peroxo-iron and hydroperoxy-iron species were detected recently via "cryo-reduction" methods.[2a] The iron-oxo intermediate was not detected at low temperature in the presence of substrate,[2a] but evidence for its production in the absence of substrate has been reported.[2b] If the second protonation occurs on the proximal oxygen, the product is iron-complexed hydrogen peroxide.

The iron-oxo species of P450 has long been thought to be the active electrophilic oxidant,[1] but studies from our laboratories resulted in the controversial conclusion that P450-catalyzed oxidations can involve a second electrophilic oxidant, the hydroperoxy-iron species or iron-complexed hydrogen peroxide. One set of evidence came from studies with sensitive probes that implicated formation of cationic species that could arise from insertion of the elements of OH^+, which is not possible for the iron-oxo species.[3,4] Another set of studies involved pairs of wild-type and mutant P450s in which a highly conserved active-site threonine was mutated to alanine with the result that the wild-type and mutant pairs displayed widely different regioselectivities in oxidations of alkenes[5] and in oxidations of substrate **1**.[6] Jones and co-workers reported similar changes in regioselectivity with another pair of wild-type and mutant P450s,[7a] and Jin et al. recently reported evidence that an oxidant other than iron-oxo can effect epoxidation reactions.[7b]

Growing evidence indicates that multiple reaction channels occur in P450-catalyzed oxidations,[8] but an alternative to the "two-oxidants" model for multiple reaction pathways exists. Shaik, Schwarz, and co-workers computed that iron-oxo should have accessible high- and low-spin states that, in principle, could react with different regioselectivity.[9] We report here kinetic isotope effect (KIE) studies of P450-catalyzed oxidations that support the two-oxidants model in contrast to Shaik's two-states model.

We studied P450-catalyzed oxidations of highly enantiomerically enriched isotopomers of substrate **1** with dideuterated methyl groups. Three products are formed in the oxidations of **1**: unrearranged alcohol **2** and rearranged alcohol **3** from methyl group oxidation, and *p*-(*trans*-2-methylcyclopropyl)phenol (**4**) from phenyl oxidation. The use of enantiomers of **1** ensures that we maintained consistent kinetic parameters for any intermediate formed from the substrate while avoiding a possible convolution of diastereomeric interactions between the chiral substrates and the enzyme. Products **2**−**4** are readily separated and quantified by GC.[10,11] To determine the intramolecular KIEs, we developed an accurate and precise

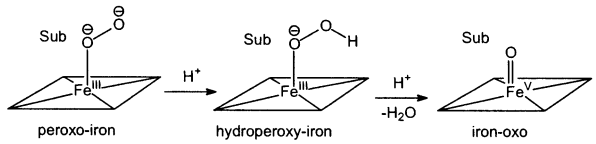


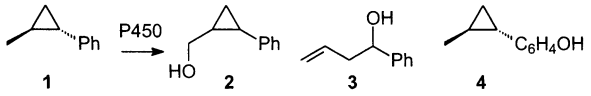
Figure 1. The iron-oxygen intermediates in P450, where the parallelogram represents heme, and "Sub" is substrate.

Table 1. Apparent KIEs in Oxidations of Substrates **1**[a]

enzyme	substrate	KIE_{app} in **2**	KIE_{app} in **3**	**2**/**3**[b]
2B1	(*R*,*R*)-**1**-d_2	7.81 ± 0.01	7.11 ± 0.01	5
	(*S*,*S*)-**1**-d_2	8.43 ± 0.01	7.83 ± 0.01	5
Δ2E1	(*R*,*R*)-**1**-d_2	7.80 ± 0.01	6.72 ± 0.01	19
	(*S*,*S*)-**1**-d_2	7.97 ± 0.02	6.45 ± 0.06	10
Δ2E1 T303A	(*R*,*R*)-**1**-d_2	7.46 ± 0.01	8.05 ± 0.02	7
	(*S*,*S*)-**1**-d_2	7.44 ± 0.04	6.49 ± 0.08	7

[a] Apparent KIEs are statistically corrected ratios of d_2 to d_1 products. Values are weighted averages (4−5 runs) with errors at 1σ. [b] Approximate ratio of product **2** to product **3**.

protocol for the analysis of products **2** and **3** using chemical ionization GCMS of acetate derviatives and integration of selected ion channels (Supporting Information). Previously, microsomal P450 oxidation of isotopomers of **1** was studied, but only the KIEs for product **2** were determined in that work.[11] Oxidations were conducted with expressed, purified hepatic cytochromes P450 2B1, P450 Δ2E1, and P450 Δ2E1 T303A. Reactions were run with substrate saturation concentrations at 10 °C for 30 min (Supporting Information).



Remarkable results were obtained in oxidations of the methyl-d_2 substrates (Table 1). The apparent intramolecular KIEs for the two products from oxidation of the methyl position differed in all cases with deviations that were much greater than the precision of the analytical method. With (*S*,*S*)-**1**, the KIEs for product **2** were greater than those for alcohol **3**. For the (*R*,*R*)-**1** enantiomer, the KIEs did not vary in the same direction; unrearranged product **2** had a larger KIE than **3** with two enzymes, but a smaller KIE for the other enzyme.

The seeming randomness of the apparent KIEs cannot be explained in the context of a single process, and the results add to the evidence that multiple oxidation pathways exist for P450-catalyzed hydroxylations. If **2** and **3** were formed from a common pathway, the observed KIEs for the two products should be the same in each individual reaction or possibly slightly different by a constant amount due to small secondary KIEs. The large variability

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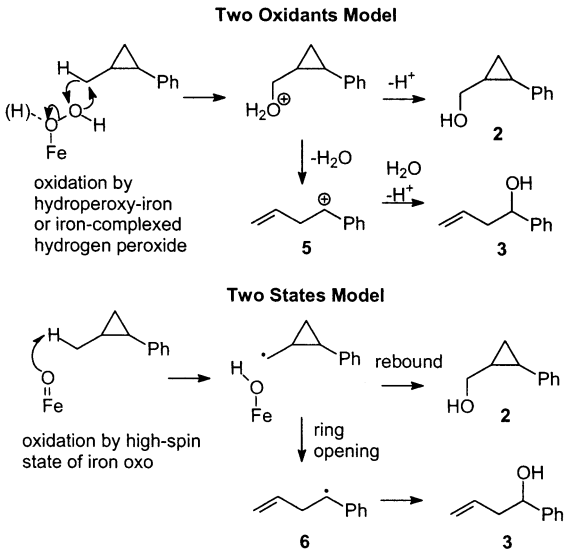
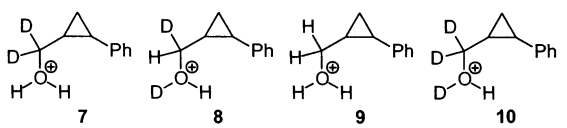


Figure 2. Bifurcated reaction pathways in the two-oxidants and two-states models.

in the apparent KIEs requires that at least two reaction channels exist and that at least one of the products was formed in both pathways.

The variable KIEs permit differentiation between the two-oxidants model and the two-states model. Both models predict that one of the two pathways will give unrearranged alcohol **2**, by insertion reactions of either the iron-oxo (two-oxidants model)[4,8] or the low-spin state of iron-oxo (two-states model).[9] Both also predict that the other hydroxylation reaction can give both products **2** and **3** (Figure 2). In the two-oxidants model, reaction of the hydroperoxy-iron species results in insertion of OH^+ into the C−H bond, and the resulting protonated alcohol can either be deprotonated to give **2** or rearrange via a solvolysis reaction to give cation **5**. In the two-states model, reaction of the high-spin state of iron-oxo gives a radical that can either be trapped to give **2** or ring open to give radical **6**.

Variable KIEs in product **2** are consistent with either model, but variable KIEs in product **3** are not. The two-states model predicts a KIE for product **3** that is greater than that for **2** at all levels of theory,[12] whereas a variable KIE in product **3** is expected in the two-oxidants model. Protonated alcohol intermediates **7** and **8** are formed by insertion of OH^+ at the methyl position of substrates **1**-d_2. In addition to the KIE in the insertion reaction, another KIE is expected in the deprotonation reactions that give cyclic product **2**. Specifically, **7** will be deprotonated faster than intermediate **8**, and this will result in the formation of **3** with a content of monodeuterated product that is disproportionately high relative to the populations of **7** and **8**. Thus, the apparent KIE for **3** will be *reduced* from the KIE in the insertion reaction by a variable amount that depends on the relative rates of deprotonation and solvolytic ring opening.



In a further test of the model, matched P450-catalyzed oxidations of **1**-d_0 and **1**-d_3 were conducted. Insertion of OH^+ would give intermediates **9** and **10**, respectively. A KIE in the proton-transfer step should result in faster deprotonation of **9** from the nondeuterated substrate and, thus, more efficient formation of cyclic product **2** from the **1**-d_0 substrates. As expected, the **2**/**3** ratio decreased from 6.5 for (*R*,*R*)-**1**-d_0 to 5.0 for (*R*,*R*)-**1**-d_3 and from 4.1 to 3.1 for (*S*,*S*)-**1**-d_0 and -d_3 substrates, respectively, in P450 2B1 oxidations. Similar results were found with P450 Δ2E1, where the **2**/**3** ratio decreased from 14 to 6 (*R*,*R*) and from 14 to 5 (*S*,*S*) when the d_0 and d_3 substrates were compared. We note that two-states model predicts the opposite effect; that is, the KIE for **3** should be greater than that for **2**, resulting in *increased* **2**/**3** ratios for the d_3 substrate.[12]

In principle, one might extract the KIEs for all three processes, both insertions and the proton transfer. For example, the results in Table 1 can be simulated with a KIE of 10 for the FeOOH reaction, a KIE of 6 for the iron-oxo reaction, and relative rates of deprotonation of **7** and **8** in the range from 1.3 to 1.8. However, this set of solutions is not unique. The conclusions we can reach are $(k_H/k_D)_{FeOOH} > 8$ and $(k_H/k_D)_{FeOOH} > (k_H/k_D)_{oxo}$.

Our studies indicate that hydroperoxy-iron or iron-complexed hydrogen peroxide is a second electrophilic oxidant in P450, in addition to iron-oxo. Mixing of two distinct hydroxylation reactions was the origin of the variable apparent KIEs we found, and this might also result in variable KIEs in P450-catalyzed oxidations of simple substrates. We note, however, that the methyl C−H bond in substrate **1** is weakened by conjugation to the cyclopropyl group,[13] and the hydroperoxy-iron oxidant might not react efficiently with stronger C−H bonds.[7b] Future studies might seek to determine the KIEs for each oxidant by isolating the individual reactions.

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Supporting Information Available: Description of experimental methods and results (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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