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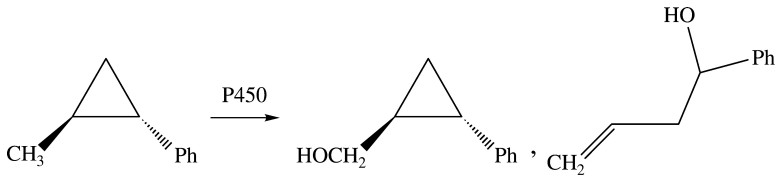
J. Am. Chem. Soc., **2003**, 125 (43), 13024-13025• DOI: 10.1021/ja036906x • Publication Date (Web): 01 October 2003



1 **2** **3**

$$KIE(\mathbf{2/3})_{TSR} = KIE(\mathbf{2/3})_{exp.}$$

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Published on Web 10/01/2003

How Does Product Isotope Effect Prove the Operation of a Two-State "Rebound" Mechanism in C−H Hydroxylation by Cytochrome P450?

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C−H hydroxylation is a fundamental process. In Nature it is catalyzed by the enzyme cytochrome P450, in a still-debated mechanism that poses a major intellectual challenge for both experiment and theory.[1] In 1976, Groves and McClusky[2] suggested that hydroxylation by P450 proceeds via a "rebound" mechanism, in which the high-valent iron−oxo species of the enzyme, so-called Compound I (Cpd I, see Figure 1), initially abstracts a hydrogen from the C−H bond. Subsequently, the so-generated organic radical rebounds on the iron−hydroxo species (e.g., $\mathbf{I}_U$ in Figure 1) to produce the alcohol product complex (e.g., **2** in Figure 1). The rebound mechanism was considered to be the consensus mechanism[3] until Newcomb et al.[4] used ultrafast radical and cationic probes to assess the mechanism. Their studies resulted in controversial lifetimes,[4] which cast doubts on the rebound mechanism and the presence of radicals in the reaction. Further, since the data suggested the presence of two "oxidizing species", Newcomb et al.[4] proposed that these "two oxidants" were Cpd I and its precursor, ferric hydroperoxide. However, subsequent evidence[5] that, for C−H hydroxylation, Cpd I is the sole oxidant, ruled out the participation of ferric hydroperoxide and further contributed to the tantalizing mechanistic puzzle. What, then, could these "two species" be that appear to be involved in the mechanism?

Density functional theoretical (DFT) calculations by our group[6] revealed that the two species are the high-spin (HS) and low-spin (LS) states of Cpd I (Figure 1), which lead to a two-state rebound mechanism with a HS component that is stepwise characterized by "normal" radical lifetimes, and an effectively concerted LS component having an ultrashort or even zero radical lifetime; *radical rearrangement thus originates only from the HS component*. Projection of the two-state model onto the experimental data provided a simple rationale for the controversy and explained trends in the experimental data.[1,6,7] Since oxygen transfer reactions by ferric hydroperoxide invariably led to very large barriers,[8] the two-state reactivity (TSR) scenario of Cpd I was deemed both necessary and sufficient to account for the experimental findings.[1] What was missing were experimentally measurable probes, characteristic of TSR. To this end, Ogliaro et al.[6b] computed the kinetic isotope effects (KIEs) for methane hydroxylation, as a model substrate, and obtained different KIEs for the HS and LS mechanisms, $KIE_{LS} < KIE_{HS}$. This result projected that *in a TSR scenario, C−H hydroxylation is predicted to exhibit a product isotope effect, namely dependence of the ratio of the rearranged and unrearranged products on isotopic substitution of the substrate*.

Recently, Newcomb et al.[9] measured the product isotope effect for *trans*-2-phenyl-methyl cyclopropane, **1** in Figure 1, which yields the unrearranged (*U*) and rearranged (*R*) alcohol products, **2** and **3**; the latter having smaller isotope effect (IE) than the former, i.e., [IE(**3**, *R*)/IE(**2**, *U*)] < 1. *This observation means that **2** and **3** are mediated by different "pathways"*. Since the measured product isotope effect was the opposite to the one computed by the TSR scenario for methane hydroxylation,[6b] Newcomb et al.[9] ruled out the TSR scenario and favored a two-oxidant scenario with Cpd I and ferric hydroperoxide. This result heightened the mechanistic controversy and underlined the urgent need to resolve the puzzle and establish the mechanism of alkane hydroxylation on more solid grounds. This is precisely the goal of the present paper, which, *in the spirit of experiment−theory synergism*, uses DFT to investigate the mechanism of C−H hydroxylation in the Newcomb probe, **1**, and to determine its product isotope effect. As shall be demonstrated, the TSR scenario predicts a product isotope effect in perfect accord with experiment.

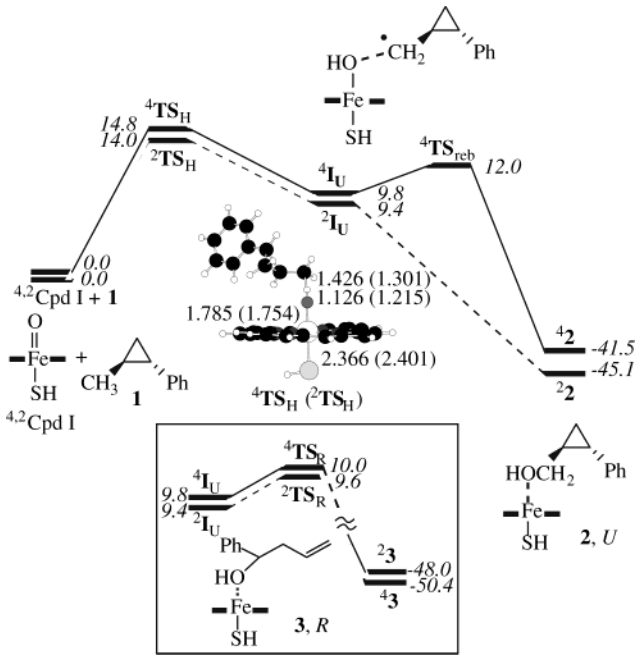

Figure 1. Two-state energy profile with zero-point correction for P450 hydroxylation of **1**, leading to unrearranged and rearranged products.

The calculations used the hybrid density functional B3LYP coupled with the double-ζ, LACVP basis set,[10] as described in the past.[6,8] Figure 1 shows the lowest-energy mechanism for C−H hydroxylation of **1**. The calculations reveal a rebound mechanism with a TSR energy profile. An initial hydrogen abstraction phase leads to radical intermediates ($^{4,2}\mathbf{I}_U$) coordinated to the heme by OH- - -C hydrogen contacts. Attempts to locate carbocation intermediates in both the gas phase and in a medium with a dielectric constant of 5.7 revealed that these intermediates are considerably higher lying. Thus, theory predicts preponderance of radical intermediates in the first step. Subsequently, the radical complexes rebound to form the alcohol product complex (^{4,2}**2**). As before,[6] the LS rebound is barrierless, while the HS rebound has a barrier.

10.1021/ja036906x CCC: $25.00

Table 1. Calculated Kinetic Isotope Effect (KIE) and Product Isotope Effect (PIE) for C−H Hydroxylation of **1**[a]

substrate	KIE_{LS}	KIE_{HS}	PIE(**2**/**3**)	PIE(**2**/**3**)[e] exptl.
1-D_2H[b]	6.20	5.72	1.083	1.143
	7.63	6.86	1.112	
	10.91	8.92	1.223	
1-D_3 (**1**-CH_3) [c]	8.06	8.55	0.943	
1-D_2H (**1**-CH_2D)[d]	6.22	5.77	1.078	

[a] PIE(**2**/**3**) and KIEs are defined in eqs 1 and 2. $KIE_{reb} = 1.01$, $KIE_R = 1.01$. As such, in eq 1 $\Phi \approx 1$ and PIE(**2**/**3**) is defined by eq 2. [b] Intramolecular KIE. The first KIE entry corresponds to a classical value and the second and third one include tunneling using the Wigner and Bell corrections, respectively. [c] Intermolecular KIE. Only the classical value is shown. Using Bell's correction PIE = 1.120. [d] Classical intermolecular KIE values (H-abstraction from **1**-D_2H vs D-abstraction from **1**-CH_2D. [e] An average value of four results from ref 9.

The inset box in the figure shows the rearrangement of the radical complexes, via the transition states $^{4,2}TS_R$, to yield the corresponding rearranged product complexes (^{4,2}**3**). The barriers for rearrangement are 0.2 kcal/mol for both HS and LS states, somewhat smaller than the calculated rearrangement barrier of the free *trans*-phenyl-cyclopropyl carbinyl radical (0.8 kcal/mol). While the HS rearrangement barrier is significantly lower than the corresponding HS rebound barrier, by contrast, the LS rearrangement barrier is finite compared with a zero LS rebound barrier. As such, the HS pathway will lead mostly to rearranged products (**3**), while the LS will lead mostly to unrearranged products (**2**). *It follows therefore that C−H hydroxylation of the Newcomb probe, trans*-2-phenyl-methyl cyclopropane, proceeds via a two-state rebound mechanism.

The product isotope effect (PIE) in TSR will be determined by interplay of the isotope effects of all the processes. Since the HS process yields mostly rearranged (*R*, **3**) product while the LS process leads mostly to unrearranged (*U*, **2**) product (the barriers with ZPE predict ca. 31% of **3** at $T = 37$ °C), we may write the following equation[6b] for the product isotope effect:

$$\mathrm{PIE}(\mathbf{2}/\mathbf{3}) = [U_H/U_D]/[R_H/R_D] = (\mathrm{KIE}_{\mathbf{LS}}/\mathrm{KIE}_{\mathbf{HS}})\cdot\Phi$$

$$\Phi = \{(1/\mathrm{KIE}_R)[(k_R{}^H/k_{reb}{}^D + \mathrm{KIE}_{reb})]/[(k_R{}^D/k_{reb}{}^D) + 1]\} \quad (1)$$

Here, KIE_{HS} and KIE_{LS} are the kinetic isotope effects of the hydrogen abstraction step, and Φ is a function that incorporates isotopic effects on the rearrangement, KIE_R, and rebound processes, KIE_{reb}. At the limit where KIE_{reb} and KIE_R are close to unity, Φ will itself be unity, and PIE(**2**/**3**) will be given simply by the ratio of the HS and LS KIEs for the hydrogen abstraction step, i.e.,

$$\mathrm{PIE}(\mathbf{2}/\mathbf{3}) \approx (\mathrm{KIE}_{\mathbf{LS}}/\mathrm{KIE}_{\mathbf{HS}}) \quad (2)$$

Table 1 shows the KIEs determined in the usual manner.[6b,11,12] Both classical and tunneling corrected values are shown.[11] The calculations show that the isotope effects on the rebound and rearrangement processes are closely unity, and as such Φ will be unity and the PIE(**2**/**3**) will be determined by the KIE ratio of the hydrogen abstraction step. At the outset, since the geometry of $^2\mathbf{TS}_H$ is close to being "central" ($r_{O\text{-}H} \approx r_{C\text{-}H}$) while that of $^4\mathbf{TS}_H$ is somewhat "late", one might expect to find that $\mathrm{KIE}_{\mathbf{LS}} > \mathrm{KIE}_{\mathbf{HS}}$, and hence, PIE(**2**/**3**) > 1, by contrast to methane hydroxylation,[6] where both $^{2,4}\mathbf{TS}_H$ structures were "late". For a direct comparison with the experimental results,[9] we calculated the isotope effect with the doubly deuterated substrate. The data of the individual KIEs and the calculated PIE (**2**/**3**) values, based on eq 2, are shown side by side. It is apparent that the theoretical value PIE(**2**/**3**) > 1 is in the direction of the experimentally determined ones, and even the absolute values are close. Thus, while Newcomb et al.[9] did not publish an error analysis to enable assessment of the inherent error of the experimental PIE(**2**/**3**) quantity, on its face value, the comparison with their results shows *that the TSR scenario provides a perfect model for interpreting the experimental results*. Thus, if the direction of experimentally derived PIE(**2**/**3**) quantity is definitely correct, *then the experiment constitutes a proof of the operation of TSR in C−H hydroxylation* in **1**. Theory predicts that if the PIE(**2**/**3**) quantity will be determined by intermolecular KIE measurement of the CH_3 viz. CD_3 substrates its value will be smaller than unity unless tunneling is important (footnote c), while measurements of the KIEs for the CD_2H/CH_2D pair will lead to PIE(**2**/**3**) >1. Finally, the results for methane,[6] propene,[11] and camphor[12] hydroxylation show that *the value of PIE will generally be substrate dependent, reflecting the structures of the **LS** vs **HS** transition states for the C−H bond activation step*. Modulation of these structures by mutations in the protein may therefore change also the PIE value. Thus, the TSR scenario appears to make ample predictions that can increase the interplay of theory and experiment in this important mechanistic area.

Acknowledgment. The research was supported by a grant by the Israeli Science Foundation (ISF) to S.S..

Supporting Information Available: Three tables and 13 figures with computational data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA036906X