Alkane Hydroxylation by Cytochrome P450: Is Kinetic Isotope Effect a Reliable Probe of Transition State Structure?

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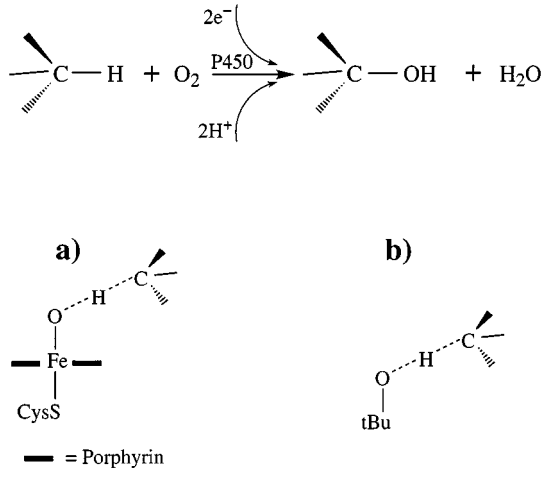
Computed kinetic isotope effect (*KIE*) values for methane hydroxylation by Cytochrome P450, in both high- and low-spin states, compare well with *KIE*'s for model hydrogen abstraction reactions, thereby demonstrating the utility of *KIE* as a mechanistic probe and supporting a two-state-rebound mechanism.

Introduction

Kinetic Isotope Effect (*KIE*) has been used extensively as a mechanistic probe in alkane hydroxylation by Cytochrome P450, Scheme 1.[1−5] In view of the importance of the process, and the questions which still surround its mechanism, it is deemed essential to establish the mechanistic significance of *KIE* by theoretical means in a manner that can be related to experimental measurements and mechanistic questions.



Scheme 1. Alkane hydroxylation by Cytochrome P450: **a**) and **b**) describe the proposed isostructural transition states based on kinetic isotope effect studies[6]

Let us first mention experimental data which form the primary motivation for this study. Thus, Dinnocenzo-Jones and co-workers have investigated the *intrinsic* $KIE_{H/D}$, for C−H vs. C−D hydroxylation[6] in a series of substrates undergoing P450 hydroxylation vis à vis hydrogen abstraction by the tertiary butoxyl radical. The $KIE_{H/D}$ data for the two processes were found to exhibit a good linear correlation as well as a virtual identity of the individual $KIE_{H/D}$ values for hydrogen abstraction and P450 oxidation of each substrate. These findings suggest, in agreement with earlier conclusions by Meunier et al.,[4] that the transition state for the bond activation step of P450 hydroxylation possesses a structure with a co-linear O−H−C moiety, as in a hydrogen abstraction process (**a** and **b**, Scheme 1). This structural feature of the transition state supports the "rebound" mechanism of alkane hydroxylation by P450.[1] However, since the rebound mechanism has been questioned by recent mechanistic investigations,[7,8] theoretical calculations are necessary to assess the mechanistic utility of the Dinnocenzo-Jones concept as a means to probe transition state structure.

To tackle this problem, we carried out density functional calculations of $KIE_{H/D}$ in the model hydroxylation reaction, Scheme 2, where methane is used as a substrate and the ferryl-oxene oxidant ($Por^{\bullet+}FeO$) carries a simplified thiolate ligand.[9,10] These $KIE_{H/D}$ values are compared with three model hydrogen abstraction processes by methoxyl and tertiary-butoxyl radicals, using methane and toluene as substrates. The mechanism for methane hydroxylation by ferryl-oxene was determined in recent work,[9,11] and the results are schematized in Figure 1. It was found that hydroxylation proceeds by a two-state-rebound mechanism of the high-spin (HS) quartet state and the low-spin (LS) doublet state, both nascent from the corresponding states of ferryl-oxene.[9,11] The processes exhibit two-phases with distinct atomic rearrangements; C−H bond activation and rebound. The bond activation takes place via two closely lying transition states ($^{4,2}TS_H$). Subsequently, the HS reaction proceeds to form an iron-hydroxo intermediate and a $CH_3^{\cdot}$ radical with a significant barrier for rebound. On the other hand, the ^2TS_H transition state collapses to alcohol products in a virtually barrier-free fashion, making the LS process effectively concerted.[9,11] In the present study we determine $KIE_{H/D}$ values for both HS and LS surfaces. These results are compared with the corresponding $KIE_{H/D}$ values of the radical abstraction reactions (Scheme 2), and enable us to assess the Dinnocenzo-Jones method,[6] and answer the title question.

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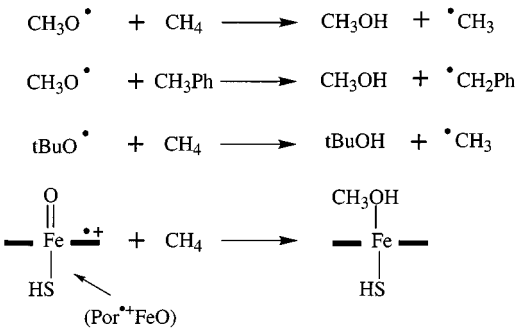


Scheme 2. Model reactions for kinetic isotope effect calculations

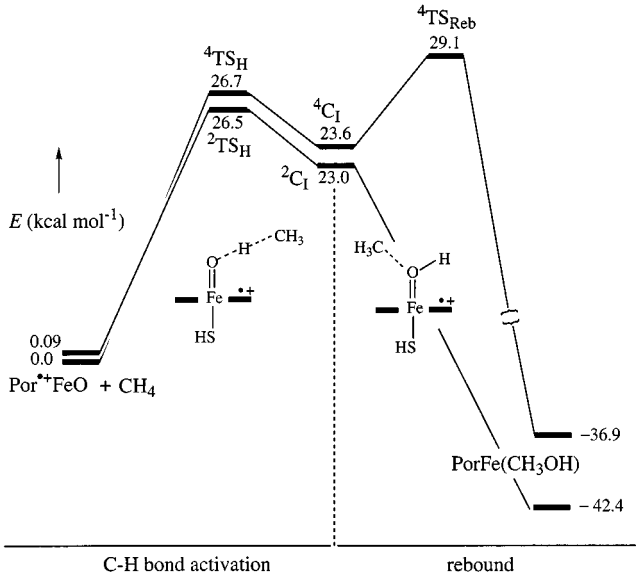


Figure 1. Calculated two-state rebound mechanism for methane hydroxylation by a model ferryl-oxene

Results and Discussion

All calculations were done with the GAUSSIAN98 package of programs.[12] The hybrid (HF/DFT) density functional UB3LYP calculations[13] was used. The Los Alamos effective core potential coupled with the double-zeta LACVP basis set[14] on iron and the 6−31G basis[12] for the rest of the atoms (hence, ECP+LACVP-6−31G). Geometries for all the species were fully optimized without symmetry constraints.[9,11] Here, the reactants, transition states, intermediates and alcohol products for all processes in Scheme 2 were ascertained by frequency calculations. Possible dependence of the KIE on the basis set was tested for the model hydrogen abstraction reaction by the alkoxyl radicals. Semiclassical *KIE* values[15] were determined from the Eyring equation using free energies of activation. Tunneling corrections[15] of the semiclassical *KIE* values were examined using three different models: the asymmetrical Eckart potential model,[15,16] the Bell model[15,16] and the Wigner model.[15,16]

Figure 2 shows essential features of the O−H−C portions of the transition states for the HS and LS bond activation, $^{4,2}TS_H$, and the model transition states for hydrogen abstraction by methoxyl and tertiary butoxyl radicals, $^2TS_{MeO}$, $^2TS_{Tol}$, and $^2TS_{tBuO}$. The latter three transition states are seen to be slightly more "central" than the P450 transition states, but otherwise the four transition states are similar. In all the structures, C−H cleavage is significant and the O−H−C portion is close to being linear. A negative spin density on the hydrogen flanked by positive densities on the heavy atoms is a mark of three-electron delocalization over three centers. The observation of such a feature in the transition states shows that all of them involve three electrons delocalized in the O−H−C portion, thereby emphasizing the common electronic structure shared by these transition states. This similarity supports the concept of Dinnocenzo-Jones and co-workers[6] that the hydrogen abstraction reaction by *t*BuO· models the bond activation in C−H hydroxylation by Cytochrome P450.

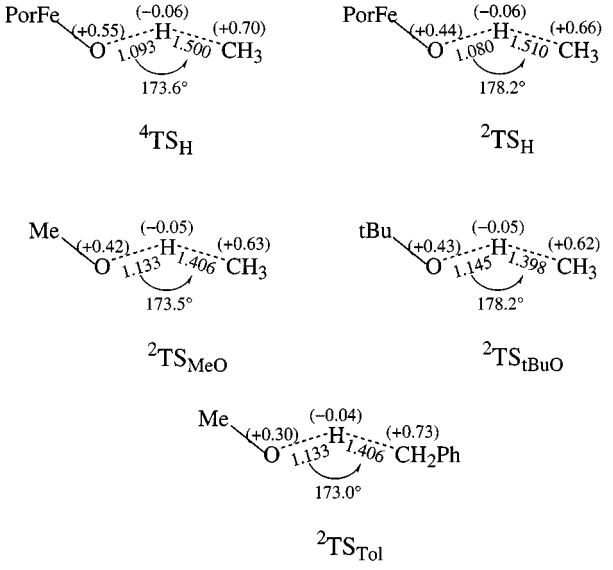


Figure 2. Transition state structures for the model reactions in Scheme 2; data in parentheses correspond to group spin densities; the spin densities on the oxygen atom P450 transition states ($^{2,4}TS_H$) are atomic spin densities and do not include the density on the rest of the species (hence the sum is not unity)

Table 1 shows computed $KIE_{H/D}$ for the four reactions; semiclassical values (3rd line), as well as tunneling corrected ones (fourth, fifth and sixth lines). As can be seen from the semiclassical values for the alkoxyl radicals, improvement of the basis set increases the $KIE_{H/D}$ values by less than 5%. Changing the substrate from methane to toluene has a small effect too (*ca* 15%). Due to the size of the problem, we cannot upgrade the frequency calculations for the P450 reactions, but the preceding comparisons indicate that the ECP+LACVP-6−31G results can be regarded with credence.

As shown in Table 1, the approximate tunneling corrections are extremely sensitive to the imaginary frequency of the transition state. All three different types of tunneling corrections agree with one another for the imaginary fre-

Table 1. Imaginary frequencies (cm^{-1}) in the transition states (TS) of the four processes (Figure 2), and calculated *KIE* values at T = 298.15 K; the Eckart correction for the doublet state is calculated with parameters for an effectively concerted process

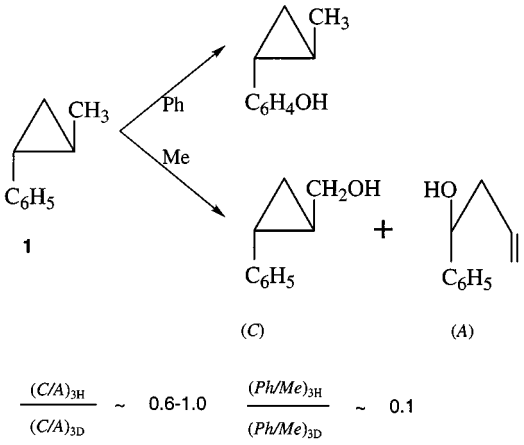
	^2TS_H [a]	^4TS_H [a]	$^2TS_{MeO}$ [a][b]	$^2TS_{tBuO}$ [a][b]	$^2TS_{Tol}$ [a]
ν_H	*i*703	*i*942	*i*1337 [*i*1648]	*i*1458 [*i*1709]	*i*1002
ν_D	*i*604	*i*764	*i*1006 [*i*1216]	*i*1090 [*i*1261]	*i*787
$KIE_{H/D}$					
semi-classical	5.10	5.87[c]	6.17 [6.36]	6.46 [6.47]	5.28
Wigner	5.57	6.97	8.51 [9.49]	9.19 [9.75]	6.51
Eckart	5.95	7.53	11.26 [21.55]	15.15 [28.22]	8.44
Bell	5.94	9.13	63.76 [2.39]	11.51 [1.04]	9.61

[a] Out of brackets are results of the ECP+LACVP-6−31G (for the P450 reactions) and 6−31G (for alkoxyl reactions) basis sets. − [b] In square brackets 6−31G* results. − [c] At T = 278 K the semiclassical $KIE_{H/D}$ for P450 is 6.95, close to the maximum limit.[4]

quencies below ca. 1000 cm^{-1}, but show strong basis set- and model dependence for higher frequencies. Apparently, such situations cannot be adequately treated by the one-dimensional tunneling corrections. Unfortunately, the size of the P450 species does not allow us to perform sophisticated calculations of the tunneling. However, for P450 situations, the tunneling correction for the three models, changes the $KIE_{H/D}$ values by less than 20% (see Table 1). Thus, tunneling has a modest effect on the $KIE_{H/D}$ values, and the semiclassical $KIE_{H/D}$ values may be used for comparison with the experiment.[6] A similar estimation of the role of tunneling in this reaction has been done by Meunier et al.[4]

The Dinnocenzo-Jones[6] $KIE_{H/D}$ values for hydrogen abstraction from toluene and *p*-xylene by *t*BuO$^{\cdot}$ are 6.0 ± 0.2 and 6.3 ± 0.3, respectively, compared with 5.9 ± 0.35 and 6.4 ± 0.90 for the corresponding P450 hydroxylations. The average computed $KIE_{H/D}$ for P450 hydroxylation is only 5−15% lower than the corresponding values for the hydrogen abstraction by alkoxyl radicals. Considering the spread of the experimental values, of 10−15%, our computed results exhibit a reasonable match between the $KIE_{H/D}$ values for hydrogen abstraction by alkoxyl groups and those for the bond activation step in P450 hydroxylation. The common features of transition state structures, discussed in Figure 2, and the similarity of $KIE_{H/D}$ values support, therefore, the mechanistic conclusion of Dinnocenzo and Jones[6] based on $KIE_{H/D}$ profiles of P450 C−H hydroxylation vis à vis a model hydrogen-abstraction reaction. The results indicate that the kinetic isotope effect can serve as a reliable probe of the transition state structure in P450 hydroxylation, and support the rebound mechanism.[1]

It is interesting to apply these results on the $KIE_{H/D}$ profiles to another situation where the deuterium substitution comes into play. Recently, Newcomb and co-workers[17−19] have shown that a substitution of the CH_3 group of **1**, Scheme 3, by CD_3, changes the regioselectivity by an order of magnitude, from a methyl to a phenyl oxidation ("metabolic switching"). However, the amount of rearrangement due to C−H hydroxylation of **1**, measured by the ratio of cyclic to acyclic products (*C*/*A*), exhibits a small isotope effect, if any.[17,18] The metabolic switching implies a large *KIE* on the C−H hydroxylation in agreement with independent measurements[1−5] of internal *KIE* values. What could then be the mechanistic significance of a small product isotope effect on the *C*/*A* ratio? Is it always going to be small, or will it change, and if so what factors govern its change?



Scheme 3. Results of oxidation of probe **1** by Cytochrome P450 [17−19]

From mutation studies, Newcomb[19] has concluded that probe **1** undergoes C−H hydroxylation mostly, if not exclusively, by ferryl-oxene. If the hydroxylation occurs by the classical rebound mechanism with a single oxidant species, where the oxidant participates with a single reactive state and produces free radicals in the first step of the reaction, a constant *C*/*A* value (subject only to secondary isotope effects) will always result. However, as already mentioned, this scenario faces difficulties to account for the trend of radical lifetimes and for the incomplete scrambling often observed.[7,8]

These difficulties can be circumvented in the two-state-rebound mechanism. In this scenario (Figure 1), rearrangement occurs only on the HS surface which exhibits a barrier for the radical rebound.[9,11] In the event where the ferryl-oxene oxidant engages primarily or exclusively in C−H hydroxylation,[20,21] the isotope effect on the *C*/*A* ratio of a probe like **1** will be gauged by the ratio of the isotope effects for the HS and LS processes.[20,21] In the extreme situations, where the kinetic isotope effects for the HS and LS processes are very different, i.e., either $(KIE_{H/D})^{HS} >> (KIE_{H/D})^{LS}$ or $(KIE_{H/D})^{HS} << (KIE_{H/D})^{LS}$, replacement of the methyl group by deuteriomethyl will considerably slow down either the HS or the LS processes, thereby causing the $(C/A)_{3D}$ ratio to either increase or decrease dramatically relative to $(C/A)_{3H}$. Thus, a significant product isotope effect is expected under such conditions. In contrast, identical $KIE_{H/D}$ values for the HS and the LS processes can lead to a constant *C*/*A* ratio (neglecting secondary isotope effect).[20] The $KIE_{H/D}$ values in Table 1 for the HS and the LS processes are not identical, however they differ by only 15% of each other. If these *KIE* results are relevant to the probe substrate **1** in Scheme 2, they would predict a small

isotope effect on the *C*/*A* ratio,[21] as is found experimentally.[17]

It is important to emphasize that the near equality, $(KIE_{H/D})^{HS} \approx (KIE_{H/D})^{LS}$, in our calculation arises due to the structural similarity of the corresponding transition-state structures for the two states (Figure 2). The HS and LS transition structures have been predicted before to depend on the donor ability of the substrate (probe) and on the binding capability of the cysteinato ligand which can introduce structural variations between the two paths.[9,11] Thus, the two-state-reactivity scenario predicts some dependence of the product isotope effect on the *C*/*A* ratio on these factors, as well as on the rates of rearrangement and rebound of the free radical clock.[20] No such dependencies will be expected when the rebound mechanism proceeds on a single state surface and with a single oxidant.

Conclusions

In summary, the combination of a small isotope effect on the *C*/*A* ratio in P450 hydroxylation, along with the match of *KIE* values to model hydrogen-abstraction reactions have jointly a clear mechanistic significance in terms of two-state reactivity.[22,23] Other scenarios are possible; for example, a large product isotope effect on the *C*/*A* ratio will indicate either a more complex mechanistic scheme of two state reactivity,[21] or two-oxidants which participate in C−H hydroxylation.[7,8] It is hoped that this work will form the incentive to further articulate the various models and elucidate the mechanistic significance of *KIE* in P450 hydroxylation.

Acknowledgments

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[21] If, however, ferryl oxene is engaged significantly also in phenyl oxidation, the kinetic expression becomes more complex. Then the $(C/A)_{3H}/(C/A)_{3D}$ ratio can be significantly different than unity, even if the *KIE's* are the same for the HS and LS path. We thank J. P. Jones for communicating this point.
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