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Kinetic Isotope Effect is a Sensitive Probe of Spin State Reactivity in C−H Hydroxylation of *N,N*-Dimethylaniline by Cytochrome P450

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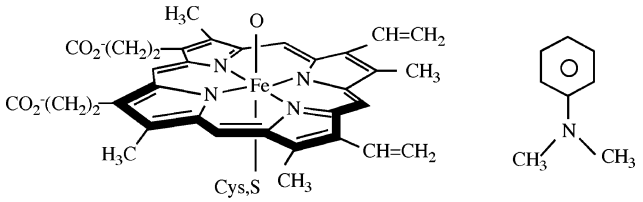
Cytochrome P450 enzymes function as oxidants that safeguard organisms against *xenobiotics*, metabolize drugs, and lead to biosynthesis of vital compounds.[1] A major transformation performed by these enzymes is C−H hydroxylation that is believed to occur by reaction of the substrate with the high-valent oxo-ferryl species, known as Compound I (Cpd I) and shown as **1** in Scheme 1.[2,3] Theoretical considerations and DFT calculations[4] revealed two major mechanistic features: (a) As originally concluded by Groves,[2] the reaction proceeds via the rebound mechanism with transition states that possess collinear O···H···C moieties; and (b) since Cpd I possesses degenerate doublet and quartet spin states, the process involves two energetically close spin state surfaces,[4] *hence*, two-state reactivity (TSR) with high-spin (HS) and low-spin (LS) components.[5] While TSR accounts for the unusual results encountered in this mechanism,[4,6] and while theory has repeatedly produced this picture,[4b,7] a lingering question remains: *is there any good experimental probe for TSR*?

The use of magnetic fields[4c] to probe the spin state scenario is an option, but these experiments are highly complex, and hence, a more accessible probe is required. As we showed recently, the intramolecular kinetic isotope effects (KIEs) for the HS and LS processes are different,[8] and hence, in principle, KIE may serve as an internal probe of the spin state reactivity. Since the identity of the primary oxidant of P450 is also in question,[9] a reaction is needed that is known to involve Cpd I. Such a reaction is the C−H hydroxylation of *N,N*-dimethylaniline (**2**, Scheme 1), which is initiated by C−H hydroxylation followed by hydrolysis to yield N-dealkylated products and formaldehyde. Recently, Dowers et al.[9] provided compelling evidence that, in different P450 enzymes, the sole active species that performs the hydroxylation is Cpd I. As such, modeling the reaction and its KIE values fits precisely the goal of this study.

The present paper uses B3LYP DFT calculations of the HS and LS mechanisms of C−H hydroxylation of *N,N*-dimethylaniline *and demonstrates* that KIE is a spin-selective probe that leads to the identification of the experimentally reactive spin states for this substrate. The calculations were carried out using the LACVP basis set, henceforth B1, as implemented in Jaguar 5.5.[10] Semiclassical and Wigner corrected KIE values[8] were derived using B1. Single point calculations, with the larger basis set (B2),[10] LACV3P+*, were carried out for energy evaluation. Since the relative energies, charges, and spin densities were almost invariant to the basis set, the B2 values were corrected using B1 zero point energies (ZPE).[11] Additional corrections were added due to effects of bulk polarity (using a solvent model with a dielectric constant, $\epsilon = 5.7$, and a probe radius of 2.72 Å) and of the NH···S hydrogen bonding to the sulfur.[8] The complete data are summarized in the Supporting Information (SI).

Scheme 1. Cpd I (**1**) and *N,N*-Dimethylaniline **2**



1, CpdI **2**

Figure 1 shows the energy profiles nascent from the LS and HS states of Cpd I. The respective B1 data (see SI) are similar; for example, the uncorrected barriers for the HS/LS processes are 9.7/8.0 (B1) versus 9.6/7.8 (B2). Furthermore, the LS-below-HS ordering of the $^{4,2}TS_H$ species was reproduced by other functionals (see SI, Table S2b). A few points are notable from Figure 1. First, the two energy barriers are small and get smaller due to the corrective terms; the ZPE is chiefly responsible for the lowering of both transition states, while the hydrogen bonding affects mostly the LS species. *These are the smallest barriers calculated so far for C−H hydroxylation.*[4b] The small barriers are derived in part from the weak C−H bond energies in **2**.[4b,12] These computational results are in accord with the experimental observation[9] that **2** is one of the most reactive substrates in P450 oxidation. Second, the two processes are effectively concerted; the radical species, $^{2,4}\mathbf{C}_I$, are shoulders on the potential energy surface, and the rebound process is barrier free. This trend follows the predictions of the model constructed for the rebound process[4a] and is in line with other data.[4,6,13] Finally, the energy difference between the HS and LS transition states increase to 3.7 kcal/mol when the environmental effects are added. This trend, which is dominated by the hydrogen bonding to the sulfur ligand of Cpd I, has been noted before for allylic hydroxylation,[14] and it typifies cases where the organic

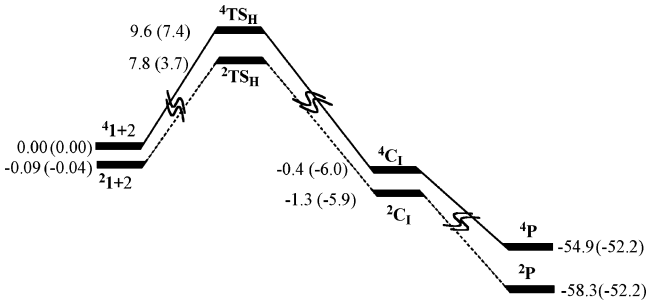


Figure 1. Energy profiles (B2//B1) for the HS and LS C−H hydroxylation of **2** by Cpd I (**1**). Values outside the parentheses are relative potential energies, while the values in parentheses involve corrections due to ZPE, bulk polarity, and NH···S hydrogen bonding ($r_{N\cdots S} = 2.66$ Å).[8]

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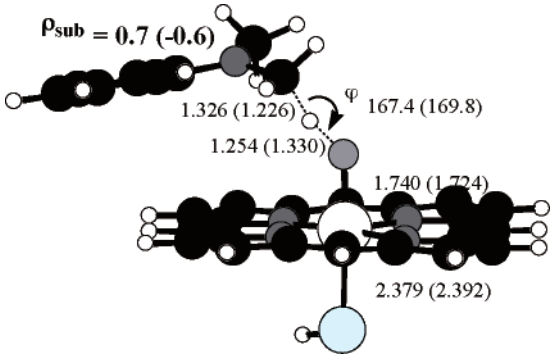


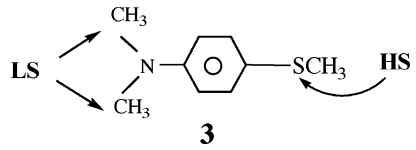
Figure 2. Optimized structures for the bond activation transitions states, ^4TS_H and ^2TS_H. Values outside the parentheses correspond to ^4TS_H, while the values in parentheses correspond to ^2TS_H. Above the structure, we note the spin densities, HS(LS), on the organic moiety (ρ_{sub}).

radical can undergo stabilization by delocalization (in the case of **2** toward the nitrogen).[11f] With this energy difference, *the reaction will proceed mostly via the LS pathway*.

The key geometric parameters of the bond activation transition state, $^{4,2}TS_H$, are displayed in Figure 2. The structures are seen to involve H-abstraction of the radical type, as is evidenced also from the spin density (ρ_{sub}) on the organic moiety (orbitals are shown in Figures S10 and S11). An electron transfer mechanism[15] was ruled out since transferring an electron from **2** to **1** (in the TS, and the reactants cluster; see Tables 10a,b in the SI) gave higher energy species compared with those in Figure 2.

To characterize spin-selective probes, we calculated KIE values for the isotopomer used by experiment,[9] that is, a *N*,*N*-dimethylaniline substrate with two CD_2H groups. The KIE values show small dependence on the conformation of the two CD_2H groups (see Tables S6 and S7 in the SI). The semiclassical values for the LS process ranged between 3.50 and 4.03 (KIE(av.) = 3.75). The Wigner-corrected values of 3.59−4.15 (KIE(av.) = 3.86) indicate that tunneling is negligible in the LS process. By contrast, the semiclassical KIE values for the HS process are in the range of 5.69−6.24 (KIE(av.) = 5.96). The Wigner-corrected KIEs are higher, ranging between 7.85 and 8.65 (KIE(av.) = 8.24) and indicating that tunneling will be important on the HS surface. This large difference between KIE_{LS} and KIE_{HS} is important because *it can serve as a probe of the reactive spin* state. The geometric data in Figure 2 show that the source of this large difference follows accepted theories[16,17] and can be attributed to the degree of C−H bond cleavage in the TS_H. Our past calculations for nine different substrates[12d] show that, generally, the degree of C−H cleavage is smaller in the LS TS (see SI, Table S9), in agreement with the generally smaller LS barrier in each case.[4b] Indeed, here too, the C−H bond length in ^4TS_H is 1.326 Å, significantly longer than the value of 1.226 Å in ^2TS_H, and the relative barriers also reflect the extent of C−H bond cleavage. As such, the LS process for **1** + **2** has a lower barrier, an "earlier" transition state, and is characterized by a smaller KIE. By contrast, the HS process has a higher barrier, a "later" transition state, and larger KIE.[9,17]

Our results in Figure 1 lead to the conclusion that *N*,*N*-dimethylaniline should be hydroxylated mostly through the LS process and should therefore exhibit a low KIE value of ca. 3.75. The experimental value is close to 3.0.[9,17] This KIE datum matches the calculated KIE_{LS} value, whereas it is much different than the calculated KIE_{HS} value. This exciting result highlights the conclusion that in some cases *the observed KIE can serve as a probe of the reacting spin state of Cpd I*, and that in the present system the change in KIE from a small to a large value will indicate the switch in spin state reactivity from LS to HS. Achieving this switch is a challenge. In cases where both HS and LS states participate and lead to different products, there will be a product isotope effect (given by the ratio KIE_{LS}/KIE_{HS}) that can probe TSR.[8] Finally, this finding supports the hypothesis of Jones et al.[18] regarding the reactivity of P450 enzymes toward *p*-methylsulfoxy-*N*,*N*-dimethylaniline, **3**, that undergoes competitive sulfoxidation and N-dealkylation. On the basis of our recent study, *sulfoxidation*[19] *occurs only by the HS state of Cpd I, while the present study shows that N-dealkylation via initial C−H hydroxylation proceeds via the LS state*. To the best of our knowledge, this substrate is the first case that reacts with P450 *by spin-selective regiochemistry*.



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Supporting Information Available: Additional tables (12), figures (13), and full ref 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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