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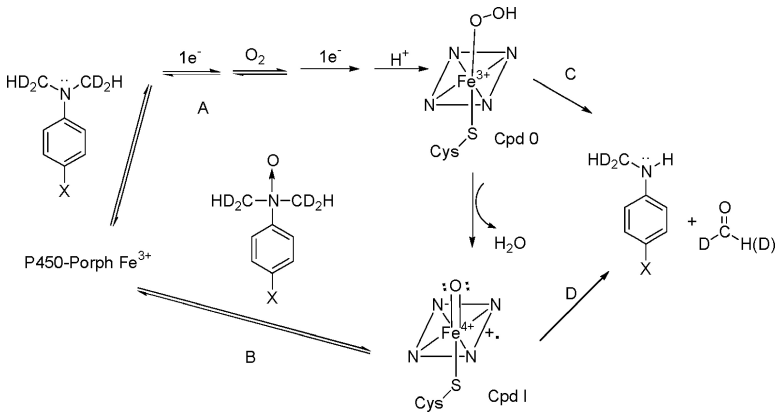

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Kinetic Isotope Effects Implicate the Iron−Oxene as the Sole Oxidant in P450-Catalyzed N-Dealkylation

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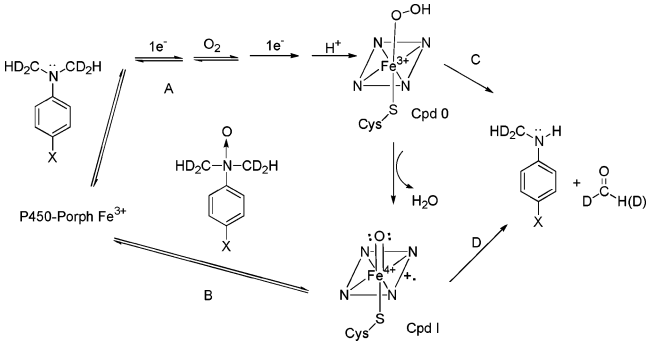
The ubiquitous cytochrome P450 (P450) enzymes are a superfamily of monooxygenases that function to metabolize both endogenous and exogenous compounds, including the majority of clinically relevant drugs.[1] A great deal of effort has been expended toward probing the mechanism by which P450 enzymes catalyze the oxidation of various organic functional groups. In the past, an electrophilic iron−oxene (Cpd I) was thought to be the sole active oxidant involved in P450-mediated reactions.[2−5] This mechanism was first elaborated by Groves and co-workers as being an initial hydrogen atom abstraction followed by recombination to produce an alcohol, in the case of aliphatic substrates.[5] However, the P450 catalytic cycle produces multiple iron−oxygen intermediates, each of which could potentially create alternate oxidative pathways (path A, Scheme 1). Furthermore, many recent publications specifically implicate an iron−hydroperoxy species (Cpd 0) as a second electrophilic oxidant (path C, Scheme 1).[6−10] Herein, we report results on *N*-dealkylation, one of the most facile reactions mediated by P450 enzymes. We have employed the *N*-oxides of a series of para-substituted $^{13}C^{2}H_2$-labeled *N*,*N*-dimethylanilines (Cpd 1, Figure 1) to function as both substrates and surrogate oxygen atom donors for P450cam and P4502E1. This *N*-oxide system requires the substrate to donate its oxygen atom to the iron−porphyrin complex to directly produce the iron−oxene (path B, Scheme 1). Kinetic isotope effect (KIE) profiles obtained using the *N*-oxide system were found to closely match the profiles produced using the normal NAD(P)H/NAD(P)-P450 reductase/O_2 system. The results are consistent with oxidation occurring via Cpd I (path D, Scheme 1).

While most mechanistic studies are consistent with a single iron−oxene oxidant, other work has indicated that two distinct oxidants may play a role in oxidation. One explanation of these results that has been proposed by Shaik and co-workers is a two-state reactivity model in which Cpd I has two accessible spin states that can behave like two different oxidants. This is known as the two-state reactivity model or TSR.[11] Another explanation is that Cpd 0 (path C, Scheme 1) is responsible for some oxidations. Chandrasena et al. recently concluded that Cpd 0 was the predominant oxidant effecting aliphatic hydroxylation of the substrates used in their study.[6] Through site-directed mutatgenisis, Vaz et al. concluded that the hydroperoxy−iron was a preferential epoxidizing agent in comparison to hydroxylation.[7] Volz et al. suggested that Cpds 0 and 1, or two different spin-state iron−oxenes (TSR), are involved in sulfoxidation versus *N*-dealkylation.[8] Sharma et al. used density functional theory to conclude "...that sulfoxidation and *N*-dealkylation proceed largely via different spin states of Cpd I" consistent with the conclusions of Volz.[12] In a separate study, Hutzler and co-workers recently concluded that two oxidants may be involved in *N*-demethylation and *O*-demethylation.[10]

Other studies have come to conclusions about the energetics of Cpd 0. Jin et al. concluded that the iron−hydroperoxy intermediate could epoxidize alkenes but not hydroxylate the high energy C−H bond in camphor.[9] Chandrasena et al. concluded that Cpd 0 could hydroxylate an easier-to-oxidize methyl group on a cyclopropyl ring but not the methyl group of straight-chain aliphatic compounds such as octane.[6] Shaik and co-workers used density functional theory to conclude that the hydroperoxy−iron species is a very weak oxidant in comparison with the iron−oxene species.[11] The combination of these findings suggests that, if the hydroperoxy−iron is a viable oxidant, it might only be expected to effect P450-mediated reactions with lower activation energies, and not reactions that have high energy barriers such as primary aliphatic oxidations. Therefore, we aimed to determine if *N*-dealkylation, a P450-mediated reaction possessing a low activation energy, occurs via Cpd I or Cpd 0 (paths C and D, respectively in Scheme 1).

Scheme 1. Cytochrome P450 Catalytic Cycle Using the Normal NAD(P)H/NAD(P)-P450 reductase/O_2 (Path A) and the *N*-oxide system (Path B)[21] [a]



[a] The *N*-oxide compound produces the *N*,*N*-dimethylaniline substrate after donation of the oxygen atom to the P450 enzyme to create Cpd I.

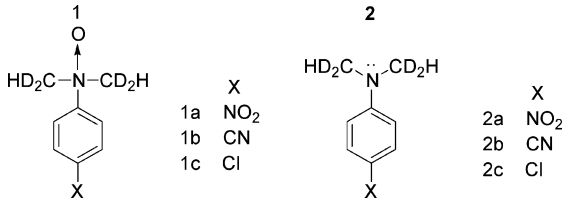


Figure 1. Kinetic isotope effect profiles were obtained with substrates **1a**−**c** and **2a**−**c**.

We have employed compounds **1a**−**c** (Figure 1) to function as surrogate oxygen atom donors for two P450 enzymes, P450cam and P4502E1. These two enzymes have been used in a number of the studies mentioned above. Oxygen donation by an *N*-oxide was originally used by Heimbrook et al. to probe the mechanism of *N*-dealkylation.[13] Donation of oxygen from each *N*-oxide compound to the P450 iron−porphyrin complex directly produces Cpd I, while simultaneously creating the corresponding *N*,*N*-dimethylaniline analogue (**2a**−**c**) to serve as a substrate for *N*-demethylation (path B, Scheme 1). This method eliminates any possibility for the formation of Cpd 0. KIE profiles obtained using the *N*-oxide system (**1a**−**c**) were compared to the profiles produced using the complete

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Table 1. Intramolecular KIE Values for the *N*-Demethylation of *N,N*-Dimethylaniline *N*-Oxide (**1a**−**c**) and *N,N*-Dimethylaniline (**2a**−**c**) Analogues by P450cam and P4502E1[20] [a]

enzyme:	P450cam		P4502E1	
system:	*N*-oxide[21]	NADH	*N*-oxide	NADPH
X[b]				
NO_2	3.07 (5)	3.1 (1)	3.66 (6)	3.65 (4)
CN	2.77 (5)	2.77 (3)	2.95 (3)	3.23 (4)
Cl	2.53 (5)	2.23 (3)	2.87 (5)	2.92 (4)

[a] The first number of each entry is the average of 3−6 independent determinations; the number in parentheses is the standard deviation of the last significant figure. The intramolecular KIE values were determined by dividing the dimedone adduct of formaldehyde-$O^{13}CD_2$ produced in each enzymatic incubation by the formaldehyde-$O^{13}CDH$ produced, multiplying by two after subtracting out the theoretical abundance of derivatized background formaldehyde. Incomplete incorporation of the isotopes used in each substrate was not accounted for in the KIE values since it was assumed to have the same relative effect on each value. [b] This column denotes the substituent attached para to the nitrogen for each substrate.

NAD(P)H/NAD(P)-P450 reductase/O_2 system with substrates **2a**−**c** (Table 1). The expected results were either that substrates **1a**−**c** and **2a**−**c** would give different isotope effect profiles, indicating Cpd 0 participates in *N*-dealkylation, or that the profiles would match, indicating *N*-dealkylation is mediated solely by Cpd I.

The KIE profiles obtained for the *N*-oxide system closely match the profiles produced using the NAD(P)H/NAD(P)-P450 reductase/O_2 system with both of the enzymes used. This indicates that the isotopically sensitive step in the two systems proceeds through the same mechanistic pathway. Since the *N*-oxide system bypasses the preceding intermediates to directly form Cpd I, the results provide compelling evidence for Cpd I as the active oxidant for P450-mediated *N*-dealkylation. A reasonable assumption can be made that the Cpd 0 species would not produce the same KIE profiles based on the Melander−Westheimer principle, which states that the magnitude of the isotope effect is dependent on the symmetry of the transition state.[14] An example of this phenomenon is observed in the KIE profiles obtained in the present work. The substrates with a more electron-withdrawing substituent attached para to the nitrogen atom produce higher isotope effects (e.g., NO_2 > CN > Cl). The electron-withdrawing group lowers the reactivity toward hydrogen atom abstraction and effectively increases the symmetry of the transition state. It has been established that oxidation of the terminal methyl group of octane has a reasonably symmetrical reaction coordinate based on observed primary and secondary isotope effects.[15] *N*-dealkylation reactions are relatively more exothermic and show smaller isotope effects than aliphatic oxidation reactions.[16] Thus, Cpd 0, which has been established to be less reactive than Cpd I, [6,8,11] would react more endothermically and produce higher isotope effects. Obviously this work does not provide information about the reactivity of Cpd 0.

In conclusion, the isotope effect profiles obtained in this work provide compelling evidence for P450-mediated *N*-dealkylation occurring solely through the iron−oxene species. While this work does not rule out the potential participation of the hydroperoxy−iron species in other P450-mediated reactions, it provides support for two-state reactivity for reactions that are thought to occur by two oxidants and that have higher barriers than *N*-dealkylation.

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References

(1) Higgins, L.; Korzekwa, K. R.; Rao, S.; Shou, M.; Jones, J. P. *Arch. Biochem. Biophys.* **2001**, *385*, 220−230.
(2) Auclair, K.; Hu, Z. B.; Little, D. M.; de Montellano, P. R. O.; Groves, J. T. *J. Am. Chem. Soc.* **2002**, *124*, 6020−6027.
(3) Guengerich, F. P.; Macdonald, T. L. *FASEB J.* **1990**, *4*, 2453−2459.
(4) Ortiz de Montellano, P. R. *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Plenum: New York, 1995.
(5) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. *J. Biochem. Biophys. Res. Commun.* **1978**, *81*, 154−160.
(6) Chandrasena, R. E. P.; Vatsis, K. P.; Coon, M. J.; Hollenberg, P. F.; Newcomb, M. *J. Am. Chem. Soc.* **2004**, *126*, 115−126.
(7) Vaz, A. D. N.; McGinnity, D. F.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3555−3560.
(8) Volz, T. J.; Rock, D. A.; Jones, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 9724−9725.
(9) Jin, S. X.; Makris, T. M.; Bryson, T. A.; Sligar, S. G.; Dawson, J. H. *J. Am. Chem. Soc.* **2003**, *125*, 3406−3407.
(10) Hutzler, J. M.; Powers, F. J.; Wynalda, M. A.; Wienkers, L. C. *Arch. Biochem. Biophys.* **2003**, *417*, 165−175.
(11) Ogliaro, F.; de Visser, S. P.; Cohen, S.; Sharma, P. K.; Shaik, S. *J. Am. Chem. Soc.* **2002**, *124*, 2806−2817.
(12) Sharma, P. K.; De Visser, S. P.; Shaik, S. *J. Am. Chem. Soc.* **2003**, *125*, 8698−8699.
(13) Heimbrook, D. C.; Murray, R. I.; Egeberg, K. D.; Sligar, S. G.; Nee, M. W.; Bruice, T. C. *J. Am. Chem. Soc.* **1984**, *106*, 1514−1515.
(14) Melander, L.; Saunders: W. H. *Reaction Rates of Isotopic Molecules*; Robert E. Krieger Publishing Company: Malabar, 1987.
(15) Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171−2173.
(16) Manchester, J. I.; Dinnocenzo, J. P.; Higgins, L. A.; Jones, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 5069−5070.
(17) Ortiz de Montellano, P. R. O.; De Voss, J. J. *Nat. Prod. Rep.* **2002**, *19*, 477−493.
(18) Cymerman Craig, J.; Purushothaman, K. K. *J. Org. Chem.* **1970**, *35*, 1721−1722.
(19) French, K. J.; Strickler, M. D.; Rock, D. A.; Rock, D. A.; Bennett, G A.; Wahlstrom, J. L.; Goldstein, B. M.; Jones, J. P. *Biochemistry* **2001**, *40*, 9532−9538.
(20) Karki, S. B.; Dinnocenzo, J. P.; Jones, J. P.; Korzekwa, K. R. *J. Am. Chem. Soc.* **1995**, *117*, 3657−3657.
(21) The mechanism proposed in Scheme 1 (path B) is one possibility. Alternatively, it is possible that the transfer of the oxygen to the iron generates a ferryl (FeVI═O) complex and a nitrogen radical cation.[17] However, this does not change the conclusion that a single atom oxygen species, not Cpd 0, mediates this reaction.
(22) Substrates **2a**−**c** were gifts from Dr. Dinnocenzo at the University of Rochester (Rochester, NY). Compounds **1a**−**c** were synthesized from compounds **2a**−**c** and purified according to literature procedures.[18] P450cam, putidaredoxin reductase, and putidaredoxin were expressed and purified as described previously.[19] Incubations with P450cam and substrates **2a**−**c** were performed as described previously,[19] using 500 μM substrate, 1 mM NADH, and 0.04 mg/mL catalase. Incubations with CYP2E1 and substrates **2a**−**c** were performed using RECO system CYP2E1 purchased from Invitrogen (Carlsbad, CA). These incubations consisted of 300 μL of RECO system CYP2E1 enzyme mix, 500 μM substrate, 1 mM NADPH, and 0.04 mg/mL catalase. Enzymatic incubations with *N*-oxide substrates were performed using 4 μM P450cam or 3 μM purified CYP2E1, purchased from Invitrogen, and 2 mM *N*-oxide substrate. All incubations were brought to a total volume of 500 μL with 10 mM phosphate buffer, pH 7.4, with the exception of RECO system CYP2E1, in which the RECO system buffer mix was used. The samples were incubated in a shaker bath for 40 min at 30 °C for P450cam and 37 °C for CYP2E1. The reactions were quenched and derivatized as described by Karki et al.,[20] and the resulting residue was dissolved in methanol (200 μL). Enzymatic products were analyzed by liquid chromatography/mass spectrometry (LC/MS) using electrospray ionization monitoring positive ions. LC/MS analysis was performed using a Thermo-Finnigan LCQ Advantage mass spectrometer coupled to a ThermoQuest high performance liquid chromatography (HPLC) system equipped with an Agilent Hypersil BDS-C18 column (2.0 mm × 125 mm). The column method began at 40:60 methanol (0.1% acetic acid)/water (0.1% acetic acid). After 1 min, the methanol solution was ramped to 80% over 4 min. The methanol solution was then ramped to 95% over 15 min and remained at 95% for another 10 min. The solvent flow rate was 100 μL/min.
(23) A relatively small amount of autocatalysis occurred with the *N*-oxide incubations. This was accounted for by subtracting out the average amounts of products produced in three control incubations containing no enzyme for each *N*-oxide used. Warfarin was used as an internal standard, and 2 nmol was added to each sample prior to extraction.

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