# A New Mechanistic Probe for Cytochrome P450: An Application of Isotope Effect Profiles

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Cytochromes P450 are a family of heme-enzymes that bind and reduce molecular oxygen, producing what is believed to be a triplet-oxygen species coordinated to the heme iron.[1] This highly reactive oxygen intermediate affords P450 with the ability to oxidize a wide variety of substrates. P450 enzymes mediate aliphatic and aromatic hydroxylation, *N-*, *O-*, and *S*-oxidation and dealkylation, and olefin epoxidation. This enzyme family is primarily involved in detoxification and excretion of xenobiotics, but ironically, it is also responsible for the bioactivation of protoxins and procarcincogens.[2] Over 300 different P450s have been characterized, X-ray crystal structures of four enzymes have been solved,[3−6] and numerous biomimetic systems have been developed to model the action of P450.[7−18] Yet, many of the mechanistic details of P450 remain obscure. Particularly unclear are the details of the product-determining step catalyzed by the short-lived iron−oxo species.[19] To probe this step, we recently determined kinetic deuterium isotope effects (KDIE) for *N*-demethylation of a series of substituted dimethylanilines by four different P450 enzymes and compared them to the isotope effects for H-atom abstraction by the *tert*-butoxy radical ($\cdot$OBu^t).[20] The magnitude of the isotope effect for each substrate was nearly identical in all of the P450s and
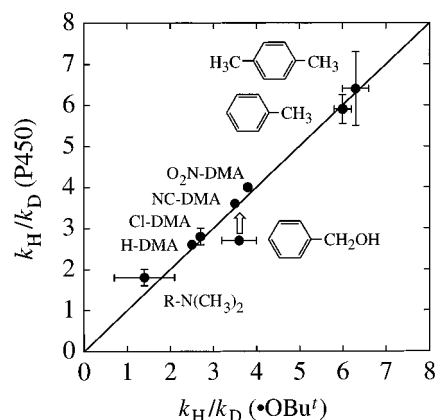
Figure 1. Kinetic ^{2}H isotope effects for H-atom abstraction from eight substrates by $\cdot$OBu^t and for their P450 enzyme-catalyzed oxidations.

for $\cdot$OBu^t, indicating that $\cdot$OBu^t might serve as a simple chemical model for the C−H bond cleavage step in P450 oxidations.

To further test this hypothesis, we extended the comparison of KDIEs to the reaction of $\cdot$OBu^t and P450 with *p*-xylene, toluene, benzyl alcohol, and a tertiary trialkylamine. Absolute rate constants for H-atom abstraction by $\cdot$OBu^t were measured using benzhydrol as a radical monitor as described by Scaiano and co-workers.[21,22] Substrates were dissolved in 2:1 di-*tert*-butyl peroxide/benzene, purged with argon, and irradiated at 340 nm with a pulsed nanosecond laser[23] to produce $\cdot$OBu^t. Using a fixed concentration of benzhydrol (50−200 mM), pseudo-first-order rate constants (k_{obsd}) for formation of the diphenyl ketyl radical ($\lambda_{max} = 540$ nm) were measured at four or more substrate concentrations.[24] Isotope effects were determined from the ratios of the second-order rate constants for reaction of undeuterated substrates divided by those for substrates selectively deuterated at the site of H-atom abstraction. The reported KDIEs are an average of at least three experiments conducted on different days.

Figure 1 is a plot of the kinetic deuterium isotope effects determined for reaction of substrates with $\cdot$OBu^t against those measured for several P450 enzymes. Intramolecular isotope effects were determined for P450 by product analysis[25,26] following incubation; the remaining isotope effects are from the literature (see legend in Table 1). In general, KIEs observed in different P450 enzymes have been found to be very similar,[20,27] suggesting that their active species are very similar. Therefore, where isotope effects for a single substrate were available for multiple P450 enzymes, an average of these values is presented in the plot, and the standard deviation is taken as an estimate of error. The eight substrates examined give a r^2 value of 0.95 against the line of perfect correlation (unit slope and intercept at the origin).

As discussed by Karki and Dinnocenzo,[28] isotope effect *profiles* are a subtle tool for distinguishing enzyme mechanisms. Thus, while the magnitude of a single isotope effect conveys only limited information about a reaction mechanism, an isotope

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Table 1. Kinetic Deuterium Isotope Effects for Reaction of Various Substrates with $\cdot OBu^t$ and Cytochrome P450

	k_H/k_D	
substrate	$\cdot OBu^t$	P450
p-xylene	$6.3 \pm 0.3^{a,b}$	$6.4 \pm 0.9^{a,c,d}$
toluene	$6.0 \pm 0.2^{a,b}$	$5.9 \pm 0.35^{a,e}$
p-nitro-*N,N*-dimethylaniline	3.8 ± 0.1^{f}	4.0 ± 0.1^{f}
p-cyano-*N,N*-dimethylaniline	3.5 ± 0.1^{f}	3.6 ± 0.1^{f}
p-chloro-*N,N*-dimethylaniline	2.7 ± 0.1^{f}	2.8 ± 0.2^{f}
N,N-dimethylaniline	2.5 ± 0.1^{f}	2.6 ± 0.1^{f}
benzyl alcohol	$3.6 \pm 0.4^{a,g}$	2.7 ± 0.1^{h}
trimethylamine/phentermine	1.4 ± 0.7^{i}	1.8 ± 0.2^{j}

^{a} This work. ^{b} Substrates were of the highest purity available and used as received (Aldrich). ^{c} Reference 38. ^{d} Reference 26. ^{e} Reference 37. ^{f} Reference 20. ^{g} PhCH$_2$OH (Aldrich, 99+%) was passed over a basic alumina column and distilled. PhCD$_2$OH was prepared by reduction of methyl benzoate with LiAlD$_4$ and purified as above. Preparations were stored under Ar at 4 °C in the dark until use. ^{h} Reference 39. ^{i} Reference 33. ^{j} Reference 31.

effect profile determined for a *series* of substrates is a much more powerful probe. One problem with the determination of isotope effects in enzymatic reactions is that the isotopically-sensitive, chemical step (e.g., C−H bond cleavage) is not always the only step in the reaction sequence that contributes to rate limitation. This can lead to masking or attenuation of the so-called intrinsic isotope effect.[29] Masking can be minimized by use of intramolecular isotope effects, where the ratios of labeled to unlabeled products are used to measure KDIEs.[30] However, in this type of experiment, positional exchange of ^{1}H and ^{2}H must be rapid relative to the isotopically sensitive step; otherwise, masking will still occur. For these reasons, the enzymatic isotope effects used in the present work were determined from intramolecular experiments in which the ^{1}H and ^{2}H *are present on the same methyl or methylene group* except for *N,N*-dimethylphentermine where a literature value for P450 demethylation of the *N*-methyl-*N*-trideuteriomethyl derivative was used.[30] This experimental design provides the closest estimate of the intrinsic isotope effect.[25] It should be noted that the isotope effect for benzyl alcohol in the P450 system is not a true intramolecular isotope effect since the hydrogen atom abstraction is from a prochiral carbon and as such represents a lower limit for the intrinsic isotope effect for this P450-mediated reaction.[31]

Our initial finding that reaction of substituted *N,N*-dimethyl-anilines with $\cdot OBu^t$ gave KDIEs that are identical to those for P450-mediated *N*-dealkylation provided evidence that the isotopically sensitive steps for these reactions proceed by similar mechanisms. The reaction of the amines with $\cdot OBu^t$ was shown to be consistent with a simple hydrogen atom transfer mechanism; an alternative electron/proton transfer was rigorously excluded.[20,28] A preponderance of experimental evidence suggests that the other substrates studied here also react with $\cdot OBu^t$ by a hydrogen atom transfer mechanism.[20−22,28,32−34] Therefore, the fact that the P450 isotope effects show such a good correlation with the $\cdot OBu^t$ isotope effects has several

important implications. Most obviously, *it is consistent with all of these P450 reactions proceeding by a common hydrogen atom transfer mechanism.*

Consistent with this conclusion, previous studies have suggested that hydroxylation reactions of alkylbenzenes by P450 proceed by a hydrogen atom transfer mechanism.[35] Thus, the fact that the P450 oxidation of amines falls on the same line as the alkylbenzenes in Figure 1 can be taken as evidence that the amine oxidations also proceed by a hydrogen atom transfer mechanism. It is important to point out that this latter interpretation conflicts with the widely held belief that P450 oxidizes amines by an electron/proton transfer mechanism.[36] In our view, the present results provide an even stronger argument against an electron/proton transfer mechanism for amine oxidation from that previously advanced[28] since (1) these results unify amine dealkylations with the hydroxylation of substrates that are much more difficult to oxidize by a single electron process; (2) xylene, toluene, and benzyl alcohol oxidations are all thought to proceed by a hydrogen atom transfer mechanism in P450-mediated reactions;[37−39] and (3) all of the $\cdot OBu^t$-mediated reactions are thought to proceed by hydrogen atom abstraction and the P450 isotope effects are in excellent agreement, with a single slope.

The fact that the correlation of P450 and $\cdot OBu^t$ isotope effects for a variety of substrates shows a *unit* slope is remarkable. It suggests that reactions of $\cdot OBu^t$ may provide a useful model for P450 reactions. In particular, the regioselectivity of P450-mediated reactions for substrates that are not strongly bound by the enzyme might be predictable based on relative rate constants for $\cdot OBu^t$ reactions, many of which are known. Alternatively, when correlations are found to be absent, they may indicate significant enzyme−substrate interactions. This interpretation could explain the different regioselectivities recently reported for the oxidations of methylcubane by P4502B1 and by $\cdot OBu^t$.[32] However, enzyme−substrate interactions should not cause a difference in isotope effects for the two systems, as long as the compared values are intrinsic isotope effects.

In conclusion, the isotope effect correlation described here provides a new screen for P450 hydroxylation mechanisms. For the substrates studied in the present work, the correlation provides strong evidence for a common, hydrogen atom transfer mechanism. Importantly, the method also has the potential for identifying reactions that do not proceed by a hydrogen atom transfer mechanism, since $\cdot OBu^t$/P450 KDIEs that fall far from the line in Figure 1 provide evidence for a change in mechanism. Finally, the correlation holds promise for a remarkably simple biomimetic model for P450 oxidations.

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