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Kinetic Deuterium Isotope Effects in Cytochrome P450 Oxidation Reactions

F. Peter Guengerich^{a,*}

^aDepartment of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146, USA

Abstract

Cytochrome P450 (P450) enzymes account for ~ 75% of the metabolism of drugs. Most of the reactions catalyzed by P450s are mixed-function oxidations, and a C-H bond is (usually) broken. The rate-limiting nature of this step can be analyzed using the kinetic isotope effect (KIE) approach. The most relevant type of KIE is one termed intermolecular non-competitive, indicative of rate-limiting C-H bond breaking. A KIE *vs.* k_{cat} for several P450s showed a correlation coefficient (r^2) of 0.62. Deuterium substitution has been considered as a potential means of slowing drug metabolism or redirecting sites of metabolism in some cases, and several general points can be made regarding the potential for application of deuterium in drug design/development based on what is known about P450 KIEs.

Keywords

Cytochrome P450; oxidations; drugs; bond energy; kinetics; isotope effects; dealkylation reactions

Introduction

Cytochrome P450 (P450) enzymes are heme proteins, found among most forms of life. Of the 57 human P450s, five account for ~ 90% of the metabolism of drugs and new chemical entities being tested as drugs.^{1,2} Most of the reactions catalyzed by P450s are mixed-function oxidations, and the general mechanism is shown in the catalytic cycle in Fig. 1. This is a complex cycle, and some of the high-valent iron intermediates have only been observed in a few cases with bacterial P450s. In Step 7 a C-H bond is (usually) broken, and the rate-limiting nature of this step can be analyzed using the kinetic isotope effect (KIE) approach with heavier atom derivatives of protium, i.e. deuterium and tritium.

*Address correspondence to: Prof. F. Peter Guengerich, Department of Biochemistry, Vanderbilt University School of Medicine, 638 Robinson Research Building, 2200 Pierce Avenue, Nashville, Tennessee 37232-0146, USA, Telephone: 1 (615) 322-2261, FAX: 1 (615) 322-4349, f.guengerich@vanderbilt.edu.

Conflict of Interest

The author did not report any conflict of interest.

KIE Concepts and Relevance to P450s

The subject of KIEs is complex,³ especially as applied to enzymes. A simplistic synopsis is that if a primary KIE exists and is expressed (i.e. generally >2) in the overall reaction, then the C-H bond breaking step is at least partially rate-limiting in the overall reaction. The terminology of Northrop^{4,5} will be used here, with Dk indicating the intrinsic KIE (i.e., the one for the chemical C-H bond breaking step), DV indicating the ratios of k_{cat} for the C-H and C-D substrates, and $^D(V/K)$ indicating the ratio of k_{cat}/K_m for the C-H and C-D substrates.

The different types of KIEs can be confusing but have been discussed in our work.⁶ Several approaches can be used to estimate Dk , which is necessary for comparisons,⁵⁻⁷ in which k_{cat} and K_m are determined in separate sets of experiments with C-H and C-D substrates to calculate DV and $^D(V/K)$.⁶ The so-called intramolecular competitive and intermolecular competitive experiments⁶ can be utilized in estimating the extents of tumbling and exchange.

History of P450 KIEs

The nature of P450 chemical reactions led to early forays into the use of KIEs as a mechanistic tool.⁸ However, many of the early studies were difficult to interpret in that (i) the studies contained mixtures of P450s and (ii) a variety of experimental designs were used.^{8,9} However, a high (intrinsic) Dk was one of the points Groves *et al.*¹⁰ utilized in developing the “oxygen rebound” mechanism generally accepted now (Fig. 1). Miwa and Lu observed significant KIEs with several P450 reactions¹¹⁻¹³ and occasionally in microsomes.¹⁴ Gillette considered theoretical aspects of P450 KIEs.^{15,16} Sligar used KIE studies to show that C-H bond breaking was not rate-limiting in the oxidation of camphor by the classic bacterial P450_{cam} system.¹⁷ However, a KIE of 3.8 was observed with that enzyme and the substrate norcamphor.¹⁸ In the latter work, a shift in the site of oxidation to a new carbon was observed, as in work with mammalian P450s,^{12,13} and termed “metabolic switching.”

Although many of the earlier studies yielded only low KIEs for reactions catalyzed by mammalian P450s,^{11,13} subsequent investigations of our own have yielded relatively large non-competitive intermolecular KIEs for alkyl hydroxylation reactions, indicative of rate-limiting C-H bond breaking.¹⁹⁻²¹ A plot of the (intermolecular non-competitive) KIE vs. k_{cat} for several P450s studied in our group is shown in Fig. 2. A correlation coefficient (r^2) of 0.62 was found, indicating that the majority of the variation in KIEs is (inversely) related to k_{cat} . The concept is that in the faster reactions, C-H bond breaking is more facile and then other steps in catalysis (Fig. 1) become rate-limiting. One of the fastest and most efficient reactions catalyzed by a mammalian P450 is cholesterol 7 α -hydroxylation (P450 7A1) ($k_{\text{cat}} \sim 190 \text{ min}^{-1}$).²⁶ No KIE was observed, and the conclusion was that Fe^{3+} reduction, not C-H bond breaking, is rate-limiting.²⁶

KIEs for Amine *N*-Dealkylation

Miwa and Hollenberg²⁷ reported very high KIEs (8.6-10.1) for amine *N*-dealkylation reactions catalyzed by peroxidases. However, the KIEs for such reactions are low for P450s,

usually 2. An explanation was provided in that amine dealkylations by both P450s and peroxidases proceed via initial 1-electron abstraction (Fig. 3), but P450s use FeO^{2+} as a base to catalyze α -proton transfer while peroxidases do not (utilizing heme edge electron transfer instead), leaving α C-H bond breaking uncatalyzed and allowing the aminium radicals to accumulate.^{29,30}

KIEs for Aryl Hydroxylation

Overall KIEs are usually not observed for P450-catalyzed aryl hydroxylations. The mechanism is generally considered to involve electrophilic addition opposed to direct C-H bond breaking (Fig. 4).^{31,32} However, 1,2-shifts of hydrogen (labeled *H in Fig. 4) and some other elements are seen due to the classic “NIH Shift,”³³ which has its origins in the chemical KIE seen for the rearrangement of the carbonyl intermediate into a phenol. Thus, retention of labels is seen in many aromatic hydroxylations due to this phenomenon.

KIEs Reflected in K_m

In general, KIEs are seen in the $^D V$ term. There has been a report of a KIE only on K_m in the *N*-demethylation of *N*-nitrosodimethylamine³⁴ but this was not confirmed.²⁵ However a KIE on K_m was observed in the oxidation of ethanol by human P450 2E1²² and is explained by a KIE on C-H bond-breaking but with a rate-limiting step following product formation, i.e.

where S is the substrate, E the enzyme (and $[E]_t$ the total enzyme concentration), and P the product. Then

$$\begin{aligned} k_{\text{cat}} &= \frac{k_3 k_5}{k_3 + k_5} [E]_t \\ K_m &= \frac{k_5 (k_2 + k_3)}{k_1 (k_3 + k_5)} \end{aligned}$$

with K_m having units of molarity. Therefore,

$$^D V = \frac{^D k_3 + k_3 / k_5}{1 + k_3 / k_5}$$

$$\text{and } ^D (V / K) = \frac{^D k_3 + k_3 / k_2}{1 + k_3 / k_2}$$

so thus the isotopically sensitive step is included in k_3 . The value $^D (V / K)$ is a reflection of $^D k_3$ when k_3 / k_2 approaches zero, i.e., $k_2 \gg k_3$. Because $^D V \approx 1$, then $^D k_3 + k_3 / k_5 \approx 1 + k_3 / k_5$. This is valid only if $k_3 \gg k_5$. In fact, for the results presented for ethanol oxidation by human P450 2E1,²² k_3 (the rate of product formation) is much faster than k_5 (the rate of product release). Hence, the above expressions for k_{cat} and K_m are reduced to

$$k_{\text{cat}} \approx k_5[E]_{\text{T}}$$

$$K_{\text{m}} \approx \frac{k_5 k_2}{k_1 k_3}$$

Thus, the effect of changing the rate of C-H bond breaking is seen in K_{m} but not k_{cat} .

Relevance of KIEs to the Use of Deuterium in Drug Discovery and Development

Although metabolism problems have been diminished as a problem in attrition of drug candidates there are still some issues.³⁵ Deuterium substitution has been considered as a potential means of slowing drug metabolism in some cases. One application is to enhance the exposure to a drug (i.e., decreased clearance and enhance area-under-the-curve). Alternatively, the production of metabolic products with unwanted side effects might be avoided, as in an example of deuterium substitution of paroxetine in the methylene hydrogens of the methylenedioxyphenyl moiety.³⁶ Several companies have been developed for the purpose of developing deuterated drugs, and patents have been filed.³⁶

The practical application of deuterium substitution is dependent upon the expression of a major primary KIE, generally $D(V/K)$, *in vivo*. Further, even if expressed *in vivo*, the KIE must have an effect on a pharmacokinetic parameter of some interest in order to make deuterium substitution useful. Relatively few *in vivo* KIEs have been documented, although this may be result of a paucity of studies. One comes from our own work with a nifedipine metabolite.³⁷ Others are related to the toxicity of chloroform³⁸ and the drug efavirenz,³⁹ where deuterium substitution and an *in vivo* KIE were invaluable in elucidation of the mechanism of kidney damage. A classic example is the effect of deuterium substitution of *N*-nitrosodimethylamine and the remarkable effect in reducing the incidence of liver cancer in rats.^{24,25,40}

Several general points can be made regarding the potential for application of deuterium in drug design/development, based on what is known about P450 KIEs:

- (i) Because amine *N*-dealkylation and aromatic hydroxylation reactions have low KIEs for mechanistic reasons (*vide supra*) (Fig. 3), these are generally not good candidates for application. However, dealkylations of ethers and amides are different, due to the much higher oxidation potentials, and high KIEs are often observed.
- (ii) Measuring a KIE *in vitro* is strongly suggested prior to *in vivo* studies.
- (iii) Other pharmacokinetic parameters (e.g. blood flow) may limit drug removal.
- (iv) In some cases, a change in the rate of elimination of drug may not result but “metabolic switching” (*vide supra*) may have an effect, e.g. blocking a reaction leading to toxicity.³³

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Figure 1.
Generalized catalytic cycle for P450 reactions.

Figure 2. Relationship between KIE (intermolecular, non-competitive) and k_{cat} for a number of P450 reactions, mainly with human P450s. Data points are from the indicated references.^{6,7,20-26}

Figure 3.
Comparisons of mechanisms of carbon hydroxylation and *N*-dealkylation.²⁸

Figure 4. General mechanisms of aryl hydroxylation.^{31,32} *H indicates a labeled hydrogen atom, i.e. deuterium or tritium. Alternate pathways (a, solid line; b, broken line) for the collapse of the tetrahedral iron-oxygen intermediate lead to the carbonyl and to the epoxide, respectively, and both can form the phenol.