

Kinetic Isotope Effects and 'Metabolic Switching' in Cytochrome P450-Catalyzed Reactions

Gerald T. Miwa and Anthony Y. H. Lu

Summary

The mechanistic significance of a kinetic isotope effect on a cytochrome P-450-catalyzed reaction depends, fundamentally, on the nature of the interaction of the substrate with the active site of the enzyme as well as on the nature of the chemistry of the reaction catalyzed. Consequently, kinetic isotope effects can be used to extract information on the topology of the enzyme and the mechanism of substrate oxidation. Kinetic isotope effect studies are sometimes accompanied by 'metabolic switching' or a change in metabolic pathway, catalyzed either by the same enzyme or by a different enzyme. For the specific case where 'metabolic switching' gives rise to a change in regional specificity for the cytochrome P-450 catalyzed metabolism of a compound, this change can be explained in terms of the topological constraints on substrate binding imposed by the active site of the enzyme.

Kinetic Isotope Effects

The C-²H bond is stronger than the corresponding C-¹H bond in a molecule and, consequently, many reactions involving the cleavage of this bond are retarded by deuterium substitution. This reduction in rate, or kinetic isotope effect, provides useful information about the importance of this step in the overall reaction and the nature of the transition step.¹ Kinetic isotope effects have traditionally been employed to extract the mechanistic details of chemical and enzymatic reactions. Since many cytochrome P-450-catalyzed reactions involve the oxidative cleavage of a C-H bond, kinetic isotope effect measurements offer the potential of providing information on some of the details of the chemical reactions catalyzed by these enzymes. For example, isotope effects on V_m have been used to document that the C-H bond cleavage step is at least partially rate-limiting in some reactions catalyzed by cytochrome P-450 systems.^{2,3}

The magnitude of primary isotope effects yields additional information on the details of the chemical mechanism. Large primary isotope effects ($Dk > 10$), accompanied by epimerization at the carbon undergoing oxidation, provided some of the strongest evidence for the hydrogen abstraction mechanism for aliphatic oxidation⁴ and recently, the magnitude of this isotope effect has been confirmed by intramolecular isotope effect studies.⁵ Similarly, V_m isotope effect measurements have provided support for α -carbon hydrogen abstraction during aromatic O-dealkylation reactions,⁶ the 'NIH-shift' mechanism of the oxidation of aromatic compounds,⁷ and the one-electron nitrogen oxidation during oxidative N-dealkylation reactions^{8,9} catalyzed by this enzyme system.

Mechanistic conclusions based on the magnitude of the primary kinetic isotope effect require an accurate estimation of this effect. In enzyme catalyzed reactions, the kinetic expression of this effect is often attenuated by pre- and post-catalytic steps not involving the cleavage of the C-H bond. Thus, Miwa and co-workers⁷ found comparable, large kinetic isotope effect values ($Dk = 12-14$) for two purified isozymes of cytochrome P-450 but considerably smaller isotope effect values on product formation ($Dv = 2$ and 4).

Two techniques have been employed to estimate the actual or intrinsic isotope effect associated with cytochrome P-450-catalyzed reactions. From measurements of deuterium and tritium isotope effects, the intrinsic isotope effect can be calculated according to the method described by Northrop.¹⁰ The major advantage of this technique is that it is generally applicable to a diverse number of substrates. The major disadvantage is that calculation of the intrinsic isotope effect depends on two measured parameters and an exponential function. Consequently, small errors in the accu-

racy of measuring both isotope effects can lead to very large errors in the estimation of the intrinsic isotope effect. Nevertheless, useful mechanistic information on O-dealkylation and ethanol oxidation reactions have been obtained by this technique.^{6,11}

For symmetrical substrates containing two or more equivalent hydrogens, the substitution of deuterium for one of these hydrogens permits an alternative means for estimating the intrinsic isotope effect. Intramolecular competition between the hydrogen isotopes serves to kinetically unmask the intrinsic isotope effect, D_k . This method can often be applied to cytochrome P-450-catalyzed reactions because of the broad substrate specificity of the enzyme. For example, intrinsic isotope effects have been estimated by this technique for benzylic oxidation,¹² $Dk > 10$, O-dealkylation,¹³ $Dk = 10$, N-demethylation,^{8,9,14,15} $Dk < 1.5$, and for aliphatic oxidation,^{4,5} $Dk > 10$.

In most enzymic reactions involving a C-H bond cleavage step, the reduction in product formation for the deuterated substrate is accompanied by a concomitant decrease in substrate metabolism. The tight complimentary binding between the substrate and the enzyme active site places severe restrictions on alternative orientations of the substrate, thus limiting metabolism to only a single site. In contrast, an unexpected change in regional selectivity was observed when the products of cytochrome P-450-catalyzed reactions were carefully determined.¹⁶ Horning has coined the term 'metabolic switching' to describe this change in selectivity during the metabolism of deuterated substrates. In this essay, 'metabolic switching' is defined more generally and refers to the increase in substrate flux through alternative pathways, catalyzed by either cytochrome P-450 or alternate enzymes, when the primary pathway for metabolism is reduced by a hydrogen isotope or other substituents.

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The Basis for 'Metabolic Switching'

The substitution of the hydrogen isotopes ^2H and ^3H for the hydrogen on a carbon atom that undergoes oxidation by the hepatic cytochrome P-450 system often results in the reduction of this pathway for metabolism and 'metabolic switching' or a compensatory increase in the metabolism to another site on the substrate molecule. The family of cytochrome P-450 enzymes have long been known for their broad substrate specificity.¹⁷ Thus, cytochrome P-450 can catalyze a diverse array of oxidative reactions with a large number of structurally unrelated substrates. In contrast, a very high degree of stereoselectivity has been reported for a number of reactions catalyzed by this enzyme. This apparent anomaly between the promiscuous substrate specificity and the often observed high stereoselectivity of these enzymes can be explained by the topology of the active site.

The orientation of the substrate within the active site of the enzyme is governed by the most favorable complementary relationship between the substrate and topography of the protein domain defining the active site. The substrate-binding sites of a few of the cytochrome P-450 isozymes have been investigated and recently reviewed.¹⁸ Evidence indicates that it is asymmetrically oriented over the porphyrin prosthetic group of the enzyme. The capacity of the site permits the accommodation of a number of structurally unrelated substrates while the asymmetry of the active site defines the stereochemical control exhibited by this enzyme. Consequently, depending on the relationship between the structure of the substrate and the topology of the enzyme, one or more orientations of the substrate are possible which can potentially result in different regio- and stereoisomeric products. A barrier to oxidation at the preferred site, such as with deuterium substitution, could, therefore, result in a compensatory increase in metabolism at an alternate site. Thus, 'metabolic switching' may be explained in terms of the relationship between the topology of the enzyme's active site and the structure of the substrate.

We have conducted a number of studies with the substrate 7-ethoxycoumarin to define more fully the basis for 'metabolic switching'. The intrinsic isotope effect, $^{13}\text{C}k = 14.6$, was estimated by the Northrop method¹⁹ for this

reaction catalyzed by a purified preparation of cytochrome P-450c. Cytochrome P-450c is the major isozyme induced in the rat liver by 3-methylcholanthrene. The intrinsic isotope effect is the actual reduction in O-deethylation rate caused by deuterium substitution. However, the expression of this isotope effect in terms of the O-deethylated product, 7-hydroxycoumarin, was strongly suppressed since the observed isotope effect on product formation, $^{13}\text{C}v = 1.9$, was considerable smaller.

The basis for this attenuation was investigated by a visiting scientist in our laboratory, Dr Nobuhiro Harada.¹⁹ From very careful stoichiometry studies, Dr Harada was able to demonstrate that although deuterium substitution had reduced the rate of O-deethylation, it had not retarded the rate of substrate, O_2 , and NADPH metabolism. Since the latter parameters were measures of the enzyme's overall oxidative activity, these results led to the puzzling conclusion that deuterium substitution dramatically reduced production of the 7-hydroxycoumarin product without affecting the capacity of the enzyme to undergo catalytic turnover. Some detective work by Dr Harada revealed that a previously undescribed metabolite, arising from oxidation of the aromatic ring at C_6 , was formed in much higher quantities from the deuterated substrate. This was a clear example of 'metabolic switching' in that deuterium substitution had caused a change in regioselectivity of this purified enzyme.

John Walsh in our laboratory extended these studies to determine the mechanism by which this new metabolite was formed.²⁰ By preparing a derivative of the deuterated substrate bearing another deuterium at C_6 on the aromatic ring, he was able to demonstrate that hydroxylation at this site occurred with migration of this label to C_5 . This 'NIH-shift' of the C_6 proton clearly implicated a $\text{C}_5\text{-C}_6$ epoxide intermediate during the formation of this new metabolite.

Viewing these results in terms of the substrate binding site of this isozyme, (previously mapped out by Jerina and co-workers²¹) provided a physical explanation for the 'metabolic switching' observed for this substrate. Fig. 1 illustrates our current concept of the origin of 'metabolic switching'. Jerina and co-workers had shown that epoxidation of a number of polycyclic aromatic hydrocarbons occurred with a very high degree of stereoselectivity.

That is, almost all regioisomeric epoxides were formed by attack of oxygen on only one of the two faces of the polycyclic substrates. Overlaying a number of these products so that all the epoxide moieties were superimposed permitted the remaining planar hydrocarbon structure of the molecules to define the minimum 2-dimensional perimeter of the active site. The protein domain defined by Jerina's group is depicted as the area extending beyond the perimeter outlined by the polycyclic hydrocarbon framework in Fig. 1.

Panel A depicts the only fashion in which 7-ethoxycoumarin can be oriented in this active site framework within the constraints of placing the α -carbon nearest the active iron-bound oxygen atom and retaining the coumarin structure within the framework of the active site. Fixed in this way, the pro-S hydrogen is oriented towards the oxygen and would be predicted to undergo abstraction during catalysis.

Dr Robert Tullman, then a post-doctoral fellow in our laboratory, tested this prediction by synthesizing ethoxycoumarin isomers containing tritium in either the S or R positions. With

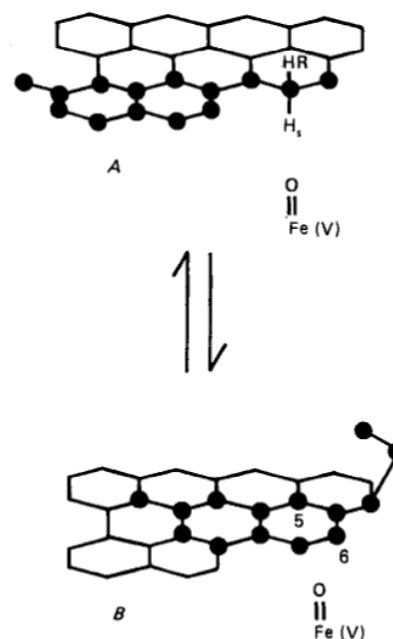
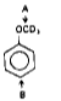
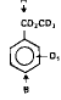
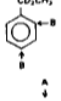
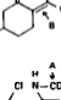
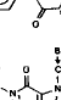
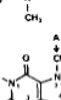
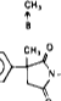
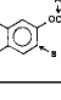
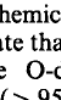


Fig. 1. Depiction of protein boundary surrounding cytochrome P-450c active site.²¹ The minimum perimeter of the active site is defined by the extended polycyclic framework shown. Panel A depicts the substrate, 7-ethoxycoumarin, in the orientation that minimizes its steric interaction with the perimeter of the active site. The heme iron is shown transferring the 'activated oxygen' from below the plane of this protein boundary towards the pro-S hydrogen on the substrate. Panel B depicts the second most favourable orientation of 7-ethoxycoumarin which places the $\text{C}_5\text{-C}_6$ double bond in a position favorable to oxygen attack.

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Table I. Examples of 'metabolic switching' associated with deuterium isotope effects on cytochrome P-450-catalyzed reactions

Deuterated substrate	Enzyme system	Path A and observed isotope effect	'Metabolic switching'		Ref.
			Path B	% increase	
	Rat liver microsomes	O-demethylation $k_H/k_D = 7.4$	4-hydroxylation	43%	26
	<i>Methylosinus trichosporium</i>	Benzylic hydroxylation $k_H/k_D = 4.5$	4-hydroxylation	25%	27
	Mouse liver S9 fraction	4-methyl hydroxylation $k_H/k_D = 1.69$	4-hydroxylation	68%	28
	Purified rabbit P-450LM ₂	1-phenylethanol $k_H/k_D = 1.27$	2-phenylethanol 2-ethylphenol 4-ethylphenol	1083% > 1000% 642%	29
	Mouse liver microsomes	Hydroxylation $k_H/k_D = 1.51$	Epoxidation	76%	30
	Rat liver microsomes	N-demethylation $k_H/k_D = 1.38$	6-hydroxylation	200%	31
	Rats, in vivo	N ₁ -demethylation decreases	N ₇ -demethylation	Increase	32
	Rats, in vivo	N ₇ -demethylation decreases	N ₅ -demethylation	Increase	32
	Rats, in vivo	N-demethylation decreases	Aromatic hydroxylation	Increase	32
	Purified rat P-448	O-deethylation $k_H/k_D = 1.90$	6-hydroxylation	490%	19

these stereochemical labels, he was able to demonstrate that cytochrome P-450c catalyzed the O-deethylation with a remarkable (> 95%) stereoselectivity for the pro-S hydrogen.¹⁸ Since 'metabolic switching' was observed for this single purified isozyme of cytochrome P-450, the alternate attack we had observed at the C₅-C₆ π -bond, when this pro-S hydrogen had been substituted with deuterium, could not have resulted from catalysis by another isozyme. Consequently, the less favorable reorientation of the substrate, as depicted in panel B, must have occurred in order to accommodate the observed oxygen addition to the C₅-C₆ π -bond.

From the intrinsic isotope effect value we were also able to estimate the energy barrier required for this reorientation to be approximately 1.5 kcal/mol.¹⁸ This value was confirmed by the independent

calculation based on the ratio of O-deethylated/ring hydroxylated products for the unlabelled and deuterium labelled substrates. Consequently, the energy barrier to substrate reorientation, imposed by the protein domain defining the active site, normally limits the substrate to the lowest energy position in the active site. For some mammalian cytochrome P-450 isozymes, the active site appears to be commodious enough to pose a relatively small energy barrier (< 1.5 kcal/mol) which is surmounted when deuterium substitution imposes a barrier to catalysis comparable to or greater than the net energy required for reorientation. Thus, current evidence indicates that 'metabolic switching' arises as a consequence of the additional energy barrier to oxidation caused by the stronger C-²H bond.

There is, however, some evidence that the active site topology must differ among the various forms of cytochrome P-450 since 'metabolic switching' was not observed for the 7-ethoxycoumarin substrate in incubations with hamster liver microsomes.²² Moreover, no change in NADPH, O₂ consumption or H₂O₂ production was observed with the deuterium labelled substrate. The reduction in O-deethylation rate, without effect on the other enzyme turnover parameters, also supports the strong commitment to catalysis of the 'active oxygen' form of cytochrome P-450 and suggests the interesting possibility that this species must degrade to still unknown oxygen metabolites. Indeed, Gorsky and co-workers²³ concluded that the difference in material balance between O₂ utilization and the oxygen bearing products, such as the oxidized

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substrate and H_2O_2 for the rabbit liver cytochrome P-450 LM₂-catalyzed oxidations of benzene and 1-pentanol, were compatible with the production of H_2O as the end product of this 'activated oxygen'.

Examples and Potential Implications of 'Metabolic Switching'

There are a growing number of documented examples of 'metabolic switching' in the literature. Table I summarizes some of these studies. The primary site of oxidation on a molecule is indicated with the letter 'A' while the secondary site exhibiting a higher rate of metabolism in the deuterated substrate is indicated by the letter 'B'. The majority of examples are probably reactions catalyzed by cytochrome P-450 enzymes although in many cases this has not been rigorously established.

The potential for and ramifications of 'metabolic switching' depends on the interactions between substrate and enzyme and on the relative biological properties of the metabolites. For some drugs, isotope substitution may reduce the rate of drug metabolism and, consequently, also reduce the clearance of the drug from the body. This will improve the duration of action and the peak plasma levels of the drug. Although usually not done with deuterium, the strategy of adding substituents, at metabolically labile sites on the drug is often utilized for reducing the metabolism-dependent clearances of drugs.

For other substrates, isotope substitution may not give rise to significant isotope effects in metabolism or clearance. This can result from either: (1) the lack of contribution of C-H bond cleavage in the overall rate of metabolism or (2) 'metabolic switching' resulting in the potentiation of alternative metabolic pathways. In the latter case, the pharmacological and toxicological consequences of 'metabolic switching' will depend on the relationship between these properties for the metabolites normally produced by the unlabelled compound and those enhanced by isotopic labelling. Two recent studies illustrate the potential for 'metabolic switching' in either potentiating or inhibiting toxic metabolic pathways.

Ethylene dibromide undergoes both cytochrome P-450-dependent oxidative metabolism and nucleophilic displacement of bromide by glutathione (Fig. 2). The displacement reaction,

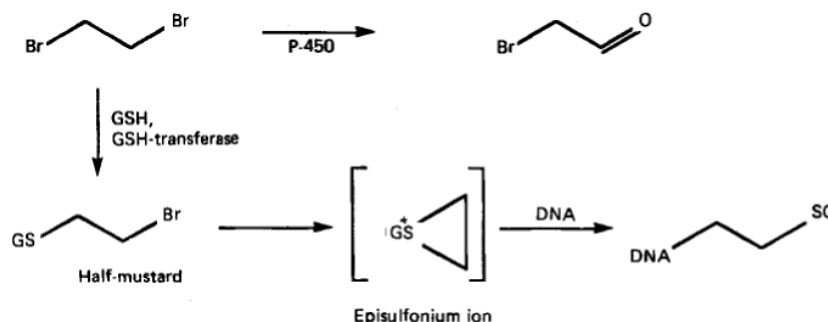


Fig. 2. Proposed mechanism for the metabolism and activation of ethylene dibromide.^{24, 25}

catalyzed by GSH-transferase, leads to a sulphur half-mustard. Recent studies by Ozawa and Guengerich²⁴ and White and co-workers²⁵ imply that DNA-alkylation and damage occurs through the episulfonium ion formed through intramolecular nucleophilic displacement of the glutathione-half mustard. Tetradeuterio ethylene dibromide is oxidized more slowly through the cytochrome P-450-catalyzed pathway but is accompanied by an increase in DNA damage and hepatotoxicity in mice.²⁵ Thus, the evidence suggests that 'metabolic switching' away from the cytochrome P-450 leads to an increase in substrate flux through GSH-transferase and the potentiation of toxic metabolic pathways.

Pulegone (Fig. 3, I) is one of the constituents of the abortifacient and hepatotoxin pennyroyal oil. The major routes of metabolism are through the epoxide, II, the alcohol, III, and the furan, IV. The furan requires net cleavage of two C-H bonds during formation and has been suspected to be a toxic metabolite of this agent. Gordon³⁰ has recently conducted studies on the effect of deuterium substitution on the two vinyl methyl groups on the hepatotoxicity of pulegone. At a

dose of 400 mg/kg, pulegone caused the death of 50% of the test animals, a marked degree of hepatic necrosis in the surviving animals and a very high elevation of plasma GTP levels. In contrast, the same dose of pulegone-D₆ resulted in only 10% lethality, no evidence of hepatic necrosis in the surviving animals and an eighteen-fold lower level in plasma GPT. This inverse isotope effect on these toxicity indices was not associated with an observed alteration in the overall metabolism of pulegone. However, an isotope effect of approximately 2-fold was observed in the formation of the furan, IV, and an inverse isotope effect of approximately 0.5 was observed in the formation of the epoxide, II, and in the major stereoisomer of the alcohol, III. Thus, the isotope effect observed in the formation of the hepatotoxic furan resulted in 'metabolic switching' to the less toxic alcohol and epoxide metabolites.

Conclusions

Deuterium substitution provides a means for delivering an energy barrier of known magnitude to the drug metabolizing cytochrome P-450 enzymes. This capability and the accompanying 'metabolic switching' provides a means for estimating the energy barrier for substrate orientation in the active site of cytochrome P-450 enzymes. This appears to be the only means currently described for measuring the energy barrier imposed by the topological constraints of the protein domain defining the active site of these enzymes. It is this energy barrier that is responsible for the reorientation of the substrate on the active site of the enzyme and the resulting change in regional selectivity as well as the increases in substrate flux through other drug metabolizing enzymes.

The growing number of examples of 'metabolic switching' associated with kinetic isotope effects on cytochrome P-450-catalyzed reactions illustrates the

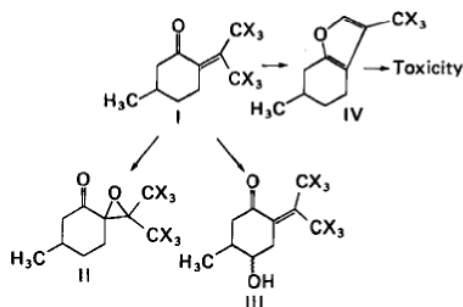


Fig. 3. Partial representation of the known pathways for pulegone (I) metabolism. Studies by Gordon and Nelson³⁰ of pulegone ($X = \text{H}$) and pulegone-D₆ ($X = {}^2\text{H}$) implicate the furan metabolite, IV, in the hepatotoxicity of this agent. Deuterium substitution reduces the formation of IV and toxicity while increasing the formation of the less toxic metabolites II and III.

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generality of this phenomena in reactions catalyzed by these enzymes. The expression of these effects on biological parameters such as drug activity or toxicity depends on the relative abundance and properties of the normal metabolites to those formed through the alternate pathways arising from 'metabolic switching'. Indeed, in certain cases, relatively small isotope effects can produce profound differences in biological indices such as illustrated by the dramatic reduction in toxicity of pulegone and the potentiation of genotoxicity for ethylene dibromide cited above. These observations suggest the possibility of achieving less toxic therapeutic agents through the conservative strategy of deuterium substitution on carbon atoms susceptible to the cytochrome P-450 oxidation critical to the genesis of toxic metabolites. Since the expression of 'metabolic switching' may be more generally applied to any measurable biological response and may be employed with substituents other than hydrogen isotopes, this phenomenon may yield a more generally useful means for unraveling the role of various metabolic pathways in the biological action of chemical agents.

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GERALD T. MIWA is at the Department of Drug Metabolism, Glaxo, Inc., Research Triangle Park, NC 27709, USA and ANTHONY Y. H. LU is at the Department of Animal Drug Metabolism, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065, USA.

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