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Theory for the Observed Isotope Effects from Enzymatic Systems That Form Multiple Products via Branched Reaction Pathways: Cytochrome P-450†

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ABSTRACT: By use of cytochrome P-450 as the prototype, kinetic descriptions are derived for the observed isotope effects for several models of enzymatic systems which are capable of generating multiple products from single substrates. The models include rapid and slow equilibria between enzyme–substrate orientations as well as multiple simultaneous and multiple sequential isotope effects. When an equilibrium is established between enzyme–substrate complexes that are responsible for the oxidation of different positions of the substrate, the kinetics can be represented by competing pathways from the same intermediate. When direct interchange between the complexes does not occur, the alternate pathway mimics the presence of a competitive inhibitor in the substrate solution. In general, the presence of alternate pathways in competition with the isotopically sensitive step will tend to unmask the intrinsic isotope effect.

One of the important techniques used for unraveling the mechanism of a chemical reaction, particularly in determining transition-state structure, is the measurement of the isotope effect (most commonly deuterium) associated with the reaction. Indeed, the technique has been successfully employed by chemists for more than half a century to study the mechanisms of many common homogeneous reactions. Its application to biochemical systems, however, has not been as straightforward because the interpretation of the observed isotope effect for an enzymatically catalyzed reaction is invariably complicated by the multistep nature of such reactions. Fortunately, in approximately the last decade the kinetics involved and the interpretation of the observed isotope effects in enzymatically mediated reactions have been elucidated and described (Northrop, 1978, 1981; Cleland, 1982).

In general, the experimental design used for isotope effect experiments can be divided into three catagories: noncompetitive intermolecular, competitive intermolecular, and in-

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tramolecular experiments. In the noncompetitive approach, the kinetic parameters V_{max} and K_m for the natural and isotopically substituted substrates are measured independently. Differences observed in these values are interpreted as isotope effects on V_{max} or on the ratio of V_{max} to K_m, V/K. The competitive isotope effect approach, in which both labeled and unlabeled substrates are present in the incubation mixture, can be divided further into two subcategories, one in which trace labels are used, such as most tritium or ^{14}C enrichment or dilution experiments, and the other in which intentionally prepared mixtures, e.g., a 50:50 mixture of protio and deuterio substrates, are used. In both subcategories, changes in the amount of label in either the substrate or the product can be used to calculate the isotope effect on V/K. The advantage of the competitive experiment is that only the substrate and the product isotopic composition need to be measured, and thus, experimental errors in such studies tend to be small. However, this approach cannot be used to measure the individual isotope effects on V_{max} and K_m.

The observed isotope effects on V_{max} and V/K are functions of the isotope effects for the various isotopically sensitive steps and the rate constants in the enzymatic cycle that govern the flux through the system. As a consequence, the intrinsic isotope effect for the bond-breaking step can be masked by these other steps. Thus, the observed isotope effect in general lies between the intrinsic isotope effect and 1.0. To eliminate these masking factors, the strategy of utilizing intramolecular isotope effect experiments was introduced. In this experimental design, a substrate must have a minimum of two symmetrical sites that can be metabolized. One or more (but not all) of these sites are isotopically substituted, and the rates of reaction at the substituted positions are measured relative to the rates at the unsubstituted positions. If the rate of interchange between the enzyme–substrate orientations from which the substituted and unsubstituted positions are metabolized is fast relative to that of the isotopically sensitive step, the intrinsic isotope effect will be observed.

The cytochromes P-450 are a family of membrane-bound isozymic monooxygenases that have the capacity to oxidize a wide variety of both exogenous and endogenous compounds. Because of their primary importance in the biotransformation of xenobiotics and in chemically induced toxicity, their individual spectrum of activities and mechanisms of reaction have been subjects of intense interest to workers in the field. The application of isotope effects to study the cytochromes P-450 has only recently gained momentum, and it is already apparent that as a group they have some unusual properties that can profoundly influence the magnitude of an observed isotope effect associated with the oxidative event. While some of the isozymes responsible for the oxidation of a particular endogenous substrate have narrow substrate specificities, e.g., aromatase (Osawa et al., 1987), many appear to have evolved to function as a means of processing and eliminating xenobiotics. In order to accomplish such a task with a limited number of isozymes, broad substrate specificities are required. The relatively weak and nonspecific interactions for a given isozyme–substrate complex can also result in broad regioselectivity, i.e., multiple sites for potential oxidation of the same substrate. When describing the enzyme kinetics for these systems, this "flexibility" may result in an "activated enzyme–substrate complex" which is actually a complex function of rate constants between several general enzyme–substrate orientations. As a general rule, however, kinetic descriptions that take into account broad regioselectivity will contain additional branched pathways in competition with the isotopically sensitive step. The general effects of such branching on intramolecular isotope effects have been discussed for the cytochrome P-450 mediated hydroxylation of octane (Jones et al., 1986).

In addition to catalyzing the formation of different metabolites from the same substrate, these enzymes can also function as NADPH oxidases in which the active oxygen species is further reduced to water (Gorsky et al., 1984). As shown by Sligar et al. (1987), water formation is in direct competition with substrate oxidation, and thus can affect the observed isotope effect.

These alternate pathways tend to decrease the masking of the intrinsic isotope effect caused by other partially rate-limiting steps in the enzymatic cycle. In general, the purpose of this paper is to provide a theoretical basis for the interpretation of the observed isotope effects for enzymatically catalyzed processes which can generate more than one product from a single substrate. Specifically, the enzymatic cycle of the cytochromes P-450 will be utilized to provide the kinetic models for the development of the theory.

Theory

The exact kinetic description of any cytochrome P-450 oxidation will depend on the binding characteristics of each substrate/isozyme pair. In order to provide a general discussion of the kinetics involved with these systems, the analysis will begin with a simple enzymatic cycle and progressively include those unusual characteristics frequently encountered with the cytochrome P-450 enzymes. Although several steps are involved in oxygen activation by the cytochrome P-450 isozymes (two one-electron reductions, etc.), the sum of these steps is represented as a single irreversible step. Product formation is considered irreversible, and conditions resulting in negligible product inhibition are assumed. All equations were derived for noncompetition with either the net rate constant method (Cleland, 1975) or the determinant method (King & Altman, 1956). A simple system with a single irreversible step prior to substrate transformation can be described by model I.

model I

$$E + S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES \xrightarrow{k_{23}} ES^* \xrightarrow{k_{34}} EP \xrightarrow{k_{41}} E + P$$

In this model, the enzyme–substrate complex (ES) undergoes an irreversible step to form the substrate–bound active oxygenating species (ES*), which then proceeds to the enzyme–product complex (EP) with a rate constant k_{34} (the isotopically sensitive step). The kinetics of this system has been previously described (Northrop, 1978, 1981; Cleland, 1982), and the following relationships have been derived:

$$V_{max} = \frac{[E_{tot}]}{1/k_{23} + 1/k_{34} + 1/k_{41}} \quad (1)$$

$$V/K = \frac{[E_{tot}]k_{12}k_{23}}{k_{21} + k_{23}} \quad (2)$$

where V_{max} is the rate of product formation at saturating substrate concentrations and V/K is approached as the substrate concentrations are decreased. The equations for the observed isotope effects are

$$^{D}V = \frac{k_{34H}/k_{34D} + k_{34H}(1/k_{23} + 1/k_{41})}{1 + k_{34H}(1/k_{23} + 1/k_{41})} \quad (3)$$

$$^{D}V/K = 1 \quad (4)$$

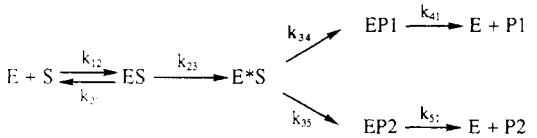
Although these and the following equations correspond to deuterium substitution, they obviously apply to any isotopic substitution. The terms ${}^{D}V$ and ${}^{D}V/K$ are those described by Northrop (1977) and represent V_H/V_D and $(V/K)_H/(V/K)_D$, respectively.

The term $k_{34H}(1/k_{23} + 1/k_{41})$ in eq 3 is called the V ratio and will tend to mask the intrinsic isotope effect. The masking becomes marked when k_{34H} is large relative to k_{23} and k_{41}. The effects on V_{max} may be rationalized in terms of the concentrations of the various enzyme species (E, ES, ES*, and EP). At saturating concentrations of the substrate, the concentration of the unbound species [E] becomes virtually zero, and the concentrations of the complexes will be functions of the rate constants to and from each of the other species (Cleland, 1975). For model I, when k_{34} is small relative to both k_{23} and k_{41}, most of the enzyme will be in the form ES*, and the observed isotope effect will approach the intrinsic isotope effect. When either k_{23} or k_{41} is small relative to k_{34}, most of the enzyme will be in the form ES or EP, respectively, and the intrinsic isotope effect will be masked.

No isotope effect will be observed for V/K with this system because an irreversible step precedes the isotopically sensitive step and there is no branching. After the species ES* is formed, the complex is committed to product formation; i.e., the rate of formation of the product will be equal to the rate of formation of ES*. The term V/K describes the rate of product formation when the concentration of the substrate is small relative to its K_m. Under these conditions, when an irreversible step precedes the isotopically sensitive step, any change in the rate constant k_{34} will be compensated for by an equal and opposite change in the steady-state concentration of ES*; i.e., $(ES_H^*)k_{34H} = (ES_D^*)k_{34D}$. Thus, no isotope effect will be observed as long as there is no isotope effect on the binding of the substrate to the enzyme or the activation rate constant, k_{23}.

Next, we will include an alternate pathway in competition with the isotopically sensitive step, shown in model II. For the cytochrome P-450 reactions, the competing pathway, k_{35}, can represent oxidation at an alternate site on the substrate for a given ES* complex. In model II, changes in the orientation of the substrate in the form ES* are assumed to occur rapidly relative to the rate of substrate oxidation. Since more than one product is being formed, several observable isotope effects may be described. These include the isotope effects on the formation of the individual products (P1 and P2), the isotope effects associated with total substrate metabolism (P1 + P2), and the isotope effects on the ratios and percentages of the products formed.

model II



For the formation of P1

$$ {}^{D}V_{P1} = \frac{k_{34H}/k_{34D} + \dfrac{k_{34H}(1/k_{23} + 1/k_{41})}{k_{35}(1/k_{23} + 1/k_{51}) + 1}}{1 + \dfrac{k_{34H}(1/k_{23} + 1/k_{41})}{k_{35}(1/k_{23} + 1/k_{51}) + 1}} \quad (5) $$

$$ {}^{D}V/K_{P1} = \frac{k_{34H}/k_{34D} + k_{34H}/k_{35}}{1 + k_{34H}/k_{35}} \quad (6) $$

Equation 5 shows that the extent of masking of the intrinsic isotope effect depends on the term $k_{34H}(1/k_{23} + 1/k_{41})/[k_{35}(1/k_{23} + 1/k_{51}) + 1]$. When rate constant k_{35} is large compared to k_{34H}, the term will be small, and the value of the observed isotope effect will approach the intrinsic isotope effect. An isotope effect will cause a change in the relative amounts of P1 and P2 formed, irrespective of any other rate-limiting steps. This can be understood when one considers the effect on the concentration of ES*. Since most of ES* is converted to EP2, differences between k_{34H} and k_{34D} will not cause as marked a difference in the steady-state concentrations of ES* as it would in model I. Thus, the observed isotope effect on the formation of P1 will depend predominantly on the difference between k_{34H} and k_{34D}.

With the addition of the branched pathway, the isotope effect on V/K for the formation of P1 can now be observed. The intrinsic isotope effect is masked solely by the branching ratio k_{34H}/k_{35}. In this situation, the alternate pathway k_{35} provides a means of keeping the concentrations virtually equal. In model II, $[ES_H](k_{34H} + k_{35}) = [ES_D](k_{34D} + k_{35})$, and as k_{35} becomes large, any change in k_{34} will be compensated for by a change in the flux through k_{35}. It can also be seen that as k_{23} becomes small eq 5 approaches eq 6. Thus, for the cytochrome P-450 system, if the generation of the active oxygenating species is rate limiting and product release is fast, ${}^{D}V = {}^{D}V/K$.

Although product release does not affect ${}^{D}V/K$, slow product release associated with branched pathways can have diverse influences on ${}^{D}V$. As expected, slow release of the product from the isotopically sensitive pathway (i.e., when k_{41} is small) will decrease the observed isotope effect, since much of the enzyme will exist as EP1. However, if the release of P2 is rate limiting (i.e., when k_{51} is small), ${}^{D}V$ will tend to be unmasked. Even if the amount of branching is small, the intrinsic isotope effect can be approached if the release of the second product is sufficiently slow. In general, the above relationships show that the presence of a nonisotopically sensitive pathway in competition with the isotopically sensitive step will tend to unmask the intrinsic isotope effect, especially when $k_{35} > k_{34}$ and $k_{51} < k_{41}$.

In addition, the isotope effect associated with the formation of P1 can result in an observed isotope effect for the formation of P2. This may be described quantitatively by

$$ {}^{D}V_{P2} = \frac{k_{34D}/k_{34H} + \dfrac{k_{35}(1/k_{23} + 1/k_{51}) + 1}{k_{34H}(1/k_{23} + 1/k_{41})}}{1 + \dfrac{k_{35}(1/k_{23} + 1/k_{51}) + 1}{k_{34H}(1/k_{23} + 1/k_{41})}} \quad (7) $$

$$ {}^{D}V/K_{P2} = \frac{k_{34D}/k_{34H} + k_{35}/k_{34H}}{1 + k_{35}/k_{34H}} \quad (8) $$

The observed isotope effect associated with the non isotopically sensitive alternate pathway will be a function of the inverse of the isotope effect for the isotopically sensitive pathway. Again the observed isotope effect will be modified by rate constants which will mask the intrinsic value. However, the factors are the inverse of those in eq 5 and 6. When k_{34H} is large relative to k_{35}, the observed isotope effect will approach the reciprocal of the intrinsic isotope effect, i.e., k_{34D}/k_{34H}. Equations 5–8 show that the effect of branching on the observed isotope effects will be greatest for the least preferred pathway.

The isotope effect on P2 formation provides the explanation for the unmasking seen for ${}^{D}V_{P1}$ when the release of P2 is slow. If k_{51} is slow relative to the other steps in the cycle, a substantial amount of the enzyme will exist as EP2. If, for ex-

ample, P2 formation is the least preferred pathway, a normal isotope effect associated with P1 formation can result in an increase in P2 formation (eq 7). If P2 release is slow, the concentration of EP2 will increase and the concentration of all other enzyme species will decrease, thereby masking the inverse isotope effect for P2 formation and unmasking the normal isotope effect for P1 formation.

Despite the complexities introduced by branching into the standard ways of interpreting observed isotope effects, branching can offer unique advantages in determining intrinsic isotope effects (Jones et al., 1986). This becomes apparent when the isotope effects on ratios and percentages are considered. If model II correctly describes the enzyme–substrate interactions, both products are formed from the same species, and the relative amounts of P1 and P2 that are formed depend solely on the rate constants k_{34} and k_{35}. (Note that P1/P2 = [ES*]k_{34}/[ES*]k_{35} = k_{34}/k_{35}.) The intrinsic isotope effect can obtained directly from the product ratios for the protio and deuterio substrates:

$$\frac{P1_H/P2_H}{P1_D/P2_D} = k_{34H}/k_{34D} \tag{9}$$

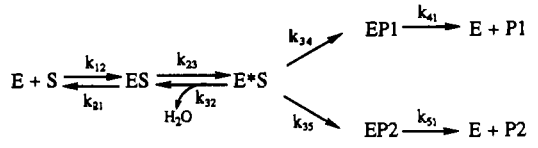
It can also be shown that the isotope effect associated with the percent P1 formed is determined by the same expression as that for $^DV/K$:

$$\frac{P1_H/(P1_H + P2_H)}{P1_D/(P1_D + P2_D)} = \frac{k_{34H}/k_{34D} + k_{35}/k_{34H}}{1 + k_{35}/k_{34H}} = {}^DV/K \tag{10}$$

Thus, according to model II, the intrinsic isotope effect can be obtained directly from the protio and deuterio product ratios or from percentages. Also, since $^DV/K$ is observable solely because of the presence of the branched pathway (see eq 6 and 8), determination of the branching ratio and $^DV/K$ can also provide the intrinsic isotope effect.

For the cytochrome P-450 isozymes, the above relationships can be used to describe the competition between oxidation at two sites on the same substrate. In some cases, however, the oxidation of the substrate is also in competition with the reduction of the active oxygen in ES* to water. Model III may be used to describe this situation.

model III



$$^DV_{P1} = \frac{k_{34H}/k_{34D} + \dfrac{k_{34H}(1/k_{23} + 1/k_{41})}{k_{35}(1/k_{23} + 1/k_{51}) + k_{32}/k_{23} + 1}}{1 + \dfrac{k_{34H}(1/k_{23} + 1/k_{41})}{k_{35}(1/k_{23} + 1/k_{51}) + k_{32}/k_{23} + 1}} \tag{11}$$

$$^DV/K_{P1} = \frac{k_{34H}/k_{34D} + \dfrac{k_{34H}(1/k_{23} + 1/k_{21})}{k_{35}(1/k_{23} + 1/k_{21}) + k_{32}/k_{23}}}{1 + \dfrac{k_{34H}(1/k_{23} + 1/k_{21})}{k_{35}(1/k_{23} + 1/k_{21}) + k_{32}/k_{23}}} \tag{12}$$

Since the new term introduced into eq 12 (k_{32}) is in the denominator, it also serves to unmask the isotope effect. This is expected since it is really another branch from ES*. The equation for $^DV/K$ in model III is more complicated than that for the previous models because the formation of water is mathematically equivalent to a reversible reaction between ES and ES*. However, the effects of the alternate pathways remain the same; i.e., they all tend to unmask the intrinsic isotope effect.

It should be noted that when water formation is significant, isotope effects on both V and V/K may be observed even when the substrate is converted to a single metabolite (i.e., k_{35} = 0). In this case, the standard equations (Northrop, 1978, 1981; Cleland, 1982) apply:

$$^DV_{P1} = \frac{k_{34H}/k_{34D} + \text{cvf}}{1 + \text{cvf}} \tag{13}$$

$$^DV/K_{P1} = \frac{k_{34H}/k_{34D} + \text{cf}}{1 + \text{cf}} \tag{14}$$

where cvf and cf are the V ratio and commitment to catalysis, respectively, and are defined as

$$\text{cvf} = \frac{k_{34H}(1/k_{23} + 1/k_{41})}{k_{32}/k_{23} + 1}$$

$$\text{cf} = (k_{34H}/k_{32})(k_{23}/k_{21} + 1)$$

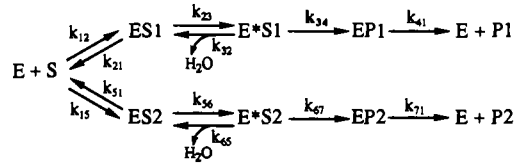
Again, it can be seen from eq 11–14 that as k_{23} becomes rate limiting and an alternate pathway exists, the observed isotope effects for V and V/K will approach the same value:

$$^DV_{P1} = {}^DV/K_{P1} = \frac{k_{34H}/k_{34D} + k_{34H}/(k_{32} + k_{35})}{1 + k_{34H}/(k_{32} + k_{35})} \tag{15}$$

As with model II, the relationships describing the observed isotope effects on P2 are of the same form as those for P1 but contain the inverse of the intrinsic isotope effect modified by the inverse of the constant terms in eq 11, 12, and 15. Moreover, the intrinsic isotope effect may also be obtained from the product ratios (eq 9). However, the isotope effect for the relative percentages of product formation will be described by eq 10 rather than eq 15, and is therefore not identical to $^DV/K$. Thus, the isotope effect on the percent of product formation is "unmasked" only by the branching ratio k_{34}/k_{35}, and water formation does not affect the observed isotope effect calculated in this way.

Equations 11–15 can be used to describe cytochrome P-450 oxidations, including water formation and multiple sites of oxidation when the rates of interconversion between the various enzyme–substrate orientations are fast. It is useful to consider the opposite limit to fast equilibrium: the complete lack of orientation interconversion. Model IV will be used to describe this situation.

model IV



This model describes a situation where two enzyme–substrate orientations are possible, but the substrate must debind from ES1 or ES2 in order to change orientations. For this model, the kinetics are analogous to the presence of a competitive inhibitor. The effect of a competitive inhibitor in the situation where the ratio of the concentrations of inhibitor to substrate remains constant has been previously discussed (Grimshaw & Cleland, 1980). It was shown that the inhibitor has no effect on $^DV/K$. Therefore, the pathways for the formation of P2 can be ignored, and eq 14 applies. In addition,

since there are no branches from ES1*, if $k_{32} = 0$, ${}^{D}V/K = 1$. It was also shown that, as the concentration of the inhibitor increases relative to substrate, ${}^{D}V$ approaches ${}^{D}V/K$. Equation 16 describes ${}^{D}V$ for model IV:

$$ {}^{D}V_{P1} = \frac{k_{34H}/k_{34D} + \dfrac{k_{34H}[1/k_{41} + 1/k_{23} + (k_{21} + k_{23})/k_{12}k_{23}K_2]}{1 + k_{32}/k_{23} + k_{21}k_{32}/k_{12}k_{23}K_2}}{1 + \dfrac{k_{34H}[1/k_{41} + 1/k_{23} + (k_{21} + k_{23})/k_{12}k_{23}K_2]}{1 + k_{32}/k_{23} + k_{21}k_{32}/k_{12}k_{23}K_2}} \quad (16) $$

where

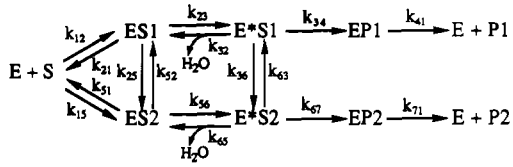
$$ K_2 = \frac{1/k_{15} + (k_{51}/k_{15}k_{56})(1 + k_{65}/k_{67})}{k_{65}/k_{67}k_{56} + 1/k_{56} + 1/k_{67} + 1/k_{71}} $$

In this equation, K_2 is the K_m for P2 formation if the ES1 pathway did not exist. If K_2 is small, i.e., the lower pathway predominates, the terms that do not contain K_2 become insignificant, and ${}^{D}V$ approaches ${}^{D}V/K$ (eq 14). If K_2 is large, i.e., the lower pathway is unimportant, then eq 16 approaches eq 13, the standard relationship for ${}^{D}V$ with no branching. Thus, while alternate enzyme–substrate orientations will not affect ${}^{D}V/K$, they can either mask or unmask ${}^{D}V$, depending on which commitment term, cf or cvf (eq 13 and 14), is larger.

It can also be shown that the isotope effect on product ratios, $(P1_H/P2_H)/(P1_D/P2_D)$, is equal to ${}^{D}V/K$. This suggests that some informative relationships can be observed if the isotope effects on both P1 and P2 are measured. When $k_{32} = 0$, i.e., no water is formed from ES*, the product ratio isotope effects will be 1.0, and ${}^{D}V_{P2}$ will be equal to ${}^{D}V_{P1}$. ${}^{D}V_{P1}$ will depend on the rate-limiting nature of the isotopically sensitive step and the value of K_2. If k_{34} is rate limiting, as K_2 becomes large (the P2 pathway becomes less significant), both ${}^{D}V_{P1}$ and ${}^{D}V_{P2}$ will approach the intrinsic isotope effect. This is expected since the increase in ES1* due to isotopic substitution will also decrease the flux through the minor pathway (P2 formation). As k_{32} becomes significant, both ${}^{D}V_{P1}$ and ${}^{D}V/K_{P1}$ can vary between 1.0 and the intrinsic isotope effect. If ${}^{D}V_{P1}$ is 1.0, ${}^{D}V_{P2}$ will be the inverse of ${}^{D}V/K_{P1}$, and ${}^{D}V/K_{P2}$ will be 1.0. If ${}^{D}V/K_{P1} = 1.0$, ${}^{D}V_{P2}$ will be the inverse of ${}^{D}V_{P1}$, and ${}^{D}V/K_{P2}$ will be 1.0. Thus, depending on the relative importance of k_{32}, ${}^{D}V_{P2}$ can vary between ${}^{D}V$ and inverse of either ${}^{D}V_{P1}$ or ${}^{D}V/K_{P1}$.

As the rates of interconversion between orientations become significant, model V would have to be considered.

model V

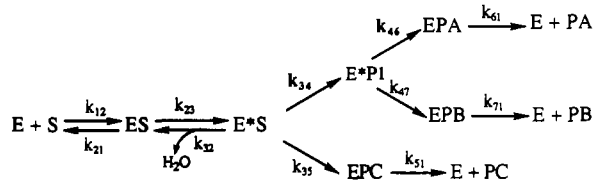


In this model, when k_{25}, k_{52}, k_{36}, and k_{63} are very large relative to the other rate constants from the ES* species, kinetic relationships analogous to those for model III would result; i.e., rapid equilibrium approximates single ES and ES* species. The introduction of the exchange rate constants results in much more complicated equations for ${}^{D}V$ and ${}^{D}V/K$, since the ES and ES* species can be formed by multiple pathways. The equations for ${}^{D}V$ and ${}^{D}V/K$ have been derived with the determinant method (King & Altman, 1956) and can be arranged to obtain the standard form, $(k_H/k_D + C)/(1 + C)$. However, the constant terms are very complicated and will not be presented here.

Multiple Isotope Effects

Sequential Isotope Effects. Due to the nonconcerted nature of the proposed cytochrome P-450 mechanisms, sequential isotopically sensitive pathways are possible. These mechanisms include aliphatic hydroxylation, which proceeds via hydrogen abstraction followed by recombination (Groves et al., 1978), and the addition–rearrangement pathways proposed for alkene (Hanzlik & Shearer, 1978; Ortiz de Montellano & Correia, 1983), alkyne (Ortiz de Montellano & Komives, 1985), and aromatic oxidations (Tomezweski et al., 1975; Bush & Trager, 1985; Korzekwa et al., 1985). Model VI will be used to describe the general situation of sequential isotope effects in an enzymatic system.

model VI



In this model, the branch from ES* represents the initial oxidation of different positions on the substrate, e.g., different tetrahedral intermediates formed during aromatic oxidation [see Korzekwa et al. (1989)], and the branch from EP1* represents further rearrangements to the products. Since all P-450-mediated oxidations are thought to be multistep in nature, an intermediate step will also be involved in PC formation. However, this step will be considered to be fast, irreversible, and isotopically insensitive. Thus, the entire pathway can be represented by a single rate constant. Again, the observed isotope effects on ${}^{D}V$ and ${}^{D}V/K$ are complicated but can be simplified by measuring the isotope effects on the ratios or precentages of products formed. When the model is valid, the intrinsic isotope effects can be obtained from the product ratios:

$$ k_{34H}/k_{34D} = \frac{(P_{AH} + P_{BH})/P_{CH}}{(P_{AD} + P_{BD})/P_{CD}} \quad (17) $$

$$ k_{46H}/k_{46D} = \frac{P_{AH}/P_{BH}}{P_{AD}/P_{BD}} \quad (18) $$

The percent PA isotope effects will be the product of the two isotope effects modified by their branching ratios:

$$ \frac{P_{AH}/(P_{AH} + P_{BH} + P_{CH})}{P_{AD}/(P_{AD} + P_{BD} + P_{CD})} = \frac{k_{34H}/k_{34D} + k_{34H}/k_{35}}{1 + k_{34H}/k_{35}} \frac{k_{46H}/k_{46D} + k_{46H}/k_{47}}{1 + k_{46H}/k_{47}} \quad (19) $$

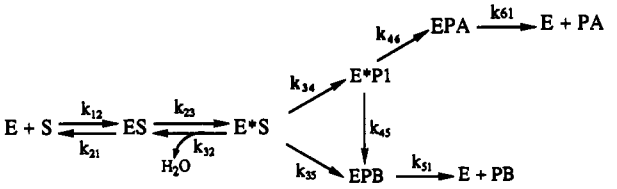
Again, since water formation is not included in the products, k_{32} does not enter into the equations. If k_{32} is negligible, ${}^{D}V/K$ can also be described by eq 19. As k_{32} becomes important (i.e., the commitment to catalysis decreases), the isotope effect on V/K is further unmasked and can be described by the product of eq 14 and the second branching isotope effect. Therefore, the percent PA isotope effect can be considered a minimum value for ${}^{D}V/K$, since the involvement of k_{32} will always tend to unmask the the intrinsic isotope effect.

${}^{D}V$ for model VI is more complicated since the degree of masking is dependent on the rate-limiting nature of the isotopically sensitive steps. Thus, the observable amount of each individual isotope effect is a function of the other isotopically sensitive rate constants, and the ratio of the rate equations can no longer be rearranged to the standard form, $(k_H/k_D + C)/(1$

+ C). A graphical analysis of the complete system has resulted in the following observations. In most situations, the ^{D}V will be larger than the observed isotope effect expected from branching alone, i.e., the percent PA isotope effect, since any rate-limiting nature of the isotopically sensitive steps will further unmask the observed value. However, an exception is seen if product release is slow. As with model II, slow release of PA will tend to mask ^{D}V, and slow release of PC will tend to unmask the isotope effect. If release of PB is slow, the first isotope effect will be masked, and the second will be unmasked.

A second model (model VII) with sequential isotope effects will be considered since it applies to the addition–rearrangement pathways for aromatic oxidation considered in the following paper (Korzekwa et al., 1989).

model VII



In model VII, k_{34} and k_{35} again represent different positions of initial oxygen attack but EP1* can proceed to either PA or PB. The isotope effects on V and V/K will be essentially the same as described for model VI. However, the intrinsic isotope effects for each branch can no longer be obtained from the product ratios since the fluxes through EP1* and EPB can no longer be determined by the ratio of PA to PB. Nevertheless, the isotope effect on the percent PA is still the product of the isotope effects for each step modified by their branching ratios (eq 19, k_{47} replaced by k_{45}).

Simultaneous Isotope Effects. We next consider isotope effects associated with both branches eminating from the same intermediate, e.g., models II and III with both k_{34} and k_{35} isotopically sensitive. This could be seen if two positions on a substrate are isotopically sensitive to the initial oxygen attack or if a chemical intermediate formed during oxidation can rearrange to two or more products via isotopically sensitive pathways. As with sequential isotope effects, isotopically sensitive rate constants are involved in the factors that mask ^{D}V and $^{D}V/K$, and these expressions become complicated. Again, these expressions can be simplified either if $^{D}V/K$ is measured for a system with an irreversible step prior to the isotopically sensitive branch (model II) or if the percent product isotope effect is measured (model II and model III). For these models the following relationship can be derived:

$$\frac{P_{1H}/(P_{1H} + P_{2H})}{P_{1D}/(P_{1D} + P_{2D})} = \frac{(k_{34H}/k_{34D})(k_{35D}/k_{35H}) + k_{34H}/k_{35H}}{1 + k_{34H}/k_{35H}} \quad (20)$$

Obviously, the individual intrinsic isotope effects cannot be obtained from the product ratios since both isotope effects will influence the observed branching ratio, but the product of the intrinsic isotope effect for P1 formation and the inverse of the intrinsic effect for P2 formation will be observed.

Discussion

It might be expected that a family of isozymes which has evolved to metabolize xenobiotics would have some unusual characteristics. While most enzymes appear to perform certain limited functions extremely well, many of the cytochromes P-450 appear to do the opposite: they oxidize a variety of substrates by several different mechanisms at relatively slow rates (turnover numbers <50 min^{-1}). The broad substrate specificity is most likely accomplished by relatively nonspecific interactions with a variety of apoproteins. The different kinds of oxidative products produced (e.g., alcohol from alkanes, epoxides from alkenes, etc.) can be rationalized if one considers that the catalytic step is the formation of the active oxidizing species. Rather than a plurality of catalytic mechanisms, only a versatile oxidant is necessary to achieve diversity in oxidative function.

Given the involvement of the cytochromes P-450 in the metabolism of both endogenous and exogenous compounds, a wide range of substrates and regiospecificities is expected. For those substrates which are converted to one product, standard enzyme isotope effect kinetics should apply (e.g., eq 13 and 14). For those enzyme–substrate complexes that produce multiple metabolites, two limits can be discussed.

The first limit is if the different ES* orientations can equilibriate very rapidly. In this case, the equations presented which describe branching from ES* are applicable. The general effect of an alternate pathway is to unmask the intrinsic isotope effect. $^{D}V/K$ is generally masked by the term cf, the commitment to forward catalysis. For a nonbranched system, this term is the ratio of the isotopically sensitive step to the net rate constant for release of the substrate (Cleland, 1982), $k_{34H}/[k_{32}k_{21}/(k_{23} + k_{21})]$ for model III, when $k_{35} = 0$. The presence of an alternate pathway decreases the commitment of ES* to P1 formation, unmasking the isotope effect. For this reason, the generalization that no V/K isotope effect can be seen if an irreversible step precedes the isotopically sensitive step does not apply to the branched systems that we have discussed.

For ^{D}V, branching assures isotopic discrimination from ES* even if the isotopically sensitive step is not significantly rate limiting. Also, while slow product release will generally mask ^{D}V, slow release of the alternate product will allow ^{D}V to approach the intrinsic isotope effect (instead of $^{D}V/K$ as in the case of a competitive inhibitor, model IV).

In the other extreme, each product is formed from a certain enzyme–substrate orientation, and interchange between orientations does not occur (model IV). In this case, the alternate ES species (the lower pathway in model IV) will act as if a competitive inhibitor is present in the substrate mixture. It has been reported previously (Grimshaw & Cleland, 1980) that the alternate pathway has no effect on $^{D}V/K$, and ^{D}V approaches $^{D}V/K$ as the alternate pathway becomes more important. Although eq 16 provides the general description for $^{D}V_{P1}$ (and $^{D}V/K_{P1}$ as K_2 becomes small), more information concerning reversible enzyme activation (water formation) may be available if $^{D}V_{P2}$ is also measured. If water formation is insignificant, any isotope effect on V_{max} will be seen for both P1 and P2 formation. This is in contrast with models in which the enzyme–substrate orientations reach virtual equilibrium and give inverse relationships between $^{D}V_{P1}$ and $^{D}V_{P2}$ (e.g., eq 7 and 8). If water formation is significant, $^{D}V_{P2}$ can vary between 1 and the inverse of ^{D}V or $^{D}V/K$.

Between these two limiting situations, there exists a complete spectrum of intermediate possibilities. Rate constants for the equilibrium of enzyme–substrate orientations can be of the same order of magnitude as the other kinetically important rate constants. It is also plausible that one or more of the products are formed from two or more different orientations, each with different branching ratios. For situations such as these, exact kinetic descriptions would be unique to each isozyme–substrate pair. It may even be that there are no well-defined enzyme–substrate orientations (potential energy minimums), but instead regions on a potential energy surface

from which the substrate is oxidized. A broad, shallow, low-energy region (e.g., nonspecific hydrophobic binding) may be expected to approximate the rapid equilibrium models, whereas more specific enzyme–substrate interactions would result in slower equilibration. Irrespective of the exact nature of the ES* species, the same general tendencies apply. Kinetically important branches from a productive enzyme–substrate orientation can unmask the intrinsic isotope effect.

Cytochrome P-450 mediated aliphatic hydroxylation and O-dealkylation reactions are known to proceed via hydrogen atom abstraction followed by hydroxy radical recombination (Groves et al., 1978; Lindsay Smith & Sleath, 1983). The transition states are thought to be relatively symmetrical, resulting in large normal intrinsic isotope effects (~9 excluding secondary isotope effects due to multiple deuterium substitution) (Jones & Trager, 1987). For these reactions, isotope effects have been reported which suggest that rapid exchange between enzyme–substrate orientations can occur. The first example of this was discovered (Groves et al., 1978) when the isotope effect on the ratio of 2-exo to 2-endo hydroxylation of norbornane by P-450cam was found to be 11.5. In fact, the large observed value and the stereochemistry of the product provided the evidence that aliphatic hydroxylation proceeds by a hydrogen abstraction mechanism. Also, Lindsay Smith and Sleath (1983) and White et al. (1985) have reported the effects of deuterium substitution on the product ratios for anisole and ethylbenzene oxidation, respectively. If eq 9 or 10 is applied to their data, large intrinsic isotope effects result. It can be shown that if some of the alternate product, P2, is formed from a pathway not in competition with the isotopically sensitive step or if the rate of equilibrium is not fast relative to the isotopically sensitive step, the intrinsic isotope effect will be underestimated by eq 9 and 10. Therefore, if the intrinsic isotope effect were known, the validity of the rapid equilibrium models could be tested. However, these equations alone should not be used to determine transition-state structure unless the upper limit of the isotope effect is observed. The situation is less ambiguous when branching occurs from a chemical intermediate, as in the proposed addition–rearrangement pathways for aromatic oxidation discussed in the following paper. Since the pathways are in direct competition, the fraction of the intrinsic isotope effect that can be observed due to branching alone (e.g., eq 10) will be the lower limit of the observed isotope effect. Values for ^{D}V will only be further unmasked due to any partial rate-limiting nature of the isotopically sensitive step, and $^{D}V/K$ will be further unmasked by low commitments to forward catalysis.

In summary, the involvement of branching in cytochrome P-450 catalyzed oxidations, while complicating the associated kinetic descriptions, can provide a means to unmask intrinsic isotope effects. While the enzymatic cycle is very complicated and determination of the relative rates and reversibility of the various steps may not be possible, the models presented here should provide a means of interpreting the observed isotope effects. Also, unless the observed isotope effects on rates or ratios approach the maximum theoretical values, quantitation of all products including water may be necessary for the determination of both the oxidative mechanism and the nature of the other steps in the enzymatic cycle.

Acknowledgments

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