Isotopically Sensitive Branching and Its Effect on the Observed Intramolecular Isotope Effects in Cytochrome P-450 Catalyzed Reactions: A New Method for the Estimation of Intrinsic Isotope Effects

Jeffrey P. Jones, Kenneth R. Korzekwa, Allan E. Rettie, and William F. Trager*

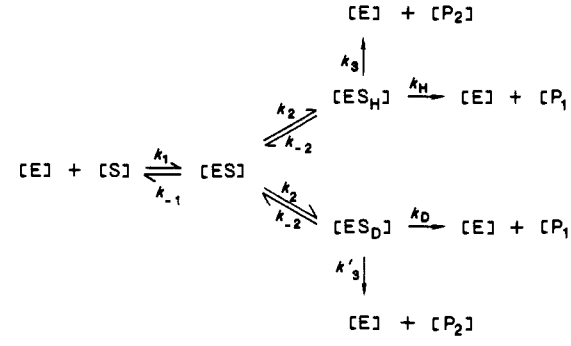
Contribution from the Department of Medicinal Chemistry, BG-20, University of Washington, Seattle, Washington 98195. *Received December 6, 1985*

Abstract: Two selectively deuterated *n*-octanes (octane-*1*-$^2H_3$ and octane-*1,2,3*-$^2H_7$) were synthesized and subjected to hydroxylation by phenobarbital-induced rat liver microsomes and purified cytochrome P-450b. The results of these experiments provide evidence which clarifies the interplay between a branched reaction pathway and the equilibration of an enzyme–substrate complex, in determining the magnitude of an observed isotope effect. An equation is derived that allows limits to be placed on the intrinsic isotope effect. The equation is based on the observed isotope effect and the regioselectivity of a branched reaction pathway, catalyzed by an enzyme that forms two products via a single enzyme–substrate complex. The intrinsic isotope effect for the formation of 1-octanol was determined by this equation to lie between *9.5* and *9.8*.

Isotope effects have played a major role in the determination of reaction mechanisms and in the elucidation of transition-state structure in organic chemistry. Their application to enzymatically mediated systems, however, has proven to be less successful because of the complexity of the multistep reaction sequences that describe many of these processes. In general the ambiguities arising from these systems have restricted the use of isotope effects to estimating the rate of the bond-breaking step relative to the rates of all other steps in the kinetic scheme, e.g., substrate binding and dissociation, product release, and other steps that may mask the intrinsic isotope effect. However, in recent years, Northrop[1] has provided a fundamental basis for interpreting isotope effects in enzymatic systems by clarifying the relationship that exists between an observed isotope effect and the intrinsic isotope effect that is associated with the bond-breaking step.

To circumvent the problems associated with these complicated schemes and the attenuation or "masking" of the intrinsic isotope effect, which make transition-state structure determination difficult, many workers in the field have employed a technique which uses intramolecular isotope effects.[2-5] In such an experiment, a molecule that has two positions that are equivalent in all respects except for isotopic substitution is used as the substrate. The observed isotope effect then reflects the intramolecular competition between the two otherwise equivalent sites. In most cases an intramolecular isotope effect more nearly approximates the intrinsic isotope effect since it depends primarily upon the product-determining step rather than other potential rate-limiting steps,[5] e.g., the magnitude of an observed intramolecular isotope effect will be independent of product release but the magnitude of an intermolecular isotope effect will not. The magnitude of an isotope effect measured by an experiment of intramolecular design, that proceeds via an *irreversible linear* reaction pathway,[6] is inherently less sensitive to masking effects. However, it is dependent upon (1) the rate of rotation between the two isotopically distinct sites within the substrate molecule and/or (2) the rate of dissociation of the enzyme–substrate complex to give free substrate and enzyme. If these rates are slow or are of the same order of magnitude as the bond-breaking step, $[ES_H]$ will not equal $[ES_D]$ (see Scheme I), and the intrinsic isotope effect will be masked. Recently Harada et al.[7] have shown that within a *branched* reaction sequence, i.e., one in which more than one product can arise from an enzyme–substrate complex (Scheme I, k_3' and $k_3 \neq 0$), another factor termed "metabolic switching" can affect the magnitude of the isotope effect that will be observed. Metabolic switching or isotopically sensitive branching[8] can be defined as a change in the relative ratios of products, due to isotopic substitution, arising from the same intermediate in the case of a chemical system or from the same enzyme–substrate complex[9]

Scheme I. Kinetic Model for Isotopically Sensitive Branched Reaction Pathways[a]



[a] The model assumes that there is no isotope effect associated with binding (i.e., a single rate constant k_2 describes the fractionation of [ES] to $[ES_H]$ and $[ES_D]$ and a single rate constant k_{-2} describes the formation of [ES] from $[ES_H]$ and $[ES_D]$) and that product formation is irreversible.

(1) Northrop, D. B. *Biochemistry* **1975**, *14*, 2644.
(2) Miwa, G. T.; Garland, W. A.; Hodshon, B. J.; Lu, A. Y. H.; Northrop, D. B. *J. Biol. Chem.* **1980**, *255*, 6049.
(3) Gelb, M. H.; Heimbrook, D. C.; Malkonen, P.; Sligar, S. G. *Biochemistry* **1982**, *21*, 370.
(4) Hjelmeland, L. M.; Aronow, L.; Trudell, J. R. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 541.
(5) Lindsay Smith, J. R.; Nee, N. E.; Noar, J. B.; Bruice, T. C. *J. Chem. Soc., Perkin Trans. 2* **1984**, 255.
(6) Such a sequence is represented in Scheme I, when k_3 and $k_3' = 0$.
(7) Harada, N.; Miwa, G. T.; Walsh, J. S.; Lu, A. Y. H. *J. Biol. Chem.* **1984**, *259*, 3005.
(8) Isotopically sensitive branching may be a better term than metabolic switching since this effect is not restricted to enzymatic systems. For example, Melander and Saunders (*Reaction Rates of Isotopic Molecules*; Melander, L., Ed.; Wiley: New York, 1980; pp 293–297) have described the kinetics that pertain to an analogous chemical system. Metabolic switching has also been used in a more general way to denote switching from one enzymatic pathway to another due to isotopic substitution; see: Horning, M. G.; Haegele, K. D.; Sommer, K. R.; Nowlin, J.; Stafford, M.; Thenot, J. P. Proceedings of the Second International Conference on Stable Isotopes, National Information Service, US Department of Commerce, Springfield, VA, 1976; p 41. Switching of this type is fundamentally different to that described above since it involves another enzyme or isozyme. This would not effect the observed isotope effect at saturating conditions since the two products would arise from different enzyme–substrate complexes.
(9) A branched reaction pathway implies separate enzyme–substrate complexes, leading to the formation of two distinct products. However, if one assumes that a rapid equilibrium exists between these two complexes, they become kinetically indistinguishable and thus can by treated as a single enzyme complex. The proximity of a C-1 hydrogen to a C-2 hydrogen in octane justifies this assumption.

0002-7863/86/1508-7074$01.50/0

in the case of an enzymatic system.

The regioselectivity of the hydroxylation reactions catalyzed by cytochrome P-450 is very broad and has been shown to be a function of both the inherent chemical reactivity of the substrate and the apoprotein structure of the enzyme.[10] In many cases, the cytochrome P-450 contained in microsomal preparations has been shown to hydroxylate *n*-alkyl chains to several regioisomeric alcohols. Whether the various isomers are the product of a single or different isozyme remains in question.[11-16] Data from purified enzymes would appear to suggest that a single isozyme can carry out a number of regioisomeric oxidations on the same substrate.[17] Conversely, work with antibodies and mechanism-based inhibitors indicate that different isozymes have different regioselectivity,[15,16] suggesting that a single isozyme catalyzes the formation of a single product. Thus, some doubt remains as to the homogeneity of some purified enzyme preparations.[18]

The oxidation of octane to 1-octanol by cytochrome P-450 microsomal preparations has been shown previously to exhibit a "significant" isotope effect.[19] Moreover, the oxidation of octane by cytochrome P-450 is also carried out at the C-2 and C-3 positions (vide infra). In this paper a kinetic model for the effect of branched pathways on isotope effects will be developed and evaluated by using the oxidation of octane to various regioisomeric alcohols by cytochrome P-450. The kinetic model will be used to predict an intrinsic isotope effect for the hydroxylation of octane at the C-1 position, and data will be presented that conclusively implicate a single isozyme in the production of at least two regioisomers.

Experimental Section

Materials. Heptafluorobutyric anhydride and acetonitrile were obtained from Pierce Chemical Co., diethyl ether was obtained from J. T. Baker, and pentane was purchased from Burdick & Jackson. All other organic chemicals were obtained from Aldrich Chemical Co., while all biochemicals were obtained from Sigma Chemical Co. All materials were used as received unless stated otherwise.

Instrumentation. Gas chromatography was performed by using an HP 5840A gas chromatograph modified for use with a J & W DB-5 or DB-1 capillary column. Difference spectra were recorded on an HP 8451A UV spectrophotometer. GC/MS analysis of the product alcohols was performed on a VG 7070H mass spectrometer in the selected ion recording mode, interfaced to a HP-5710A GC fitted with a J & W DB-5 fused silica capillary column. The derivatized metabolites were cold trapped at 40 °C, and then the temperature was ramped at 20 °C/min to 90 °C followed by isothermal elution. Mass spectral parameters were as follows: dwell, 5 ms; ionizing voltage, ca. 70 eV; source temperature, 200–205 °C. The deuterium incorporation in each substrate was determined by bleeding each compound into the source of the mass spectrometer at a steady rate through the reference inlet and monitoring the ion current of the various isotopically substituted species using selected ion recording of the molecular ion. The mass spectrometric parameters for the substrate were the same as those for the analysis of the product alcohols except the dwell time was increased to 50 ms. The measured intensity of each ion monitored was corrected for the natural isotopic abundance of ^{2}H, ^{13}C, ^{17}O, ^{18}O, ^{29}Si, and ^{30}Si.

Synthesis and Incorporation of Substrates. Octane-*1*-$^{2}H_3$. Methyl-octanoate (0.016 mol) in diethyl ether was added dropwise to lithium aluminum deuteride (0.016 mol) suspended in ether. The reaction was stirred at room temperature for 6 h. Then 0.5 mL of water, 1 mL of 15% NaOH, and finally another 1 mL of water were added. The solution was filtered and then extracted with pentane. The pentane–ether layer was dried over sodium sulfate and evaporated to yield 1-octanol-*1*-$^{2}H_2$ (2.05 g). The 1-octanol-*1*-$^{2}H_2$ (0.016 mol) was reacted with tosyl chloride (0.016 mol) in ca. 150 mL of dichloromethane containing 6 mL of triethylamine. The reaction solution was washed with water followed by saturated sodium bicarbonate. The organic phase was dried over anhydrous sodium sulfate and filtered and the solvent evaporated in vacuo. The residue was dissolved in ether and was reduced with lithium aluminum deuteride (0.014 mol) without further purification to yield octane-*1*-$^{2}H_3$ (4 mmol after chromatography and evaporation of the solvent). The octane-*1*-$^{2}H_3$ was purified via column chromatography using silica gel (60 Å, 230–240 mesh) with pentane as the eluant. The resulting octane-*1*-$^{2}H_3$ was greater than 99.8% pure by GC with a deuterium enrichment of 96.94% ± 0.01% octane-*1*-$^{2}H_3$, 2.24% octane-*1*-$^{2}H_2$, and 0.82% octane-*1*-$^{2}H_1$.

Octane-*1,2,3*-$^{2}H_7$. 2-Octanone (0.063 mol) was added to methanol-^{2}H (ca. 20 mL) in which sodium metal (0.002 mol) had been dissolved. The mixture was refluxed for 14 h, and the octanone was isolated via pentane–D_2O extraction and then analyzed for deuterium enrichment. This procedure was repeated until greater than 94% of the octanone was composed of octanone-*1,3*-$^{2}H_5$ (yield 0.034 mol). The ion corresponding to $C^{2}H_3C{=}O^{+}$ was used to determine incorporation of deuterium at the C-1 position, and the ion corresponding to $CH_3(CH_2)_4C^{2}H_2C{=}O^{+}$ was used to determine incorporation of deuterium at the C-3 position. The apparent deuterium enrichment at the C-1 position was 96%, while at the C-3 position it was found to be 95%. The ketone (0.034 mol) was then reduced to 2-octanol-*1,2,3*-$^{2}H_6$ (0.029 mol) with lithium aluminum deuteride (0.017 mol). The incorporation at the C-1 position was determined to be about 96.5% based on fragment ions from the Me_3Si derivative of the alcohol. 2-Octanol-*1,2,3*-$^{2}H_6$ was reacted with tosyl chloride and then reduced with lithium aluminum deuteride to yield octane-*1,2,3*-$^{2}H_7$ (0.8 mmol after purification) which was purified as octane-*1*^{2}-H_3. The final compound was greater than 99.8% pure and had deuterium enrichment of 88.7% octane-*1,2,3*-$^{2}H_7$, 8.8% octane-*1,2,3*-$^{2}H_6$, and 2.5% octane-*1,2,3*-$^{2}H_5$.

Octane-*1,8*-$^{2}H_2$. 1,8-Octanediol was reacted with tosyl chloride and the resulting ditosylate reduced with lithium aluminum deuteride in THF to yield octane-*1,8*-$^{2}H_2$. Reaction conditions and purification procedure were similar to those for octane-*1*-$^{2}H_3$. The final compound was greater than 99.8% pure and had a deuterium enrichment of 98.3% octane-*1,8*-$^{2}H_2$ and 1.7% octane-*1*-$^{2}H_1$.

Microsomal Preparation and Incubation Conditions. Microsomal reaction mixtures were prepared as described previously by Porter et al.[20] The incubations were run from 20 to 25 min and contained between 7 and 8 nmol of P-450, as determined by the method of Omura and Sato,[21] 12.3 μmol of NADPH, and 2.47 μmol of octane diluted to a volume of 2 mL with Trizma buffer (0.2 M, pH 8.2 at 25 °C). Each incubation was terminated by addition of 5 mL of pentane and stored at −70 °C until workup.

Purified P-450b Preparation and Incubation Conditions. The purification of the major phenobarbital inducible form of P-450 was accomplished by using the procedure of Waxman and Walsh.[22] NADPH-dependent cytochrome P-450 reductase was purified as described by Shepard et al.[23] The incubations were carried out in closed scintillation vials for 10 min. Each incubation contained 1 nmol of P-450, 1 nmol of P-450 reductase, 0.6 μmol of NADPH, and 0.5 nmol of phosphotidylcholine diluted to a final volume of 2 mL with 100 mM Trizma buffer, pH 8.2. Each reaction was initiated by addition of 2.4 μmol of the appropriate octane in 5 μL of methanol. Upon termination each incubation was extracted with (2×) 5 mL of pentane.

Derivitization of Octanols for GC/MS. The pentane extracts were dried over sodium sulfate and evaporated until ca. 30 μL remained. To the remaining volume, 25 μL of acetonitrile was added. Prior to analysis, pyridine (1 μL) and BSTFA (4 μL) were added to each sample.

Gas Chromatographic Analysis of *n*-Octane Hydroxylation Products. When protio octane was subjected to hydroxylation by either P-450b- or phenobarbital-induced microsomes, three metabolites were obtained which corresponded to authentic 1-octanol, 2-octanol, and 3-octanol by their gas chromatographic and mass spectral characteristics. The ratios of 1-octanol to 2-octanol to 3-octanol based on nine determinations were

(10) White, R. E.; McCarthy, M. B.; Egeberg, K. D.; Sligar, S. G. *Arch. Biochem. Biophys.* **1984**, *228*, 493.
(11) Frommer, U.; Ullrich, V.; Standinger, H.; Orrenius, S. *Biochem. Biophys. Acta* **1971**, *280*, 487.
(12) Albro, P. W.; Chae, K.; Philpot, R.; Corbett, J. T.; Schroeder, J.; Jordan, S. *Drug Metab. Disp.* **1984**, *12*, 742.
(13) Ichihara, K.; Kusonose, E.; Kusonose, M. *Biochem. Biophys. Acta* **1969**, *176*, 713.
(14) Bjorkhem, I. Proceedings of the Second International Conference on Stable Isotopes, National Information Service, US Department of Commerce, Springfield, VA, 1976; p 32.
(15) Nashlund, B. M. A.; Halpert, J. *J. Pharmacol. Exp. Ther.* **1985**, *231*, 16.
(16) Ortiz de Montellano, P. R.; Reich, N. O. *J. Biol. Chem.* **1985**, *259*, 4136.
(17) Jensen, I.; Mole, J.; Schenkman, J. *J. Biol. Chem.* **1985**, *260*, 7084.
(18) Bansal, S. K.; Love, J. H.; Gurtoo, H. L. *Eur. J. Biochem.* **1985**, *146*, 23.
(19) Shapiro, S.; Piper, J. U.; Caspi, E. *J. Am. Chem. Soc.* **1982**, *104*, 2301.
(20) Porter, W. R.; Branchflower, R. V.; Trager, W. F. *Biochem. Pharmacol.* **1977**, *26*, 549.
(21) Omura, T.; Sato, K. *J. Biol. Chem.* **1964**, *239*, 2370.
(22) Waxman, D. J.; Walsh, C. *J. Biol. Chem.* **1982**, *257*, 10446.
(23) Shephard, E. A.; Pike, S. F.; Rabin, B. R.; Phillips, I. R. *Anal. Biochem.* **1983**, *129*, 430.

Table I. Fraction of Deuterium Present in Substrate, $Sn/(Sn + Sn - 1)$, and the Fraction of Deuterium Remaining in the 1-Octanol Product, $dn/(dn + dn - 1)$

substrate	$Sn/(Sn + Sn - 1)$[a]	$Sn - 1/(Sn + Sn - 1)$	$dn/(dn + dn - 1)$	
			microsomes	P-450b
octane-*1*-$^2H_3$	0.9774 ± 0.0001 (3)[b]	0.0226 ± 0.0001 (3)	0.8849 ± 0.0077 (8)[c]	0.9025 ± 0.0009 (5)
octane-*1,2,3*-$^2H_7$	0.9097 ± 0.0055 (3)	0.0903 ± 0.0055 (3)	0.7408 ± 0.0058 (5)	0.7517 ± 0.0026 (5)
octane-*1,8*-$^2H_2$	0.9830 ± 0.0002 (3)	0.0170 ± 0.0002 (3)	0.9196 ± 0.0018 (5)	0.9224 ± 0.0080 (5)

[a] n equals the number of deuterium atoms. [b] The mean ± the standard deviation for ( ) separate determinations. [c] The mean ± the standard deviation for ( ) separate incubations.

1:5:0.6 for the microsomal preparations and 1:23:7 for the purified preparations. These ratios were measured via gas chromatography of the corresponding heptafluorobutyrate ester derivatives.

Calculation of Isotope Effects. In theory the isotope effect, k_H/k_D, should be directly related to the ratio of the ion intensities measured for metabolites arising from the hydroxylation at the protio site vs. that at the deuterio site. For example, for the substrate octane-*1*-$^2H_3$, k_H/k_D should be equal to the ratio of 1-octanol-*8*-$^2H_3$ to 1-octanol-*1*-$^2H_2$ which can be obtained by measuring the ratio of the ion intensities of a suitable ion for the two products. The M–CH_3 peak of the trimethylsilyl derivative would be a suitable ion for this measurement provided the substrate was 100% octane-*1*-$^2H_3$.

Under these circumstances the isotope effect can be calculated from eq 1 where d_3 is the ion intensity of the product 1-octanol-*8*-$^2H_3$ arising from hydroxylation of a terminal carbon hydrogen bond and d_2 is the ion

$$(k_H/k_D)_{obsd} = d_3/d_2 \quad (1)$$

intensity of the product, 1-octanol-*1*-$^2H_2$, arising from hydroxylation of a terminal carbon–deuterium bond.

In practice, absolute incorporation is essentially impossible to achieve and the incompletely labeled substrate must be taken into account. The ion intensity at d_2 will be contaminated with ion intensity from 1-octanol-*1*-$^2H_2$ and 1-octanol-*8*-$^2H_2$ arising from incompletely deuterated substrate, and this value must be subtracted from the measurement. The fraction of ion intensity due to this contaminant, F_2, is given by eq 2

$$F_2 = Sd_2/(Sd_3 + Sd_2)[d_2/(d_2 + d_1)] \quad (2)$$

where $Sd_2/(Sd_3 + Sd_2)$ is the fraction of octane-*1*-$^2H_2$ present in total substrate and $d_2/(d_2 + d_1)$ is the fraction of 1-octanol-*1*-$^2H_2$ plus 1-octanol-*8*-$^2H_2$ over the total 1-octanol formed by hydroxylation of the octane-*1*-$^2H_2$ contaminant. It is important to realize that this fraction, $d_2/(d_2 + d_1)$, is simply the fraction of deuterium in the 1-octanol derived from the octane-*1*-$^2H_2$ contaminant in the octane-*1*-$^2H_3$ substrate and reflects the isotope effect (k_H/k_D) associated with hydroxylation of this substrate times a statistical factor. This statistical factor accounts for the fact that in this substrate there are twice as many carbon–hydrogen bonds available for hydroxylation as there are equivalent carbon–deuterium bonds.

The amount of contamination that must be subtracted from the intensity d_2 is equal to d_2F_2 and eq 1 becomes eq 3.

$$(k_H/k_D)_{obsd} = d_3/(d_2 - d_2F_2) \quad (3)$$

The value of F_2 was obtained by determining the fraction of octane-*1*-$^2H_2$ contaminating the octane-*1*-$^2H_3$ substrate and multiplying it by $d_2/(d_2 + d_1)$ which was obtained directly from the ion intensities of 1-octanol-*1,8*-$^2H_2$ and 1-octanol-*1*-^2H when octane-*1,8*-$^2H_2$ was used as substrate. Octane-*1,8*-$^2H_2$ is an appropriate substrate for calculation of the correction factor since its hydroxylation would be accompanied by the same isotope effect and since it contains the same number of deuterium atoms as the octane-*1,1*-$^2H_2$ contaminant.[24]

An analogous equation to eq 3 can also be used to determine the isotope effect associated with terminal hydroxylation of octane-*1,2,3*-$^2H_7$ except the form of the correction factor F changes since the octane-$^2H_6$ arising from incomplete deuteration is actually composed of three distinct species in which the hydrogen is contained at either the C-1, C-2, or C-3 position (eq 4). The ratio $d_2/d_2 + d_1$ was again taken from octane-

$$F_6 = (Sd_6/Sd_6 + Sd_7)_1(d_2/d_2 + d_1) + (Sd_6/Sd_6 + Sd_7)_{2,3} \quad (4)$$

1,8-$^2H_2$ since it contains the statistically correct number of hydrogens vs. deuteriums at the two carbons (C-1 and C-8) being hydroxylated. The fraction of protium present at the C-2 and C-3 carbons in the substrate $(S_6/S_6 + S_7)_{2,3}$ was obtained by subtracting the value 0.05 for $(S_6/S_6 + S_7)_1$ from the fraction 0.09 representing the total amount of S_6, i.e., $[S_6/(S_6 + S_7)]_{1,2,3}$ present in the octane-*1,2,3*-$^2H_7$ substrate, Table I.

Since the absolute ion intensities of d_7, d_6, d_3, and d_2 vary from experiment to experiment depending upon mass spectral conditions, they are best represented as fractions, e.g., the value of $d_6/d_7 + d_6$ should be invariant from experiment to experiment. The values for the fraction of deuterium present in substrate and retained in the product are given in Table I. The isotope effects can be calculated directly from the values in Table I if the form of eq 3 is changed to eq 5. Eq 5 lends itself to the propagation of errors such that the reliability of the measurements can readily be assessed.

$$(k_H/k_D)_{obsd} = \frac{dn/(dn + dn - 1)}{(dn - 1/(dn + dn - 1))(1 - Fn)} \quad (5)$$

Theory

A kinetic model that describes the formation of multiple products from a single substrate and enzyme by branched reaction pathways is shown in Scheme I. In this scheme the products P_1 and P_2 are assumed to arise from kinetically indistinguishable enzyme–substrate complexes, the combination of which can be expressed by $[ES_H]$. An equation expressing the effect of branching on the isotope effect was derived from Scheme I, assuming steady-state kinetics, as follows. Equation 6 expresses the effect of the concentrations of ES_H and ES_D on the observed isotope effect. Equations 7 and 8 express the steady-state con-

$$(k_H/k_D)_{obsd} = (k_H/k_D)[ES_H]/[ES_D] \quad (6)$$

centrations of the two enzyme–substrate complexes. The concentrations of ES_H and ES_D obtained by rearrangement of eq 7 and 8 can be substituted into eq 6 and rearranged to yield eq 9.

$$d[ES_H]/dt = 0 = k_2[ES] - [ES_H](k_{-2} + k_3 + k_H) \quad (7)$$

$$d[ES_D]/dt = 0 = k_2[ES] - [ES_D](k_{-2} + k_3' + k_D) \quad (8)$$

$$(k_H/k_D)_{obsd} = \frac{k_H/k_D + k_H/(k_{-2} + k_3')}{(k_{-2} + k_3)/(k_{-2} + k_3') + k_H/(k_{-2} + k_3')} \quad (9)$$

Analysis of the limits of this model for the two substrates, octane-*1*-$^2H_3$ and octane-*1,2,3*-$^2H_7$, provides insight into how branching can effect $[ES_H]$ and $[ES_D]$ and thus the observed isotope effect.

Consider the substrate octane-*1*-$^2H_3$. If P_1 is taken to be the product 1-octanol and P_2 is taken to be the product 2-octanol (see Scheme I), the effect of branching to product P_2 and in turn its affect on the observed isotope effect for formation of product P_1 can be evaluated. For this substrate the rate of C-2 hydroxylation on the protio half of the molecule will be identical with the rate of C-2 hydroxylation on the deuterio half of the molecule. That is $k_3 = k_3'$, assuming negligible β secondary isotope effects. Thus, eq 9 can be rewritten as eq 10.

$$(k_H/k_D)_{obsd} = \frac{k_H/k_D + k_H/(k_{-2} + k_3)}{1 + k_H/(k_{-2} + k_3')} \quad (10)$$

(24) The correspondence between octane-*1,8*-$^2H_2$ and octane-*1,1*-$^2H_2$ assumes rapid rotation of a given methyl group in the active site and negligible secondary isotope effects. Contamination of d_1 by contributions from incompletely deuterated substrate, i.e., octane-*1*-$^2H_2$, was corrected for by measuring the fraction of octane-*1*-^2H present in the substrate (octane-*1,8*-$^2H_2$), multiplying it by d_1, and subtracting the product from d_1. Since this contaminant contains a single deuterium atom, the effect of isotope discrimination on the value of d_1 was ignored.

(25) This statement is true for $k_3' \gg$ or $\ll k_H$. However, if $k_3/k_3' < k_H/k_D$, it is theoretically conceivable that the two isotope effects for the two regioisomers could differ by less than 2-fold if $k_3' < k_H$. From the GC analysis of the protio substrate (see Experimental Section), it is known that $k_H/k_3 = 1/23$; thus, if $k_3/k_3' < k_H/k_D$, then k_3' must be greater than k_H. Therefore, given this system, k_3/k_3' must be at least 2-fold smaller then k_H/k_D if the boundaries are to be satisfied.

Table II. Observed Intramolecular Isotope Effects for the Hydroxylation of Selectively Deuterated Octanes

substrate	isotope effect	
	microsomes	P-450b
octane-*1*-$^2H_3$	7.85 ± 0.76[a]	9.45 ± 0.15
octane-*1,2,3*-$^2H_7$	3.12 ± 0.17	3.30 ± 0.10

[a] The mean ± standard deviation as calculated by propagation of error.

Evaluation of the limits of eq 10 leads to the conclusion that if $k_{-2} \gg k_H$, $(k_H/k_D)_{obsd}$ approaches the intrinsic isotope effect. However, if $k_{-2} \ll k_H$, the value of $(k_H/k_D)_{obsd}$ will depend upon the magnitude of k_3'. That is, as k_3' increases, $(k_H/k_D)_{obsd}$ will approach the intrinsic isotope effect. Thus, the overall effect of k_3 is to *unmask* the intrinsic isotope effect. In effect the branching pathway k_3' siphons-off "excess" $[ES_D]$ and tends to keep the ratio of the concentrations of the two enzyme–substrate complexes closer to unity.

Consider the substrate octane-*1,2,3*-$^2H_7$. For this substrate, in contrast to octane-*1*-$^2H_3$, the rate of C-2 hydroxylation on the protio half of the molecule will clearly be different than the rate of C-2 hydroxylation on the deuterio half of the molecule provided an isotope effect is associated with 2-octanol formation, i.e., $k_3 \neq k_3'$ in Scheme I. Hence, eq 9 is needed to evaluate the limits. If the ratio of the rate constants for C-1 hydroxylation relative to C-2 hydroxylation on the protio half of the molecule k_H/k_3 is assumed to be approximately equal to the ratio of rate constants for the analogous reactions on the deuterio half of the molecule, k_D/k_3', i.e., the isotope effect at the C-1 position is equal to the isotope effect at the C-2 position, then the following conclusions can be reached: (a) if $k_{-2} \gg k_H$ and k_3, $[ES_H]$ approaches $[ES_D]$ and $(k_H/k_D)_{obsd}$ approaches the intrinsic isotope effect; (b) if $k_{-2} \ll k_H$ and k_3, $(k_H/k_D)_{obsd}$ approaches 1. For the first limit, $(k_H/k_D)_{obsd}$ is totally independent of both k_3 and k_3' because both branching rate constants are too small relative to k_{-2} to have an effect. However, in the second limit, or if $k_{-2} \cong$ or $> k_H$, the isotope effect for C-2 hydroxylation, k_3/k_3', will *mask* the intrinsic isotope effect for C-1 hydroxylation. The masking of the isotope effect associated with C-2 hydroxylation is a result of an effective increase in concentration of $[ES_D]$ relative to $[ES_H]$.

Results and Discussion

The observed isotope effect for the C-1 hydroxylation of octane-*1*-$^2H_3$ is 7.9 for the microsomal system and 9.5 for the P-450b preparation (see Table II). By contrast the corresponding values for octane-*1,2,3*-$^2H_7$ are 3.1 and 3.3, respectively. The difference in $(k_H/k_D)_{obsd}$ for octane-*1*-$^2H_3$ in microsomes vs. P-450b undoubtedly is due to the fact that microsomes are a complex mixture of a number of different isozymes in which more than a single isozyme may be active in catalyzing the formation of 1-octanol and/or 2-octanol. Thus, the observed value would represent an average value of the various activities.

Of more interest to the present discussion is the large differnce in the observed isotope effects for the C-1 hydroxylation of octane-*1*-$^2H_3$ vs. octane-*1,2,3*-$^2H_7$. Based on the analysis of the limits of the kinetic model these results would be inconsistent with $k_{-2} \gg k_H$ and k_3, since under these conditions both substrates would give the same $(k_H/k_D)_{obsd}$, and this value would approach the intrinsic value. If $k_{-2} \ll k_H$ and k_3 and $k_3/k_3' = k_H/k_D$, then the isotope effect for octane-*1*-$^2H_3$ should be close to the intrinsic value and the isotope effect for octane-*1,2,3*-$^2H_7$ should approach 1. While the large value of 9.5 for octane-$^2H_3$ (from P-450b) is consistent with these boundaries for the rate constants, the value of 3.3 for the octane-*1,2,3*-$^2H_7$ is not. However, these boundaries might still be correct if the assumption that k_H/k_D is equal to k_3/k_3' is incorrect. If the latter were true the isotope effect at the C-2 position would have to be 2-fold less than the isotope effect at the C-1 position.[23] Although such a large difference in the isotope effects for the hydroxylation at a primary vs. a secondary carbon might seem unrealistic in a simple chemical system, such a difference is not necessarily unreasonable for an enzymatic system. Finally if $k_{-2} \cong k_H$ and k_3, the kinetic model readily accounts for the difference in observed isotope effects for the two substrates.

As predicted by eq 10, the effect of a branched pathway on octane-*1*-$^2H_3$ would be to unmask the intrinsic isotope effect. This prediction is supported by the large observed isotope effect of 9.5 (Table II). The isotope effect associated with this substrate can also be used to predict the limits for the intrinsic isotope effect. An expression for the intrinsic isotope effect can be obtained by rearrangement of eq 10 to eq 11. Evaluation of the limits of eq

$$k_H/k_D = (k_H/k_D)_{obsd} + [(k_H/k_D)_{obsd}k_H/(k_{-2} + k_3')] - k_H/(k_{-2} + k_3') \quad (11)$$

11 leads to the following conclusions. If k_{-2} approaches infinity the observed isotope effect equals the intrinsic isotope effect, i.e., $(k_H/k_D)_{obsd} = k_H/k_D$. Thus, the lower limit of the intrinsic isotope effect is 9.5. The upper limit is approached as k_{-2} approaches 0. To determine this limit, the value of k_H/k_3' must be known. An estimation of k_H/k_3' can be obtained as follows. For the substrate, octane-*1*-$^2H_3$, k_3' will be approximately equal to k_3 provided that any β secondary isotope effects are small. Thus, $k_H/k_3 \cong k_H/k_3'$ which in turn should equal the product ratio, 1-octanol/2-octanol, obtained from the nondeuterated substrate, i.e., octane. The product ratio was measured by gas chromatography and was found to be 1-octanol/2-octanol = 1/23. If this value is substituted in eq 11, the upper limit of the intrinsic isotope effect is found to be 9.8.

In the Experimental Section, it was reported that the incubation of octane with purified P-450b led to the formation of 3-octanol in addition to 1- and 2-octanol. Gas chromatography indicated that the ratio of 3-octanol to 1-octanol was 7:1. If deuteration of C-1 leads to an increase in the formation of 3-octanol, this process would also tend to deplete $[ES_D]$. If this is the case, k_H/k_3' would equal 1/30. However, substitution of this ratio into eq 11 does not significantly change the calculated value for the upper limit of the intrinsic isotope effect. Work pertaining to whether branching to multiple positions is directly additive is presently in progress.

The increase in the observed isotope effect for the P-450b preparation relative to the microsomal preparation, Table II, can be attributed to one or more of the following processes: (1) an increase in the rate of interchange between ES_H and ES_D, (2) an inherently larger intrinsic isotope effect associated with the purified enzyme preparation and no branching effects, or (3) a purified enzyme capable of branching; i.e., more than one product is formed by a *single enzyme*. If either of the first two processes were responsible for the difference, the isotope effects for both substrates should be increased by the same relative amount. The fact that this is not observed indicates that a single enzyme catalyzes the formation of the two products.

On first analysis the isotope effect associated with the P-450b hydroxylation of octane-*1*-$^2H_3$ would generally be taken to represent the "true" intramolecular isotope effect associated with the aliphatic hydroxylation of octane. Moreover, the large value that was obtained for this substate (9.5) would seem to imply that the rate of interchange between $[ES_H]$ and $[ES_D]$ in Scheme I is fast, and thus the observed isotope effect is close to the intrinsic isotope effect. However, this interpretation ignores any possible affects branching may have on the magnitude of the observed isotope effect. In fact, the observation of a large isotope effect, as has been shown, is due to the unmasking of the intrinsic isotope effect by the branched pathway (k_3') and *not* to rapid interchange of the two methyl groups (C-1 and C-8) at the active site. It is important to note however that in either case, i.e., fast rate of interchange or branching, if $[ES_H]$ equals $[ES_D]$ the full intrinsic isotope effect will be observed.

In conclusion, this analysis has important implications as it suggests that an estimate of the intrinsic isotope effect can be made for a given reaction which is one component of a branched pathway without necessarily resorting to the use of Northrop's treatment.[1] Moreover, the effects of isotopically sensitive branching must be considered and evaluated in those systems to which it applies if

serious misinterpretation of the kinetic factors that are influencing the observed isotope effect is to be avoided.

Acknowledgment. We are grateful to Dr. Robert Hanzlik for his helpful comments. This work was supported in part by the National Institute of Health (Grants G.M. 25136 and 36922).

Supplementary Material Available: Experimental and calculational details (1 page). Ordering information is given on any current masthead page.

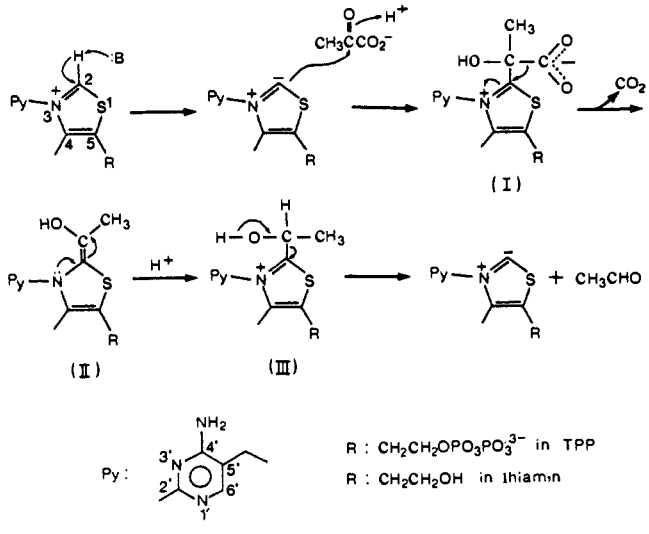
Crystal Structure of Thiamin Thiazolone: A Possible Transition-State Analogue with an Intramolecular N-H···O Hydrogen Bond in the V Form

Whanchul Shin* and Young Chang Kim[1]

Contribution from the Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151, Korea. *Received April 23, 1986*

Abstract: The pyrophosphate ester of thiamin thiazolone (TT) has been proposed to be a transition-state analogue for the thiamin pyrophosphate dependent enzymes due to its high affinity for the apoenzyme and its structural resemblance to the metastable enamine which is assumed to be the immediate product of decarboxylation of the pyruvate adduct of thiamin. TT, $C_{12}H_{16}N_4O_2S$, crystallizes in the monoclinic space group $P2_1/n$ with $a = 4.634$ (2) Å, $b = 12.591$ (6) Å, $c = 22.291$ (10) Å, $\beta = 95.20$ (4)°, and $Z = 4$. The structure was solved by direct methods and refined to an R value of 0.041 for 987 observed reflections measured with Cu Kα radiation on a diffractometer. The molecular conformation of TT is quite different from the S conformation which is characteristic of other C(2)-substituted thiamins. Instead, TT assumes a V conformation ($\phi_T = 104°$, $\phi_P = -74°$) that has previously been observed only in oxythiamin which is a strong antagonist of thiamin. An intramolecular hydrogen bond between the 4′-amino group and C(2) oxygen stabilizes the V form. This is the first crystal structure showing that the 4′-amino group, whose functional role in thiamin catalysis is not well established, is involved in an intramolecular interaction. The structure of TT suggests that the active conformation of free thiamin in the holoenzyme may be the V form.

Thiamin (vitamin B_1), in the form of the pyrophosphate ester TPP, is a coenzyme in a number of enzyme systems that catalyze decarboxylation of α-keto acids and the transfer of aldehyde or acyl groups.[2] Although as shown in the following scheme Breslow's mechanism[3] depicts the essential features of thiamin catalysis, many details of the enzymatic reactions remain to be



elucidated. One of the intriguing questions is the function of the 4′-amino group which is absolutely required for the enzymatic reactions. Schellenberger[4] proposed that the 4′-amino group actively participates in the catalytic reaction, acting as an acid and a base alternatively, and that thiamin and its C(2) adducts be in the V form[5] in which the 4′-amino group is close to the C(2) active center. Sable et al. also proposed the stable V form of free thiamin from the NMR studies on the model compounds.[6] However, none of the crystal structures of either free thiamin or C(2)-substituted thiamin have revealed the proposed V form. Instead, two other basic conformations have been observed.[7] Free thiamin assumes, with minor exceptions, the F conformation, and C(2)-substituted thiamin has the S conformation. In both conformations, the 4′-amino group is quite distant from the C(2) active center. Even free thiamin compounds that show deviations from the F form do not assume the V conformation but the S conformation. The V form occurs only in oxythiamin, a strong antagonist in which the 4′-amino group is substituted with an oxo group.[8] Undoubtedly the structural characteristics should cor-

(1) Present address: Department of Crystallography, University of Pittsburgh, Pittsburgh, PA 15260.
(2) (a) Krampitz, L. O. *Annu. Rev. Biochem.* **1969**, *38*, 213-240. (b) Gallo, A. A.; Mieyal, J. J.; Sable, H. Z. *Bioorg. Chem.* **1978**, *4*, 147-177.
(3) Breslow, R. J. *J. Am. Chem. Soc.* **1958**, *80*, 3719-3726.
(4) (a) Schellenberger, A. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 1024-1035. (b) Schellenberger, A. *Ann. N. Y. Acad. Sci.* **1982**, *378*, 51-62.
(5) The conformation of the thiamin molecule is best expressed in terms of the two torsion angles, ϕ_T and ϕ_P, about the bonds from the methylene bridge carbon to the thiazolium and pyrimidine rings, respectively. The torsion angles ϕ_T = C(5′)-C(3,5′)-N(3)-C(2) and ϕ_P = N(3)-C(3,5′)-C(5′)-C(4′). A V conformation is specified by $\phi_T \simeq \pm 90°$ and $\phi_P \simeq \mp 90°$. An F conformation is specified by $\phi_T \simeq 0°$ and $\phi_P \simeq \pm 90°$. An S conformation is specified by $\phi_T \simeq \pm 100°$ and $\phi_P \simeq \pm 150°$. For more details, see: footnote 13 in ref 28 and footnote 4 in ref 8a.
(6) Biaglow, J. E.; Mieyal, J. J.; Suchy, J.; Sable, H. Z. *J. Biol. Chem.* **1969**, *244*, 4054-4062.
(7) Shin, W.; Pletcher, J.; Blank, G.; Sax, M. *J. Am. Chem. Soc.* **1977**, *99*, 3491-3499.
(8) (a) Shin, W.; Pletcher, J.; Sax, M.; Blank, G. *J. Am. Chem. Soc.* **1979**, *101*, 2462-2469. (b) Shin, W.; Pletcher, J.; Sax, M. *Acta Crystallogr., Sect. B* **1981**, *B37*, 1719-1724.

0002-7863/86/1508-7078$01.50/0