Effect of Commitments to Catalysis on the Degree of Curvature in Proton Inventories of the Kinetic Parameters for Enzyme-Catalyzed Reactions: Application to Tryptophan Indole-Lyase

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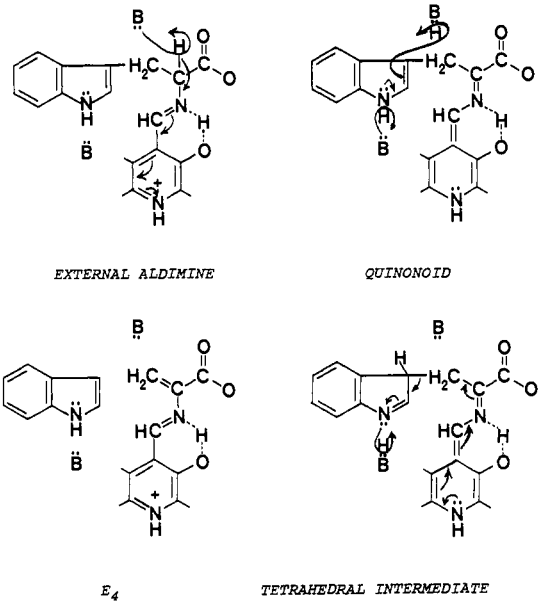
Abstract: A relatively simple method for obtaining ^{D}k, the intrinsic solvent deuterium isotope effect for an enzyme-catalyzed reaction, is presented and discussed. Steady-state and pre-steady-state determinations of the solvent deuterium and substrate deuterium isotope effects, the later obtained in both H_2O and D_2O, enable the development of a catalytic mechanism. The interrelatedness of the isotope effect equations for the enzyme system allows calculation of ^{D}k. As a result, the variation of $1/^{D}k_n$, the reciprocal of the partial intrinsic solvent deuterium isotope effect, with $n(D_2O)$, the atom fraction of deuterium in mixed isotopic waters, has been determined for the tryptophan indole-lyase enzyme-catalyzed reaction, and is compared to the variation of $(V/K)_n/(V/K)_0$ with $n(D_2O)$. Comparison of the proton inventories indicates that without knowledge of the commitments to catalysis underestimates of the number of protons calculated from a fit of the kinetic parameter data to the Gross–Butler equation can occur. A series of curves is presented which demonstrate that the degree of curvature in a bowl-shaped proton inventory decreases as the commitment to catalysis increases, and that ultimately, as the relative size of the commitment continues to increase, a dome-shaped proton inventory will result.

Introduction

Almost from its inception, the proton inventory technique[1] has been applied to enzyme systems that exhibit significant solvent deuterium isotope effects[2] in an attempt to determine the number of protons involved in the chemistry of the reaction. An approach to the analysis of enzymic solvent deuterium isotope effects which takes into account the kinetic complexity of enzyme catalysis is the concept of the virtual transition state developed by Schowen[3a] and Stein.[3b] The concept has been used extensively by Quinn[3c,d] and co-workers in their solvent deuterium isotope effect studies on cholesterol esterase catalyzed hydrolysis of *p*-nitrophenyl esters and acetylcholinesterase.[3e-h] Thus, the above researchers were the first to recognize that the degree of curvature in the proton inventories of the kinetic parameters for an enzyme-catalyzed reaction was not just the result of multiple protons being involved in the chemical transition state for the reaction.

For enzyme-catalyzed reactions, isotope effects are measured separately on the fundamental kinetic parameters, V and V/K (for deuterium, usually written as ^{D}V and $^{D}(V/K)$).[4] The kinetic parameters are then determined in mixed isotopic waters to obtain V_n and $(V/K)_n$. The experimentally determined values of the observed isotope effects, $^{D}V_n$ and $^{D}(V/K)_n$ as a function of $n(D_2O)$ are then considered in terms of the Gross–Butler equation.[5] However, the Gross–Butler equation was derived for simple chemical systems where a single step determines the overall rate of the reaction. Thus, when the equation is applied to enzyme systems, the interpretation of the results in terms of transition-state theory is subject to certain constraints.[1,6] The kinetic complexity of both V and V/K for the reaction[7] is one such constraint, and a measure of the kinetic complexity of any enzyme system is the commitments to catalysis.[8] As will be shown in this paper for

Chart I



(1) (a) Schowen, R. L. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, 1977; p 64. (b) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. G., Schowen, R. L., Eds.; Plenum: New York, 1978; p 225. (c) Schowen, K. B. J.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551. (d) Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1984**, *17*, 1. (e) Alverez, F. J.; Schowen, R. L. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsvier: New York, 1987; Vol. 7, p 1.

(2) (a) Thornton, E. K.; Thornton, E. R. In *Isotope Effects in Chemical Reactions*; Collins, C. J., Bowman, N. S., Eds.; Van Nostrand: New York, 1970; p 213. (b) Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; Wiley-Interscience: New York, 1980; p 202.

(3) (a) Schowen, R. L. In *Transition States of Biochemical Processes*; Gandour, R. G., Schowen, R. L., Eds.; Plenum: New York, 1978, p 77. (b) Stein, R. L. *J. Org. Chem.* **1981**, *46*, 3328. (c) Quinn, D. M. *Chem. Rev.* **1987**, *87*, 955. (d) Quinn, D. M. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, 1991; Chapter 3. (e) Sutton, L. D.; Quinn, D. M. *J. Am. Chem. Soc.* **1990**, *112*, 8404. (f) Acheson, S. A.; Barlow, P. N.; Lee, G. C.; Swanson, M. L.; Quinn, D. M. *J. Am. Chem. Soc.* **1987**, *109*, 246. (g) Barlow, P. N.; Acheson, S. A.; Swanson, M. L.; Quinn, D. M. *J. Am. Chem. Soc.* **1987**, *109*, 253. (h) Acheson, S. A.; Quinn, D. M. *Biochim. Biophys. Acta* **1990**, *1040*, 199.

(4) Northrop, D. B. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, 1977; p 122.

(5) (a) Kresge, A. J. *Pure Appl. Chem.* **1964**, *8*, 243. (b) Gold, V. *Adv. Phys. Org. Chem.* **1969**, *7*, 259.

(6) (a) Klinman, J. P. *Adv. Enzymol.* **1979**, *46*, 415. (b) Ray, B. J. *Biochemistry* **1983**, *22*, 4625.

(7) (a) Cleland, W. W. *Bioorg. Chem.* **1987**, *15*, 283. (b) Cleland, W. W. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsvier: New York, 1987; Vol. 7, p 61. (c) Cleland, W. W. In *Investigation of Rates and Mechanisms of Reactions*; Bernasconni, C., Ed.; Wiley: New York, 1987; Vol. 6, p 791.

0002-7863/91/1513-8499$02.50/0

the enzyme-catalyzed tryptophan indole-lyase reaction, without knowledge of the commitments to catalysis one may seriously underestimate the number of protons when either $^{D}V_n$ or $^{D}(V/K)_n$ data are fit to the Gross–Butler equation. However, with knowledge of the commitments to catalysis, as will also be shown in this paper, one can substantially reduce the kinetic complexity of the enzyme system, and obtain a more accurate accounting of the number of protons involved in the reaction.

The enzyme tryptophan indole-lyase (deaminating) catalyzes a pyridoxal phosphate dependent β-elimination reaction to form indole, pyruvate, and ammonia. A recent study of the catalytic mechanism for the enzyme suggests that there are two enzymic groups that directly participate in the chemistry of the reaction.[9a] As shown in Chart I, after the substrate undergoes transimination, an enzyme group exhibiting a pK of 7.6 abstracts the α-proton to form a quinonoid. A second enzymic moiety (pK 6.0) abstracts the ring nitrogen proton, which activates the carbon–carbon bond between C-3 and C-4. The α-proton is then transferred to the C-4 position of the substrate to form a tetrahedral intermediate (indolenine-like tautomer) which in turn collapses to form indole and an enzyme-bound aminoacrylate. It has been postulated that the enzyme undergoes a conformational change associated with C-3–C-4 bond cleavage.[9]

For tryptophan indole-lyase, the primary deuterium and solvent deuterium isotope effects have been determined in both the steady and pre steady states.[9] In the steady state, the substrate deuterium isotope effects for α-proton abstraction and the solvent deuterium isotope effects on V and V/K_{trp} are known, as well as the substrate deuterium isotope effects in D_2O. All of the above isotope effects are pH independent, and in conjunction with the pH dependence of the steady-state kinetic parameters, suggest tryptophan is not sticky; i.e., there is no significant external commitment factor.[10] Further, in D_2O, V/K_{trp} decreases *below* two pK values of 8.1 and 6.5 and the +0.5 pK_a shift for both enzymic groups indicates that neither is a sulfhydryl residue.[9a] While significant solvent deuterium isotope effects are observed with tryptophan as the substrate, there are no observable solvent deuterium isotope effects with *S*-methyl-L-cysteine as the substrate, suggesting interaction of the indole moiety of the substrate with the enzyme is mediated by water, and thus the cause of the observed isotope effect. Also, from pre-steady-state data, the intrinsic[11] deuterium isotope effect for α-proton abstraction has been determined to be 3.6 in either H_2O or D_2O.[9b]

The above isotope effect data for tryptophan indole-lyase, and the relatively large solvent deuterium isotope effects of 3.8 and 2.8 on V and V/K, respectively,[9a] make this an ideal enzyme system to conduct a proton inventory study.[1b] In this paper, data on the variation of the solvent deuterium isotope effect on both of the kinetic parameters, V_n and $(V/K_{trp})_n$, as a function of $n(D_2O)$ are reported. Also included are the equations which allow one to calculate, from existing data in the literature, the commitments to catalysis, and thus, the intrinsic solvent deuterium isotope effect, ^{D}k. From the variation of the solvent deuterium isotope effect on V/K_{trp} as a function of $n(D_2O)$, the partial intrinsic solvent deuterium isotope effect, $^{D}k_n$, is calculated. As a result, the proton inventory, $1/^{D}k_n$ vs $n(D_2O)$, is presented and discussed.

Nomenclature

The modified nomenclature of Northrop[4,12] will be used in which isotope effects on the kinetic or thermodynamic parameters are indicated by a leading superscript. Thus, the substrate deuterium isotope effects on the kinetic parameters, V and V/K, are indicated as ^{d}V and $^{d}(V/K)$, respectively. However, a leading upper case D superscript will be used to designate the solvent deuterium isotope effects, ^{D}V and $^{D}(V/K)$. Also, for the solvent deuterium isotope effects, a following subscript, n, will be used to designate the apparent isotope effect at any atom fraction of deuterium in the mixed isotopic waters; i.e., $^{D}V_n$ and $^{D}(V/K)_n$ are the solvent deuterium isotope effects on the maximum velocity and V/K, respectively, at n atom fraction of deuterium in mixed isotopic waters; e.g., $^{D}V_n = V_0/V_n$, where V_0 is the V determined in H_2O, and V_n is the apparent V determined in $n(D_2O)$. Thus, when n equals 1, $^{D}V_n = {}^{D}V$, and $^{D}(V/K)_n = {}^{D}(V/K)$.

For an isotope effect on a single rate constant in an enzymatic reaction, the following general equations will always be applicable when one varies the concentration of substrate[7] or atom fraction of deuterium in mixed isotopic waters:

$$^{D}(V/K)_n = \frac{^{D}k_n + C_f + C_r(^{D}K_{eq})_n}{1 + C_f + C_r} \quad (1)$$

$$^{D}V_n = \frac{^{D}k_n + R_f/E_f + C_r(^{D}K_{eq})_n}{1 + R_f/E_f + C_r} \quad (2)$$

In eqs 1 and 2, n is the atom fraction of deuterium in the mixed isotopic waters, $^{D}k_n$ is the partial intrinsic solvent deuterium isotope effect[11] at any n, and $^{D}(K_{eq})_n$ is the partial equilibrium solvent deuterium isotope effect,[13a] also at any n; C_f and C_r are the forward and reverse commitments, respectively; R_f and E_f are the catalytic ratio and equilibrium preceding catalysis, respectively.[8] The commitments, catalytic ratio, and equilibration preceding catalysis are mechanism dependent, and therefore, must be defined in context to the enzyme system being studied. For the trytophan indole-lyase system, C_f, C_r, and R_f/E_f will be defined in the later text (see eqs 5 and 6 and Discussion). Moreover, while $^{D}k_n$ and $^{D}(K_{eq})_n$ are dependent on $n(D_2O)$, C_f, C_r, and R_f/E_f are independent of $n(D_2O)$.[13b] Thus, when n is equal to 1, $^{D}k_n = {}^{D}k$ (see eq 8), and $^{D}(K_{eq})_n = {}^{D}K_{eq}$.[13a] The same equations apply for substrate isotope effects, since one simply changes the leading superscript from upper case D to lower case d. Thus, for ^{d}V and $^{d}(V/K)$, the substrate deuterium isotope effects are $^{d}K_{eq}$ and ^{d}k, which represent the equilibrium substrate deuterium isotope effect and the intrinsic substrate deuterium isotope effect, respectively, for the isotopically sensitive step.

Kinetic Mechanism and Equations

For tryptophan indole-lyase, a minimal kinetic mechanism can be written that is consistent with all the data so far acquired. The stepwise nature of the primary deuterium isotope effects for α-proton abstraction and the solvent deuterium isotope effects has been previously established since both ^{d}V and $^{d}(V/K_{trp})$ decrease in D_2O.[9a] Therefore, any mechanism for the enzyme must take these observations into account. Scheme I depicts the catalytic

Scheme I

$$E \underset{k_2}{\overset{k_1(A)}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \xrightarrow{k_7} E_5$$

(8) Northrop, D. B. *Biochemistry* **1981**, *20*, 4056.
(9) (a) Kiick, D. M.; Phillips, R. S. *Biochemistry* **1988**, *22*, 7339. (b) Phillips, R. S. *J. Am. Chem. Soc.* **1989**, *111*, 727.
(10) Commitments are the sum of internal and external components. The external component of the commitment is the partition ratio that includes the rate constant for reactant release, while the internal commitment consists of those partition ratios which do not include the rate constant for reactant release; for a more exhaustive definition, see refs 7, 8, and 12.
(11) An intrinsic isotope effect for an enzyme-catalyzed reaction is the isotope effect on the forward rate constant for the isotope-sensitive step.[4,7,8,12]
(12) (a) Cook, P. F.; Cleland, W. W. *Biochemistry* **1981**, *20*, 1790. (b) Cleland, W. W. *CRC Crit. Rev. Biochem.* **1982**, *13*, 385.

(13) (a) Equilibrium isotope effects are the result of isotopic substitution perturbing the equilibrium constant for the isotopically sensitive step.[4,8] A simplified quantative expression can be written as follows:

$$^{D}(K_{eq})_n = \frac{(1 - n + n/\sqrt[x]{^{D}k_r})^x}{(1 - n + n/\sqrt[x]{^{D}k_f})^x}$$

In the above expression, $^{D}k_f$ and $^{D}k_r$ are the isotope effect on the forward and reverse rate constants, respectively, for the solvent deuterium sensitive step in the mechanism, where x is the number of protons contributing equally to the isotope effect and n is the atom fraction of deuterium in the isotopic waters. Thus, when both x and $n = 1$, $^{D}(K_{eq})_n = {}^{D}K_{eq} = {}^{D}k_f/{}^{D}k_r$. (b) Changes in the viscosity of the solvent medium as a result of isotopic substitution are assumed to be insignificant. However, viscosity changes may not be trivial and research in this area is much needed.

pathway. E represents the internal aldimine on free enzyme, and k_1 represents binding of tryptophan (A), and transimination. It has been shown that transimination is relatively fast and kinetically indistinguishable from the binding of substrate.[9b] Therefore, the two steps are combined into one. The external aldimine, E_2, undergoes α-proton abstraction (substrate deuterium sensitive) to form the quinonoid, E_3. The solvent deuterium sensitive steps, k_5 and k_6, are for the postulated conformational change associated with formation of the tetrahedral intermediate prior to the elimination of indole to form the enzyme-bound aminoacrylate, E_4. Release of indole and breakdown of the aminoacrylate to pyruvate and ammonia are represented by k_7.

On the basis of the above mechanism, the equations for V/K_{trp} can easily be derived using the net rate constant method,[14] and are shown in eqs 3 and 4. Equation 3 is written in terms of k_3,

$$V/K = \frac{k_3k_1/k_2}{1 + k_3/k_2 + (k_4/k_5)(1 + k_6/k_7)} \quad (3)$$

$$V/K = \frac{k_5k_3k_1/k_4k_2}{1 + (k_5/k_4)(1 + k_3/k_2) + k_6/k_7} \quad (4)$$

which is the substrate deuterium sensitive step. However, V/K_{trp} may also be defined in terms of k_5, the solvent deuterium sensitive step (eq 4). Equations 3 and 4 are used to derive eqs 5 and 6 for the substrate and solvent deuterium isotope effects. However, binding of substrate and transimination are very fast;[9b] therefore, only the transition states that are isotope sensitive are considered to be significant; i.e., both k_2 and k_7 are infinite.[15] The equations for the isotope effects follow:

$$^d(V/K_{trp}) = \frac{^dk_3 + {}^dK_{eq3}(k_4/k_5)}{1 + k_4/k_5} \quad (5)$$

$$^D(V/K_{trp}) = \frac{^Dk_5 + k_5/k_4}{1 + k_5/k_4} \quad (6)$$

For eqs 5 and 6, the equilibrium deuterium isotope effect for substrate is given by $^dK_{eq3} = k_{3h}k_{4d}/k_{3d}k_{4h}$, and the intrinsic deuterium isotope effects for substrate and solvent are $^dk_3 = k_{3h}/k_{3d}$ and $^Dk_5 = k_{5H}/k_{5D}$, respectively. The commitments are readily defined from the above equations. For the substrate deuterium isotope effects (eq 5), the reverse commitment is the partition ratio, k_4/k_5. The forward commitment factor for the solvent deuterium isotope effects (eq 6) is k_5/k_4, which it should be noted, is the reciprocal of the reverse commitment for the substrate deuterium isotope effects. The above relationships will be used to calculate $^Dk_5$, the intrinsic solvent deuterium isotope effect for tryptophan indole-lyase, as described in the Theoretical Approach.

Theoretical Approach

In a recent pre-steady-state kinetic study on tryptophan indole-lyase carried out at pL 9.1, the intrinsic substrate deuterium isotope effect for α-proton abstraction was determined to be 3.6.[9b] The proposed catalytic mechanism for tryptophan indole-lyase requires formation of a pyridoxal phosphate stabilized α-carbanion intermediate which exhibits a very sharp and relatively intense absorption peak at 506 nm. As a result, formation of this intermediate can be monitored spectrophotometrically in the pre steady state. Thus, Phillips[9b] has shown there are three distinct processes occurring in the formation of the intermediate for the tryptophan indole-lyase reaction, and has assigned the first of these to be formation of the quinonoid intermediate, which is not only substrate sensitive, but also substrate deuterium sensitive. Also, it should be noted, the first relaxation is not solvent deuterium sensitive, and there is no change in the value of the substrate deuterium isotope effect when determined in D_2O. The two subsequent relaxations observed by Phillips[9b] in the pre steady state both exhibit a solvent deuterium isotope effect, and are both insensitive to the tryptophan concentration. The invariance of the substrate deuterium isotope effect as observed at 506 nm in the pre steady state to a change in solvent isotope is consistent with the isotope effects determined in the steady state,[9] and suggest the intrinsic isotope effect, $^dk_3$, for α-proton abstraction is 3.6.

Table I. Deuterium Isotope Effects and Commitments for the Tryptophan Indole-Lyase Reaction

parameter	value ± SD	ref	parameter	value ± SD	ref
$^d(V/K_{trp})$	2.8 ± 0.2	9a	k_5/k_4	2.3 ± 0.8	15a
^dV	2.5 ± 0.1	9a	$^D(V/K_{trp})$	2.8 ± 0.1	9a
$^dk_3$	3.6 ± 1.2	9b	^DV	3.8 ± 0.3	9a
k_4/k_5	0.44 ± 0.15	15a	$^Dk_5$	6.9 ± 2.3	

Knowing the value of the intrinsic substrate deuterium isotope effect, $^dk_3$, as well as the isotope effects on the kinetic parameters as described by eqs 5 and 6, ultimately allows one to calculate the intrinsic solvent deuterium isotope effect, $^Dk_5$. From eq 5, calculation of k_4/k_5 for the tryptophan indole-lyase reaction can be accomplished because $^d(V/K_{trp})$ has been determined.[9a,16] Thus, since the value of $^D(V/K_{trp})$ is known, the intrinsic solvent deuterium isotope effect, $^Dk_5$, can be calculated from eq 6. The results from the above calculations are listed in Table I.

The values of the observed solvent isotope effects, ^DV and $^D(V/K_{trp})$, are relatively large, and therefore, amenable to the proton inventory technique.[1b] Generally, the above isotope effects are determined as a function of $n(D_2O)$ and the data are fit to the Gross–Butler equation in an attempt to determine the number of protons involved in the reaction.[1,17] Shown in eq 7 is a simplified form of the Gross–Butler equation written for analysis of $^D(V/K)$, where x is the number of protons, each of which is contributing *equally* to the observed isotope effect,[18] and n is the atom fraction of deuterium in the mixed isotopic water. Thus, at $n = 1$, $^D(V/K)$ is the observed deuterium isotope effect, while $(V/K)_n$ is the V/K at any n, and $(V/K)_0$ is the V/K determined in H_2O. That being the case, $(V/K)_n/(V/K)_0$ will be equal to $1/^D(V/K)_n$, the reciprocal of the observed isotope effect on V/K when $x = 1$. By substituting V for V/K in eq 7, an expression similar to that shown for V/K can also be written for V_{max}.

$$(V/K)_n/(V/K)_0 = (1 - n + n/\sqrt[x]{^D(V/K)})^x \quad (7)$$

Observed kinetic isotope effects on V and V/K are described by eqs 1 and 2. Upon inspection of eqs 1 and 2, it is apparent that the values of the observed kinetic solvent deuterium isotope effects (^DV_n and $^D(V/K)_n$) are the result of a number of different factors. What is not so obvious is the fact that the commitments, catalytic ratio, and equilibration preceding catalysis are derived relative to H_2O, and are independent of, and therefore unaffected by, the amount of deuterium present in mixed isotopic water.[13b] It is the number of solvent protons, however, each contributing equally, that affects the size of the intrinsic solvent deuterium isotope effect, ^Dk. Therefore, only the partial intrinsic solvent deuterium isotope effect, ^Dk_n, will be directly affected by the amount of deuterium present in mixed isotopic water.[19] Therefore,

(14) Cleland, W. W. *Biochemistry* **1975**, *14*, 3220.

(15) (a) Anderson, V. E. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, 1991; Chapter 16. (b) Under initial velocity conditions, release of indole will essentially be irreversible.

(16) The calculations can only be performed if one assumes $^dK_{eq3}$ is not significantly different from unity. If $^dK_{eq3}$ were not unity, only small variations would occur.[15a] In similar systems, values for $^dK_{eq}$ usually range from 0.8 to 1.2.[7] However, values in this range do not significantly alter the calculated value of k_5/k_4; i.e., variation in k_4/k_5 can be 0.4–0.5.

(17) In order to use the simplified form of the Gross–Butler equation (eqs 7 and 8), the fractionation factors for the active site residues are assumed to be unity. As discussed by Klinman, this may not always be the case.[6a] However, for tryptophan indole-lyase, from the pL dependence of the solvent deuterium isotope effects, it does not appear that a sulfhydryl residue is involved in the chemistry for the reaction.[9a]

(18) (a) The assumption that each proton is contributing equally to the overall size of the isotope effect, and that the rule of the geometric mean is not violated, must be made in order to carry out the analysis.[18b-e] (b) Bigeleisen, J. *J. Chem. Phys.* **1955**, *23*, 2264. (c) Bigeleisen, J. *J. Chem. Phys.* **1958**, *28*, 694. (d) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15. (e) Ishida, T.; Bigeleisen, J. *J. Chem. Phys.* **1976**, *64*, 4775.

since the commitments are independent of the atom fraction of deuterium present, if values of the commitments are known, the partial intrinsic isotope effect as a function of atom fraction deuterium can be calculated. As described in the previous text, the commitments for the tryptophan indole-lyase reaction are known, and when the above calculations are performed, allow one to plot $1/{}^{D}k_n$ as a function of $n(D_2O)$. The transformed data are fitted to the following form of the Gross–Butler equation:

$$1/{}^{D}k_n = (1 - n + n/\sqrt[x]{{}^{D}k})^x \quad (8)$$

As will be shown, significant differences occur in the degree of curvature observed, and thus the number of protons calculated from a fit of the data to the Gross–Butler equation in this manner.

Experimental Section

Chemicals and Enzymes. The L-tryptophan and pyridoxal phosphate used in this study were obtained from Sigma. The DTT and NADH were purchased from Boehringer Mannheim. Both the D_2O and KOD were 99 atom % D and were purchased from Cambridge Isotopes. The Good's Buffers were from Research Organics, and all other reagents and chemicals obtained from commercial sources were of the highest quality available.

Tryptophan indole-lyase from *Escherichia coli* B/lt17-A was purified as previously described.[9,20] At pH 8, 25 °C, with L-tryptophan as the substrate, the enzyme specific activity was determined to be 7 U/mg. The rabbit muscle LDH used in the coupled assays was purchased as a lyophilized powder from Sigma.

Solutions. Mixtures of H_2O–D_2O were obtained by volumetrically combining solutions of the required reagents, substrates, and buffers previously prepared in either H_2O or D_2O (99 atom % D). Differences in the density between H_2O (d = 0.997 g/mL) and D_2O (d = 1.1044 g/mL) at 25 °C, as well as isotopic dilution of the L_2O solutions by protons released by the reagents, etc., were considered in calculating the final n values reported. The pL's of the reaction mixtures were measured with a Radiometer PHM 82 pH meter with a combined microelectrode before and after sufficient data were collected for determination of initial velocities. Negligible pL changes were observed before and after the reaction.[21]

Substrate Calibration. The concentration of L-tryptophan was determined and standardized from UV absorbance measurements at 278 nm using an extinction coefficient of 5.55 mM^{-1} cm^{-1}.[22] The concentration determinations were in agreement within 1%.

Initial Velocity Studies. Tryptophan indole-lyase was assayed spectrophotometrically on an OLIS modernized Cary 14 monochromator interfaced to a Compaq 386S computer using an OLIS 4300S Operating System for data acquisition and calculation of initial velocities. All assays were carried out at 25 °C, and reaction rates were measured by coupling the pyruvate produced to the oxidation of NADH in the presence of excess LDH.[9a] The temperature was maintained with a circulating water bath with the capacity to heat and cool both the cell holder and the cell compartment of the Cary 14. Reaction cuvettes were 1 cm in path length, and 1 mL in volume. The temperature of the assays was routinely monitored with a YSI telethermometer while the cuvette was still in the cell compartment. A typical assay contained 0.1 M Taps or Ches buffer,[23] pL 9.1, 0.2 M KCl, 1 mM DTT, 0.2 mM NADH, 100 units/mL LDH, and variable concentrations of L-tryptophan (ca. $0.1K_{trp}$ to $10K_{trp}$).

Velocity as a function of enzyme concentration was determined in both H_2O and D_2O and found to be linear, which demonstrates that the LDH coupling reaction does not contribute to the observed rate determinations. All assays reflected initial velocity conditions with less than 10% of the reactant used over the time course. Initial velocity data obtained in H_2O with enzyme dissolved in D_2O were found to be virtually identical to those obtained with enzyme dissolved in H_2O.

Data Analysis. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations, and all double reciprocal plots were linear. Data were analyzed using the FORTRAN program of Cleland written for the following equation:[24]

$$v = VA/(K_a + A) \quad (9)$$

In eq 9, A is the reactant concentration, K_a is the Michaelis constant, and V is the maximum velocity extrapolated to infinite substrate concentration. The kinetic parameters, or calculated values of the observed intrinsic solvent deuterium isotope effect determined as a function of $n(D_2O)$, were fitted to the Gross–Butler equation (eq 7 or 8) using R. G. Duggleby's nonlinear regression analysis in a BASIC87 program written by D. B. Northrop, School of Pharmacy, University of Wisconsin—Madison.

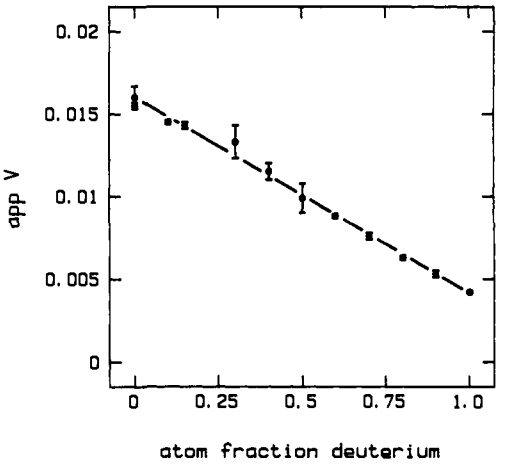


Figure 1. Dependence of the maximum velocity for the tryptophan indole-lyase reaction on atom fraction deuterium in mixed isotopic waters. The points represent values and standard errors for V_{max} calculated from a fit of initial velocity data (obtained as a function of tryptophan concentration; see Experimental Section) to eq 9. The calculated V_{max} values were fitted to eq 7, resulting in the curve shown.

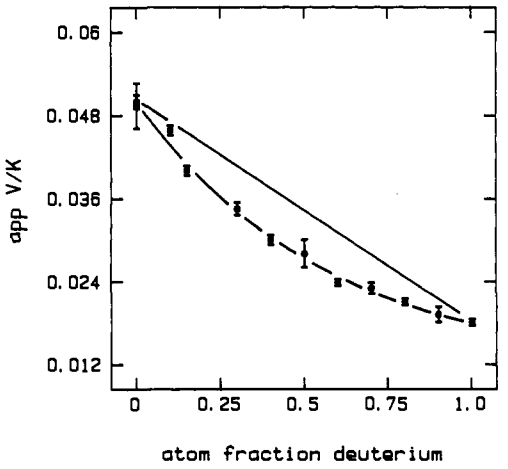


Figure 2. Dependence of the tryptophan V/K for the tryptophan indole-lyase reaction on atom fraction of deuterium in mixed isotopic waters. The points represent values and standard errors for V/K_{trp} calculated from a fit of initial velocity data (obtained as a function of tryptophan concentration; see Experimental Section) to eq 9. The calculated V/K_{trp} values were fitted to eq 7, resulting in the curve shown. For reference purposes, a straight line has been drawn between the data points at $n(D_2O)$ = 0 and 1.

Results

The dependence of V_{trp} on the atom fraction of deuterium in the reaction mixtures for tryptophan indole-lyase is shown in Figure 1. It is obvious the apparent V is linearly dependent on n, and from a fit of the data shown in Figure 1 to the simplified

(19) The ${}^{D}(K_{eq})_n$ will also be dependent on the atom fraction of deuterium in mixed isotopic water,[13a] and if C_i is significant, must also be taken into account. However, except for the SH group of cysteine, most amino acid side chains have ϕ's $\cong$ 1, which results in ${}^{D}K_{eq} \cong 1$ and simplifies the analysis in most cases.

(20) Phillips, R. S.; von Tersch, R. L.; Miles, E. W.; Ahmed, S. A. *Biochemistry* **1987**, *26*, 4163 (Abstract).

(21) The pL of the 99 atom % D_2O solutions was calculated by adding 0.41 to the pH meter reading, whereas linear interpolation (0–0.41) was used to calculate the pL from the pH meter reading of the assays in mixed isotopic waters.[1c]

(22) Morton, R. A. *Biochemical Spectroscopy*; Halsted: New York, 1975; Vol. 1, p 184.

(23) The same buffers were used in the D_2O solvent isotope effect studies but at a range 0.5 pL units higher than in H_2O. The higher range was used because of the equilibrium isotope effect on the acid dissociation constant of the buffers.[1a]

(24) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

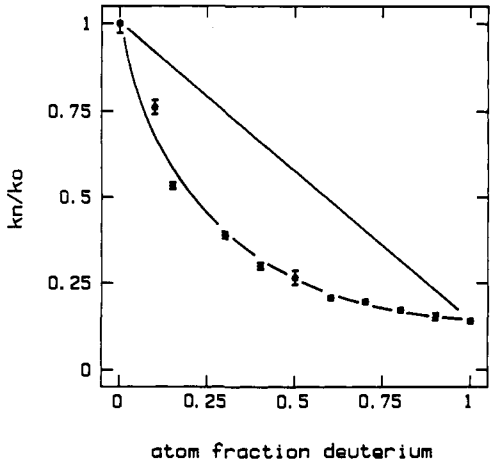


Figure 3. Proton inventory for the intrinsic solvent deuterium isotope effect, $1/^{D}k_n = k_n/k_0$, for tryptophan indole-lyase. The k_n/k_0 values were calculated from $(V/K)_n/(V/K)_0$ initial velocity data for tryptophan indole-lyase as described in the text (see Theoretical Approach). The calculated values were fitted to eq 8, resulting in the curve and standard errors for the points shown. For reference purposes, a straight line has been drawn between data points at $n(D_2O) = 0$ and 1.

form of the Gross–Butler equation (eq 7), the calculated number of protons is 1.03 ± 0.06.

The dependence of V/K_{trp} on the atom fraction of deuterium in the reaction mixtures for tryptophan indole-lyase is shown in Figure 2. In contrast to Figure 1, the curve shown in Figure 2 is clearly bowl-shaped. However, when the data are transformed into log form, and plotted vs $n(D_2O)$, a linear curve, as expected, does not result; the ln V/K_{trp} vs atom fraction of deuterium in the reaction mixtures is bowl-shaped (data not shown). The data in Figure 2 can also be used to calculate partial intrinsic solvent deuterium isotope effects (see Theoretical Approach). Figure 3 shows the results when the calculated values of $1/^{D}k_n = k_n/k_0$ are plotted vs $n(D_2O)$; note the degree of curvature is even more severe than observed in Figure 2. Again, when the data are transformed into log form, and ln $1/^{D}k_n$ is plotted vs $n(D_2O)$, a linear curve does not result (data not shown).

Discussion

As shown in eqs 1 and 2, while solvent deuterium isotope effects on both V and V/K for an enzyme-catalyzed reaction are direct results of changes in the rate of the isotope-sensitive step(s) upon isotopic substitution (the intrinsic solvent deuterium isotope effect), significant partition ratios, i.e., commitments, of the reaction pathway will also contribute to the overall size of the observed effects. The net effect of having finite and significant commitments in an enzyme system is that the observed isotope effect on the kinetic parameter, ^{D}V or $^{D}(V/K)$, will be much less than the size of the intrinsic solvent deuterium isotope effect; i.e., ^{D}V or $^{D}(V/K)$ will vary inversely with the size of the commitments, catalytic ratio, or equilibration preceding catalysis. Moreover, since C_f, C_r, and R_f/E_f are defined relative to H_2O, they are independent of the atom fraction of deuterium in mixed isotope waters, and it follows, therefore, that only changes in the intrinsic solvent deuterium isotope effect in mixed isotope waters will accurately reflect the number of protons in the system. Thus, one might ask the following question: What effect will finite commitments have on the shape of and therefore the number of protons calculated from a proton inventory performed on either of the kinetic parameters for an enzyme-catalyzed reaction?

From data for the tryptophan indole-lyase enzyme system, a minimal mechanism can be written which defines the partition ratios, i.e., commitments, for the system (see Kinetic Mechanism). For the system developed, the values of the commitments are used to calculate the partial intrinsic solvent deuterium isotope effect as a function of $n(D_2O)$, resulting in the proton inventory displayed in Figure 3. A large degree of curvature is observed in the proton inventory for $1/^{D}k_n = k_n/k_0$, which suggests that multiple protons

Table II. Effect of Commitments on the Variation of $1/^{D}(V/K)_n$ with $n(D_2O)$ for an Enzyme-Catalyzed Reaction

commitment	$^{D}(V/K)$	$1/^{D}(V/K)$	$^{D}(V/K)_{0.5}$	$1/^{D}(V/K)_{0.5}$
0	10[a]	0.1	2.5	0.39
0.1[b]	9.2	0.11	2.4	0.42
0.2[b]	8.5	0.12	2.3	0.44
0.5	7.0	0.14	2.0	0.49
1.0	5.5	0.18	1.8	0.56
2.0	4.0	0.25	1.5	0.66
3.0	3.3	0.31	1.4	0.72
5.0	2.5	0.4	1.3	0.8
10	1.8	0.55	1.1	0.88

[a] The isotope effect is assumed to be due to three protons. Therefore, when the commitment is 0, $^{D}k = 2.154^3 = {}^{D}(V/K)$. [b] Associated curves for the calculated values of $1/^{D}(V/K)$ and $1/^{D}(V/K)_{0.5}$ are not shown in Figure 4.

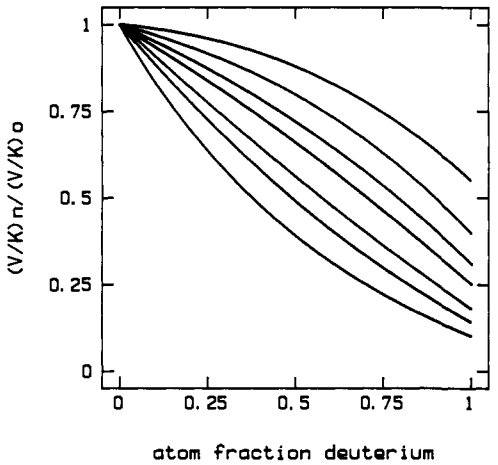


Figure 4. Apparent proton inventory curves of the relative $(V/K)_n/(V/K)_0$ for an enzyme-catalyzed reaction where the intrinsic solvent deuterium isotope effect due to three protons is 10. For the lower most curve, $^{D}(V/K)_n = {}^{D}k_n$, while each subsequent curve is a reflection of an increasing commitment from 0.5, 1, 2, 3, 5, and 10. The curves were drawn from the values listed in Table II which were calculated as described in the text (see Discussion).

contribute to the isotope effect. The difference in the degree of curvature after correction for the commitments in the system, as shown in Figures 2 and 3, suggests that as the value of the commitments increase the degree of curvature, and thus the number of protons calculated, decreases.

In order to further understand the above result, a series of proton inventory curves, a plot of $1/^{D}(V/K)_n$ vs $n(D_2O)$, were constructed for a hypothetical enzyme system with an intrinsic solvent deuterium isotope effect of 10 ($1/^{D}k = 0.1$). The overall size of the intrinsic solvent deuterium isotope effect is assumed to be due to three protons (each individual isotope effect equal to 2.154). Changes in $1/^{D}k_n$ as a function of $n(D_2O)$ were calculated using eq 8.[1b] The calculated value(s)[25a] of the partial intrinsic solvent deuterium isotope effect as a function $n(D_2O)$ was then used to calculate the change in the isotope effect on the kinetic parameter, $^{D}(V/K)_n$, as a function of the size of the commitment. The commitment effects were calculated using a simplified form of eq 1:[26]

$$^{D}(V/K)_n = \frac{^{D}k_n + C}{1 + C} \qquad (10)$$

For eq 10, C represents the commitment to catalysis. Variation in the isotope effect on the kinetic parameter due to an increase in the size of the commitment can thus be calculated. The results

(25) (a) To determine the relative degree of curvature, it is only necessary to calculate $1/^{D}k_n$ at $n = 0.5$; i.e., $1/^{D}k_{.5} = 0.393$.[1b,25b,c] (b) Albery, W. J.; Davies, M. H. *J. Chem. Soc., Faraday Trans. 1* **1972**, *68*, 167. (c) Albery, W. J.; Gelles, J. S. *J. Chem. Soc., Faraday Trans. 1* **1982**, *78*, 1569.

(26) By using eq 14, C_r is assumed to be zero, and therefore $^{D}(K_{eq})_n$ is not a factor in the analysis.

of the above calculations are listed in Table II.

When the resulting $1/^{D}(V/K)_n$ values are plotted as a function of $n(D_2O)$, a series of curves result as shown in Figure 4. It is apparent that as the size of the commitment increases the degree of curvature decreases. Clearly, without knowledge of the commitments, an underestimate of the number of protons in the system will occur if $1/^{D}V_n$ and/or $1/^{D}(V/K)_n$ are fit directly to the Gross–Butler equation. Moreover, as Figure 4 shows, and as previously described by Alvarez and Schowen,[1e] the proton inventory curve will become dome-shaped as the relative size of the commitment(s) becomes larger. Since there are a number of mechanistic models that can accommodate a dome-shaped proton inventory,[1,3] it becomes exceedingly important to know the value(s) of the commitment(s) in the system before attempts are made to model an enzyme-catalyzed reaction.

The shapes of the curves shown in Figure 1 and 3 are obviously different. On the one hand, the V_n/V_0 proton inventory is linear. However, the proton inventory for ^{D}k is curved, which suggests multiple proton catalysis. A number of different factors could be contributing to the above result. V/K includes all steps from binding of substrate to the enzyme through, and including, the first irreversable step in the mechanism (indole release). However, V is a reflection of breakdown of the already formed Michaelis complex to regenerate free enzyme. Consider the mechanism shown in Scheme I, $R_f/E_f = (k_5/k_3)/(1 + k_4/k_3) = 2.13/1.93$; a value of $R_f/E_f = 1.1$ will not account for the degree of curvature shown in Figure 3 because correction of the $^{D}V_n$ data yields a $1/^{D}k_n$ proton inventory with a calculated number of protons of 3.2 ± 0.9. An inherent property of the mechanism shown in Scheme I is that either proton inventory for the intrinsic solvent deuterium isotope effect, whether derived from the $^{D}V_n$ or $^{D}(V/K)_n$ data, should exhibit the same degree of curvature (in both cases, $1/^{D}(k_5)_n$ as a function of $n(D_2O)$ is plotted). That the curves are different suggests the system must be more complex than the one proposed in Scheme I, and thus, there must be two solvent deuterium sensitive steps along the reaction pathway for tryptophan indole-lyase. This aspect of the catalytic mechanism could not have been differentiated without the proton inventory for the intrinsic solvent deuterium isotope effect for the enzyme. Recently, Rebholz and Northrop[27] have used proton inventory data to distinguish that more than one step in the catalytic mechanism for porcine pepsin is solvent deuterium sensitive. Changes in the degree of curvature of the proton inventory as a result of altered substrate structure have been observed for porcine pepsin, and the researchers attribute the differences to a change in the rate-limiting step for the enzyme. Thus, for porcine pepsin, while peptide bond cleavage is solvent deuterium sensitive, there is also a solvent deuterium sensitive reprotonation of the enzyme after hydrolysis and the release of the first product.[27]

For tryptophan indole-lyase with tryptophan as the substrate, pre-steady-state data indicate binding and transimination of the substrate are relatively fast, and thus essentially at equilibrium in the steady state. Moreover, in the steady state, the decrease in the substrate deuterium isotope effects on V and V/K_{trp} when measured in D_2O indicates α-proton abstraction is not solvent deuterium sensitive. Consistent with the above is the fact that there is no observable solvent deuterium isotope effect with *S*-methyl-L-cysteine, which also indicates that breakdown of the aminoacrylate (structurally the same for both substrates) is not solvent deuterium sensitive. Thus, the solvent deuterium sensitive step(s) in the mechanism appears to be formation of the tetrahedral intermediate and the associated postulated conformational change, or the elimination of indole. The extreme curvature observed in the V/K proton inventories, which indicate that multiple protons contribute to the isotope effect, is consistent with the postulated conformational change associated with formation of the tetrahedral intermediate. Thus, the isotope effect observed on V/K can be attributed to a slower enzyme conformational change upon deuteration of the solvent.

The solvent deuterium isotope effect observed on V_{max} may be integrated into the mechanism for tryptophan indole-lyase by expanding k_7 (see Scheme I) as shown in Scheme II. k_7 now

Scheme II

$$E_4 \xrightarrow{k_7} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \xrightarrow{k_{11}} E$$

represents release of indole, while E_5 is the enzyme-bound aminoacrylate, which undergoes hydrolysis (k_9 and k_{10}) to form E_6. The rate constant k_{11} is conversion of the enzyme to a form that is capable of sustaining another round of catalysis. Since indole release is irreversible, the equations for V/K remain unchanged.

For tryptophan indole-lyase, since no solvent deuterium isotope effect is observed on V or V/K with *S*-methyl-L-cysteine as the substrate, hydrolysis of the aminoacrylate cannot account for the observed ^{D}V with tryptophan as the substrate. However, it is possible that, after the release of products, solvent reorganization in the indole binding domain of the enzyme is necessary for another round of catalysis to occur on the enzyme. The pH dependence of the kinetic parameters for tryptophan indole-lyase is known, and the maximum velocity for both tryptophan and *S*-methyl-L-cysteine has been shown to be pH independent. The aforementioned result indicates that substrates bind only to the correctly protonated form of the enzyme. Since V/K reflects catalytic activity at very low concentrations of substrate, the reorganization of water in the enzyme active site will be at equilibrium, and thus no kinetic isotope effect will be expressed on V/K_{trp}. However, the solvent deuterium isotope effect observed on V may be the result of deuteration of the solvent slowing down the reorganization of water in the active site of the enzyme, which limits the overall reaction in D_2O. This is quite conceivable given the chemistry of the reaction and the hydrophobicity of indole.

So that the enzyme can accommodate the indole moiety of tryptophan, the indole binding pocket ideally should be void of charge, and thus probably contains aromatic or other hydrophobic amino acid residues. However, because of the chemistry of the reaction, the enzymic group with a pK of 6.5 abstracts the proton of the ring nitrogen of the substrate. Distribution of the charge on the enzyme group may be accomplished by having a number of water molecules present. Thus, it is only correctly protonated enzyme with the proper orientation of water in the active site that can bind substrate and facilitate catalysis. It is also possible that abstraction by the enzyme of the ring nitrogen proton of tryptophan, which facilitates formation of the tetrahedral intermediate, is mediated by water, i.e., a solvation catalytic proton bridge.[1] Unfortunately, until the magnitude of R_f/E_f and/or C_r is/are known, the number of water molecules forming the putative bridge remains unknown. However, kinetic studies of the reverse reaction for tryptophan indole-lyase should provide data which can be used to determine R_f/E_f and/or C_r, and thus, calculation of both the intrinsic and partial intrinsic solvent deuterium isotope effects can be accomplished. The number of protons involved in the solvation proton catalytic bridge can then be determined from the proton inventory for the intrinsic solvent deuterium isotope effect.

Acknowledgment. I thank Professors V. E. Anderson and D. B. Northrop for many insightful and helpful discussions during the course of the study, and also Karen L. Rebholz for assistance in curve fitting the proton inventory data and suggesting the idea of doing simulations as a means of illustrating the commitment effect. This research was supported by a National Institutes of Health grant (GM43307), the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the University Physicians Fund, University of Tennessee, Memphis.

(27) Rebholz, K. L.; Northrop, D. B. *Biochem. Biophys. Res. Commun.* **1991**, *in press*.