Product Dependence of Deuterium Isotope Effects in Enzyme-Catalyzed Reactions†

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ABSTRACT: Theory for enzyme-catalyzed reactions is developed for the dependence on product concentration of deuterium isotope effects on V and V/K. Generally, a product that decreases the off-rate for a second product to zero causes the isotope effect on V/K to decrease to ${}^{D}K_{eq}$ and that on V to decrease to a value between 1 and ${}^{D}K_{eq}$. If the second product off-rate is decreased to a finite value, ${}^{D}V$ and ${}^{D}(V/K)$ will decrease to a value greater than ${}^{D}K_{eq}$, while if there is no effect on the off-rate for the second product, ${}^{D}V$ and ${}^{D}(V/K)$ will not change. Interestingly, for a ping-pong mechanism, the presence of the product that provides a reversible connection between the isotope-sensitive step and the isotope-insensitive half-reaction will give an isotope effect on V/K for the latter. (In the absence of the product, the isotope effect on V/K for the isotope-insensitive half-reaction will be unity.) Theory is supported with data for alcohol and lactate dehydrogenases. For lactate dehydrogenase, ${}^{D}(V/K_{pyruvate})$ decreases from 1.93 ± 0.02 at zero to 1.16 ± 0.02 at infinite lactate concentration, while ${}^{D}V$ decreases from a value of 1.75 ± 0.03 at zero to a value of 0.93 ± 0.05 at infinite lactate concentration. Thus, it appears that the pathway in which lactate is released first is greatly preferred, but the pathway in which NAD^+ is released before lactate is observed at high lactate concentration. For alcohol dehydrogenase, ${}^{D}(V/K_{acetaldehyde})$ decreases from a value of 2.8 ± 0.1 at zero to 1.8 ± 0.1 at infinite concentration of ethanol, while ${}^{D}V$ remains unchanged and equal to unity. The value of unity observed for ${}^{D}V$ is suggestive of a Theorell–Chance mechanism, but the finite value of ${}^{D}(V/K)$ obtained at high ethanol concentrations indicates the presence of the pathway in which NAD^+ is released prior to ethanol. Data for alcohol dehydrogenase are consistent with antagonism of binding between NAD^+ and ethanol, that is ethanol and NAD^+ increase the off-rates for NAD^+ and ethanol from the E:NAD:ethanol ternary complex.

Through the use of isotope effects, information can be obtained at virtually all levels of enzyme mechanism, including the kinetic (Cook & Cleland, 1981a), regulatory (Ahn & Klinman, 1983; Cook, 1982; Parkin & Schramm, 1984a,b; Parmentier et al., 1992), and chemical (Hermes et al., 1982; Belasco et al., 1983; Weiss et al., 1986, 1991; Gavva et al., 1991) mechanisms. Information can be obtained on the order of addition of reactants making use of the dependence of isotope effects on the concentration of reactants and the differential expression of deuterium isotope effects on the kinetic parameters V and V/K (Cook & Cleland, 1981a). Isotope effect data measured as a function of substrate concentration(s) yield both qualitative and quantitative information on the relative rates of release of reactants from the Michaelis complex. Information is seldom obtained, however, concerning the binary (EA and EB) complexes. A review of the above can be found in Cook (1991a).

The present paper outlines theory for enzyme-catalyzed reactions on the dependence of ${}^{D}V$ and ${}^{D}(V/K)$ on the concentration of added products of the enzyme-catalyzed reaction. Experiments of this type complement those carried out for the substrate dependence of isotope effects and further allow one to obtain information on the rates of dissociation of binary enzyme–product complexes. Theory is applied to experimental data obtained for alcohol and lactate dehydrogenases.

MATERIALS AND METHODS

Chemicals and Enzymes. Pyruvic acid was from Fisher Scientific, while acetaldehyde was from Aldrich. Acetaldehyde was freshly distilled before use. Ethanol was from Midwest Solvent Company. Rabbit skeletal muscle lactate dehydrogenase and NADH were from Boehringer Mannheim, while L-(+)-lactic acid and yeast alcohol dehydrogenase were from Sigma. The A-side NADD was prepared and purified according to Viola et al. (1979).

Oxalate Inhibition. Oxalate was used as an analog of lactate in the direction of lactate oxidation by lactate dehydrogenase. The enzyme was assayed in this direction by monitoring the appearance of absorbance at 340 nm. Assays were carried out at pH 7.1, 150 mM Tris-HCl with lactate fixed at 75 mM and NAD^+ varied from 0.125 to 1.25 mM at different oxalate levels from 0 to 3 mM; reaction was initiated by the addition of 0.3 units of enzyme. A unit is defined as the amount of enzyme required to reduce 1 μmol of pyruvate in 1 min at 25 °C and pH 7. The high reactant concentrations and the presence of Tris made unnecessary the addition of equilibrium perturbants such as hydrazine.

Deuterium Isotope Effects. The reduction of acetaldehyde by yeast alcohol and pyruvate by rabbit muscle lactate dehydrogenases was assayed by monitoring the disappearance of NADH at 340 nm. Isotope effects were measured by direct comparison of initial velocities. The reduced dinucleotide (labeled or unlabeled) was maintained saturating, and the

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reactant (acetaldehyde for YADH[1] or pyruvate for LDH) was varied at different concentrations of the product (ethanol for YADH and lactate for LDH). All assays were carried out at pH 7, 100 mM Mops, and 25 °C. The concentrations of the NADH and NADD solutions were determined enzymatically using 1 unit of lactate dehydrogenase at pH 7, 100 mM Mops, with 5 mM pyruvate.

Data Processing. Reciprocal initial velocities were plotted vs reciprocal substrate concentrations and all plots were linear. Data were fitted using the appropriate rate equation and Fortran programs developed by Cleland (1979). The K_i for product inhibition was obtained by fitting eq 1 to the product inhibition data.

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (1)$$

Initial velocities obtained by varying deuterium-labeled or unlabeled substrate concentration at a saturating nucleotide concentration were fitted using eqs 2 and 3.

$$v = VA/[K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (2)$$

$$v = VA/[(K_a + A)(1 + F_iE_v)] \quad (3)$$

The dependence of the deuterium isotope effects on product concentration was fitted using eq 4.

$$Y = A(1 + X/K_{in})/(1 + X/K_{id}) \quad (4)$$

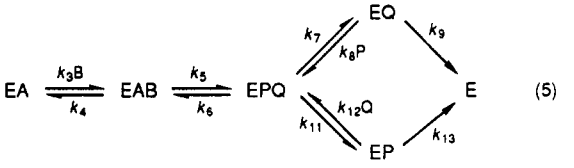
In eqs 1–3, A and I represent the concentration of reactant and inhibitor, respectively; K_a is the Michaelis constant for A, and K_{is} and K_{ii} are slope and intercept inhibition constants. In eqs 2 and 3, F_i is the fraction of deuterium label in substrate; while E_V, $E_{V/K}$, and E_v are the isotope effects minus 1 for V, V/K, and both, respectively. In eq 4, Y is the observed value of the isotope effect at any concentration of added product, A is the value of the isotope effect in the absence of product, K_{id} is the concentration of product that gives half of the change in the isotope effect, K_{in} is a ratio of rate constants that causes the value of the isotope effect to level off at a finite value, and AK_{id}/K_{in} is the value of the isotope effect at infinite product concentration. In all cases, the best fit of the data was chosen on the basis of the lowest values of the standard errors of the fitted parameters and the lowest value of σ. σ is defined as the square root of the sum of the squares of the residuals divided by the degrees of freedom, where degrees of freedom is equal to the number of points minus the number of parameters (Cleland, 1979).

THEORY

Bireactant Mechanisms. Theory has been developed for the dependence of isotope effects on the kinetic parameters V and V/K for each reactant in a bireactant enzyme-catalyzed reaction (Cook & Cleland, 1981a; Cook, 1991b). Basically, the isotope effect on V/K for the first reactant in an ordered mechanism is 1 since this parameter is effectively the on-rate for reactant and thus in most cases is insensitive to isotopic substitution at the position of bond cleavage. In a random mechanism, however, isotope effects are likely to be expressed on the V/K for both reactants because of the capability of switching from one pathway to another. The substrate dependence of isotope effects in no instance generates information on the formation or decomposition of the binary enzyme–reactant complexes, and, in addition, limited information may be obtained on the decomposition of the enzyme–product complex(es) from a comparison of the isotope effects on V and V/K.

One must bear in mind that in order to use the theory discussed above or that discussed below, an isotope effect must be observed. Observation of an isotope effect is not always possible in enzyme-catalyzed reactions because of kinetic masking of the isotope-sensitive step. That is, the isotope-sensitive step may not be slow or, if it is, may not completely limit the reaction, so that the full intrinsic isotope effect is not observed. The intrinsic isotope effect is the isotope effect on the isotope-sensitive step if it is the only slow step along the reaction pathway. [For a more comprehensive treatment, the reader is referred to the recent review by Northrop (1991).] Under conditions where either a small isotope effect or a value of 1 is measured, the magnitude of the isotope effect can be enhanced by the use of slow substrates or changing reaction conditions such as pH (Cook, 1991c).

In the presence of added product, isotope effects in enzyme-catalyzed reactions may either decrease with increasing product concentration or remain constant, and this will depend on the kinetic mechanism of the enzyme studied. Below, theory is developed for isotope effects in bireactant enzyme-catalyzed reactions in the presence of added product. Consider mechanism 5:



(5)

where A, B, P, and Q are reactants and products, k_3 and k_4 reflect formation and decomposition of the Michaelis complex, k_5 and k_6 represent the bond-breaking step and will be isotope dependent, and k_7/k_8 and k_{11}/k_{12} represent dissociation constants for P and Q from EPQ while k_9 and k_{13} represent the dissociation rate constants for EP and EQ, respectively. Conditions are such that either of the reactants (A or B) in a random mechanism or the first reactant to add in an ordered mechanism is maintained at a saturating concentration (A in mechanism 5) and the second reactant is varied in the absence and presence of several different concentrations of product including zero. As is the case for obtaining the substrate dependence of isotope effects (Cook & Cleland, 1981a), the label can be present in either A or B. Thus, isotope effects are obtained on the kinetic parameters V and V/K as a function of product concentration. The overall equations for ^DV and $^D(V/K)$ will adhere to the following general expressions used previously (Northrop, 1975; Cook & Cleland, 1981a).

$$^DV = [^Dk_5 + c_{Vf} + c_r(^DK_{eq})]/[1 + c_{Vf} + c_r] \quad (6)$$

$$^D(V/K) = [^Dk_5 + c_f + c_r(^DK_{eq})]/[1 + c_f + c_r] \quad (7)$$

Because of the presence of k_6, a reverse commitment to catalysis c_r is obtained in addition to a c_f [$^D(V/K)$] and $c_{Vf}(^DV)$.[2] The term $^DK_{eq}$ reflects the isotope effect on the equilibrium constant for the reaction (Cleland, 1980). Mechanistic differences will be reflected in the isotope effects on both V and V/K, and thus both types of effects will be considered for each of the mechanisms discussed.

Ordered Mechanisms (k_{11}, k_{12}, and k_{13} Are Not Present in Mechanism 5). Expressions for V and V/K_b are dependent

[1] Abbreviations: YADH, yeast alcohol dehydrogenase; LDH, rabbit skeletal muscle lactate dehydrogenase; Mops, 4-morpholinepropanesulfonic acid.

on the product added, and are given below with each of the two products, P and Q, considered in turn.

When kinetic parameters and isotope effects are obtained at different added concentrations of P (maintaining Q at zero concentration), the expressions for V and V/K_b derived by the method of net rate constants (Cleland, 1975) are given by eqs 8 and 9, while expressions for the isotope effect are given by eqs 6 and 7.

$$V/E_t = k_5/[1 + c_{Vf} + c_r] \quad (8)$$

$$V/K_bE_t = (k_3k_5/k_4)/[1 + c_f + c_r] \quad (9)$$

where c_f, c_{Vf}, and c_r are given by eqs 10–12.

$$c_f = k_5/k_4 \quad (10)$$

$$c_{Vf} = k_5[1/k_7 + 1/k_9(1 + k_8P/k_7)] \quad (11)$$

$$c_r = (k_6/k_7)(1 + k_8P/k_9) \quad (12)$$

Note that eqs 11 and 12 depend on the concentration of P. The c_r term appears in the numerator and denominator of both eqs 6 and 7, while c_{Vf} and c_f appear in the numerator and denominator of eqs 6 and 7, respectively, but only c_{Vf} and c_r are affected by the presence of P. With P at zero concentration the c_r term is k_6/k_7 and c_{Vf} is $k_5(1/k_7 + 1/k_9)$, and finite values of ^DV and $^D(V/K_b)$ will be observed as long as the values c_f, c_r, and c_{Vf} are not large with respect to $^Dk_5$. As the concentration of P is increased to infinity, the expressions for ^DV and $^D(V/K_b)$ become equal to $(^DK_{eq} + k_5/k_6)/(1 + k_5/k_6)$ and $^DK_{eq}$, respectively. Thus, for ^DV, a value between $^DK_{eq}$ and 1 will be obtained with $^DK_{eq}$ observed when the equilibrium constant k_5/k_6 is less than 1 while a value of 1 will be obtained when the reaction is for all practical purposes irreversible. The expression for the concentration of P that gives half the change in the V (or V/K) isotope effects was obtained by setting eq 6 (or 7) at zero P equal to eq 6 (or 7) at infinite P and solving for P. The expressions are given by eqs 13 and 14 for ^DV and $^D(V/K_b)$, respectively.

$$[P_{0.5}] = k_7/k_8(1 + k_9/k_5)/(1 + k_6/k_5) + k_9/k_8 \quad (13)$$

$$[P_{0.5}] = k_7/k_8[(k_9/k_6)[1 + (k_5/k_4)]] + k_9/k_8 \quad (14)$$

In eq 13, k_6/k_5 is the equilibrium constant in the direction of formation of A and B. In the case of these isotope effects, the concentration of P that gives half the change in the isotope effect will be equal to k_7/k_8 (the dissociation constant for EPQ to EQ and P) only under a very specific set of conditions. These conditions require that the EPQ to EQ equilibrium be isolated so that addition of P to EQ generates only EPQ. This can be accomplished for ^DV by (1) having the equilibrium between central complexes favor EPQ, thus resulting in essentially no change in the concentration of EPQ as a result of conversion to EAB, that is, by letting k_6/k_5 be small; and (2) slowing the release of Q from EQ compared to either conversion of EAB to EPQ (if interconversion of the central complexes were slow EQ could not build up) or addition of P to EQ, that is, by letting k_9/k_5 and k_9/k_8 be small. An alternative scenario requires a balance between conversion of EAB to EPQ and disappearance of EQ (k_9/k_5 equal to 1) and an equilibrium constant equal to 1 (this will keep EQ/EPQ constant) along with a slow release of Q from EQ with respect to addition of P to EQ (k_9/k_8 equal to zero). Under conditions where the above criteria are not met (likely the majority of cases), the concentration of P that gives half the change in the isotope effect will be much greater than k_7/k_8.

Similar logic can be used for $^D(V/K_b)$ (eq 14). Under conditions where B is not sticky, that is, k_5/k_4 is zero, the concentration of EPQ will be controlled by addition of P to EQ. In addition, k_9/k_6 must equal 1 so that there is a balance between the conversion of EPQ to EAB and the release of Q from EQ resulting in no net change in the EQ/EPQ ratio. Lastly, as is also the case for eq 13, k_9/k_8 must equal zero, that is, the addition of P to EQ must be faster than release of Q from EQ. Finite values for k_5/k_4 and/or k_9/k_8 and a value other than 1 for k_9/k_6 will result in a value either larger or in some cases smaller (if $k_9/k_6 < 1$ and k_5/k_4 and k_9/k_8 are not large) than k_7/k_8.

Isotope effects can also be obtained at different concentrations of Q (maintaining P at zero concentration). The product Q competes with A for free E. Under V conditions, both A and B are saturating, while A is also saturating under V/K_b conditions. As a result there will be no effect of Q on either V or V/K_b.

The above will apply to steady-state ordered mechanisms including Theorell–Chance, except that in the latter the isotope effect on V will equal unity in the absence of added P. In a rapid equilibrium ordered mechanism, the rate of interconversion of the central complexes limits the overall reaction, and thus P will not inhibit since no significant EQ is available in the steady state. As a result, initial velocity patterns alone will serve to distinguish this mechanism from others. No product inhibition by P will be observed and Q will be competitive against both A and B.

Random Mechanisms (All Rate Constants of Mechanism 5 Apply). When kinetic parameters and isotope effects are obtained at different concentrations of P (maintaining Q at zero concentration) the expressions for V and V/K_b derived using the method of net rate constants for branched pathways (Cleland, 1975) are given by eqs 8 and 9, while isotope effect expressions are again given by eqs 6 and 7, and the expressions for c_f, c_{Vf}, and c_r are given by eqs 10, 15, and 16, respectively.

$$c_{Vf} = k_5[1 + k_{11}/k_{13} + 1/(Pk_8/k_7 + k_9/k_7)]/[k_{11} + k_9/(Pk_8/k_7 + k_9/k_7)] \quad (15)$$

$$c_r = (k_6)/[k_{11} + k_9/(Pk_8/k_7 + k_9/k_7)] \quad (16)$$

Thus eqs 6 and 7 depend on the concentration of P. The c_r term again appears in the numerator and denominator of eqs 6 and 7, while c_{Vf} and c_f appear in the numerator and denominator of eqs 6 and 7, respectively. As for the ordered mechanism, only the c_{Vf} and c_r terms represented by eqs 15 and 16 contain the concentration of P. The limiting values of c_{Vf} and c_r with P at zero (initial velocity conditions with no P added) are $k_5[k_9(k_{11} + k_{13}) + k_7k_{13}]/k_9k_{13}(k_{11} + k_7)$ and $(k_6)/(k_{11} + k_7)$, respectively, while those at infinite P are $k_5(1/k_{13} + 1/k_{11})$ and (k_6/k_{11}), respectively. If the values

[2] The term c_f and c_r are called forward and reverse commitments to catalysis, respectively, while c_{Vf} is called the catalytic ratio (Northrop, 1991). The c_f reflects substrate stickiness where stickiness refers to the partitioning of EAB toward products as opposed to EA and B, while c_r is identical except that it reflects partitioning of EPQ to reactants as opposed to EQ and P (or EP and Q if the mechanism is random). The c_{Vf} term is the sum of the ratios of the rate constant for the isotope-sensitive step to each of the unimolecular net rate constants in the forward reaction direction. If steps prior to the isotope-sensitive step but after substrate binding exist, the concentration of the enzyme form undergoing the isotope-sensitive step must be corrected for these by multiplying the above sum by the partition ratio(s) leading to the isotope-sensitive step.

of the commitment factors are not large with respect to ${}^{D}k_5$, finite isotope effects will be observed on V and V/K_b. As long as ${}^{D}V$ and ${}^{D}(V/K_b)$ are finite and greater than ${}^{D}K_{eq}$ at infinite P, good evidence is provided for a random mechanism.

When the isotope effects are obtained at different concentrations of Q (maintaining P at zero concentration), the expressions for V, V/K_b, ${}^{D}V$, ${}^{D}(V/K_b)$, and c_f are given by eqs 8, 9, 6, 7, and 10, respectively, while those for c_{Vf} and c_r are given by eqs 17 and 18, respectively.

$$c_{Vf} = k_5[1 + k_7/k_9 + 1/(Qk_{12}/k_{11} + k_{13}/k_{11})]/[k_7 + k_{13}/(Qk_{12}/k_{11} + k_{13}/k_{11})] \quad (17)$$

$$c_r = (k_6)/[k_7 + k_{13}/(Qk_{12}/k_{11} + k_{13}/k_{11})] \quad (18)$$

While the expression for c_f is the same for all sequential mechanisms considered, those for c_{Vf} and c_r differ and depend on the concentration of Q. Note that although some of the rate constants differ, the equations are symmetrically equivalent to those derived for varying the concentration of P. The explanation used above for added P will also apply to added Q considering the bottom pathway of mechanism 5 where Q adds to the EP complex to give EPQ. As above, as long as ${}^{D}V$ and ${}^{D}(V/K_b)$ are finite and greater than ${}^{D}K_{eq}$ at infinite Q, good evidence is provided for a random mechanism.

The finite value of the isotope effects at infinite concentration of a given product predicted by the above theory for random mechanisms is a result of the inability of one product to trap the other on enzyme. As the concentration of one product increases, the other can still be released from the central complex, and as a result the isotope effects will be finite as long as the product is not too sticky. (Sticky here indicates a preference for the enzyme–product Michaelis complex to partition toward reactants as opposed to dissociating to give enzyme and product.) The predominant pathway for release of product can be distinguished by a comparison of the magnitudes of the isotope effects observed at infinite concentrations of P and Q. As a product concentration becomes high, the release of products is forced through the pathway in which the other product is released first, and thus the larger isotope effect at infinite product concentration indicates the stickier product. For example, if isotope effects of 1.5 and 2 are obtained at infinite concentrations of P and Q, respectively, Q is the stickier product. Increasing Q to infinity causes P to be released first, while increasing P to infinity causes Q to be released first. This is true of the isotope effects on V and V/K.

The above will apply to the steady-state random mechanism but not to the rapid equilibrium random mechanism. In a rapid equilibrium mechanism, the rate of interconversion of the central complexes limits the overall reaction, and thus neither P nor Q will inhibit in their normal capacity as products, since no significant EQ or EP will accumulate in the steady state. Both products may be capable of forming dead-end EAP and EBQ complexes, but these will not influence the observed isotope effects. However, unlike the rapid equilibrium ordered case, initial velocity patterns alone will not serve to distinguish this mechanism from others. The product dependence of isotope effects will allow a distinction between the two possible random mechanisms and ordered mechanisms.

Ping-Pong Mechanisms. As opposed to the sequential mechanisms considered above, the predictions are quite different in the case of a ping-poing mechanism. An isotope effect on V/K for the substrate of a half-reaction is observed only if that half-reaction is sensitive to deuterium substitution, and only the product of that half-reaction will affect this V/K value. The V/K for the substrate of the other half-reaction will not show an isotope effect if this half-reaction is not sensitive to deuterium substitution. However, the product of both half-reactions will potentially affect an isotope effect on V if one is observed. Consider mechanism 19:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} FP \underset{k_6P}{\overset{k_5}{\rightleftharpoons}} F \underset{k_8}{\overset{k_7B}{\rightleftharpoons}} FB \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EQ \underset{k_{12}Q}{\overset{k_{11}}{\rightleftharpoons}} E \quad (19)$$

where k_3 and k_4 are deuterium sensitive but k_9 and k_{10} are not. Other rate constants represent addition and release of reactants and products. Under conditions where P and Q are maintained at zero concentration there is an irreversible step resulting from the release of P, and thus if deuterium is lost in the first half-reaction (a common occurrence for primary deuterium isotope effects in ping-pong reactions), an isotope effect of unity would result. The addition of P, however, generates a reversible connection between the addition of B and the isotope-sensitive step (k_3) representing the interconversion of EA and FP. As a result, an isotope effect could be observed on V/K_b that is a function of the equilibrium isotope effect for the first half-reaction. The equations for V, V/K_a, and V/K_b derived using the method of net rate constants (Cleland, 1975) are given as eqs 20–22, while the isotope effects are given in eqs 23–25.

$$V = k_3/[1 + k_3[1/k_5 + 1/k_{11} + 1/k_9(1 + k_{10}/k_{11})] + k_4/k_5] \quad (20)$$

$$V/K_a = (k_1k_3/k_2)/[1 + k_3/k_2 + k_4/k_5] \quad (21)$$

$$V/K_b = (k_3k_7/k_4)/[1 + k_8/k_9(1 + k_{10}/k_{11})] \times [k_3/k_4 + (1 + k_3/k_4)Pk_6/k_5] \quad (22)$$

$${}^{D}V = \frac{{}^{D}k_3 + k_3[1/k_5 + 1/k_{11} + 1/k_9(1 + k_{10}/k_{11})] + (k_4/k_5){}^{D}K_{eq,1}}{1 + k_3[1/k_5 + 1/k_{11} + 1/k_9(1 + k_{10}/k_{11})] + k_4/k_5} \quad (23)$$

$${}^{D}(V/K_a) = [{}^{D}k_3 + k_3/k_2 + (k_4/k_5){}^{D}K_{eq,1}]/[1 + k_3/k_2 + k_4/k_5] \quad (24)$$

$${}^{D}(V/K_b) = [K_{eq,1} + ({}^{D}K_{eq,1} + K_{eq,1})Pk_6/k_5]/[K_{eq,1} + (1 + K_{eq,1})Pk_6/k_5] \quad (25)$$

In the above expressions, $K_{eq,1}$ (k_3/k_4) and ${}^{D}K_{eq,1}$ (${}^{D}k_3/{}^{D}k_4$) refer to the equilibrium constant for the first half-reaction and the isotope effect on that parameter, respectively. Note that ${}^{D}V$ and ${}^{D}(V/K_a)$ are independent of the product P, due to the competitive nature of B and P (both bind to F in mechanism 19) and the fact that both V and V/K_a are obtained under conditions of saturating B. The V and V/K_a isotope effect equations have the same form as those discussed above for sequential mechanisms. In these equations, c_f, c_r, and c_{Vf} are k_3/k_2, k_4/k_5, and $k_3[1/k_5 + 1/k_{11} + 1/k_9(1 + k_{10}/k_{11})]$, respectively. The form of eq 25 is unique, being a function of only the equilibrium isotope effect. As stated above, when the concentration of P is zero, ${}^{D}(V/K_b)$ will be 1, since V/K_b does not contain the isotope-sensitive step (k_3). However, when the product P is added, a reversible connection is established between FP and F and, as a result, ${}^{D}(V/K_b)$ may reflect the equilibrium isotope effect for the first half-reaction under the following circumstances. As P increases to infinity,

eq 25 reduces to eq 26.

$$^{D}(V/K_b) = [K_{eq,1} + {}^{D}K_{eq,1}]/[1 + K_{eq,1}] \quad (26)$$

Whether an isotope effect is observed depends on the value of $K_{eq,1}$. If the equilibrium between EA and FP is far toward FP, the addition of P will result in an isotope effect on V/K_b of 1. The only effect of P under these conditions is competition with B for F. If the equilibrium is toward EA, however, the addition of P will result in $^{D}(V/K_b)$ being equal to $^{D}K_{eq,1}$.

The ping-pong mechanism is not symmetric with respect to the effects of added P and Q. This is a result of an isotope sensitive step being present in only one of the two half-reactions. When Q is added, the expressions for V and ^{D}V are identical to those in eqs 20 and 23, while V/K_b is identical to eq 22 at P equal zero [$V/K_b = (k_7k_9/k_8)/(1 + k_9/k_8 + k_{10}/k_{11})$], and thus the isotope effect on V/K_b is 1. The reason for the independence of V and V/K_b on the concentration of Q is again that A is saturating to obtain these parameters and A and Q are competitive. The expression for V/K_a is given in eq 27.

$$V/K_a = (k_1k_3/k_2)/[(1 + k_3/k_2 + k_4/k_5)[1 + Qk_{11}/k_{12}(1 + k_{10}/k_9)]] \quad (27)$$

The dependence on Q is evident, but it is a multiplier for the entire denominator and as a result is not observed in the isotope effect equation which is identical to eq 24. Although Q does establish a reversible connection with the second half-reaction, there is no isotope-sensitive step in this portion of the reaction. If there were another isotope effect manifested in the second half-reaction, e.g., a secondary effect, then an equation similar to eq 25 would be obtained for $^{D}(V/K_a)$ reflecting that effect.

Terreactant and Higher Order Mechanisms. A simple extension of the treatment used above for bireactant mechanisms can be used for higher order reactions. The experiments are carried out with A and B maintained saturating and C varied so that isotope effects on V and V/K_c are obtained. In terreactant mechanisms the c_r term is more complex, with three terms in the denominator of the expression, one for each reactant released from the EPQR complex. As for the substrate dependence of isotope effects discussed above, the terreactant product release mechanism is reduced to a bireactant one by maintaining one of the products constant and varying one at a time of the remaining two products. Thus, there are six possible experiments to be carried out. If the varied product decreases ^{D}V but not the $^{D}(V/K)$, it is either not the first product released from EPQR or the mechanism is random. As an example, consider an ordered terreactant mechanism with products released in the order P, Q, and R. With R held at a fixed concentration, Q will have an effect only on V since P is released between the addition of Q and the catalytic step(s), but P will decrease both ^{D}V and $^{D}(V/K)$ to $^{D}K_{eq}$. With Q held at a constant concentration, R will have no effect since it binds to E making E unavailable (A and B are saturating), while P will again affect both but at lower concentrations than when R is held constant (the Q present increases the amount of EQR to which P binds). With P held constant, R will still have no effect but Q will now affect both ^{D}V and $^{D}(V/K)$ since the presence of P has established a reversible connection between the catalytic step(s) and addition of Q. In all cases, the isotope effect on V/K will decrease to $^{D}K_{eq}$ when a change is observed. In a steady-state random mechanism there is a possibility that all products affect $^{D}(V/K)$ but the isotope effect will usually decrease to a finite value greater than $^{D}K_{eq}$. If none of the products have an effect, the mechanism is rapid equilibrium. The varied product that gives the highest value of $^{D}(V/K)$ is the stickiest. Thus, again preferred pathways as well as obligatory order can be determined using isotope effects.

The minimal mechanisms considered for the product dependence of isotope effects on V, V/K_a, and V/K_b are necessarily more complex than those considered for the substrate dependence of isotope effects since they must also account for the reversal of the reaction that occurs upon the addition of product. However, the information obtained using the approach outlined here, in conjunction with that obtained from the substrate dependence of isotope effects, allows one to develop a more complete picture of the overall kinetic mechanism. The dependence of isotope effects on product concentration had been alluded to by Northrop (1975), but the theory had not been developed. As a result, theory has been developed in detail herein.

Summary. Generally, the following can be stated concerning the dependence of ^{D}V and $^{D}(V/K)$ on the concentration of product.

(1) The terms affected by the presence of product are c_r and c_{Vf}.

(2) For a steady-state ordered mechanism, $^{D}(V/K)$ decreases to become equal to $^{D}K_{eq}$, while ^{D}V decreases to a value between 1 and $^{D}K_{eq}$ at infinite concentration of P. The last product released will have no effect on either of the isotope effects. If the mechanism is Theorell–Chance, that is, the off-rate for Q completely limits the overall reaction, ^{D}V will not be affected by increasing the concentration of P. The only exception to the above is observed for an ordered reaction that has a very high K_{eq} or an irreversible catalytic step. In this case P will have no effect on $^{D}(V/K)$ and ^{D}V will decrease to a value of 1.

(3) For a steady-state random mechanism, ^{D}V and $^{D}(V/K)$ will decrease to a finite value greater than $^{D}K_{eq}$ whether P or Q is increased. The product that gives the highest value of $^{D}(V/K)$ at infinite concentration is the stickiest.

(4) A rapid equilibrium mechanism is obtained when neither product affects ^{D}V or $^{D}(V/K)$.

(5) For a ping-pong mechanism, the presence of the product that provides a reversible connection between the isotope-sensitive step and the non-isotope-sensitive half-reaction will give an isotope effect on the V/K for the latter. (In the absence of product, this V/K will have an isotope effect of 1.) The magnitude of the isotope effect will depend on the equilibrium constant for the isotope-sensitive half-reaction and the equilibrium isotope effect. This will be the only effect of added product.

RESULTS

To demonstrate the utility of the dependence of deuterium isotope effects on the concentration of added product in enzyme-catalyzed reactions, data were collected for the lactate (LDH) and alcohol dehydrogenase (YADH) reactions. Both enzymes are thought to have steady-state ordered kinetic mechanisms [YADH (Dickinson & Monger, 1973; Ganzhorn & Plapp, 1988); LDH (Zewe & Fromm, 1965)]. Deuterium isotope effects were measured by direct comparison of initial velocities with saturating NADH or A-side NADD varying the concentration of the other substrate using several concentrations of lactate (for LDH) or ethanol (for YADH) including zero.

Lactate Dehydrogenase. An apparent K_i for lactate of 40 mM was obtained from a Dixon analysis with NADH and pyruvate fixed at 300 and 170 μM, respectively (data not shown). Isotope effect data are shown in Figure 1A with both

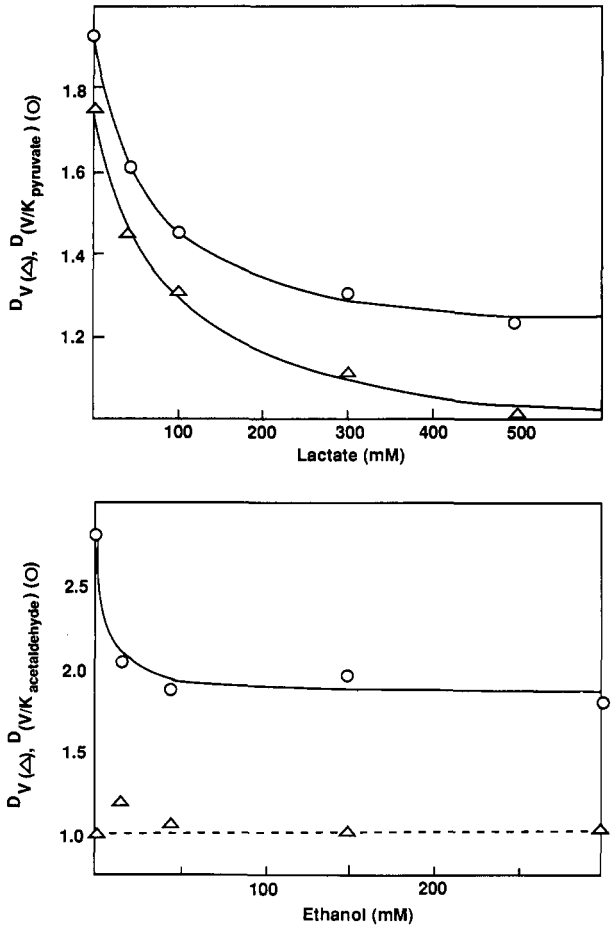


FIGURE 1: Dependence of V and V/K deuterium isotope effects obtained for the LDH (A, top) and yeast ADH (B, bottom) reactions on the concentration of products. (A) ^{D}V and $^{D}(V/K_{pyruvate})$ as a function of lactate concentration. (B) ^{D}V and $^{D}(V/K_{acetaldehyde})$ as a function of ethanol concentration. The points are experimental values, while the solid curves are from a fit of the data using eq 4.

^{D}V and $^{D}(V/K_{pyruvate})$ obtained as a function of lactate concentration. A fit of the equation for a hyperbola to the average $^{D}(V/K)$ values shown in Figure 1A gives a $^{D}(V/K_{pyruvate})$ of 1.93 ± 0.02 at zero lactate, which decreases to a value of 1.16 ± 0.02 at infinite lactate. The lactate concentration that gives half the change in the isotope effect is 60 ± 6 mM. The isotope effect on V also decreases hyperbolically from 1.75 ± 0.03 at zero lactate to 0.93 ± 0.05 at infinite lactate. The lactate concentration that gives half the change in the isotope effect is 80 ± 20 mM.

The above data suggest a random release of lactate and NAD. Additional information on the randomness of the kinetic mechanism in the direction of lactate oxidation can be obtained from dead-end inhibition patterns. Inhibition by the dead-end analog oxalate was determined for LDH with NAD as the variable substrate and lactate fixed at a concentration of 75 mM. The inhibition pattern (Figure 2) is noncompetitive with K_{is} and K_{ii} values of 2 ± 1 mM and 140 ± 25 μM, respectively. A fit using the equation for uncompetitive converges and in fact gives a K_{ii} value similar to that obtained from the noncompetitive fit. However, the σ value is larger by 25%, and the residuals are all negative for the uncompetitive fit, suggesting that noncompetitive inhibition better describes the data.

Yeast Alcohol Dehydrogenase. Isotope effect data have been collected for the yeast alcohol dehydrogenase-catalyzed reduction of acetaldehyde by NADH or A-side NADD (Figure 1B). This enzyme was reported to have a steady-state ordered

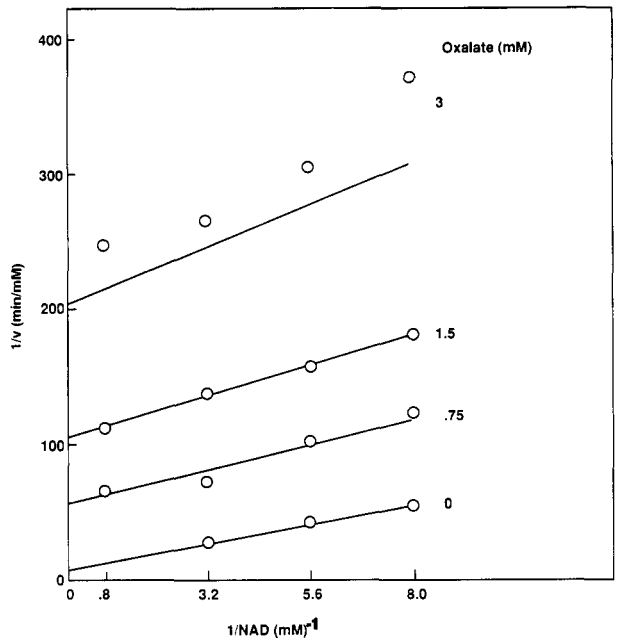


FIGURE 2: Dead-end inhibition by oxalate, as an analog of lactate. Initial velocity data were collected at nonsaturating lactate (75 mM), varying the concentrations of NAD and oxalate as indicated at pH 7.1, 150 mM Tris-HCl, and 25 °C. The points are experimental values, while the solid lines are from a fit of eq 1 to the data.

mechanism by Dickinson and Monger (1973) and Ganzhorn and Plapp (1988), although the isotope effect data of the latter authors in the direction of ethanol oxidation conform to a steady-state random mechanism [^{D}V, 1.8; $^{D}(V/K_{NAD})$, 1.8; $^{D}(V/K_{ethanol})$, 3.2]. An apparent K_i of 15 mM for ethanol was obtained from a Dixon analysis with NADH and acetaldehyde maintained at a saturating concentration and ethanol concentration increased (data not shown). Ethanol was found to be noncompetitive vs NAD^+. The isotope effects were then obtained as a function of added ethanol concentration. The isotope effect on V is within error 1 over the entire range of ethanol concentrations, in agreement with a Theorell–Chance mechanism. However, the isotope effect on $V/K_{acetaldehyde}$ decreases from a value of 2.8 ± 0.1 at zero ethanol to a value of 1.8 ± 0.1 at infinite ethanol concentration (Figure 2B). The concentration of ethanol that gives half the change in the isotope effect is 3.7 ± 1 mM.

DISCUSSION

Lactate Dehydrogenase. The finite isotope effect observed for $^{D}(V/K_{pyruvate})$ at infinite lactate concentration indicates that NAD can be released from the E:NAD:lactate ternary complex. The presence of lactate at high concentrations results in an increase in the reverse commitment factor (c_r, given by eq 16 where P is lactate) in the direction of lactate formation. As can be seen from the limits of eq 16, the change in c_r when lactate is very high compared to that obtained at zero lactate is the elimination of the k_7 term reflecting release of P prior to Q in mechanism 5. Since the isotope effect is larger when P is at zero concentration, the rate of release of P from EPQ must be greater than that of Q from EPQ, and the reaction mechanism for product release approximates ordered release of P prior to Q. (The latter will always apply when there is a significant difference between the rates of release of P and Q, that is, the flux will proceed through the pathway with the fastest rates.) However, at high concentrations of added P, when the only pathway available requires release of Q prior to P (NAD prior to lactate), there is still a finite flux since

the isotope effect has not become equal to $^{D}K_{eq}$ (0.85; Cook & Cleland, 1981a).

One can calculate the change in commitment factor resulting from forcing the flux through the less preferred pathway in which NAD is released prior to lactate using the general equation for the isotope effect on V/K (eq 7), and replacing $^{D}k_5$ with $^{D}k_5'$, where the latter is ($^{D}k_5 + c_f$). (The latter is allowed since $^{D}k_5'$ is independent of the concentration of added lactate.) Using the maximum observed isotope effect of 1.9 at zero lactate and the value of 1.16 observed at infinite lactate for $^{D}k_5'$ and $^{D}(V/K)$, respectively, a value of 2.5 is calculated for the change in c_r as the lactate concentration is increased from zero to infinity. Thus, the rate of release of NAD from E:NAD:lactate is ≥2.5 times slower than the release of lactate from the same complex. (A better estimate would be obtained if the other experiment in which the isotope effect were obtained as a function of NAD concentration were performed.)

A random kinetic mechanism proposed for the lactate dehydrogenase may be somewhat surprising and appear to contradict results in the literature. However, most of the information concerning this enzyme has been obtained in the direction of pyruvate reduction. For example, tritium isotope effects on V/K_{NADH} have been measured as a function of pyruvate concentration to determine whether the kinetic mechanism is ordered (Duggleby & Northrop, 1989). An isotope effect of 2.6 is measured at low pyruvate concentration ([pyruvate] < $K_{pyruvate}$), which decreases to a value of 1 at infinite pyruvate. These data indicate a steady-state ordered addition of NADH prior to pyruvate (Cook et al., 1980).[3]

Initial velocity data in both oxidative and reductive reaction directions were obtained by Zewe and Fromm (1965), who suggested a modified Theorell–Chance mechanism with isomerization of E:NAD. The proposed order of addition of NAD prior to lactate was based on an uncompetitive inhibition pattern with oxalate as a dead-end analog and NAD as the variable substrate. In the direction of pyruvate reduction, however, oxalate is noncompetitive vs NADH. The authors suggest that at the higher concentrations of oxalate used for the inhibition patterns in the direction of pyruvate reduction an E:oxalate complex is allowed. That this complex is also allowed in the lactate oxidation direction using a similar oxalate concentration range is shown by the noncompetitive inhibition pattern in Figure 2. Thus, it appears that oxalate, and by analogy pyruvate and oxalate, bind to free enzyme. The only question then concerns the productivity of an E:lactate complex, that is, whether a productive E:NAD:lactate Michaelis complex is formed. Data are available from isotope exchange at equilibrium (Silverstein & Boyer, 1964) that bear on this question. The NAD to NADH exchange still occurs even at very high concentrations of the lactate/pyruvate pair, indicating an alternative pathway that proceeds via the addition of lactate and pyruvate prior to NAD and NADH, respectively. The rate of the latter pathway, however, is only 0.01 that of the predominant pathway in which nucleotides add to enzyme first. In order that the isotope exchange data be consistent with the isotope effect data presented here, there are two possibilities. First, lactate could be released 100 times faster than NAD from E:NAD:lactate. The value of 100-fold sets the upper limit for the relative values of k_7/k_{11} estimated above to have a lower limit of 2.5. If the above ratio were 100, c_r [$k_6/(k_{11} + k_7)$] would reduce to k_6/k_7 at low lactate concentrations and become k_6/k_{11} at high lactate concentrations, and the calculated change in the commitment factor would be 100 (not 2.5) as the flux switched from one pathway to another. Since a value much lower than 100 is estimated, k_6/k_7 must be close to zero. Second, the low value of the NAD to NADH exchange rate at high pyruvate and lactate concentrations may be a result of a very slow release of NADH from E:NADH:pyruvate. Third, the discrepancy may be a combination of the two. Of these, the second would be in agreement with the data of Palm (1965) and Duggleby and Northrop (1989), who showed an effectively ordered addition of NADH prior to pyruvate.

Neither of the above possibilities can be completely eliminated on the basis of the present data. However, it is clear that although the kinetic mechanism for LDH is predominantly ordered, the pathway in which NAD is released prior to lactate is allowed and occurs at a rate 2.5–100 times slower than the preferred pathway. Finally, since the isotope effect on V becomes 1 at infinite lactate, a further contribution must be present from the c_{Vf} term. On the basis of the above logic, the release of NAD from E:NAD:lactate must be slow, and thus the k_5/k_{11} term likely provides the additional contribution. These data illustrate the power of the product dependence of deuterium isotope effects.

Yeast Alcohol Dehydrogenase. Isotope effect data for the yeast ADH-catalyzed reduction of acetaldehyde as a function of ethanol concentration also suggest a steady-state random kinetic mechanism. At zero ethanol, the isotope effect of 2.8 on $V/K_{acetaldehyde}$ suggests a significant rate limitation by hydride transfer from NADH to acetaldehyde, while the isotope effect of 1 on V is consistent with the proposed Theorell–Chance mechanism (Dickinson & Monger, 1973).

The decrease in $^{D}(V/K_{acetaldehyde})$ from a value of 2.8 at zero ethanol to a value of 1.8 at infinite ethanol indicates a finite release of NAD from the E:NAD:ethanol ternary complex. As above for LDH, a lower limit for the ratio of the flux through the two pathways, that is, release of ethanol first and release of NAD first, can be estimated as 1. The estimated equality of the fluxes through the two pathways is inconsistent with the Theorell–Chance nature of the kinetic mechanism, and there may be another explanation for the alternative release of NAD prior to ethanol observed at high ethanol concentrations. A clue concerning the alternative explanation comes from work on equine liver ADH using cyclohexanol as a reactant (Dalziel & Dickinson, 1966; Cook & Cleland, 1981b). These authors documented substrate activation by cyclohexanol at high concentrations as a result of an increase in the off-rate for NADH from the E:NADH:cyclohexanone complex compared to the E:NADH complex. This is likely also the reason for the observed randomness of the yeast ADH reaction in the direction of ethanol oxidation, that is, an increase in the off-rate for NAD from the E:NAD:ethanol complex compared to E:NAD.

However, it should be noted that the value of the isotope effect of 1.8 measured at infinite concentration of ethanol agrees perfectly with that measured on V/K_{NAD} by Ganzhorn and Plapp (1988). Thus, the YADH mechanism may not be ordered but random in the direction of alcohol oxidation. Finally, the isotope effect of 1 on V even at high ethanol concentrations must indicate that dissociation of the E:ethanol complex is also slow.

[3] Interestingly, this experiment was also carried out by Palm (1965) with identical results, but the data were misinterpreted to suggest a rate-limiting dissociation of E:NAD:pyruvate. (It should be noted that these data were collected well before any theory regarding isotope effects in enzyme mechanism was proposed.)

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