Consumption of Hydrogen Ions in Rapid-Equilibrium Enzyme Kinetics[†]

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Enzyme-catalyzed reductase reactions in particular are characterized by large changes in the binding of hydrogen ions $\Delta_r N_H$. This is a thermodynamic property of the reaction that is catalyzed. For example, in the ferredoxin−nitrite reductase reaction, there is an increase of eight in the binding of hydrogen ions for every molecule of nitrite reduced to ammonia H_2O. If these hydrogen ions are consumed in the rate-determining reaction, the limiting velocity is proportional to $[H^+]^8$. This would make it practically impossible to determine the kinetic parameters. This article shows that when n hydrogen ions are consumed in reactions preceding the rate-determining reaction the limiting velocity is not proportional to $[H^+]^n$ and may only vary with pH according to the pK's of the enzyme−substrate complex that produces products. Rapid-equilibrium rate equations for ordered A + B → products are derived for two mechanisms in which a single hydrogen ion is consumed prior to the rate-determining reaction. Rate equations are tested by calculating velocities for the minimum number of velocity measurements required to estimate the kinetic parameters and using these velocities to estimate the kinetic parameters.

Introduction

There is a good deal of information on the change in binding of hydrogen ions in enzyme-catalyzed reactions because $\Delta_r N_H$ can be calculated[1] by taking the derivative of the apparent equilibrium constant K' with respect to the pH. $\Delta_r N_H$ is a thermodynamic

$$\Delta_r N_H = -d \log K'/d\text{pH} \qquad (1)$$

property of the reaction being catalyzed. Values of $\Delta_r N_H$ have been calculated for over 200 enzyme-catalyzed reactions at 298.15 K, pH's 5, 6, 7, 8, and 9, and 0.25 M ionic strength.[1,2] $\Delta_r N_H$ can also be calculated[2,3] from the average binding of hydrogen atoms $\bar{N}_H(i)$ by the reactants at a specified pH.

$$\Delta_r N_H = \Sigma \nu_i' \bar{N}_H(i) \qquad (2)$$

The stoichiometric numbers for the reactants in the biochemical equation are represented by ν_i'. The change in binding of hydrogen ions $\Delta_r N_H$ usually varies with the pH because of the pK's of the substrates.

It is important to distinguish between $\Delta_r N_H$ and the number n of hydrogen ions consumed in a reaction in a mechanism for an enzyme-catalyzed reaction. The number n of hydrogen ions consumed in a reaction in a mechanism is an integer. When a hydrogen ion is consumed in a reaction, the convention in chemical thermodynamics is that its stoichiometric number is negative. However, in this article, n is considered to be a positive integer.

$\Delta_r N_H$ can be especially large for oxidoreductase reactions like ferredoxin-nitrite reductase

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$$\text{Nitrite} + 6 \text{ ferredoxin}_{red} = \text{ammonia} + 6 \text{ ferredoxin}_{ox} + 2H_2O \qquad (3)$$
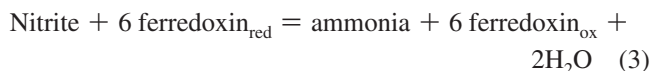
The values of $\Delta_r N_H$ for this reaction[1] range from 7.99 at pH 5 to 7.64 at pH 9. The kinetic effects of the consumption of eight hydrogen ions in the rate-determining reaction for reaction 3 have been discussed.[4] Calculations on this mechanism show that when $n = 8$ going from pH 5.1 to 5.0 increases the limiting velocity V_{fexp} by a factor of 5 at pH 5. The factors at pH 6, 7, 8, and 9 are 5.1, 5.8, 7.0, and 8.0. Thus, if eight hydrogen ions are consumed in the rate-determining reaction, it would not be practical to determine kinetic parameters without much better control of pH. Similar calculations[5,6] have been made for ordered A + B = ordered P + Q, and the relationship between $\Delta_r N_H$ and n has been discussed for this mechanism. However, hydrogen ions can be consumed in reactions prior to the rate-determining reaction, and this article is about these possibilities.

The rapid-equilibrium assumption[7−9] of enzyme kinetics is especially useful in discussing the effects of pH on rates of enzyme-catalyzed reactions. *Mathematica* is an especially suitable computer application for discussing rapid-equilibrium kinetics because Solve can be used to derive the rapid-equilbrium rate equation for a mechanism, and Solve can also be used to estimate the values of kinetic parameters from the minimum number of velocity measurements.[10,11]

Rapid-Equilibrium Rate Equation for A + B → Products When No Hydrogen Ions Are Consumed
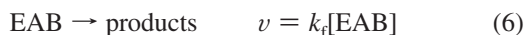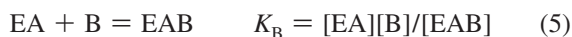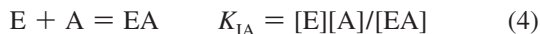
The mechanism at a specified pH is

TABLE 1: Estimation of Four Kinetic Parameters in Mechanisms [9−,11] from Three Velocity Measurements at $\{[A],[B],[H^+]\} = \{100,100,10^{-5}\}, \{1,100,10^{-5}\}, \{100,1,10^{-5}\}$

	$k_f[E]_t$	K_{IA}	K_B
no errors	1.00	10.00	20.00
1.05 × v1	1.06	10.29	21.25
1.05 × v2	1.00	9.16	20.15
1.05 × v3	0.99	10.59	18.69

$$E + A = EA \qquad K_{IA} = [E][A]/[EA] \qquad (4)$$

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \qquad (5)$$

$$EAB \rightarrow products \qquad v = k_f[EAB] \qquad (6)$$

The rate equation derived by use of Solve in *Mathematica* from two equilibrium equations and one conservation equation is

$$v = \frac{k_f[E]_t[A][B]}{[A][B] + K_B[A] + K_B K_{IA}} \qquad (7)$$

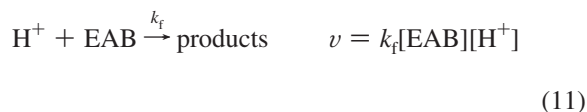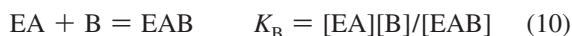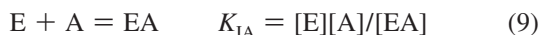This rapid-equilibrium rate equation can be rearranged to

$$v = \frac{k_f[E]_t}{1 + \dfrac{K_B}{[B]}\left(1 + \dfrac{K_{IA}}{[A]}\right)} = \frac{V_{fexp}}{1 + \dfrac{K_B}{[B]}\left(1 + \dfrac{K_{IA}}{[A]}\right)} \qquad (8)$$

where $V_{fexp} = k_f[E]_t$ is the experimental limiting velocity that may be a function of the hydrogen ion concentration. This equation is well-known and is given as background.

When one hydrogen ion is consumed in the forward reaction for ordered $A + B \rightarrow$ products, there are three possibilities: (1) The hydrogen ion is consumed in the rate-determining reaction. (2) The hydrogen ion is consumed in the reaction preceding the rate-determining reaction. (3) The hydrogen ion is bound by EA. Further acid dissociations of E, EA, EAB, A, and B can be introduced in this mechanism. In the rest of this paper, mechanisms are written in terms of species, but many possible species are omitted so that new roles for the consumption of hydrogen ions can be clarified.

Consumption of One Hydrogen Ion in the Rate-Determining Reaction for Ordered A + B → Products

The mechanism for the first possibility is

$$E + A = EA \qquad K_{IA} = [E][A]/[EA] \qquad (9)$$

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \qquad (10)$$

$$H^+ + EAB \xrightarrow{k_f} products \qquad v = k_f[EAB][H^+]$$

$$(11)$$

The rapid-equilibrium rate equation derived using Solve is

$$v = \frac{k_f[E]_t[A][B]([H^+])}{[A][B] + K_B[A] + K_B K_{IA}} =$$
$$\frac{V_{fexp}[A][B]}{[A][B] + K_B[A] + K_B K_{IA}} \qquad (12)$$

where the limiting velocity V_{fexp} is given by $k_f[E]_t[H^+]$. The Michaelis constants in eq 12 are equilibrium constants. The determination of K_{IA} and K_B at two or more temperatures makes it possible[1] to calculate the standard transformed Gibbs energy

of reaction $\Delta_r G'^\circ$, the corresponding standard transformed enthalpy of reaction $\Delta_r H'^\circ$, and the corresponding standard transformed entropy of reaction $\Delta_r S'^\circ$.

The consumption of one hydrogen ion in the rate-determining reaction causes the limiting velocity V_{fexp} to increase by a factor of 10 for each decrease in the pH by 1. This effect extends over the whole pH range considered. When more hydrogen ions are consumed in the rate-determining reaction, as described in connection with reaction 3, the limiting velocity is given by $V_{fexp} = [H^+]^n k_f[E]_t$.

The values of the three kinetic parameters in eq 12 at a specified pH can be determined by measuring three velocities and solving three simultaneous polynomial equations using Solve; programs for doing this have been published.[10−12] The velocities at $\{[A],[B],[H^+]\} = \{100,100,10^{-5}\}$, $\{1,100,10^{-5}\}$, $\{100,1,10^{-5}\}$ are 81.967, 31.25, and 4.3478. These velocities give the correct values of the kinetic parameter, but it is necessary to test for the effects of experimental errors. That can be done by multiplying the velocities by 1.05, one at a time. The results are given in Table 1.
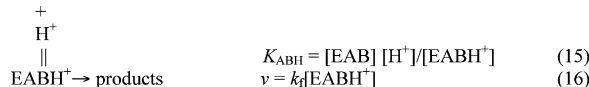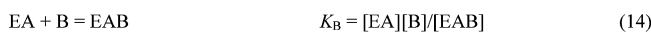
The same values are obtained at other pHs.

Consumption of One Hydrogen Ion in the Reaction Preceding the Rate-Determining Reaction for Ordered A + B → Products

The mechanism for the second possibility is

$$E + A = EA \qquad K_A = [E][A]/[EA] \qquad (13)$$
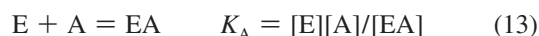
This way of writing the mechanism is consistent with the usual

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \qquad (14)$$
$$+$$
$$H^+$$
$$\|$$
$$EABH^+ \rightarrow products \qquad K_{ABH} = [EAB][H^+]/[EABH^+] \qquad (15)$$
$$v = k_f[EABH^+] \qquad (16)$$

way of introducing acid dissociations in enzyme kinetics. The rate equation derived for this mechanism by use of Solve is

$$v =$$
$$\frac{[H^+][A][B]k_f[E]_t}{[H^+][A][B] + [A][B]K_{ABH} + [A]K_{ABH}K_B + K_A K_{ABH} K_B} \qquad (17)$$

There are four kinetic parameters and four terms in the denominator. The values of these pH-independent parameters can be estimated by measuring four velocities. Equation 17 can be rearranged into the following form that shows the pH dependencies of the more familiar pH-dependent kinetic parameters shown in eq 8

$$v = \frac{k_f[E]_t/(1 + K_{ABH}/[H^+])}{1 + \dfrac{K_B(1 + K_A/[A])}{[B](1 + [H^+]/K_{ABH})}} \qquad (18)$$

According to mechanisms 13−16, K_{IA} in eq 8 is independent of pH, and V_{fexp} and K_B in eq 8 are given by

$$V_{fexp} = \frac{k_f[E]_t}{1 + K_{ABH}/[H^+]} \quad (19)$$

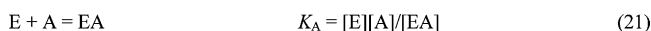$$K_B' = \frac{K_B}{1 + [H^+]/K_{ABH}} \quad (20)$$

The important point here is that V_{fexp} is no longer proportional to $[H^+]$, even though one hydrogen ion is consumed in the mechanism. The reason that this is important is that maybe V_{fexp} for enzyme-catalyzed reactions like eq 3 will not be of the form $V_{fexp} = k_f[E]_t[H^+]^n$.

The four kinetic parameters in mechanisms 13−16 can be calculated from four velocity measurements by using Solve in *Mathematica*. As a test, arbitrary values of kinetic parameters are assigned so that four velocities can be calculated using eq 17: $k_f[E]_t = 1$, $K_A = 10$, $K_B = 20$, and $K_{ABH} = 10^{-7}$. The velocities calculated at $\{[A],[B],[H^+]\}$ are $v(100,100,10^{-6}) = 0.89127$, $v(0.5,100,10^{-6}) = 0.65790$, $v(100,100,10^{-8}) = 0.075758$, and $v(100,0.5,10^{-8}) = 0.18182$. Only the relative values of these velocities are important because $k_f[E]_t$ is arbitrary. These triplets of concentrations were chosen so that $\{100,100,10^{-6}\}$ targets $k_f[E]_t$, $\{0.5,100,10^{-6}\}$ targets K_A, $\{100,100,10^{-8}\}$ targets K_{ABH}, and $\{100,0.5,10^{-6}\}$ targets K_B. The correct values of $k_f[E]_t$, K_A, K_B, and K_{ABH} are obtained by solving the four simultaneous polynomial equations, as expected. However, experimental errors have to be taken into account. This can be done by raising the velocities 5%, one at a time. The results of these calculations are summarized in Table 2.

Consumption of One Hydrogen Ion by EA in Ordered A + B → Products
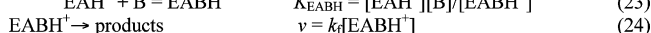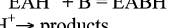
The mechanism for the third possibility is

The rapid-equilibrium rate equation derived using Solve in

$$
\begin{array}{lll}
E + A = EA & K_A = [E][A]/[EA] & (21) \\
\quad + & & \\
\quad H^+ & & \\
\quad \parallel & K_{EAH} = [EA][H^+]/[EAH^+] & (22) \\
EAH^+ + B = EABH^+ & K_{EABH} = [EAH^+][B]/[EABH^+] & (23) \\
EABH^+ \rightarrow products & v = k_f[EABH^+] & (24)
\end{array}
$$

Mathematica is

$$v = \frac{[A][B]k_f[E]_t[H^+]}{[A][B][H^+] + [A][H^+]K_{EABH} + [A]K_{EABH}K_{EAH} + K_AK_{EABH}K_{EAH}} \quad (25)$$

This equation can be rearranged to

$$v = \frac{k_f[E]_t}{1 + K_{EABH}/[B] + K_{EABH}K_{EAH}/[B][H^+] + K_AK_{EABH}K_{EAH}/[A][B][H^+]} \quad (26)$$

The important point about eq 26 is that the limiting velocity is not a function of $[H^+]$. The four kinetic parameters can be calculated from four velocity measurements by using Solve. As a test, arbitrary values of kinetic parameters are assigned so that four velocities can be calculated using eq 25: $k_f[E]_t = 1$,

TABLE 2: Estimation of Four Kinetic Parameters in Mechanisms [13−16] from Four Velocity Measurements at $\{[A],[B],[H^+]\} = \{100,100,10^{-6}\}$, $\{0.5,100,10^{-6}\}$, $\{100,100,10^{-8}\}$, and $\{100,0.5,10^{-6}\}$

	$k_f[E]_t$	K_A	K_{AB}	K_{ABH}
no errors	1.00	10.00	20.00	1.00×10^{-7}
$1.05 \times v1$	1.06	11.34	19.94	1.06×10^{-7}
$1.05 \times v2$	1.00	8.04	20.36	1.00×10^{-7}
$1.05 \times v3$	0.99	10.00	21.36	9.30×10^{-8}
$1.05 \times v4$	1.00	10.70	18.44	1.01×10^{-7}

TABLE 3: Estimation of Four Kinetic Parameters from Four Velocity Measurements When One Hydrogen Ion Is Bound by EA at $\{[A],[B],[H^+]\} = \{10,10,10^{-6}\}$, $\{0.5,100,10^{-6}\}$, $\{100,100,10^{-8}\}$, $\{100,0.5,10^{-6}\}$

	$k_f[E]_t$	K_A	K_{EAH}	K_{EABH}
n errors	1.00	10.00	1.00×10^{-7}	20.00
$1.05 \times v1$	1.06	11.30	1.02×10^{-7}	21.21
$1.05 \times v2$	1.00	7.91	1.02×10^{-7}	20.00
$1.05 \times v3$	1.00	10.89	9.11×10^{-8}	20.16
$1.05 \times v4$	0.99	10.00	1.06×10^{-7}	18.71

$K_A = 10$, $K_{EABH} = 20$, and $K_{EAH} = 10^{-7}$. The calculated velocities at $\{[A],[B],[H^+]\}$ are $v(100,100,10^{-6}) = 0.8183$, $v(0.5,100,10^{-6}) = 0.6173$, $v(100,100,10^{-8}) = 0.2941$, and $v(100,0.5,10^{-8}) = 0.02203$. Correct values are obtained by solving the four simultaneous polynomial equations, as expected. However, experimental errors have to be taken into account. This is done by raising the kinetic parameters 5%, one at a time. These calculations are summarized in Table 3.

Discussion

Rapid-equilibrium rate equations have been derived for three mechanisms for ordered A + B → products with three different routes for the consumption of one hydrogen ion. These rate equations have been tested to show that the kinetic parameters can be calculated using the minimum number of velocity measurements. The mechanisms have been kept as simple as possible to clarify the effects of the consumption of hydrogen ions on the limiting velocity and on the number of hydrogen atoms in the enzyme−substrate complex that dissociates to form products.

These mechanisms can be extended in several directions. For applications to specific enzyme-catalyzed reactions, more pK's need to be added. This approach can be applied to random A + B → products. Reverse reactions can be included so that the Haldane relation can be derived. The number n of hydrogen ions consumed can be increased, and reactions of the type A + mB → products can be treated. The effects of errors in the hydrogen ion concentration can be calculated.

When hydrogen ions are consumed in the rate-determining reaction, the limiting velocity is of the form $V_{fexp} = [H^+]^nV_f$, where V_f brings in the pK's of the enzyme−substrate complex that dissociates to yield products. The consumption of hydrogen ions prior to the rate-determining reaction does not affect the limiting velocity. The consumption of hydrogen ions prior to the formation of the rate-determining complex increases the number of hydrogen atoms in the enzyme−substrate complex that dissociates to products; see EABH$^+$ in eq 16 and eq 24. This will affect the change in binding of hydrogen ions $\Delta_r N_H$ of the biochemical reaction that is catalyzed.

The number n of hydrogen ions consumed in a reaction in a mechanism can be determined by identifying the mechanism by use of rate measurements. $\Delta_r N_H$ is a property of the

biochemical reaction that is catalyzed and is not affected by the mechanism. The consumption of hydrogen ions is especially important in reductase reactions.

When hydrogen ions are consumed prior to the rate-determining reaction, they affect the pH dependencies of the Michaelis constants, but they do not affect V_{fexp}.

References and Notes

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