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Effects of pH in Biochemical Thermodynamics and Enzyme Kinetics

Robert A. Alberty

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

E-Mail: alberty@mit.edu

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Abstract

In biochemical thermodynamics, the apparent equilibrium constants of enzyme-catalyzed reactions have been represented by $K' = K_{ref}10^{n\mathrm{pH}}f(\mathrm{pH})$, where K_{ref} is a reference chemical reaction, n is the number of hydrogen ions in the reference reaction, and f(pH) is a function of pH that brings in the pKs of the substrates. This equation suggests that hydrogen ions are involved in two different ways in biochemical thermodynamics. If this is true in thermodynamics, it has to be true in kinetics. However, the choice of reference reaction in thermodynamics is arbitrary, and so n cannot be determined from equilibrium measurements. However, when hydrogen ions are consumed in the rate-determining reaction, the experimental limiting velocity of the forward reaction is given by $V_{fexp} = 10^{n\mathrm{pH}}V_f$. V_f is the limiting velocity in the forward direction when $n = 0$, or V_f can be calculated from experimental data using $V_f = 10^{n\mathrm{pH}}V_{fexp}$. V_f brings in the pKs of the enzyme-substrate complex that reacts in the rate-determining reaction. When hydrogen ions are consumed in the rate-determining reaction, the Haldane equation yields $K' = K_{ref}10^{n\mathrm{pH}}f(\mathrm{pH})$. Since n can be -8 (EC 1.7.7.1), the effects of pH on kinetic and thermodynamic properties can be very large. webMathematica can provide the thermodynamic properties of enzyme-catalyzed reactions that are difficult to calculate and require a database without having Mathematica® in a personal computer or knowing how to use it.

Introduction

Since 1992 I have been concentrating on biochemical thermodynamics, and so when I attended ESCEC 2005 I looked at it as a biochemical thermodynamist. But that conference got me interested in enzyme kinetics again; I say "again" because I had worked on enzyme kinetics in 1950 – 1966. My recent experience in biochemical thermodynamics has made me look at enzyme kinetics in a very different way than I did in 1950 – 1966. As a bridge between biochemical thermodynamics and enzyme kinetics, the first thing I did was to write a paper "Relations between Biochemical Thermodynamics and Enzyme Kinetics" [1]. Since I was interested in pH effects, it was natural to use the rapid-equilibrium assumption because so many p*K*s and chemical equilibrium constants have to be included in the derivation of the rate equation. My respect for enzyme kinetics grew when I realized that enzyme kinetics includes all of biochemical thermodynamics as a special case, that is, when the reaction rate is zero.

Now I want to tell you about some new ideas in enzyme kinetics that in a sense have their origin in biochemical thermodynamics. When I was working on the kinetics of the conversion of fumarate to malate a long time ago, we recognized that the pH dependence of the apparent equilibrium constant for the fumarase reaction is given by $K' = K_{ref} f(\mathrm{pH})$, where K_{ref} is the equilibrium constant for a chemical reaction written in terms of species and f(pH) brings in the p*K*s of fumarate and malate. Later, when I became interested in the thermodynamics of the hydrolysis of ATP to ADP, I recognized that the pH dependence of the apparent equilibrium constant for that reaction is described by

$$K' = K_{ref} 10^{\mathrm{pH}} f(\mathrm{pH}) \qquad (1)$$

where the reference reaction is $ATP^{4-} + H_2O = ADP^{3-} + H_2PO_4^- + H^+$ [2]. This equation can be generalized to $K' = K_{ref} 10^{n\mathrm{pH}} f(\mathrm{pH})$, where n is an integer: positive if produced, negative if consumed. This type of expression for an apparent equilibrium constant has been used many times. Tewari and Goldberg used it in 1988 [3] for the conversion of penicillin G to 6-aminopenicillanic acid in 1988. Athel Cornish-Bowden and I used it in an article about pH effects in *Trends Biochem. Sci*. in 1993 [4].

But I want to caution you about this equation. In thermodynamics, the choice of reference reaction is arbitrary. Therefore, you can use the reference reaction $H^+ + ATP^{4-} + H_2O = HADP^{2-} + H_2PO_4^-$, which leads to $K' = K_{ref} 10^{-\mathrm{pH}} f(\mathrm{pH})$, rather than equation 1. Thus, if n means anything in kinetics, it has to be determined by rate measurements. Thermodynamics is independent of mechanism, and so it cannot be used to determine n in a mechanistic sense. However, the equation $K' = K_{ref} 10^{n\mathrm{pH}} f(\mathrm{pH})$ is important because it suggests that there are two different ways that hydrogen ions can be involved in biochemical thermodynamics and enzyme kinetics. This is especially important because the $10^{n\mathrm{pH}}$ effect is huge, since npH is in the exponent and the effect extends over the whole range of pH [5].

Derivation of Rate Equations when Hydrogen Ions are Consumed in the Rate-determining Reaction

Now I want to show that when the rapid-equilibrium assumption is used, *n* can be determined from rate measurements. One of the reasons this can be done is that enzyme kinetics is something like electrochemistry because you can use the idea of half reactions. In rapid-equilibrium enzyme kinetics, there is a forward half reaction and a reverse half reaction. I developed this point of view in writing a recent article for the *Journal of Chemical Education* [6] on a faster way to derive rapid-equilibrium rate equations (incidentally, that article contains rapid-equilibrium rate equations and Haldane equations fifteen different mechanisms). In studying kinetics of the forward reaction, you are learning about the properties of half reactions.

When a hydrogen ion is consumed in a reduction reaction, the hydrogen ion shows up in the chemical reference reaction. For example, consider the chemical reference reaction for EC 1.1.1.1.

$$H^+ + NAD_{red}(H_{27})^{2-} + \text{acetaldehyde}(H_4) = \text{ethanol}(H_6) + NAD_{ox}(H_{26})^- \quad (2)$$

In enzyme kinetics this hydrogen ion has to be consumed in the rate-determining reaction because its pH effects extend over the whole pH range of interest.

The following mechanism for ordered A + B = ordered P + Q includes two p*K*s for each reactant, the enzymatic site, and each enzyme-substrate complex. The equal signs indicate reactions that are assumed to be equilibrated rapidly and ↔ indicates the rate-determining reaction where a single hydrogen ion is consumed.

$$\begin{array}{ccccc} E^- & & HA^- & & H_2EA^- \\ \| & & \| & & \| \\ HE & + & H_2A & = & H_3EA \\ \| & & \| & & \| \\ H_2E^+ & & H_3A^+ & & H_4EA^+ \end{array} \quad (3)$$

$$\begin{array}{ccccc} H_2EA^- & & B^- & & H_3EAB^- \\ \| & & \| & & \| \\ H_3EA & + & HB & = & H_4EAB \\ \| & & \| & & \| \\ H_4EA^+ & & H_2B^+ & & H_5EAB^+ \end{array} \quad (4)$$

$$\begin{array}{ccc} H_3EAB^- & & H_4EPQ \\ \| & & \| \\ H^+ + H_4EAB & \underset{k_r}{\overset{k_f}{\rightleftharpoons}} & H_5EPQ^+ \\ \| & & \| \\ H_5EAB^+ & & H_6EPQ^{2+} \end{array} \tag{5}$$

$$\begin{array}{ccccc} H_4EPQ & & HEQ & & H_2P^- \\ \| & & \| & & \| \\ H_5EPQ^+ & = & H_2EQ^+ & + & H_3P \\ \| & & \| & & \| \\ H_6EPQ^{2+} & & H_3EQ^{2+} & & H_4P^+ \end{array} \tag{6}$$

$$\begin{array}{ccccc} HEQ & & E^- & & Q \\ \| & & \| & & \| \\ H_2EQ^+ & = & HE & + & HQ^+ \\ \| & & \| & & \| \\ H_3EQ^{2+} & & H_2E^+ & & H_2Q^+ \end{array} \tag{7}$$

The expressions for the pH dependencies of the four Michaelis constants for this mechanism are quite complicated (according to this mechanism, there are seven kinetic constants for each Michaelis constant, but the effects due to the p*K*s of substrates can be taken out. The p*K*s of substrates can be determined by using acid titrations of the substrates, and taking them out of the pH dependencies of the Michaelis constants simplifies the determination of the other p*K*s in the mechanism.

The rapid-equilibrium rate equation for this mechanism is

$$v = \frac{\dfrac{10^{-pH}V_f[A][B]}{K_{IA}K_B} - \dfrac{V_r[P][Q]}{K_PK_{IQ}}}{1 + \dfrac{[A]}{K_{IA}} + \dfrac{[A][B]}{K_{IA}K_B} + \dfrac{[Q]}{K_{IQ}} + \dfrac{[P][Q]}{K_{IQ}K_P}} = \frac{\dfrac{V_{fexp}[A][B]}{K_{IA}K_B} - \dfrac{V_r[P][Q]}{K_PK_{IQ}}}{1 + \dfrac{[A]}{K_{IA}} + \dfrac{[A][B]}{K_{IA}K_B} + \dfrac{[Q]}{K_{IQ}} + \dfrac{[P][Q]}{K_{IQ}K_P}} \tag{8}$$

This is a new rate equation because of the 10^{-pH}. This factor is a result of the consumption of one hydrogen ion in the rate-determining reaction. I use the symbol V_{fexp} in the second form of the rate equation because V_{fexp} is the property obtained in making a Lineweaver-Burk plot at a specified pH. Equation 8 shows that the experimental limiting velocity in the forward direction is $V_{fexp} = 10^{-pH}V_f$, where

$$V_f = \frac{k_f[E]_t}{1 + 10^{pH - pK1EAB} + 10^{pK2EAB - pH}} \tag{9}$$

Note $pK_1 > pK_2$. V_{fexp} and the other kinetic parameters can be obtained from rate measurements, but V_f has to be calculated using $V_f = 10^{pH} V_{fexp}$ for this mechanism.

According to this mechanism, V_f yields a bell-shaped plot, but V_{fexp} increases as the pH is reduced, as shown by $V_{fexp} = 10^{-pH} V_f$. Mechanism 3 to 7 can be generalized by replacing 10^{-pH} with 10^{npH} so that one, or more hydrogen ions can be consumed in the rate-determining reaction.

Use of Mathematica to Derive Rapid-equilibrium Rate Equations

Mathematica® [7] is very useful for deriving equations for various kinetic properties and making plots and tables. An example of a program is **derordAB** (see Appendix) for the forward reaction for the ordered mechanism of A + B → *products* when hydrogen ions are consumed in the rate-determining reaction [8]. This program is for a reaction like alcohol dehydrogenase where none of the reactants have p*K*s in the range pH 5 – 9. The required input is specified, and the program derives the expressions for V_{fexp}, V_f, K_{IA}, and K_B as functions of pH. It also derives the initial velocity v as a function of [A], [B], and the pH. The pH dependencies for V_f/K_B, and $V_f/K_{IA}K_B$ that are also produced by the program are useful for making bell-shaped plots of experimental data for the determination of kinetic parameters.

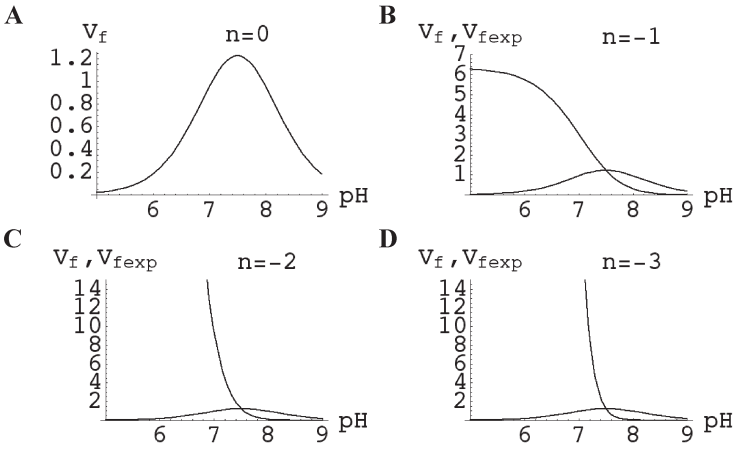

Figure 1. (A) Plot of V_f when $pK_{1EAB} = 8$, $pK_{2EAB} = 7$, $k_f[E]_t = 2$, and $n = 0$. (B) Composite plot of V_f and V_{fexp} when n=-1. (C) Composite plot of V_f and V_{fexp} when n=-2. (D) Composite plot of V_f and V_{fexp} when n=-3.

Figure 1 shows the pH dependencies of V_f and V_{fexp} when n= 0, -1, -2 and -3. V_f produces a bell-shaped plot with pK_{1EAB}= 8 and pK_{2EAB}= 7. The calculation of this figure has used $V_{fexp}= 10^{n(pH-7.5)}V_f$ so that the curves cross at pH 7.5; otherwise the two functions have very different magnitudes. Changing the 7.5 does not alter conclusions about the pH dependence of V_{fexp}.

Equation 8 shows that n can be determined from the initial rate of the forward reaction. If hydrogen ions are produced in the rate determining reaction, they do not affect the rate equation for the initial forward velocity.

The Haldane equation obtained from equation 8 is

$$K' = \frac{[P][Q]}{[A][B]} = \frac{10^{-pH}V_f K_P K_{IQ}}{V_r K_{IA} K_B} = \frac{V_{fexp} K_P K_{IQ}}{V_r K_{IA} K_B}$$
$$= \frac{K_{ref}10^{-pH}(1+10^{pH-pK1P}+10^{pK2P-pH})(1+10^{pH-pK1Q}+10^{pK2Q-pH})}{(1+10^{pH-pK1A}+10^{pK2A-pH})(1+10^{pH-pK1B}+10^{pK2B-pH})} \quad (10)$$

This shows the form of f(pH) that occurs in equation 1. It also shows that for this mechanism n=-1. This is a new type of Haldane equation. It can be generalized by replacing 10^{-pH} with 10^{npH}. The Haldane equation yields an expression for K' that is of the form of K' $=K_{ref}10^{npH}f$(pH).

When V_{fexp}, V_r, K_{IA}, K_B, K_P, and K_{IQ} are determined using the initial reaction rates of the forward and reverse reactions at a specified pH, the right value of K' is obtained at each pH. However, to determine the pKs and $k_f[E]_t$ in the expression for V_f, it is necessary to use $V_f= 10^{npH}V_{fexp}$. This is the way that n can be obtained from kinetic measurements.

An Ordered Mechanism for a Type of Oxidoreductase Reaction

The pH effects due to the consumption of hydrogen ions can be very large for some oxidoreductase reactions as, for example, the nitrite-ferredoxin reductase reaction:

$$\text{EC 1.7.7.1 Nitrite + 6ferredoxin}_{red} = \text{ammonia + 6ferredoxin}_{ox} + 2\,H_2O \quad (11)$$

This reaction consumes 8 hydrogen ions (4 to make ammonia at pHs below about 9 and 4 to make 2 H_2O). Apparent equilibrium constants for this reaction have been calculated using existing tables of standard Gibbs energies of formation of species [9, 10]. The apparent equilibrium constant for reaction 11 decreases extremely rapidly with increasing pH. At pH

5, it is 1.2×10^{91} and at pH 9 it is 1.9×10^{59}. The change in binding of hydrogen ions calculated using $\Delta_r N_H = -d\log K'/dpH$ is essentially 8 across this range of pH. The mechanism for this and related oxidoreductase reactions can be represented by [11]

$$E + mR = ER_m \qquad K^m_{IRm} = \frac{[E][R]^m}{[ER_m]} \tag{12}$$

$$ER_m + O = ER_mO \qquad K_O = \frac{[ER_m][O]}{[ER_mO]} \tag{13}$$

$$ER_mO \rightarrow \text{products} \tag{14}$$

where R is the reductant and O is the oxidant. Equal signs indicate equilibria that are adjusted rapidly, and so the Michaelis constants K_{IRm} and K_O are apparent equilibrium constants that are functions of pH. The Michaelis constant K_{IRm} is used, rather than K^m_{IRm} because it has the units of a concentration. The rapid-equilibrium rate equation for the forward reaction is

$$v = \frac{V_{fexp}}{1 + \frac{K_O}{[O]} + \frac{K^m_{IRm} K_O}{[R]^m [O]}} = \frac{10^{-8pH} V_f}{1 + \frac{K_O}{[O]} + \frac{K^m_{IRm} K_O}{[R]^m [O]}} \tag{15}$$

Note that the pH factor is 10^{-40} at pH 5 and 10^{-72} at pH 9. This sensitivity to pH will make it almost impossible to determine the kinetic parameters for reaction 11.

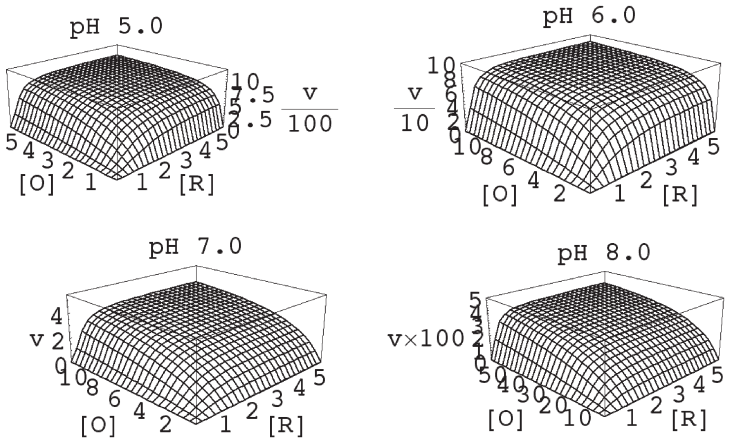

Figure 2. Three-dimensional plots of initial reaction velocities for O + 2R → *products* versus reactant concentrations at specified pHs for arbitrary constants. For this ordered mechanism n is -2 and m=2. The scale of the ordinate has been changed by 10^4 between pH 5 and pH 9. Each plot is made up of 20 Michaelis plots at constant [A] and 20 Michaelis plots at constant [B].

Mathematica® is very useful for displaying initial reaction velocities as functions of the reactant concentrations and the pH. A computer program was written to derive the initial rate equation for mechanism 12 – 14. The chemical equilibrium constants and the value of *n* have to be specified. This rate equation can be treated like an actual reaction system in the sense that the initial velocity *v* can be calculated at various [R], [O], and pH. Using Mathematica®, *v* can be presented as a surface in a 3-dimensional plot at a specific pH [11]. Figure 2 shows these plots for the catalyzed reaction O + 2R → *products* as functions of [O] and [R] at four pHs, for *n*=-2 and arbitrary p*K*s and other constants.

Note that the plots of *v* versus [R] are sigmoid. A sigmoid plot of *v* versus the concentration of a reactant is usually taken to be an indication of allosterism. This can arise when there is positive cooperativity between active sites of a polymeric enzyme. But a sigmoid plot can have other origins. In this case, it is caused by the stoichiometric number of R in the biochemical equation for the forward reaction O + 2R → *products*.

Three-dimensional Lineweaver-Burk Plots for the Forward Reaction Ordered A + B

Mathematica® can also be used to plot reciprocal velocities 1/*v* versus 1/[A] and 1/[B]. This is illustrated for the ordered mechanism for A + B → *products* [12]. These plots can be called three-dimensional Lineweaver-Burk plots. These plots can be made for various pHs and for *n*= 0, -1, -2, ... They are given in Figure 3 only for arbitrary constants and pHs 5, 6, 7, and 8, with *n*=-1.

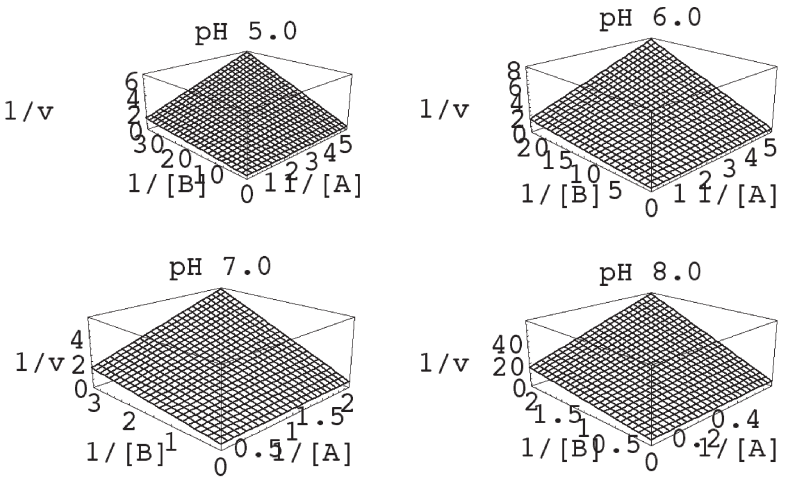

Figure 3. Reciprocal initial velocities for the ordered mechanism for A + B → *products* versus 1/[A] and 1/[B] at pHs 5, 6, 7, and 8 for *n*=-1 and arbitrary constants.

The plots in Figure 3 are referred to in mathematics as ruled surfaces, and quite a bit has been written about ruled surfaces. Each of the surfaces in Figure 3 is characterized by three constants, the experimental limiting velocity V_{fexp}, and Michaelis constants K_{IA} and K_B at the specified pH. These surfaces are not planes, but the lines are all straight. When I first made one of these plots, I recognized that each of these surfaces is really determined by the ordinates of three of the corners: one corner can be taken a reference because the concentration of the enzyme is arbitrary. And the velocities depend on three kinetic constants. Therefore, you should be able to calculate the three kinetic constants from three velocity measurements.

webMathematica

webMathematica provides a way to take advantage of the computing power of Mathematica® to perform calculated calculations without having Mathematica® in your computer or knowing how to use it. An example of such a calculation is the calculation of the apparent equilibrium constant *K*' for an enzyme-catalyzed reaction at a desired temperature, pH, and ionic strength. This requires a database of species properties. BasicBiochemData3 [13, 14] provides this data. WebMathematics presents a screen with boxes for inputting the enzyme-catalyzed reaction, desired temperature, pH, and ionic strength. This information is sent to a server that has Mathematica® and a database on it so the server can calculate the apparent equilibrium constant (or other thermodynamic property) and present it on the screen of the user.

webMathematica programming requires knowledge of Mathematica® and HTML, and it has been done by Dr. Violeta Ivanova of MIT's Office of Educational Innovation and Technology. This program can be extended to calculate the change in binding of hydrogen ions in an enzyme-catalyzed reaction. BasicBiochemData3 [13, 14] makes it possible to calculate these properties for about 300 different reactions at 298.15 K. For about 100 reactions these calculations can be made at other temperatures in the range 273.15 K to about 313.15 K. webMathematica can be used to calculate other properties in biochemical thermodynamics and enzyme kinetics.

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Appendix

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derordAB[pK1e_,pK2e_,pK1ea_,pK2ea_,pK1eab_,pK2eab_,kfEt_,n_,kHEA_,kHEAB_]:=
Module[{eafactor,efactor,abfactor,vf,vfexp,kia,kb,v},(*Calculates kinetic parameters of the
forward enzyme-catalyzed reaction ordered A+B = products as functions of pH. The output
is a list of 6 functions of pH: vfexp,vf,kia,kb,vf/kb, and vf/kiakb. v is a function of [A]= a,
[B]= b, and pH. The 7.5 in (pH-7.5) can be changed because it is equivalent to changing the
value of kfEt.*)
efactor = 1+10^(pK2e-pH)+10^(pH-pK1e);
eafactor = 1+10^(pK2ea-pH)+10^(pH-pK1ea);
eabfactor = 1+10^(pK2eab-pH)+10^(pH-pK1eab);
vf = kfEt/eabfactor;
vfexp =(10^(n*(pH-7.5)))*vf;
kia = kHEA*efactor/eafactor;
kb = kHEAB*eafactor/eabfactor;
v = vfexp/(1+(kb/b)*(1+(kia/a)));
{vfexp,vf,kia,kb,vf/kb,vf/(kia*kb),v}]
```

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