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Three mechanisms and rapid-equilibrium rate equations for a type of reductase reaction

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Abstract

Rapid-equilibrium rate equations for enzyme-catalyzed reactions are especially useful when the mechanism involves a number of p*K*s, but they are also useful when some reactants have stoichiometric numbers greater than one or hydrogen ions are produced or consumed in the rate-determining step. The pH dependencies of limiting velocities, Michaelis constants, and reaction velocities for the forward reaction are discussed for two examples of reductase reactions of the type *m*R + O -> products, where R is the reductant and O is the oxidant. For the nitrate reductase reaction (EC 1.9.6.1), $m = 2$ and two hydrogen ions are consumed. For the nitrite–ferredoxin reductase reaction (EC 1.7.7.1), $m = 6$ and eight hydrogen ions are consumed. The expressions for the limiting velocities, Michaelis constants, and rate equations for the forward reaction are derived for two ordered mechanisms and the random mechanism. Three Mathematica® programs are used to make plots of kinetic parameters as functions of pH and three-dimensional plots of rapid-equilibrium velocities as functions of [O] and [R] for arbitrary sets of input parameters.

Keywords: Apparent equilibrium constants; Enzyme kinetics; Rapid-equilibrium rate equations; pH effects in kinetics; Change in binding of hydrogen ions; Reductase reactions

1. Introduction

Rapid-equilibrium rate equations are especially useful for mechanisms of enzyme-catalyzed reactions that involve a large number of p*K*s, but they are also useful for reactions with large productions or consumptions of hydrogen ions and reactions involving reactants with stoichiometric numbers greater than one. Examples of reactions of this type are

$$\text{EC } 1.7.1.4 \text{ Nitrite} + 3\text{NADP}_{\text{red}} = \text{ammonia} + 3\text{NADP}_{\text{ox}} + 2\text{H}_2\text{O} \quad (1)$$

$$\text{EC } 1.7.2.2 \text{ Nitrite} + 6\text{cytochromec}_{\text{red}} = \text{ammonia} + 6\text{cytochromec}_{\text{ox}} + 2\text{H}_2\text{O} \quad (2)$$

$$\text{EC } 1.7.7.1 \text{ Nitrite} + 6\text{ferredoxin}_{\text{red}} = \text{ammonia} + 6\text{ferredoxin}_{\text{ox}} + 2\text{H}_2\text{O} \quad (3)$$

$$\text{EC } 1.8.1.2 \text{ Sulfite} + 3\text{NADP}_{\text{red}} = \text{H}_2\text{S(aq)} + 3\text{NADP}_{\text{ox}} + 3\text{H}_2\text{O} \quad (4)$$

$$\text{EC } 1.9.6.1 \text{ Nitrate} + 2\text{cytochromec}_{\text{red}} = \text{nitrite} + 2\text{cytochromec}_{\text{ox}} + \text{H}_2\text{O} \quad (5)$$

These reactions all have large apparent equilibrium constants in the pH range 5 to 9 [1], and so only the kinetics of forward reactions are discussed here. These forward reactions are described by O + *m*R -> products, where O is the oxidant, R is the reductant, and *m* is the number of reductant molecules required to reduce the oxidant. The kinetics of these reactions have not been studied as far as I know, perhaps because of the high stoichiometric numbers of the reductants.

The apparent equilibrium constants K' of enzyme-catalyzed reactions can be represented by $K' = K_{\text{ref}}10^{n\text{pH}}f(\text{pH})$, where K_{ref} is the equilibrium constant for a reference chemical reaction, n is the number of hydrogen ions produced in a reference reaction, and $f(\text{pH})$ is the function of pH that brings in the p*K*s of the reactants [2]. The number n of hydrogen ions is positive when they are produced in the reference reaction and is negative

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when they are consumed in the reference reaction. When n is positive, the exponential term $10^{n\mathrm{pH}}$ in K' causes K' to increase with increasing pH. When n is negative, K' decreases with increasing pH, as is the case for all the enzyme-catalyzed reactions discussed here.

In biochemical thermodynamics, the choice of a reference reaction is arbitrary, but the change in binding of hydrogen ions $\Delta_r N_H$ in an enzyme-catalyzed reaction at a specified pH is not arbitrary because $\Delta_r N_H = -\mathrm{d}\log K'/\mathrm{dpH}$ [1,3–5]. The $\Delta_r N_H$ for these five reactions are all in the range 2–8, and are nearly independent of pH. Kinetic measurements can be used to determine n, even though thermodynamic measurements cannot because biochemical thermodynamic properties are independent of the catalytic mechanism.

Rapid-equilibrium rate equations are derived for the forward reactions O + mR -> products for three types of mechanisms in which the enzymatic site and enzyme-substrate complexes are assumed to have two pKs, but O and R are assumed not to have pKs in the range pH 5–9. Since the equations for the pH dependencies of the limiting velocities and Michaelis constants are quite complicated, the rate equations that include the effects of pH are quite large; and so, a Mathematica® program is used to make plots with specified values of pKs and chemical equilibrium constants. These calculations are shown only for EC 1.9.6.1 and EC 1.7.7.1, for which apparent equilibrium constants K' and changes in binding of hydrogen ions $\Delta_r N_H$ at 298.15 K and 0.25 M ionic strength are given in Table 1. These values have been calculated from the standard Gibbs energies of formation of the species involved [1,3–5]. It is the change in binding of hydrogen ions in the biochemical reaction that causes the apparent equilibrium constant to change with the pH.

These reductase reactions are especially interesting because they involve two kinds of pH effects and because the stoichiometric numbers of the reductants are greater than one. Three types of mechanisms are considered and rapid-equilibrium rate equations including pH effects are derived.

2. Ordered mechanism with *m*R binding to the enzymatic site first (Mechanism I)

The mechanism for the derivation of the rapid-equilibrium rate equation for the forward reaction is

$$\mathrm{E} + m\mathrm{R} \;=\; \mathrm{ER}_m \quad K^m_{\mathrm{IR}m} = \frac{[\mathrm{E}][\mathrm{R}]^m}{[\mathrm{ER}_m]} \tag{6}$$

$$\mathrm{ER}_m + \mathrm{O} = \mathrm{ER}_m\mathrm{O} \quad K_\mathrm{O} = \frac{[\mathrm{ER}_m][\mathrm{O}]}{[\mathrm{ER}_m\mathrm{O}]} \tag{7}$$

$$\mathrm{ER}_m\mathrm{O} -> \text{ products} \tag{8}$$

Equal signs indicate that equilibria are adjusted rapidly, and so the Michaelis constants $K_{\mathrm{IR}m}$ and K_O are equilibrium constants that are functions of pH. The Michaelis constant

Table 1
Apparent equilibrium constants K' and changes in binding of hydrogen ions $\Delta_r N_H$ for EC 1.9.6.1 and EC 1.7.7.1 at 298.15 K and 0.25 M ionic strength [3–6]

Reaction		pH 5	pH 6	pH 7	pH 8	pH 9
EC 1.9.6.1	K'	5.0×10^{10}	5.0×10^{8}	5.0×10^{6}	5.0×10^{4}	5.0×10^{2}
	$\Delta_r N_H$	2.01	2.00	2.00	2.00	2.00
EC 1.7.7.1	K'	1.2×10^{91}	1.2×10^{83}	1.2×10^{75}	1.3×10^{67}	1.9×10^{59}
	$\Delta_r N_H$	7.99	8.00	7.99	7.94	7.64

$K_{\mathrm{IR}m}$ is used because it has the units of a concentration. The rapid-equilibrium rate equation for the forward reaction is

$$v = \frac{V_{\mathrm{fexp}}}{1 + \dfrac{K_\mathrm{O}}{[\mathrm{O}]} + \dfrac{K^m_{\mathrm{IR}m}K_\mathrm{O}}{[\mathrm{R}]^m[\mathrm{O}]}} \tag{9}$$

The experimental limiting velocity in the forward direction is represented by V_{fexp} for reasons that will be given later (see Eqs. (15) and (16)).

None of the reactants on the left sides of EC 1.9.6.1 and EC 1.7.7.1 involve pKs in the pH 5–9 range, but possible pKs of the enzymatic site and enzyme-substrate complexes have to be taken into account in deriving the equation for the initial velocity v in the forward reaction that is a function of pH. In addition, the rate-determining reaction must deal with the consumption of hydrogen ions that is indicated by $\Delta_r N_H$ and the chemical reactions

$$\mathrm{NO_3^-} + 2\mathrm{Fe^{2+}} + 2\mathrm{H^+} = \mathrm{NO_2^-} + 2\mathrm{Fe^{3+}} + \mathrm{H_2O} \tag{10}$$

$$\mathrm{NO_2^-} + 6\mathrm{Fd_{red}}(z=0) + 8\mathrm{H^+} = \mathrm{NH_4^+} + 6\mathrm{Fd_{ox}}(z=+1) + 2\mathrm{H_2O} \tag{11}$$

$\mathrm{Fe^{2+}}$ and $\mathrm{Fe^{3+}}$ are used as abbreviations for cytochromec_{red} and cytochromec_{ox}. In treating the kinetics, hydrogen ions may be consumed in the rate-determining reaction; if they are consumed in other reactions in the mechanism, their effect will depend on the competition between reactions prior to the rate-determining reaction. The effects of hydrogen ions consumed in the rate-determining reaction extend over the whole pH range.

The following extension of mechanism (6)–(8) brings in two pKs each for E, ER_m, and $\mathrm{ER}_m\mathrm{O}$, and two chemical equilibrium constants (that is, reactions written in terms of species):

$$\begin{array}{ccc} \mathrm{E^-} & & \mathrm{ER}_m^- \\ \| & & \| \\ \mathrm{HE} & + m\mathrm{R} = & \mathrm{HER}_m \\ \| & & \| \\ \mathrm{H_2E^+} & & \mathrm{H_2ER}_m^+ \end{array} \quad K^m_{\mathrm{HER}m} = \frac{[\mathrm{HE}][\mathrm{R}]^m}{[\mathrm{HER}_m]} \tag{12}$$

$$\begin{array}{ccc} \mathrm{ER}_m^- & & \mathrm{ER}_m\mathrm{O}^- \\ \| & & \| \\ \mathrm{HER}_m & + \mathrm{O} = & \mathrm{HER}_m\mathrm{O} \\ \| & & \| \\ \mathrm{H_2ER}_m^+ & & \mathrm{H_2ER}_m\mathrm{O}^+ \end{array} \quad K_{\mathrm{HER}m\mathrm{O}} = \frac{[\mathrm{HER}_m][\mathrm{O}]^m}{[\mathrm{HER}_m\mathrm{O}]} \tag{13}$$

$$\begin{array}{ccc} & & \mathrm{ER}_m\mathrm{O}^- \\ & & \| \quad k_f \\ |n|\mathrm{H^+} & = & \mathrm{HER}_m\mathrm{O} -> \text{ products} \\ & & \| \\ & & \mathrm{H_2ER}_m\mathrm{O}^+ \end{array} \tag{14}$$

k_f is the rate constant for the rate-determining reaction. Since hydrogen ions are consumed, n is negative, and it is necessary to use absolute value signs in Eq. (14). These definitions for the chemical equilibrium constants are used so that $K_{\mathrm{HER}m}$ and $K_{\mathrm{HER}m\mathrm{O}}$ have units of concentrations. The two chemical equilibrium expressions in Eqs. (12) and (13) are always obeyed. It is assumed that O and R do not have pKs in the pH range of interest.

When $n = 0$, the limiting velocity of the forward reaction is given by

$$V_f = \frac{k_f[\mathrm{E}]_t}{1 + 10^{\mathrm{pH}-\mathrm{pK1ERmO}} + 10^{-\mathrm{pH}+\mathrm{pK2ERmO}}} \tag{15}$$

Note that $\mathrm{p}K_1 > \mathrm{p}K_2$. When n is not equal to zero, k_f in the rate equation is replaced with $k_f[\mathrm{H}^+]^{-n} = k_f 10^{n\mathrm{pH}}$. Thus V_{fexp} in Eq. (9) is equal to $10^{n\mathrm{pH}}V_f$ so that

$$V_{\mathrm{fexp}} = \frac{k_f 10^{n\mathrm{pH}}[\mathrm{E}]_t}{1 + 10^{\mathrm{pH}-\mathrm{pK1ERmO}} + 10^{-\mathrm{pH}+\mathrm{pK2ERmO}}} = 10^{n\mathrm{pH}}V_f \tag{16}$$

Both V_f and V_{fexp} are needed when $n \neq 0$.

In view of the importance of Eq. (16), it is worth reviewing the statement in the paragraph after Eq. (5) that when n is negative (that is when hydrogen ions are consumed), $K' = K_{\mathrm{ref}}10^{n\mathrm{pH}}\,f(\mathrm{pH})$ decreases with increasing pH. The equation $V_{\mathrm{fexp}} = 10^{n\mathrm{pH}}V_f$ shows that when n is negative, V_{fexp} decreases with increasing pH.

The initial velocity of the forward reaction (see Eq. (9)) is given by

$$v = \frac{V_{\mathrm{fexp}}}{1+\frac{K_\mathrm{O}}{[\mathrm{O}]}+\frac{K_\mathrm{O}K^m_{\mathrm{IR}m}}{[\mathrm{O}][\mathrm{R}]^m}} = \frac{10^{n\mathrm{pH}}V_f}{1+\frac{K_\mathrm{O}}{[\mathrm{O}]}\left(1+\frac{K^m_{\mathrm{IR}m}}{[\mathrm{R}]^m}\right)} \tag{17}$$

The determination of the terms in the denominator of a rate equation using Lineweaver-Burk plots has been discussed many times in the literature, but an especially clear explanation is given by Cook and Cleland [6].

The pH dependencies of the two Michaelis constants are given by

$$K^m_{\mathrm{IR}m} = \frac{K^m_{\mathrm{HER}m}\left(1 + 10^{\mathrm{pH}-\mathrm{pK1E}} + 10^{-\mathrm{pH}+\mathrm{pK2E}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{pK1ER}m} + 10^{-\mathrm{pH}+\mathrm{pK2ER}m}\right)} \tag{18}$$

$$K_\mathrm{O} = \frac{K_{\mathrm{HER}m\mathrm{O}}\left(1 + 10^{\mathrm{pH}-\mathrm{pK1ER}m} + 10^{-\mathrm{pH}+\mathrm{pK2ER}m}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{pK1ER}m\mathrm{O}} + 10^{-\mathrm{pH}+\mathrm{pK2ER}m\mathrm{O}}\right)} \tag{19}$$

V_{fexp} can be determined using Lineweaver-Burk plots of Eq. (9), and V_f can be calculated using $V_f = 10^{-\,n\mathrm{pH}}V_{\mathrm{fexp}}$. V_f can be used to calculate $\mathrm{p}K_{1\mathrm{ER}m\mathrm{O}}$, $\mathrm{p}K_{2\mathrm{ER}m\mathrm{O}}$ and $k_f\,[\mathrm{E}]_t$ from experimental data using Eq. (15) because it is convenient to determine these three parameters from bell-shaped plots.

Since the pH dependencies for K_O and $K^m_{\mathrm{IR}m}$ each involve five unknowns, it is convenient to determine the pKs and chemical equilibrium constants in Eqs. (18) and (19) by using plots of V_f/K_O and $V_f/K_\mathrm{O}K^m_{\mathrm{IR}m}$ versus pH as indicated by the following two equations:

$$\frac{V_f}{K_\mathrm{O}} = \frac{k_f[\mathrm{E}]_t}{K_{\mathrm{HER}m\mathrm{O}}\left(1 + 10^{\mathrm{pH}-\mathrm{pK1ER}m} + 10^{-\mathrm{pH}+\mathrm{pK2ER}m}\right)} \tag{20}$$

$$\frac{V_f}{K_\mathrm{O}K^m_{\mathrm{IR}m}} = \frac{k_f[\mathrm{E}]_t}{K_{\mathrm{HER}m\mathrm{O}}K^m_{\mathrm{HER}m}\left(1 + 10^{\mathrm{pH}-\mathrm{pK1E}} + 10^{-\mathrm{pH}+\mathrm{pK2E}}\right)} \tag{21}$$

The equation for the initial velocity of the forward reaction v as a function of [O], [R], and pH is not given here, but it can be obtained by substituting the expressions for V_{fexp}, K_O, and $K_{\mathrm{IR}m}$ in Eq. (9).

3. Ordered mechanism with O binding to the enzymatic site first (Mechanism II)

The mechanism for the derivation of the rapid-equilibrium rate equation for the forward reaction is given by

$$\mathrm{E} + \mathrm{O} = \mathrm{EO} \qquad K_\mathrm{IO} = \frac{[\mathrm{E}][\mathrm{O}]}{[\mathrm{EO}]} \tag{22}$$

$$\mathrm{EO} + m\mathrm{R} = \mathrm{ER}_m\mathrm{O} \qquad K^m_{\mathrm{R}m} = \frac{[\mathrm{EO}][\mathrm{R}]^m}{[\mathrm{EOR}_m]} \tag{23}$$

$$\mathrm{ER}m\mathrm{O} -> \ \text{products} \tag{24}$$

The initial velocity of the forward reaction is given by

$$v = \frac{V_{\mathrm{fexp}}}{1 + \frac{K^m_{\mathrm{R}m}}{[\mathrm{R}]^m} + \frac{K^m_{\mathrm{R}m}K_\mathrm{IO}}{[\mathrm{R}]^m[\mathrm{O}]}} = \frac{10^{n\mathrm{pH}}V_f}{1 + \frac{K^m_{\mathrm{R}m}}{[\mathrm{R}]^m}\left(1 + \frac{K_\mathrm{IO}}{[\mathrm{O}]}\right)} \tag{25}$$

The following extension of mechanism (22)–(24) brings in two pKs each for E, EO, and $\mathrm{ER}_m\mathrm{O}$, and two chemical equilibrium constants:

$$\begin{array}{ccc} \mathrm{E}^- & & \mathrm{EO}^- \\ \| & & \| \\ \mathrm{HE} & + \mathrm{O} = & \mathrm{HEO} \\ \| & & \| \\ \mathrm{H_2E}^+ & & \mathrm{H_2EO}^+ \end{array} \qquad K_\mathrm{HEO} = \frac{[\mathrm{HE}][\mathrm{O}]}{[\mathrm{HEO}]} \tag{26}$$

$$\begin{array}{ccc} \mathrm{EO}^- & & \mathrm{ER}_m\mathrm{O}^- \\ \| & & \| \\ \mathrm{HEO} & + m\mathrm{R} = & \mathrm{HER}_m\mathrm{O} \\ \| & & \| \\ \mathrm{H_2EO}^+ & & \mathrm{H_2ER}_m\mathrm{O}^+ \end{array} \qquad K^m_{\mathrm{HER}_{m\mathrm{O}}} = \frac{[\mathrm{HEO}][\mathrm{R}]^m}{[\mathrm{HEOR}_m]} \tag{27}$$

$$\begin{array}{ccc} & & \mathrm{ER}_m\mathrm{O}^- \\ & & \| \qquad\quad k_f \\ |n|\mathrm{H}^+ & + & \mathrm{HER}_m\mathrm{O} -> \ \text{products} \\ & & \| \\ & & \mathrm{H_2ER}_m\mathrm{O}^+ \end{array} \tag{28}$$

The pH dependence of V_f is given by Eq. (15). The pH dependencies of the two Michaelis constants are given by

$$K_{IO} = \frac{K_{HEO}\left(1 + 10^{pH-pK1E} + 10^{-pH+pK2E}\right)}{\left(1 + 10^{pH-pK1EO} + 10^{-pH+pK2EO}\right)} \tag{29}$$

$$K_{Rm}^{m} = \frac{K_{HERmO}^{m}\left(1 + 10^{pH-pK1EO} + 10^{-pH+pK2EO}\right)}{\left(1 + 10^{pH-pK1ERmO} + 10^{-pH+pK2ERmO}\right)} \tag{30}$$

Since the pH dependencies for K_{IO} and K_{Rm}^{m} each involve five unknowns, it is convenient to determine the kinetic parameters in Eqs. (29) and (30) by using plots of V_f/K_{Rm}^{m} and $V_f/K_{IO}K_{Rm}^{m}$ versus pH, as indicated by the following two equations:

$$\frac{V_f}{K_{Rm}^{m}} = \frac{k_f[E]_t}{K_{HERmO}^{m}\left(1 + 10^{pH-pK1EO} + 10^{-pH+pK2EO}\right)} \tag{31}$$

$$\frac{V_f}{K_{IO}K_{Rm}^{m}} = \frac{k_f[E]_t}{K_{HEO}K_{HERmO}^{m}\left(1 + 10^{pH-pK1E} + 10^{-pH+pK2E}\right)} \tag{32}$$

The equation for the initial velocity of the forward reaction v as a function of [O], [R], and pH is not given here, but it can be obtained by substituting the expressions for V_{fexp}, K_{IO}, and K_{Rm} in Eq. (25).

4. Random mechanism (Mechanism III)

In the random mechanism, there are two possible paths to ER_mO.

Path 1:

$$E + mR = ER_m \qquad K_{IRm}^{m} = \frac{[E][R]^m}{[ER_m]} \tag{33}$$

$$ER_m + O = ER_mO \qquad K_O = \frac{[ER_m][O]}{[ER_mO]} \tag{34}$$

Path 2:

$$E + O = EO \qquad K_{IO} = \frac{[E][O]}{[EO]} \tag{35}$$

$$EO + mR = ER_mO \qquad K_{Rm}^{m} = \frac{[EO][R]^m}{[ER_mO]} \tag{36}$$

But these four equilibrium constants are not independent: $K_{IRm}^{m}K_O = K_{Rm}^{m}K_{IO}$. Since they are not independent, it is necessary to omit one of these four reactions from the derivation of the rate equation. Here, the reaction EO + mR = ER_mO is arbitrarily omitted. Thus the random mechanism is taken to be

$$E + mR = ER_m \qquad K_{IRm}^{m} = \frac{[E][R]^m}{[ER_m]} \tag{37}$$

$$E + O = EO \qquad K_{IO} = \frac{[E][O]}{[EO]} \tag{38}$$

$$ER_m + O = ER_mO \qquad K_O = \frac{[ER_m][O]}{[ER_mO]} \tag{39}$$

$$ER_mO -> \text{ products} \tag{40}$$

This random mechanism yields the following equation for the initial velocity v of the forward reaction:

$$v = \frac{V_{fexp}}{1 + \frac{K_O}{[O]} + \frac{K_O K_{IRm}^{m}}{K_{IO}[R]^m} + \frac{K_{IRm}^{m}K_O}{[R]^m[O]}} = \frac{10^{npH}V_f}{1 + \frac{K_O}{[O]} + \frac{K_O K_{IRm}^{m}}{K_{IO}[R]^m}\left(1 + \frac{K_{IO}}{[O]}\right)} \tag{41}$$

In contrast with Mchanisms I and II, this mechanism leads to four terms in the denominator. O and R play similar roles, and so it is not possible to tell which binds first from experimental data, but it is possible to tell that the mechanism is random. To calculate initial velocities with this equation it is necessary to have values for K_O, K_{IO}, and K_{IRm}.

It might appear that the reaction EO + mR = ER_mO should be added to this mechanism, but in thermodynamics this is a redundant reaction, and it is thermodynamics that is used to calculate the equilibrium composition prior to the rate-determining reaction. Stated another way, there should be a single path to each enzyme-substrate complex in the calculation of the equilibrium distribution of E, ER_m, EO, and ER_mO.

Eq. (41) can be compared with equations in Cook and Cleland [6] for the initial rapid-equilibrium velocity of the reverse reaction of A = P + Q and the forward reaction A + B = products. Their Eqs. (5)–(34) for the initial rate of the forward reaction for A + B = products is

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{IA}K_B}{[A][B]}} \tag{42}$$

This equation involves the parameters V, K_A, K_B, and K_{IA}. This equation looks different from Eq. (41), but $K_{IRm}^{m}K_O = K_{Rm}^{m}K_{IO}$ can be used to change Eq. (41) to

$$v = \frac{V_{fexp}}{1 + \frac{K_O}{[O]} + \frac{K_{Rm}^{m}}{[R]^m} + \frac{K_{IRm}^{m}K_O}{[R]^m[O]}} \tag{43}$$

This equation is similar to Eq. (42). To calculate initial velocities with this equation it is necessary to have values for K_O, K_{Rm}, and K_{IRm}.

When the kinetic parameters in Eq. (41) have been determined experimentally, V_f, K_{IRm}^{m}, K_{IO}, and K_O are given as functions of

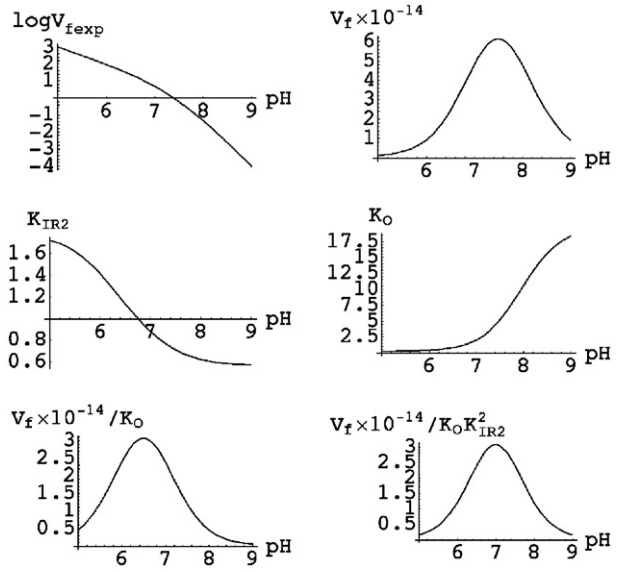

Fig. 1. Plots of pH dependencies of kinetic parameters when $m=2$ and $n=-2$, as in EC 1.9.6.1, with Mechanism I. The second plot yields pK_{1ER2O}, pK_{2ER2O}, and $k_f[E]_t$. The fifth plot yields pK_{1ER2}, pK_{2ER2}, and K_{HER2O}. The sixth plot yields pK_{1E}, pK_{2E}, and K_{HER2}. The input for **calcpropsRmO** is given in Table A. $k_f[E]_t$ has to have a very large value so that V_{fexp} has a value of the order of 1 mM/min at pH 7.5.

pH by Eqs. (15), (18), (29), and (19). Since the equations for K^m_{IRm}, K_{IO}, and K_O each involve five constants, it is convenient to make plots of V_f/K_O, $V_f/K_O K^m_{IRm}$, and $V_f K_{IO}/K_O K^m_{IRm}$ versus pH because three constants can be calculated from each. The first two of these functions of pH are given in Eqs. (20) and (21). The third function is given by

$$\frac{V_f K_{IO}}{K_O K^m_{IRm}} = \frac{k_f[E]_t K_{HEO}}{K_{HERmO} K^m_{H2ERm}\left(1 + 10^{pH-pK1EO} + 10^{-pH+pK2EO}\right)} \quad (45)$$

A plot of this function versus pH yields K_{HEO}, pK_{1EO}, and pK_{2EO}.

Since the reaction EO + mR = ER$_m$O was omitted arbitrarily, any one of the other three equilibrium constant expressions could have been omitted instead. This will lead to rate equations with different forms, but all these forms will yield the same [ER$_m$O] and the same dependence of v on the independent variables [O], [R], and pH. Thermodynamics is very different from kinetics because when the equilibrium composition of a system of reactions is calculated, this composition will satisfy ALL possible equilibrium constant expressions that can be written for the system, not simply the set of equilibrium constant expressions used in the calculation. This is very different from the situation in steady-state kinetics where there can be alternate pathways and where every possible reaction in the mechanism must be included in the rate equation.

5. Mathematica calculations for the type of reaction catalyzed by nitrate reductase by Mechanism I

A Mathematica® program calcpropsRmO (see Appendix) was written to calculate the four kinetic parameters (V_{fexp}, V_f, K_O, and K_{IRm}) for Mechanism I as functions of pH using the eleven input parameters (6 pKs, 2 chemical equilibrium constants, $k_f[E]_t$, m and n). This program also calculates the ratios V_f/K_{RmO} and $V_f/K_O K^m_{IRm}$ that are useful for calculating kinetic parameters from bell-shaped plots of experimental data. This program also derives the initial velocity v for the forward reaction as a function of [R], [O], and pH. For Mechanism I with m = 2 and n = − 2, the nine other input parameters are chosen arbitrarily to calculate the pH dependencies of the six kinetic properties that are shown in Fig. 1. The input parameters and the symbols used in the Mathematica program are summarized in the Appendix in Table 2.

The mathematical function for the initial velocity v according to Mechanism I can be treated like an actual reaction system by calculating v at various [R], [O], and pH. These "data" can be used to calculate the limiting velocities and Michaelis constants

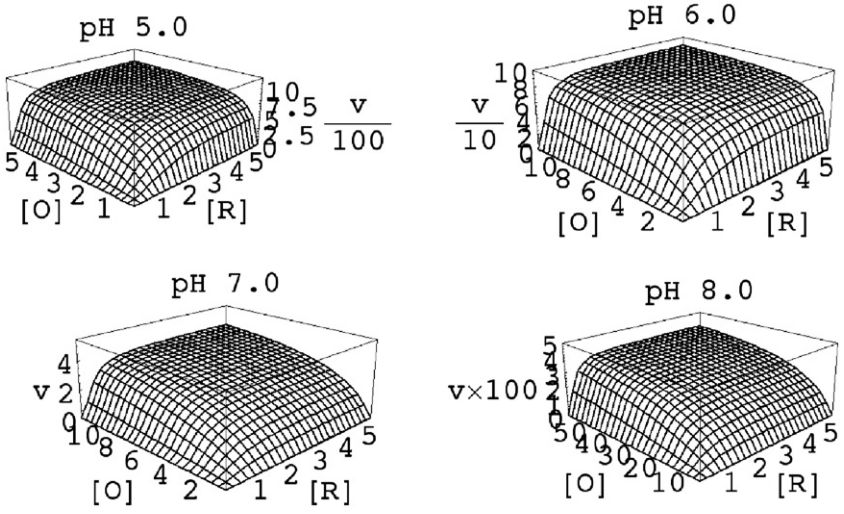

Fig. 2. Plots of initial velocities v of the catalyzed reaction O+2R -> products as functions of [O] and [R] at four pHs. The number n of hydrogen ions produced in the rate-determining reaction for this ordered mechanism is −2 and m=2. Note that plots of v versus [R] at constant [O] are sigmoid.

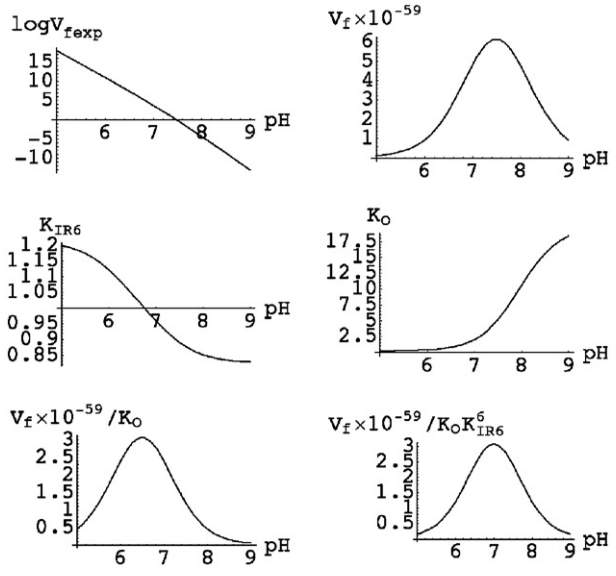

Fig. 3. Plots of pH dependencies of kinetic parameters when $m=6$ and $n=-8$, as in EC 1.7.7.1, for Mechanism I. The plot of $\log V_{fexp}$ is not exactly linear. The second plot yields pK_{1ER6O}, pK_{2ER6O}, and $k_f[E]_t$. The fifth plot yields pK_{1ER6}, pK_{2ER6}, and K_{HER6O}. The sixth plot yields pK_{1E}, pK_{2E}, and K_{ER6}. The input parameters have the same values as in Fig. 1 except that $k_f[E]_t = 10^{(8\times 7.5)}$. $k_f[E]_t$ has to have a very large value so that V_{fexp} has a value of the order of 1 mM/min at pH 7.5.

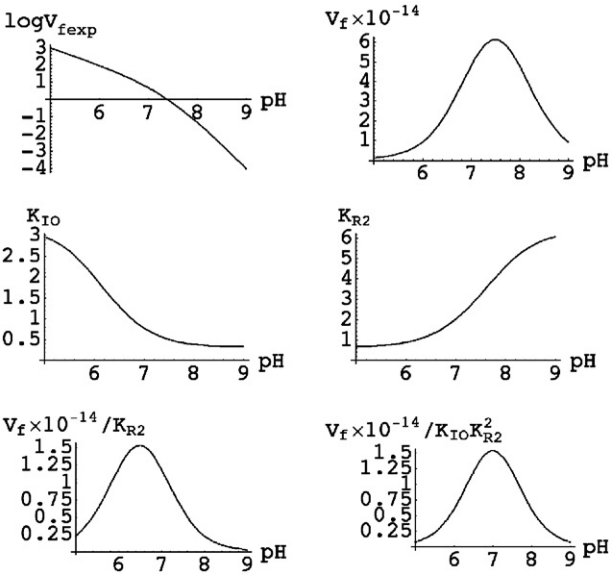

Fig. 5. Plots of pH dependencies of kinetic parameters when $m=2$ and $n=-1$ in Mechanism II, as for EC 1.9.6.1.

as functions of pH by use of Lineweaver–Burk plots. These kinetic parameters can be used to calculate the underlying input constants (pKs, chemical equilibrium constants, and k_f).

Since v is a function of [R] and [O] at a specified pH, it can be presented as a surface in a three-dimensional plot. Fig. 2 shows v as a function of [R] and [O] at four pHs when $m = 2$ and $n = -2$, as in the case for nitrate reductase. These plots show the large effect of the pH on v for given [O] and [R]. This is due to the 10^{-2pH} factor in the rate equation for the forward reaction. The scale of the ordinate changes by 10^4 between pH 5 and pH 8. The plots of v versus [R] at constant [O] have a sigmoid shape. A sigmoid plot of v versus the concentration 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 orig ns. In this case, it is caused by the stoichiometric number of R in the biochemical equation for the forward reaction: O + 2R ->. These plots show that the Michaelis constant for R does not change very much in going from pH 5 to pH 9, as shown in Fig. 2. The Michaelis constant for O increases a lot in going from pH 5 to pH 9. V_{fexp} can be obtained as a function of pH from the rate equation by setting [R] and [O] to high values. V_f is obtained using $V_f = 10^{2pH}V_{fexp}$. V_f is important because its plot versus pH makes it possible to determine the constants in Eq. (15).

Since each measurement of an initial velocity involves the pH dependence of V_{fexp}, it is of interest to calculate the error in

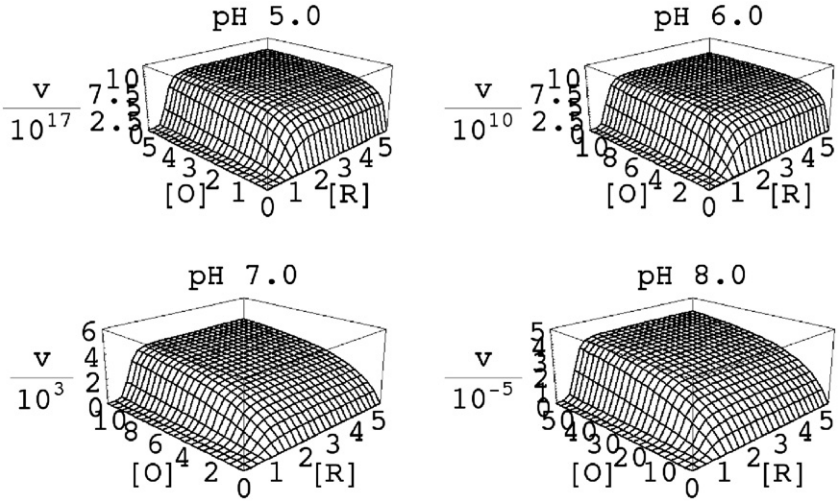

Fig. 4. Plots of initial velocities of the catalyzed reaction O+6R-> as functions of [O] and [R] at six pHs. The number n of hydrogen ions produced in the rate-determining reaction for the ordered mechanism is -8 and $m=6$. There is more sigmoid character in v versus [R] at constant [O] than in Fig. 2.

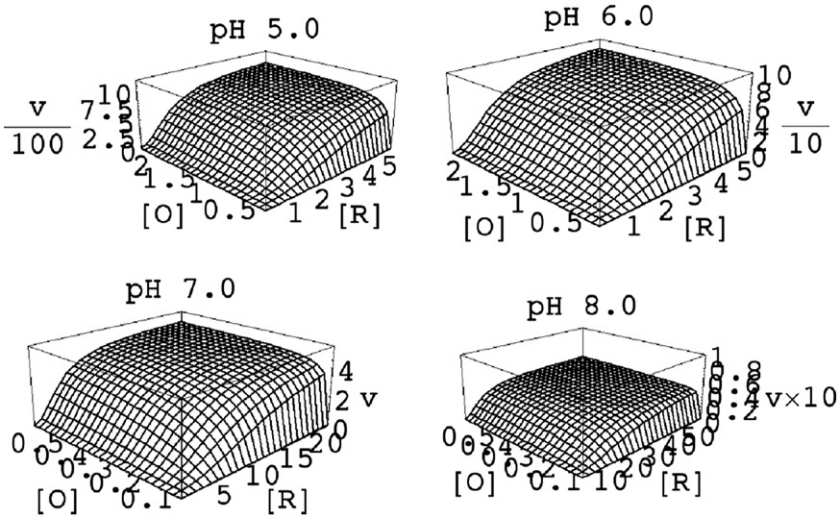

Fig. 6. Plots of initial velocities v of the catalyzed reaction 2R+O-> as functions of [O] and [R] at four pHs for Mechanism II. The number n of hydrogen ions consumed in the rate-determining reaction is −2 and m=2. Note that the plots of v versus [R] at constant [O] are sigmoid.

V_{fexp} caused by an error of 0.10 in the pH for Mechanism 1. The percentage error in V_{fexp} caused by an error of 0.1 in pH for O + 2R -> is 26% at pH 5, 29% at pH 6, 45% at pH 7, 76% at pH 8, 96% at pH 9.

6. Mathematica calculations for the type of reaction catalyzed by nitrite–ferredoxin reductase

The second application of calcpropsRmO is to nitrite–ferredoxin reductase type of reaction. For this biochemical reaction $m=6$ and $n=-8$. According to Mechanism I, the reducing power of six ferredoxin$_{red}$ has to be accumulated at the enzymatic site before nitrite can be reduced to ammonia. The pKs and chemical equilibrium constants are taken to be the same as for the nitrate reductase reaction, but $k_f[E]_t$ is taken to be $10^{8x7.5} = 10^{60}$ so that $k_f[E]_t[H^+]^8$ is of the order of 1 mM/min at pH 7.5. The program calcpropsRmO was used to plot V_{fexp}, V_f, K^6_{IR6}, K_O, V_f/K_O, $V_f/K_OK^6_{IR6}$ as functions of pH for the ferredoxin–nitrite reductase reaction. These six plots are shown in Fig. 3.

An overview of the kinetics of this reaction is given in Fig. 4. The effects of changing the pH by 0.1 are even larger here. Since the limiting velocities can be calculated from the rate equation at high concentrations of O and R, it is possible to calculate V_{fexp} at pHs 5.0 and 5.1, etc. This shows that going from 5.1 to 5.0, raises V_{fexp} by a factor of 5.0. The factors at pHs 6, 7, 8, and 9 are 5.1, 5.8, 7.0, and 8.0. Thus it would not be practical to determine kinetic parameters for this reaction without much better control of pH. The sigmoid character of the plots of v versus [R] at constant [O] is greater than in Fig. 2. The change in scale in the ordinate of Fig. 4 is 10^{22} between pH 5 and pH 8.

7. Mathematica calculations for the type of reaction catalyzed by nitrate reductase by Mechanism II

These calculations have been made with a second Mathematica^R program calcpropsORm that is not given in the Appendix, but involves a couple of changes in the earlier program calcpropsRmO, which is given in the Appendix. The input parameters are given in Table A. The rate equation is given in Eq. (25). Fig. 5 shows the pH dependencies of the kinetic parameters when Mechanism II is used for a reaction of the nitrate reductase type, that is when m = 2 and n = − 2. The first two plots are the same as the first two plots in Fig. 1, as expected. The other four plots are changed.

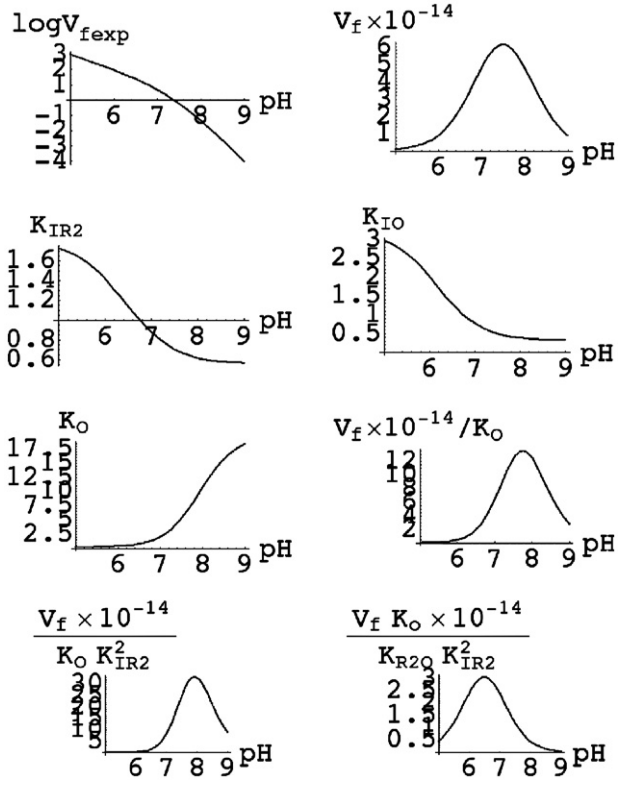

Fig. 7. Plots of dependencies of kinetic parameters for Mechanism III when m=2 and n=−2, as in EC 1.9.6.1. The second plot yields p$K1_{ER2O}$, pK_{2ER2O}, and $k_f[E]_t$. The fifth plot yields pK_{1ER2}, pK_{2ER2}, and K_{HER2O}. The sixth plot yields pK_{1E}, pK_{2E}, and K_{ER2}.

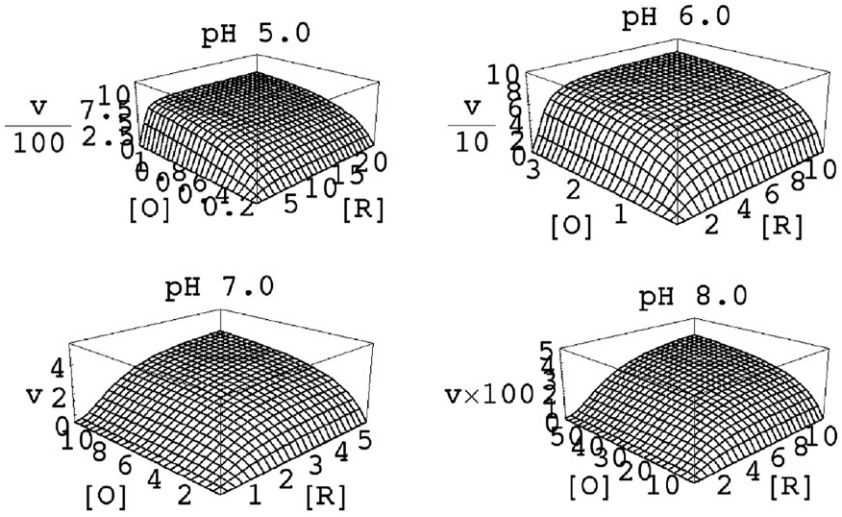

Fig. 8. Plots of initial velocities v of the catalyzed reaction 2R+O -> as functions of [O] and [R] at four pHs for Mechanism III. The number n of hydrogen ions consumed in the rate-determining reaction is −2 and m=2. Note the change in scale of the ordinate is 10^4 over the pH range 5 to 8.

An overview of the rate equation is obtained by plotting the initial reaction velocity at a specific pH versus [O] and [R] in three dimensions. Plots at pHs 5, 6, 7, and 8 are shown in Fig. 6.

Mechanism II is not applied to the nitrite reductase reaction (EC 1.7.7.1) because the sensitivity to pH is so great.

8. Mathematica calculations for the type of reaction catalyzed by nitrate reductase by Mechanism III

A Mathematica program calcpropsrandom has been written to derive the kinetic properties and rate equation for the mechanism described in Section 4. This program requires the specification of three more parameters: pK_{1EO}, pK_{2EO}, and K_{HEO}. This program has been used to plot the kinetic parameters and ratios that are useful in calculating pKs and chemical equilibrium constants that are given in Fig. 7.

The first two plots are the same as in previous figures. The plot for K_{IO} is the same as in Fig. 5. The plot for K_{IR2} is the same as in Fig. 1.

An overview of the rate equation is obtained by plotting the initial reaction velocity at a specific pH versus [O] and [R] in three dimensions. Plots at pHs, 5, 6, 7, and 8 are shown in Fig. 8.

Mechanism III is not applied to the nitrite reductase reaction (EC 1.7.7.1) because the sensitivity to pH is so great.

9. Discussion

This article emphasizes the need for both V_{fexp} and V_f. When hydrogen ions are consumed in the rate-determining reaction n is a negative integer, and the limiting experimental velocity in the forward direction V_{fexp} increases with decreasing pH as shown by Eq. (16). This has to happen because when hydrogen ions are consumed, the apparent equilibrium constant K' for the catalyzed reaction has to increase as the pH is reduced. The pH effect 10^{npH} has to be taken out of V_{fexp} to obtain V_f that makes it possible to calculate the pKs of the enzyme-substrate complex and $k_f[E]_t$.

This article is incomplete in the sense that the reverse reactions are not considered, but the apparent equilibrium constants for these reactions are very large. These calculations show that the pH effects on the kinetics for these reactions are very large and can make it nearly impossible to determine kinetic parameters. It is assumed that hydrogen ions are consumed in the rate-determining reaction rather than other reactions in the mechanism because the other reactions compete with each other. When hydrogen ions are consumed in the rate-determining reaction, they produce a powerful driving force for the forward reaction. The forward rate has to increase according to $[H^+]^{-n}$, where n is a negative integer because the apparent equilibrium constant K' has to be so much greater at acidic pHs, as shown in Table 1.

When the rapid-equilibrium assumption is used in deriving the rate equation, thermodynamics is used to calculate the equilibrium composition of the mixture of substrates, enzymatic site, and enzyme-substrate complexes. This calculation is quite different from the calculation of concentrations in steady-state kinetics. In steady-state kinetics, there can be alternate pathways and rate constants for all of these pathways must be included in the calculations. In biochemical thermodynamics at a specified pH, the equilibrium composition is obtained by solving a set of equations for apparent equilibrium constants and conservation equations for the elements, excluding hydrogen atoms. The set of equations that is solved must not include redundant equations; that is, equations that can be obtained by adding and subtracting other equations in the set. This applies to both expressions for apparent equilibrium constants and conservation equations. In practical terms this means that only apparent equilibrium constant expressions for independent "paths" can be used. Cycles of reactions are not permitted in equilibrium calculations because when there are cycles, there is a relation between equilibrium constants and one of the equilibrium constant expressions must be omitted from the calculation of the equilibrium composition.

In general, rapid-equilibrium rate equations have fewer terms in the denominator than steady-state rate equations, but often

rapid-equilibrium rate equations do represent the experimental data. When they do, they are to be preferred because the Michaelis constants are then equilibrium constants and can be interpreted using thermodynamics. Rapid-equilibrium rate equations can be derived for random mechanisms as well as ordered mechanisms and this yields more terms in the denominator of the rate equation.

Experimental data for this type of reductase reaction may not fit the equations presented here, but they are the place to start.

Acknowledgements

I am indebted to Robert N. Goldberg (NIST) for many helpful discussions. Grateful thanks to NIH for support of this research by award number 5-RO1-GM48348-11.

Appendix A

calcpropsRmO[pK1e_,pK2e_,pK1erm_,pK2erm_, pK1ermo_,pK2ermo_,kfEt_,kerm_,kermo_,m_,n_]:= Module [{efactor,ermfactor,ermofactor,vf,krm,vfexp,v}, (*This program derives kinetic parameters for the initial velocity of the forward reaction O + mR ->, where O is the oxidant and R is the reductant at specified pH. R binds first. This program also derives the rate equation for the forward reaction. o and r are the concentrations of the oxidant and reductant. m is the number of R molecules needed to reduce the oxidant. n is the number of hydrogen ions produced in the rate-determining step. The output is a list of seven functions.*)

efactor $1+10^{pK2e-pH}+10^{pH-pK1e}$;
emfactor $1+10^{pK2erm-pH}+10^{pH-pK1erm}$;
emofactor $1+10^{pK2ermo-pH}+10^{pH-pK1ermo}$;
vf kfEt/ermofactor;
vfexp (10^(*n* pH)) * vf;
krm kerm * (efactor / ermfactor)^(1/m);
krmo kermo * (ermfactor / ermofactor);
v vfexp / (1 + (krmo/o) * (1 + (krm/r)^m));
{vfexp,vf,krm,krmo,vf/krmo, vf/(krmo * krm^m),v}]

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[6] P.F. Cook, W.W. Cleland, Enzyme Kinetics and Mechanism, Garland Science, New York, 2007.

Table 2
Input for rate calculations

Input	Mathematica symbol	calcpropsRmO	calcpropsRmO	calcpropsORm	calcpropsrandom
Reaction		EC 1.9.6.1	EC 1.7.7.1	EC 1.9.6.1	EC 1.9.6.1
pK_{1E}	pK1e	7.5	7.5	7.5	7.5
pK_{2E}	pK2e	6.6	6.6	6.6	6.6
pK_{1ER_m}	pK1erm	7.0	7.0	7.0	7.0
pK_{2ER_m}	pK2erm	6.0	6.0	6.0	6.0
pK_{1ER_mO}	pK1ermo	8.0	8.0	8.0	8.0
pK_{2ER_mO}	pK2remo	7.0	7.0	7.0	7.0
$k_f[E]_t$	kfEt	10^(2*7.5)	10^(8*7.5)	10^(2*7.5)	10^(2*7.5)
K_{HER_m}	kerm	1	1	1	1
K_{HER_mO}	kermo	2	2	2	2
m	m	2	6	2	2
n	n	−2	−8	−2	−2
pK_{1EO}	pK1eo				7.0
pK_{2EO}	pK2eo				6.0
K_{HEO}	keo				1