STEADY-STATE KINETICS: TWO-SUBSTRATE, TWO-PRODUCT REACTIONS

Steady-state kinetic measurements are part of any thorough investigation of an enzyme. They describe a mechanism that is minimal, in that only those steps associated with a change in composition of an enzyme-substrate intermediate are detected. They provide a basis for interpreting fast-reaction measurements, which can detect additional steps that are isomerizations and do not involve change in composition. They provide a basis for correlating other mechanistic information, such as structural data, particularly from X-ray diffraction results, and thermodynamic data, e.g., binding of reactants to the enzyme. They provide values for kinetic constants that can be used to understand the reaction, to compare enzymes, to characterize purity of the enzyme, etc.

Kinetic data typically are measurements of the concentrations of one or more reacting species as a function of time for varied initial concentrations and experimental conditions (temperature, pH, ...). The reaction velocity can be calculated from the data, as, for example,

$$v^{observed} = \frac{d}{dt}[product]$$

A function can be constructed that expresses the relationship between the experimental values for the reaction velocity and the time and other independent experimental variables.

$$v^{observed} = f^{observed}(t,[reactants],T,pH,...)$$

The function $f^{observed}$ is the rate law for the reaction. Often initial velocities, v_o, are determined. The advantages of doing this are the time dependence of $v^{observed}$ is eliminated from the analysis and also the values apply to the starting concentrations of reactants, which are known accurately at the instant of mixing. Progress curves measured over long times of reaction can be but are not often analyzed in steady-state kinetic studies.

The aims of a kinetic analysis are:

(1) To develop a mechanism that is consistent with the kinetic data. In this context, a mechanism is a set of chemical reactions that describes the interconversions of all reacting species (substrates, products, enzyme catalyst, intermediate enzyme-substrate complexes,

(2) To characterize this set of chemical reactions quantitatively, in terms of reaction rate constants and equilibrium constants for the individual steps of the mechanism.

In order to accomplish this, one constructs a mathematical model, based on a suggested mechanism, that relates the reaction velocity to the independent experimental variables and to a set of variable parameters (in particular, rate constants and equilibrium constants) that are characteristic of the mechanism:

$$v^{model} = f^{model}(t,[reactants],T,pH,...;variableparameters)$$

For complicated mechanisms it can be difficult to construct the function f^{model}. The King-Altman method largely solves this problem.

If f^{model} has the same mathematical form as $f^{observed}$, then the mechanism upon which f^{model} is based is possible, and the values of the variable parameters of f^{model} are those of the corresponding constants of $f^{observed}$.

In practice, one need not construct a function corresponding to $f^{observed}$. Instead, the list of data points, one entry for each measurement of the dependent variable, $v^{observed}$, can be taken as constituting $f^{observed}$. The comparison of $f^{observed}$ and f^{model} then consists of fitting values of v^{model} calculated by use of f^{model} to values of $v^{observed}$. The variable parameters of f^{model} are adjusted by suitable manipulations until best fit is obtained between calculated and experimental values of the reaction rate. If an acceptable fit can be obtained with plausible parameter values, the mechanism upon which f^{model} is based is possible. Testing f^{model} against the data can be simple. For example, for a single substrate reaction, if the measured values are described by a straight line in a reciprocal plot ($1/v_o$ *vs* $1/[substrate]$, then the Michaelis-Menten mechanism is possible (as are other more complex mechanisms).

The simplest form of f^{model} for a particular mechanism generally has variable parameters that are sums and products of the rate constants for individual steps of the mechanism. Two or more mechanisms can give the same function. In such cases the variable parameters must be related differently to the constants for the individual steps of each mechanism. Extra-kinetic (e.g., thermodynamic or structural) information may help in selecting the proper mechanism.

The fitting of f^{model} to $f^{observed}$ can be done by computer or by graphical analysis. With the latter method for a two-substrate reaction catalyzed by an enzyme, steady-state reaction rates are typically measured for the concentration of one substrate, *b*, held fixed and the concentration of the other substrate, *a*, varied. Such partial data often follow simple Michaelis-Menten behavior ($1/v_o$ linear in $1/a$) and can be displayed graphically in a primary plot of $1/v_o$ *vs* $1/a$ for fixed *b*. Several such primary plots at different concentrations of the fixed substrate are obtained. The intercepts $1/V^A_{app}$ of the several primary plots are functions of the concentration, *b*, of fixed substrate. A secondary plot ($1/V^A_{app}$ *vs* $1/b$) gives values for parameters that are independent of reactant concentrations. Switching fixed and varied substrates, one applies the same procedure to data for fixed concentrations, *a*, and varied concentrations, *b*, giving values for other parameters of the rate law. It is economical if the set of concentrations *a* and *b* form a matrix, i.e., if for each different concentraton of *a* there is a rate measurement for every different concentration of *b*, and vice-versa. Then the full set of data can be used in each set of primary plots. This experimental design is convenient, even if the data are to be fit to a mechanism by use of a computer.

There are two broad divisions of enzyme-catalyzed group transfer reactions (equation references are to the Appendix):

substituted enzyme	(see eq. 1)
ternary complex	
random order	(see eq. 2)
compulsory order	(see eq. 3)

The Theorell-Chance mechanism (eq. 4 of the Appendix) is a special case of compulsory order, for which the steady-state concentrations of the ternary complexes are very low, below the level of stoichiometric significance. There are various schemes for naming mechanisms, e.g., "compulsory order" is also called "ordered bi-bi". There are various methods of diagrammatically representing mechanisms; one can write as equivalent to eq. (3) a set of chemical equations (eq. 5) or a closed figure (eq. 6b).

Equation (7) gives the initial reaction velocity as a function of the concentrations *a* and *b* of the reactants, with product concentrations *p* and *q* zero. The derivation of eq. (7) by use of the King-Altman procedure for the compulsory-order ternary-complex mechanism (eq. 6b) is shown in the section of the Appendix following eq. (7). A list below eq. (7) gives the relationship of the four variable parameters of eq. (7) to the eight step constants of the mechanism of eq. (6b). The eight step constants are determined by two such sets of four parameters, one set for the forward reaction (concentrations *a* and *b* varied, *p* and *q* fixed at zero) and another for the reverse reaction (concentrations *p* and *q* varied, *a* and *b* fixed at zero).

Other mechanisms give a function of the same form as eq. (7). A King-Altman analysis for the Theorell-Chance mechanism is given following the derivation for the compulsory-order mechanism in the Appendix. The corespondences between the four variable parameters of eq. (7) and the step constants of the Theorell-Chance mechanism are given also in a list below eq. (7). The expression of the variable parameters in terms of step constants differs according to the mechanism. The random order mechanism also gives a function of the form of eq. (7). The substituted enzyme mechanism gives eq. (7) but without the term K_m^{AB}/ab in the denominator. Note that only one parameter, $K_i^A = K_m^{AB}/K_m^B$, is simply related to an equilibrium constant, that for dissociation of the enzyme--first-substrate complex, *EA*.

The lactate dehydrogenase reaction is well described as a compulsory-order ternary-complex mechanism (eq. 3). A Theorell-Chance mechanism (eq. 4) fits much of the steady-state kinetic results for this enzyme, but some steady state and other data support the use of the more complicated model and demonstrate, also, isomerizations of the several enzyme forms (in particular, *E, EA*, and *EB*).

The following describes the manipulations of eq. (7) that are used in a graphical analysis based upon primary and secondary plots of steady-state kinetic data. The analysis gives values for the variable parameters of eq. (7) that fit the kinetic data. The example is the lactate-dehydrogenase catalyzed reduction of pyruvate by NADH to give lactate and NAD. Papers by Zewe and Fromm (1962, 1965) illustrate the method.

I. Fix concentration of pyruvate, vary concentration of NADH:

The primary plots of $1/v_o$ *vs* $1/[NADH]$ correspond to eq. (8) and are shown in the sketch accompanying eq. (8). The common point of intersection, independent of the concentration of the fixed substrate, pyruvate, determines the ratio of parameters $K_m^B/K_m^{AB} = 1/K_i^A = -1/[NADH]$ at the intersection. The intercepts $1/V_{app}^A$ are a function of the concentration of the fixed substrate, pyruvate. The secondary plot, corresponding to eq. (9), of $1/V_{app}^A$ *vs* $1/[pyruvate]$, evaluates the parameters K_m^B and V.

II. Fix concentration of NADH, vary concentration of pyruvate:

A similar treatment of the data (eqs. 10 and 11) evaluates $K_m^A/K_m^{AB} = 1/K_i^B$ from the primary plots of $1/v_o$ *vs* $1/[pyruvate]$, and V and K_m^A from the secondary plot of $1/V_{app}^B$ *vs* $1/[NADH]$.

Note that V is determined by both secondary plots and that two values of K_m^{AB} are determined, by each pair of primary and secondary plots, as $K_i^A K_m^B$ and $K_i^B K_m^A$. A total of four independent parameters are evaluated (V, K_m^A, K_m^B, K_m^{AB}).

A fit of eq. (7) to kinetic data for lactate dehydrogenase does not distinguish the compulsory order (eq. 3) from formally equivalent mechanisms, such as Theorell-Chance (eq. 4) or random order (eq. 2).

III. Inhibition by products:

Inhibition by products can distinguish between the mechanisms of eqs. (2-4). Linear inhibition can be competitive (inhibitor increases only the slope of the reciprocal plot), uncompetitive (inhibitor increases only the intercept), or noncompetitive (inhibitor increases both slope and intercept). Table I of the Appendix describes the character of the inhibition by product P or Q for the several combinations of fixed and variable substrates, A and B. The patterns of inhibition are different for the mechanisms of eqs. (2-4). For example, if for the lactate dehydrogenase reaction one observes noncompetitive inhibition by lactate at fixed concentration of NADH and variable concentration of pyruvate, then the random order and Theorell-Chance mechanisms are ruled out and a compulsory-order mechanism is favored.

The derivation of the product inhibition patterns of Table I is based upon the full rate eqs. (12) that include terms in the concentrations p and q of the products P and Q. Setting both p and q at zero in eqs. (12) gives eq. (7), which has only a and b as independent variables. Setting q at 0 gives eqs. (13) for product inhibition by P, and setting p at 0 gives eqs. (14) for product inhibition by Q. Inspection of these equations allows deduction of Table I.

One can deduce inhibition patterns simply by inspection of the mechanism and with the use of several rules concerning the effect of an inhibitor on the slope and intercept of a reciprocal plot.

There are other types of steady-state measurements that lead to refinement of and understanding of mechanism: studies of pH and temperature dependences, study of isotope exchange at equilibrium, study of kinetic isotope effects, study of inhibition by substrate analogs, such as oxamate as an analog of pyruvate.

REFERENCES

Cornish-Bowden, A., *Principles of Enzyme Kinetics*, Butterworths, London-Boston, 1976.

Cleland, W. W., "Steady State Kinetics" in *The Enzymes*, Vol. II, 3rd Edition, (P. D. Boyer, ed.), pp. 1-65, Academic Press, New York, 1970.

Zewe, V., and Fromm, H. J., "Kinetic Studies of Rabbit Muscle Lactate Dehydrogenase. II. Mechanism of the Reaction", *Biochemistry* **4**, 782-792, 1965.

Zewe, V., and Fromm, H. J., "Kinetic Studies of Rabbit Muscle Lactate Dehydrogenase," *J. Biol. Chem.* **237**, 1668-1675, 1962.