Howard, J. B., Vermeulen, M., & Swenson, R. (1980) *J. Biol. Chem. 255*, 3820-3823.
Kearney, J. F. (1984) in *Fundamental Immunology* (Paul, W. E., Ed.) pp 751-766, Raven, New York.
Kurecki, T., Kress, L. F., & Laskowski, M. (1979) *Anal. Biochem. 99*, 415-420.
Larsson, L. I., Skriver, L., Nielsen, L. S., Grondahl-Hansen, J., Kristensen, P., & Dano, K. (1984) *J. Cell Biol. 98*, 894-903.
Lin, H.-Y., Wells, B. R., Taylor, R. E., & Birkedal-Hansen, H. (1987) *J. Biol. Chem. 262*, 6823-6831.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mccartney, H. W., & Tschesche, H. (1983) *Eur. J. Biochem. 130*, 71-78.
McKerrow, J. H. (1987) *J. Biol. Chem. 262*, 5943.
Murphy, G., Reynolds, J. J., & Werb, Z. (1985) *J. Biol. Chem. 260*, 3079-3083.
Nagase, H., Cawston, T. E., De Silva, M., & Barrett, A. J. (1982) *Biochim. Biophys. Acta 702*, 133-142.
Neville, D. M., Jr. (1971) *J. Biol. Chem. 246*, 6328-6334.
Otsuka, K., Sodek, J., & Limeback, H. (1984) *Eur. J. Biochem. 145*, 123-129.
Rosenfeld, M. G., Kreibich, G., Popor, D., Kato, K., & Sabatini, D. D. (1982) *J. Cell Biol. 93*, 135-143.
Sottrup-Jensen, L., Stepanick, T. M., Jonew, C. M., Lonblad, P. B., Kristensen, T., & Wierzbicki, D. M. (1984) *J. Biol. Chem. 259*, 8293-8303.
Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607-1615.
Stricklin, G. P., Jeffrey, J. J., Roswit, W. T., & Eisen, A. Z. (1983) *Biochemistry 22*, 61-68.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1173-1178.
Wahl, L. M., Olsen, C. E., Sandberg, A. L., & Mergenhagen, S. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4955-4958.
Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M., & Teitelbaum, S. L. (1985) *J. Clin. Invest. 76*, 219-224.
Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H. J., Smith, B., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P., & Docherty, A. J. P. (1986) *Biochem. J. 240*, 913-916.
Wilhelm, S. M., Eisen, A. Z., Teter, M., Clark, S. D., Kronberger, A., & Goldberg, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3756-3760.

Calculation of Rate and Equilibrium Constants for a Ping-Pong Mechanism from Steady-State Data†

Theodore S. Jardetzky*,‡ and Mark Seville*,§

Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, and Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received February 5, 1988; Revised Manuscript Received April 26, 1988

ABSTRACT: A method for the determination of individual rate constants and equilibrium constants from simple steady-state kinetic data is presented for a simple ping-pong mechanism. This involves determining the Michaelis constants and catalytic constants for three pairs of amino acids and keto acids. The equations that relate these steady-state constants to the equilibrium and rate constants are derived. The usefulness and limitations of the treatment are discussed in terms of hypothetical case studies. The method is then used to derive the dissociation constants and rate constants for an experimental system consisting of *Escherichia coli* tyrosine aminotransferase and several of its substrates. This simple analysis will be of use in the study and comparison of enzymes generated by site-directed mutagenesis where multiple time-consuming studies become impractical.

The determination of microscopic rate constants for enzyme-catalyzed reactions provides valuable information in the detailed analysis and comparison of enzyme reaction mechanisms. It is a complicated and time-consuming process involving the use of specialized equipment, which is not available in most laboratories. Here we report a method by which individual rate constants can be estimated from simple steady-state data that can be obtained on a standard bench-top spectrophotometer. In addition, the method provides a way of calculating the enzyme–substrate equilibrium constants.

The ping-pong mechanism originally proposed by Cleland (1963) adequately describes the sequential two-substrate reaction catalyzed by aminotransferases. The transamination reaction is normally fully reversible and can be measured in either direction. Thus, the actual number of substrates that can be utilized for kinetic analysis is, in fact, four (two sets of two). For any four substrate system, which consists of two amino acids and the two corresponding keto acids, a minimum of eight rate constants are required to define the fully reversible pair of transaminations. The full complement of steady-state parameters consists of four Michaelis constants, two catalytic constants, and four inhibition constants.

Cleland (1963) has previously derived the relationship between these steady-state parameters and the individual rate constants. Unfortunately, determination of all the steady-state parameters, which can be obtained from a given four-substrate

† This work was initiated at the Department of Biophysical Chemistry, Biozentrum, Basel, Switzerland, and supported in part by Swiss National Science Foundation Grant 30.098-0.85, awarded to K. Kirschner.
* Correspondence should be addressed to either author.
‡ Harvard University.
§ University of Colorado.

0006-2960/88/0427-6758$01.50/0

set, is insufficient for the calculation of the individual rate constants (Cleland, 1963).

In this paper we show that it is possible to estimate the microscopic rate constants from a subset of the most easily obtained steady-state data (Michaelis constants and catalytic constants) by including data from an additional amino/keto substrate pair. Furthermore, a simple set of equations is derived which defines the enzyme–substrate equilibrium dissociation constants in terms of these steady-state parameters. The method is applied to tyrosine aminotransferase (Fotheringham et al., 1986) from *Escherichia coli* as an example of its practical value.

Over 60 distinct aminotransferases have been identified [see Braunstein (1973) for a comprehensive review], providing a large natural database for the elucidation of structure–function relationships. In addition, use of site-directed mutagenesis will create more enzymes to be studied (Malcolm & Kirsch, 1985; Toney & Kirsch, 1987; Seville et al., unpublished results). The information gained from the method described here will contribute to a more quantitative understanding of the kinetic differences between these enzymes and in the elucidation of structure–activity relationships.

Materials and Methods

The purification of *E. coli* tyrosine aminotransferase and the determination of the steady-state kinetic parameters will be presented in detail in a subsequent paper (Seville et al., unpublished results).

Theory

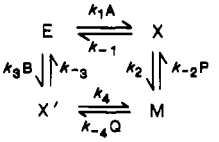
Most, if not all, aminotransferases can transaminate several of the naturally occurring amino acids and keto acids with varying degrees of specificity. The simplest scheme for the reaction [see Cornish-Bowden (1979) for a concise description] of any one amino acid and its corresponding keto acid with enzyme is shown in Scheme I, where A is the amino acid, E is the PLP[1] form of the enzyme, X is the enzyme-bound intermediate, M is the PMP form of the enzyme, and P is the keto acid. This scheme portrays a partial reaction of the enzyme.

Scheme I

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M + P$$

In order to generate a nonequilibrium steady state, amino acid A and a different keto acid Q are added in excess to the enzyme. The overall reaction scheme is then the sum of the two partial reactions (Scheme II).

Scheme II



The initial rate, v_i, in the absence of products for the enzyme with substrates A and Q is given by (Cleland, 1963)

$$v_i = \frac{V_{AQ}E_0}{\frac{K_{A(Q)}}{A} + \frac{K_{Q(A)}}{Q} + 1}$$

[1] Abbreviations: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; subscripted K, Michaelis constant; subscripted V, catalytic constant; subscripted K_d, dissociation constant; subscripted k, rate constant.

Table I: Basic Equations Relating Steady-State Parameters and Rate Constants[a]

For Substrates A, Q		
$V_{AQ} = k_2k_{-3}/(k_2 + k_{-3})$	$K_{A(Q)} = (k_{-1} + k_2)k_{-3}/[k_1(k_2 + k_{-3})]$	$K_{Q(A)} = (k_{-3} + k_4)k_2/[k_{-4}(k_2 + k_{-3})]$
For Substrates B, P		
$V_{BP} = k_4k_{-1}/(k_4 + k_{-1})$	$K_{P(B)} = (k_{-1} + k_2)k_4/[k_{-2}(k_4 + k_{-1})]$	$K_{B(P)} = (k_{-3} + k_4)k_{-1}/[k_3(k_4 + k_{-1})]$
For Substrates C, P		
$V_{CP} = k_6k_{-1}/(k_6 + k_{-1})$	$K_{P(C)} = (k_{-1} + k_2)k_6/[k_{-2}(k_6 + k_{-1})]$	$K_{C(P)} = (k_{-5} + k_6)k_{-1}/[k_5(k_6 + k_{-1})]$
For Substrates A, R		
$V_{AR} = k_2k_{-5}/(k_2 + k_{-5})$	$K_{A(R)} = (k_{-1} + k_2)k_{-5}/[k_1(k_2 + k_{-5})]$	$K_{R(A)} = (k_{-5} + k_6)k_2/[k_{-6}(k_2 + k_{-5})]$
For Substrates B, R		
$V_{BR} = k_4k_{-5}/(k_4 + k_{-5})$	$K_{B(R)} = (k_{-3} + k_4)k_{-5}/[k_3(k_4 + k_{-5})]$	$K_{R(B)} = (k_{-5} + k_6)k_4/[k_{-6}(k_4 + k_{-5})]$
For Substrates C, Q		
$V_{CQ} = k_6k_{-3}/(k_6 + k_{-3})$	$K_{C(Q)} = (k_{-5} + k_6)k_{-3}/[k_5(k_6 + k_{-3})]$	$K_{Q(C)} = (k_{-3} + k_4)k_6/[k_{-4}(k_6 + k_{-3})]$

[a] The equations were originally derived by Cleland (1963). The nomenclature used here is similar to that in Cornish-Bowden (1979).

where V_{AQ} is the catalytic constant of the enzyme with substrates A and Q, $K_{A(Q)}$ is the Michaelis constant of the enzyme for A with Q as second substrate, $K_{Q(A)}$ is the Michaelis constant of the enzyme for Q with A as second substrate, and E_0 is enzyme concentration.

In principle, one can measure the reaction in the opposite direction using P and B as the substrates. This allows for the determination of three more steady-state parameters, V_{BP}, $K_{B(P)}$, and $K_{P(B)}$. Each of these steady-state parameters can be expressed in terms of the individual rate constants shown in Scheme II.

Thus, for any two pairs of complementary substrate (A with Q or B with P), there are six known steady-state parameters but eight unknown microscopic rate constants. Therefore, the problem of defining the rate constants in terms of the steady-state parameters is underdetermined. However, if another amino/keto acid species (C/R) is substituted for the B/Q species while the A/P species is retained, then it is possible to measure another six unique steady-state parameters (three for A with R and three for C with P). We now have a total of 12 determinable steady-state parameters. However, because we have retained the A/P half-reaction, the total number of unknown rate constants is only increased by the number of rate constants describing the C/R half-reaction to 12. For clarity, the three partial reactions under discussion are shown with their corresponding rate constants (Scheme III).

Scheme III

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M + P$$

$$B + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X' \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} M + Q$$

$$C + E \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} X'' \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} M + R$$

By making steady-state measurements with all 6 possible combinations of substrate pairs defined by this system, one generates 18 steady-state parameters relating the 12 microscopic rate constants in the system. The 18 expressions, using a notation similar to that used by Cornish-Bowden (1979), are given in Table I. Note, for example, that $K_{A(Q)}$ is not equal to $K_{A(R)}$. The result is that the steady-state Michaelis constants

for a particular substrate will depend on the second substrate used.

Derivation of Equilibrium Constants. Although the set of equations described above cannot be used to solve analytically for the individual rate constants, certain collections of rate constants can be determined analytically. Of particular interest, are the k_{off}/k_{on} (i.e., the dissociation constants, K_d) for each of the substrates. The solution of the K_d values in terms of the steady-state parameters can be found in the following manner.

The six expressions for the catalytic constants (Table I) contain only the six unimolecular rate constants of the above reaction schemes. By using the expressions for V_{BR}, V_{BP}, and V_{AR} given in Table I, one can solve for k_{-1} in terms of k_2. This is done as follows: first, the expression for V_{AR} is rearranged into the form

$$k_{-5} = \frac{k_2 V_{AR}}{k_2 - V_{AR}} \tag{1}$$

and the expression for V_{BR} is rearranged to

$$k_4 = \frac{k_{-5} V_{BR}}{k_{-5} - V_{BR}} \tag{2}$$

Then eq 1 is substituted in eq 2, yielding

$$k_4 = \frac{k_2 V_{AR} V_{BR}}{k_2 V_{AR} - V_{BR}(k_2 - V_{AR})} \tag{3}$$

Then the expression for V_{BP} is arranged into the form

$$k_{-1} = \frac{k_4 V_{BP}}{k_4 - V_{BP}} \tag{4}$$

The expression for k_4 given by eq 3 is substituted into eq 4. After rearrangement this yields

$$k_{-1} = \frac{-k_2}{k_2\left(\frac{1}{V_{BR}} - \frac{1}{V_{BP}} - \frac{1}{V_{AR}}\right) + 1} \tag{5}$$

One can also solve for k_{-1} in an analogous manner using the expressions for V_{CQ}, V_{AQ}, and V_{CP} listed in Table I. This gives

$$k_{-1} = \frac{-k_2}{k_2\left(\frac{1}{V_{CQ}} - \frac{1}{V_{AQ}} - \frac{1}{V_{CP}}\right) + 1} \tag{6}$$

Setting eq 5 and 6 equal gives the following relationship for the mechanism:

$$\frac{1}{V_{BR}} - \frac{1}{V_{BP}} - \frac{1}{V_{AR}} = \frac{1}{V_{CQ}} - \frac{1}{V_{AQ}} - \frac{1}{V_{CP}} = \alpha \tag{7}$$

The quantity defined by eq 7 is abbreviated to α in subsequent equations. The above relationship is true for the ideal ping-pong mechanism.

These equations can be used to solve for the dissociation constant K_{dA} by starting with the equation for K_{AQ} from Table I in the form

$$K_{AQ} = \frac{(k_{-1} + k_2)V_{AQ}}{k_1 k_2} \tag{8}$$

By use of the relationship $K_{dA} = k_{-1}/k_1$ this can be rearranged to

$$K_{AQ} = \left(\frac{K_{dA}}{k_2} + \frac{1}{k_1}\right)V_{AQ} \tag{9}$$

Table II: Steady-State Parameters Used in the Calculation of the Rate Constants for Tyrosine Aminotransferase[a]

constant[b]	dimension	value of constant
V_{AQ}	s^{-1}	325
V_{AR}	s^{-1}	480
V_{BP}	s^{-1}	375
V_{CP}	s^{-1}	625
V_{BR}	s^{-1}	545
V_{CQ}	s^{-1}	201[c]
$K_{A(Q)}$	M	2.1×10^{-3}
$K_{P(B)}$	M	3.6×10^{-5}
$K_{Q(A)}$	M	3.1×10^{-4}
$K_{B(P)}$	M	2.3×10^{-2}
$K_{C(P)}$	M	7.5×10^{-4}
$K_{R(A)}$	M	9.0×10^{-6}

[a] All the assays were carried out in 100 mM sodium arsenate buffer, pH 7.4 at 37 °C. The initial velocity measurements with asparate were obtained by using the malate dehydrogenase coupled assay of Karmen (1955), those with glutamate were obtained by using the glutamate dehydrogenase coupled assay of Duggleby and Morrison (1978), and those with tyrosine were obtained by direct spectrophotometric measurement of the conversion of tyrosine to (4-hydroxyphenyl)pyruvate at 295 nm. [b] The substrates designated by the various subscripts are A, aspartate; P, oxaloacetate; B, glutamate; Q, α-ketoglutarate; C, tyrosine; and R, (4-hydroxyphenyl)pyruvate. The identifying subscripts correspond to the reactants in the three half-reactions shown in Scheme III. [c] Not used in the calculations.

(The parentheses have been dropped from the subscript to K_{AQ} to avoid an excessive number of symbols.) Then either eq 5 or 6 is rearranged to express k_2

$$k_2 = \frac{-1}{\alpha + \frac{1}{k_{-1}}} \tag{10}$$

and k_2 in eq 9 is substituted by the form given in eq 10, yielding

$$\frac{K_{AQ}}{V_{AQ}} = -K_{dA}\left(\alpha + \frac{1}{k_{-1}} + \frac{1}{k_1}\right) \tag{11}$$

Multiplying through by K_{dA} and using the fact that K_{dA}/k_{-1} is equal to $1/k_1$, one finds

$$K_{dA} = \frac{-K_{AQ}}{V_{AQ}\alpha} \tag{12}$$

Therefore, it is possible to calculate the dissociation constant K_{dA} from steady-state data.

One can solve for the expression for K_{dP}, where K_{dP} is equal to k_2/k_{-2}, by using the kinetic Haldane relationship

$$\frac{V_{AQ}K_{PB}}{K_{AQ}V_{BP}} = \frac{k_1 k_2}{k_{-1}k_{-2}} = \frac{K_{dP}}{K_{dA}}$$

which provides

$$K_{dP} = \frac{-K_{PB}}{V_{BP}\alpha}$$

In fact, this is the general form for the solution of all of the dissociation constants for the six substrates. The quantity α is replaced by the analogous quantities β and γ defined below.

For K_{dB} and K_{dQ}

$$\beta = \frac{1}{V_{AR}} - \frac{1}{V_{AQ}} - \frac{1}{V_{BR}} = \frac{1}{V_{CP}} - \frac{1}{V_{BP}} - \frac{1}{V_{CQ}}$$

Then

$$K_{dB} = \frac{k_{-3}}{k_3} = \frac{-K_B}{V_B\beta}$$

and

$$K_{dQ} = \frac{k_4}{k_{-4}} = \frac{-K_Q}{V_Q\beta}$$

(The second subscript to the Michaelis constant and the catalytic constant have been omitted because, in principle, the quantity K/V for any given substrate is independent of the second substrate used for the measurements.)

For K_{dC} and K_{dR} the parameter is calculated from

$$\gamma = \frac{1}{V_{AQ}} - \frac{1}{V_{CQ}} - \frac{1}{V_{AR}} = \frac{1}{V_{BP}} - \frac{1}{V_{BR}} - \frac{1}{V_{CP}}$$

Then

$$K_{dC} = \frac{k_{-5}}{k_5} = \frac{-K_C}{V_C\gamma}$$

and

$$K_{dR} = \frac{k_6}{k_{-6}} = \frac{-K_R}{V_R\gamma}$$

Therefore, the thermodynamic dissociation constants for the binding of all of the substrates can be calculated from this set of steady-state data.

Estimation of Rate Constants. The problem of determining the rate constants is not as straightforward as the equilibrium constants. Given that the steady-state parameter equations provide a set of nonlinear homogeneous equations, there are an infinite number of possible solutions that mathematically satisfy the equations. However, with two equations relating k_{-1} to k_2 (eq 5 and 6), it is possible to express all of the other rate constants in terms of k_2 from either of these equations. The problem can be reduced to that of finding all of the values for k_2 for which all other rate constants (k_i) have physically meaningful values. A set of constraints can be placed on the values that any k_i may take, which can be used to select a subset of physically meaningful solutions from the set of all possible solutions. The constraints that we chose are for unimolecular steps

$$k_i > 0 \text{ s}^{-1}$$

and for bimolecular steps

$$0 < k_i < 10^9 \text{ M}^{-1} \text{ s}^{-1}$$

I.e., all reaction steps must have positive rate constants, and the bimolecular steps cannot exceed the diffusion limit. Most enzymes are found to have association constants with substrates in the range 10^6–10^9 M^{-1} s^{-1} (Fersht, 1985). As shown below, these constraints are enough to find the set of physically meaningful solutions. A similar approach was taken by Wallin and Arion (1973) in their evaluation of rate-determining steps of glucose-6-phosphatase.

We used several sets of hypothetical data to test the limits of the method and the interpretability of the results, as well as to aid in understanding the behavior of the rate constants when using real data. We proceeded as follows: The rate constants k_1 through k_{-6} for the three half-reactions shown in Scheme III were assigned various values. The bimolecular rate constants were all set to 10^7 M^{-1} s^{-1}, and the unimolecular rate constants were either 10^3 or 5×10^4 s^{-1}. Then, from these rate constants, the steady-state parameters for the hypothetical system were calculated by using the relationships in Table I. These parameters were then entered into a simple BASIC program where all of the rate constants are solved in terms of k_2. The program then searched for values of k_2 where all of the k_i obey their respective boundary conditions. The value of k_2 was refined at its lower and upper boundary limits by 0.1 (s^{-1}) incremental steps. The solutions found for the rate constants were then compared with the values originally assigned.

The same procedure was used to calculate the rate constants from the experimental steady-state data for tyrosine aminotransferase.

Table III: Solutions for the Rate Constants and Dissociation Constants for the Reaction of Tyrosine Aminotransferase with Aspartate, Oxaloacetate, Glutamate, α-Ketoglutarate, Tyrosine, and (4-Hydroxyphenyl)pyruvate[a]

substrate	constant[b]	calcd values[c]	ratio of limits[d]
glutamate	on	2.70×10^4–4.66×10^4	1.72
aspartate	on	2.89×10^5–5.39×10^5	1.86
tyrosine	on	8.80×10^5–1.48×10^8	168
α-ketoglutarate	on	1.60×10^6–2.67×10^6	1.67
oxaloacetate	on	1.46×10^7–2.20×10^7	1.53
(4-hydroxyphenyl)-pyruvate	on	5.36×10^7–9.80×10^8	18
glutamate	off	581–1002	1.72
aspartate	off	642–1196	1.86
tyrosine	off	1376–2.30×10^5	168
α-ketoglutarate	off	546–902	1.65
oxaloacetate	off	481–737	1.53
(4-hydroxyphenyl)-pyruvate	off	1309–2.39×10^4	18
glutamate	K_d	21.5	
aspartate	K_d	2.2	
tyrosine	K_d	1.5	
α-ketoglutarate	K_d	0.34	
oxaloacetate	K_d	0.033	
(4-hydroxyphenyl)-pyruvate	K_d	0.024	

[a] Calculated from the experimental steady-state data collected in Table II. [b] The dimensions of the on-rate constants are M^{-1} s^{-1}, the off-rate constants are s^{-1}, and the dissociation constants are in mM. [c] The minimum and maximum calculated values for the rate constants are given that satisfy the boundary conditions (see text). The K_d values are analytically determined by using the relevant equations given in the text. [d] The ratio of limits defines the accuracy to which the rate constant can be determined. A value of 1.0 indicates that it is exactly determined.

Results and Discussion

Each calculated rate constant has a minimum and maximum value that corresponds to the minimum and maximum values for k_2 where all of the equations and boundary conditions are satisfied. The question, therefore, becomes one of how well-defined the range of values calculated for an individual rate constant is.

Hypothetical Test Cases. Typically, we found that the solution set for a given rate constant is better defined for those reaction steps where the rate constant is close to being rate-limiting in the overall reaction. Thus, in cases where up to 4 of the unimolecular rate constants have values approaching the values of the slowest k_i, we find that 8 of the 12 microscopic rate constants are defined to ±20%. Conversely, the rate constants for the fastest steps are rather poorly defined: Often the ratio of maximum to minimum values is greater than 100.

It is not a priori possible to say whether a particular set of steady-state data will be sufficient to define the majority of the rate constants. However, the worst case we could find involved a situation in which the rate constants for all the unimolecular steps were assigned equal values and likewise for all the bimolecular steps. This resulted in widely divergent limits for all of the calculated rate constants (a ratio of around 200). It seems very unlikely that this degenerate case would ever be encountered in an actual experimental system.

Tyrosine Aminotransferase. We have applied the method to the tyrosine aminotransferase from *E. coli* using aspartate, oxaloacetate, glutamate, α-ketoglutarate, tyrosine, and (4-hydroxyphenyl)pyruvate as substrates. The experimental steady-state data are collected in Table II, and the calculated rate constants are collected in Table III. As can be seen, 8 of the 12 rate constants are defined to within a factor of 2. These are the rate constants for the two pairs of dicarboxylic acid substrates. The rate constants for the aromatic substrate pair are more poorly defined. As discussed for the hypothetical cases above, this is because these rate constants are considerably faster.

The association rates for the substrates are ordered as follows: (4-hydroxyphenyl)pyruvate > oxaloacetate > α-ketoglutarate > aspartate > glutamate. The range of values for the rate of tyrosine association is too broad to be inserted into this sequence, but it can be said that it is at least faster than the other two amino acids.

The results indicate that the lower affinity of the enzyme for the amino acids aspartate and glutamate is due mainly to lower bimolecular association constants, relative to the corresponding keto acids. The fact that the amino acid association constants are rather slow (on the order of 10^5 M^{-1} s^{-1}) indicates that the initial binding as modeled may actually consist of more than one step, which is consistent with mechanisms proposed for related aminotransferases (Kirsch et al., 1984; Fasella & Hammes, 1967).

The off-rate constants for aspartate, glutamate, oxaloacetate, and α-ketoglutarate are all found to lie in the region of 500–1000 s^{-1}. The release of both the aromatic substrates is faster, but because of the spread of values it is not possible to say by how much.

The calculated K_d values for the substrates are also listed in Table III. Comparison of these values to the corresponding Michaelis constants provides a test of how well the Michaelis constants reflect substrate binding specificity. For the nonaromatic substrates, the Michaelis constants are very close to the K_d values. However, for tyrosine and (4-hydroxyphenyl)pyruvate, the difference is a factor of around 2-fold. Although this difference is not too large, comparison of Michaelis constants between different enzymes could easily lead to significant errors in estimations of relative affinities. In several hypothetical cases, for example, we encountered differences of 25-fold between the Michaelis and dissociation constants. The use of dissociation constants is clearly superior for comparisons of binding.

The other indirect alternative to obtaining these dissociation constants from steady-state data involves using product inhibition studies (Henson & Cleland, 1964). Determining them by the method presented here provides not only the dissociation constants but also the added information on more substrates. However, with certain enzymes it may not be possible to find three substrate pairs: For example, the enzyme may exhibit a very high degree of substrate specificity and only utilize two substrate pairs, or there may be no convenient assay for reactions involving the third substrate pair. In such situations it would be necessary to use the product inhibition technique to obtain dissociation constants.

The direct methods of determining dissociation constants include isotope exchange (Garces & Cleland, 1969) and spectrophotometric titration (Jenkins, 1964). The method and analysis we have described here is indirect. If the time and resources are available, then of course direct measurements of both equilibrium and rate constants are to be preferred. However, our method is rapid and simple. We envisage it being of use in, for example, the analysis and comparison of collections of enzymes generated by site-directed mutagenisis.

It should be pointed out that the calculated rate constants obtained by using the model in Scheme III may in reality reflect more than a single process. However, the use of a more complex model than that given in Scheme III with the explicit inclusion of more chemical intermediates would increase the number of rate constants required to define the system. Therefore, to be able to calculate the rate constants in a fashion analogous to that described above, it would be necessary to obtain steady-state data for additional substrate pairs. The kinetic model that we use is the minimal two-step model and as such is applicable to all enzymes that utilize the ping-pong mechanism, whether or not there are in practice multiple intermediates on the reaction pathway. The transamination reactions catalyzed by tyrosine aminotransferase, by analogy with mitochondrial aspartate aminotransferase (Kirsch et al., 1984), probably proceed via several intermediate complexes.

We have presented a method that allows the determination of microscopic rate constants from steady-state data using three substrate pairs. In addition, we have derived analytical expressions for the K_d values of the substrates in terms of the steady-state parameters. The minimum amount of data required is six steady-state measurements using six different substrates for the simplest ping-pong reaction scheme.

This method can supply supplementary information and insight into enzyme mechanisms and play a useful role in the characterization of substrate specificities of natural and mutant aminotransferases. Of course, the numbers obtained are a simplified substitute for detailed reaction kinetic and equilibria studies, given our assumption of such a simple mechanism, and should therefore be interpreted with due caution. However, we are confident that the method will be invaluable in the comparison of data from a large number of enzymes, for which detailed kinetic studies become unrealistic.

ACKNOWLEDGMENTS

We thank Prof. K. Kirschner for providing facilities during the initial part of this work, Drs. I. Fotheringham, M. Hunter, and M. Edwards for the generous gift of the bacterial strain overproducing tyrosine aminotransferase, Guiseppe Licci for performing some of the enzyme assays, and Dympna O'Connor for typing the manuscript.

REFERENCES

Braunstein, A. E. (1973) *Enzymes (3rd Ed.) 9*, 379–481.
Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.
Cornish-Bowden, A. (1979) in *Fundamentals of Enzyme Kinetics*, Butterworths, London.
Duggleby, R. G., & Morrison, J. F. (1978) *Biochim. Biophys. Acta 526*, 398–409.
Fasella, P., & Hammes, G. G. (1967) *Biochemistry 6*, 1798–1804.
Fersht, A. (1985) in *Enzyme Structure and Mechanism*, Freeman, New York.
Fotheringham, I. G., Dacey, S. A., Taylor, P. P., Smith, T. J., Hunter, M. G., Finlay, M. E., Primrose, S. B., Parker, D. M., & Edwards, M. E. (1986) *Biochem. J. 234*, 593–604.
Garces, E., & Cleland, W. W. (1969) *Biochemistry 8*, 633–640.
Haldane, J. B. S. (1930) *Enzymes*, Langmans Green, London [reprinted (1965) MIT Press, Cambridge, MA].
Henson, C. P., & Cleland, W. W. (1964) *Biochemistry 3*, 338–345.
Jenkins, W. T. (1964) *J. Biol. Chem. 239*, 1742–1747.

Karmen, A. (1955) *J. Clin. Invest. 34*, 131–133.
Kirsch, J. F., Eichele, G., Ford, G. F., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497–525.
Malcolm, B. A., & Kirsch, J. F. (1985) *Biochem. Biophys. Res. Commun. 132*, 915–921.
Toney, M. D., & Kirsch, J. F. (1987) *J. Biol. Chem. 262*, 12403–12405.
Wallin, B. K., & Arion, W. J. (1973) *J. Biol. Chem. 248*, 2380–2386.

Interactions of 5-Lipoxygenase with Membranes: Studies on the Association of Soluble Enzyme with Membranes and Alterations in Enzyme Activity

Angela Wong,* Shing Mei Hwang, Michael N. Cook, G. Kurt Hogaboom, and Stanley T. Crooke
Department of Molecular Pharmacology, Smith Kline & French Laboratories, King of Prussia, Pennsylvania 19406
Received February 10, 1988; Revised Manuscript Received April 18, 1988

ABSTRACT: Treatment of rat basophilic leukemia cells (RBL-1) with the calcium ionophore A23187 resulted in activation of 5-lipoxygenase, as indicated by an induction of leukotriene release [Orning, L., Hammarström, S., & Samuelsson, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2017]. The enzyme activation was accompanied by a time-dependent association of 5-lipoxygenase to the particular fraction. When cells were lysed in the presence of 0.05–10 μM $CaCl_2$, the soluble 5-lipoxygenase became associated with the particulate fraction. This was demonstrated by a decrease in immunoreactivities and enzymatic activities in the soluble fraction and a parallel increase in particulate-associated immunoreactivities. The particulate-bound enzyme was not active. Ca^{2+} induced the membrane association of 5-lipoxygenase when added into the incubation mixtures containing the membrane fraction with either the cytosolic fraction or the purified enzyme. 5-Lipoxygenase also bound to the microsomal-enriched fraction in the presence of Ca^{2+}. Maximal membrane binding was obtained after a 1-min incubation at 4 °C. When a fixed amount of isolated membranes (0.2 mg of protein) and increasing cytosolic protein (0.5–4 mg) were used, a linear increase in enzyme binding was observed. The binding became saturated at 3 mg of cytosolic protein/mg of membrane protein. 5-Lipoxygenase binding to the membrane fraction was unaffected by pretreatment of the membranes with trypsin but was inhibited by treating with phospholipase A_2, suggesting that phospholipids are involved. The membrane-bound enzyme was not extracted by treatment with a chelator (10 mM EDTA) or high salt (2 M NaCl) but was dissociated from membranes with detergents (0.5% sodium dodecyl sulfate, 10 mM Chaps, 0.5% digitonin, 0.5% Triton X-100, or 1% Brij 35). The membrane fraction when added to the cytosol, followed by subsequent addition of Ca^{2+} and substrate, stimulated 5-lipoxygenase activity. There was an increase in both the extent of product formation and the pseudo-steady-state velocity. The maximal stimulation was produced by 0.2 mg/mL membranes. In summary, these data suggest that Ca^{2+} induces a shift of 5-lipoxygenase from a soluble to a membrane-associated form which is accompanied by activation of the enzyme. Membrane phospholipids may be the elements in the membranes that bind to 5-lipoxygenase.

5-Lipoxygenases are enzymes in leukocytes and mast cells that oxidize arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5HPETE)[1] and leukotriene A_4 (LTA_4), which in turn are converted to a variety of products including leukotriene B_4 and the peptidoleukotrienes (LTC_4, LTD_4, and LTE_4) (Borgeat & Samuelsson, 1979a,b; Borgeat et al., 1976; Jakschik & Lee, 1980; Radmark et al., 1980a,b; Panossian et al., 1982; Maas et al., 1982; Maycock et al., 1982). These substances exhibit a broad range of biological actions including effects on cell migration, enzyme secretion, smooth muscle contraction in the respiratory and gastrointestinal tracts, and vascular permeability. Considerable evidence now implicates these products in a variety of acute and chronic inflammatory responses in vivo as well as in acute allergic reactions affecting the airway and skin (Samuelsson, 1983).

5-Lipoxygenase is activated by calcium and ATP (Jakschik & Lee, 1980; Jakschik et al., 1980; Ochi et al., 1983; Hogaboom et al., 1986). It has also been found that Ca^{2+} may induce the binding of 5-lipoxygenase to the membranes (Rouzer & Samuelsson, 1987). This suggests that activation of the enzyme is accompanied by a shift of the enzyme from the cytosol to the membrane within the cell. Considering the tendency of the enzyme to associate with membranes upon stimulation by Ca^{2+} and the abundance of its substrate in the plasma membrane, the translocation–activation may be a plausible regulatory mechanism. In the present studies, we demonstrate that treatment of RBL-1 cells with the calcium ionophore A23187 induces a shift of 5-lipoxygenase from the soluble to the particulate form. This is accompanied by an activation of the enzyme, as indicated by the production and release of LTC_4. The movement of the soluble enzyme to the particulate fraction occurs in physiological concentrations of Ca^{2+}. We have examined the interactions of 5-lipoxygenase

* Address correspondence to this author.

[1] Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; 5HPETE, 5-hydroperoxyeicosatetraenoic acid; LT, leukotriene; RBL cell, rat basophilic leukemia cell; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/88/0427-6763$01.50/0