Quantitative determination of the steady-state kinetics of multienzyme reactions using the algebraic rate equations for the component single-enzyme reactions

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Methods are given whereby the steady-state kinetic characteristics of multienzyme reactions consisting of individual single-enzyme reactions linked by freely diffusible intermediates can be determined quantitatively from the experimentally determined complete algebraic rate equations for the individual reactions. The approach is based on the fact that a valid steady-state rate equation for such a multienzyme reaction, in terms of the rate equations for the individual reactions, can be obtained simply from knowledge of the relative rates of the individual reactions when the multienzyme reaction is in the steady state. A number of model multienzyme reactions, which differ as to structural arrangement of the individual reactions, are examined by this approach. Simple mathematical methods which are applicable to most of these models are given for direct calculation of dependent variables. It is either pointed out or demonstrated with Mathematica that the rate equations for all of these models can be handled very easily with the aid of a personal computer equipped

with appropriate equation-solving software. Since the approach permits evaluation of all dependent variables for any specific combination of values for the kinetic parameters and independent variables, numerical values for the flux control coefficients of the individual enzymes can be obtained by direct calculation for a wide variety of conditions and can be compared with those obtained according to the methods of Metabolic Control Analysis. Several such comparisons have been made and in all cases identical results were obtained. The intuitive notion that the individual enzymes of a multienzyme reaction would be equally rate limiting if the total amount of enzyme were being used with maximum efficiency is tested and shown to be incorrect. In the course of this test the flux control coefficients for the individual enzymes were found to be appropriate indicators of relative rate limitation or control by the enzymes and to account properly for differences in specific activity among the enzymes.

INTRODUCTION

As is generally recognized, the rate behaviour of a multienzyme reaction consisting of several individual reactions linked by freely diffusible intermediates depends on the rate behaviour of the individual reactions. Therefore, a logical approach to the problem of determining the kinetics of a multienzyme reaction of this type would be to determine the kinetic characteristics of the individual reactions and use the resulting rate equations to obtain a rate equation for the multienzyme reaction. In the case of theoretical investigations, this approach is obligatory and thus has been used in all such investigations. However, all, or nearly all, such previous investigations have been conducted using approximations or abbreviated forms of the rate equations for the individual reactions. Notable exceptions may be the recent computer modelling approaches of Hofmeyr and van der Merwe [1] and Cornish-Bowden and Hofmeyr [2], which apparently provide for the use of full rate equations, but which require approximation of the concentrations of the linking intermediates by means of iterative procedures.

The present report describes an exceptionally simple means by which the steady-state kinetics of multienzyme reactions of the above type can be described using the familiar algebraic rate equations of single-enzyme kinetics without the use of approximations other than those employed in the acquisition of the single-enzyme rate equations. An important feature of the approach is that it permits easy acquisition of accurate values for the dependent variables for any combination of values for the kinetic parameters and independent variables, a consequence of which is that accurate numerical values for indicators such as flux control coefficients can be easily obtained for a wide variety of conditions.

APPROACH

Consider the multienzyme reaction:

in which a reactant A is converted into a product P by way of the freely diffusible intermediates B and C which link three individual reactions catalysed by the enzymes E_1 , E_2 , and E_3 . The overall reaction is assumed to be in a steady state by virtue of A and P being maintained at constant concentration and the constituents of the system are assumed to be uniformly distributed. It is assumed further that, when the net velocity is greater than zero, the concentrations of the reactant and intermediates are much greater than the concentrations of the enzymes, that, unless indicated otherwise, allosteric interactions among the components are absent, and that each of the individual reactions is catalysed in accordance with Haldane's generalized Michaelis-Menten rate equation.

When reaction (1) is in the steady state the net velocity v is the same for all of the individual reactions. Thus, in terminology analogous to that of Cleland [3]:

$$\begin{aligned} v &= \frac{e_1 V_{11} K_{b_1} a - e_1 V_{21} K_a b}{K_a K_{b_1} + K_{b_1} a + K_a b} \\ &= \frac{e_2 V_{12} K_{c_2} b - e_2 V_{22} K_{b_2} c}{K_{b_2} K_{c_2} + K_{c_2} b + K_{b_2} c} \\ &= \frac{e_3 V_{13} K_p c - e_3 V_{23} K_{c_3} p}{K_{c_3} K_p + K_p c + K_{c_3} p} \end{aligned} \quad (2)$$

Here the V s are maximum velocities, the K s are Michaelis constants, and the lower-case letters corresponding to the upper-case letters of reaction (1) represent concentrations. Since fractions that are equal to each other are equal to the sum of their numerators divided by the sum of their denominators, eqn. (2) is equivalent to:

$$v = \frac{e_1 V_{11} K_{b_1} a - e_1 V_{21} K_a b + e_2 V_{12} K_{c_2} b - e_2 V_{22} K_{b_2} c + e_3 V_{13} K_p c - e_3 V_{23} K_{c_3} p}{K_a K_{b_1} + K_{b_1} a + K_a b + K_{b_2} K_{c_2} + K_{c_2} b + K_{b_2} c + K_{c_3} K_p + K_p c + K_{c_3} p} \quad (3)$$

which is a rate equation for the overall reaction (1).

The question now is how to deal with eqn. (3) so as to determine the velocity of the overall reaction as a function of the independent variables e_1 , e_2 , e_3 , a , and p . As presented, this equation includes two dependent variables (b and c) other than v which must either be eliminated or evaluated if v is to be evaluated. They can be eliminated very easily by (i) solving the E_2 and E_3 components of eqn. (2) for c and setting the expressions that are equivalent to c equal to each other, and (ii) solving the resulting $E_2 E_3$ equation and the E_1 component of eqn. (2) for b and setting the expressions that are equivalent to b equal to each other. Doing this yields the implicit $E_1 E_2 E_3$ rate equation:

$$\begin{aligned} & (K_a K_{b_1} K_{c_2} K_p - K_a K_{b_2} K_{c_2} K_p + K_a K_{b_2} K_{c_3} K_p \\ & + K_{b_1} K_{c_2} K_p a + K_a K_{b_2} K_{c_3} p) v^3 \\ & + (K_a K_{b_2} K_{c_3} K_p V_{21} e_1 - K_a K_{b_2} K_{c_2} K_p V_{21} e_1 \\ & - K_{b_1} K_{c_2} K_p V_{11} e_1 a + K_a K_{b_2} K_{c_3} V_{21} e_1 p \\ & - K_a K_{b_1} K_{c_2} K_p V_{12} e_2 + K_a K_{b_2} K_{c_3} K_p V_{22} e_2 \\ & - K_{b_1} K_{c_2} K_p V_{12} e_2 a + K_a K_{b_2} K_{c_3} V_{22} e_2 p \\ & - K_a K_{b_1} K_{c_2} K_p V_{13} e_3 + K_a K_{b_2} K_{c_2} K_p V_{13} e_3 \\ & - K_{b_1} K_{c_2} K_p V_{13} e_3 a + K_a K_{b_2} K_{c_3} V_{23} e_3 p) v^2 \\ & + (K_a K_{b_2} K_{c_3} K_p V_{21} V_{22} e_1 e_2 + K_{b_1} K_{c_2} K_p V_{11} V_{12} e_1 e_2 a \\ & + K_a K_{b_2} K_{c_3} V_{21} V_{22} e_1 e_2 p + K_a K_{b_2} K_{c_2} K_p V_{13} V_{21} e_1 e_3 \\ & + K_{b_1} K_{c_2} K_p V_{11} V_{13} e_1 e_3 a + K_a K_{b_2} K_{c_3} V_{21} V_{23} e_1 e_3 p \\ & + K_a K_{b_1} K_{c_3} K_p V_{12} V_{13} e_2 e_3 + K_{b_1} K_{c_2} K_p V_{12} V_{13} e_2 e_3 a \\ & + K_a K_{b_2} K_{c_3} V_{22} V_{23} e_2 e_3 p) v \\ & - K_{b_1} K_{c_2} K_p V_{11} V_{12} V_{13} e_1 e_2 e_3 a \\ & + K_a K_{b_2} K_{c_3} V_{21} V_{22} V_{23} e_1 e_2 e_3 p = 0 \end{aligned} \quad (4)$$

Although daunting in number and complexity of terms as presented here, this equation is easy to deal with when the various kinetic parameters are represented with simpler symbols and a computer with appropriate equation-solving software is employed. As for equation-solving software, an excellent choice has proved to be Mathematica (Wolfram Research, Inc., Champaign, IL, U.S.A.), which performs a wide variety of mathematical operations and which is available for a wide variety of personal computers. Mathematica permits easy insertion of values into equations of any length and automatically simplifies them after numerical values have been inserted. For example, in dealing with eqn. (4), one need only prepare a list of numerical values for the kinetic parameters and independent variables. Upon command Mathematica inserts the list values and reduces eqn. (4) to a simple third-order polynomial equation in terms of v . Mathematica solves equations of this sort very rapidly (1–5 s with a 16 MHz IBM PS/2 Model 70 computer equipped with an Intel co-processor and 5 Mb of RAM) and provides solutions in as many significant figures as desired. Solving of equations up to ninth order has been attempted here with complete success. This means that multienzyme reactions of the type shown in reaction (1), having up to at least nine individual reactions, can be handled as

described above. If investigation of the basic kinetic characteristics of multienzyme reactions is the primary goal, use of such large systems is unnecessary because most of the funda-

mental properties can be demonstrated with systems having only three individual reactions.

It should be noted that eqn. (4) includes all the information of the individual rate equations of eqn. (2). Thus, the value of v obtained with eqn. (4) for a particular set of values for the kinetic parameters and independent variables can be used to evaluate b and c with the rate equations for the individual reactions. If mistakes have been avoided, the values obtained will satisfy eqn. (3), which is useful for checking results.

Sets of numerical values that satisfy eqn. (3) can be obtained without a computer and without eliminating dependent variables from the overall rate equation. To do so one must specify a value for v that is lower than the V_{max} of the overall reaction, which is determined in the case of reaction (1) by the individual reaction having the lowest eV_1 , and leave either a or p unspecified. For example, one could specify values for v and p and then work backwards through eqn. (2), evaluating c , b , and a in sequence using the E_3 , E_2 , and E_1 equations respectively.

RELATIVE RATE LIMITATION OR CONTROL

A matter of interest with respect to the kinetics of multienzyme reactions is the relative degrees to which the individual enzymes limit or control the rate of the overall reaction. Information of this sort may be useful in that it may give clues as to how the overall reaction is regulated or as to how one might go about modifying a multienzyme reaction to achieve a specific purpose.

In the cases of enzymic and other chemical reactions, 'rate' is generally understood to mean 'net rate' or 'net velocity'. Consequently, a detailed analysis of rate control in such reactions would be rather complex in that it would require one to distinguish between thermodynamic and kinetic control [4]. The primary concern here is determination of the relative degrees to which the individual enzymes of reaction (1) limit the rate of the overall reaction under a specific set of conditions of a , e_1 , e_2 , e_3 , and p regardless of how much of the limitation is thermodynamic and how much of it is kinetic. To do this one must have an appropriate indicator of relative rate limitation. Such an indicator is currently widely thought to be the reflection coefficient derived by Higgins [5] and subsequently applied very extensively to sequences of linked enzymic reactions by Kacser and Burns [6,7], Heinrich and Rapoport [8,9], and many others (see [10] for an excellent sampling of work in this area). This coefficient is now generally referred to as the control coefficient [11]. When used as an indicator of the relative degree to which a particular enzyme of a linked sequence of enzyme-catalysed reactions limits or controls the rate of the overall reaction, it is usually referred to as the flux control coefficient, which for E_1 of reaction (1), for example, may be defined variously as follows:

$$C_{e_1}^v = \frac{\partial v / v}{\partial e_1 / e_1} = \frac{\partial \ln v}{\partial \ln e_1} = \frac{e_1}{v} \frac{\partial v}{\partial e_1} \quad (5)$$

An important and very useful property of the control coefficient is that the coefficients for the individual components of a system always add up to unity.

If the flux control coefficient were an appropriate indicator of relative rate limitation, one could expect the flux control coefficients for the individual enzymes of reaction (1) to be equal when the enzymes are equally rate limiting. Of course a test of this expectation would require knowledge of the conditions under which the enzymes are equally rate limiting. Since increasing the concentration of a relatively non-limiting enzyme would have relatively little effect on the rate of the overall reaction, and thus would be wasteful of enzyme, one might suppose that the individual enzymes would be equally rate limiting if they were being used with maximum efficiency. In other words, one might suppose that the enzymes are equally rate limiting when their relative concentrations are such that the net velocity of the overall reaction is maximal for a given total concentration of enzyme [12]. As can readily be seen by the Lagrange method of undetermined modifiers [13], this condition exists for reaction (1) when $\partial v/\partial e_1 = \partial v/\partial e_2 = \partial v/\partial e_3$. Therefore, one might suppose that the appropriate indicator of relative rate limitation or control by the individual enzymes should be simply the relative value of $\partial v/\partial e$. As will be pointed out below, this notion is incorrect.

A test of the above notion does not require the use of undetermined modifiers. Thus, a result identical with that indicated by the Lagrange method can be obtained with eqn. (4) by (i) specifying that $e_1 + e_2 + e_3 = t$, where t is a specific total concentration of enzyme; (ii) eliminating one of the enzyme concentrations from eqn. (4) with the use of this constraint, say e_3 by setting it equal to $t - e_1 - e_2$; (iii) determining $\partial v/\partial e_1$ and $\partial v/\partial e_2$ by implicit differentiation of the e_3 -eliminated form of eqn. (4); (iv) imposing the condition maximum v by setting the expressions that are equivalent to $\partial v/\partial e_1$ and $\partial v/\partial e_2$ equal to zero; (v) forming a list of the above three equations in v , e_1 , and e_2 within Mathematica, which upon command evaluates these variables simultaneously after numerical values for a , p , t , and the kinetic parameters have been inserted into the list; (vi) evaluating e_3 from its relationship to t , e_1 , and e_2 ; and (vii) determining ∂v with respect to each of the enzymes using the unmodified form of eqn. (4) and the enzyme concentrations obtained as described above.

This last step can be achieved very easily within Mathematica by (i) inserting into eqn. (4) the appropriate numerical values for a , p , the kinetic parameters (those used to obtain v and the enzyme concentrations), and the enzyme concentrations that are to be held constant, say e_2 and e_3 ; (ii) solving the equation for e_1 ; (iii) differentiating the resulting explicit equation with respect to v ; (iv) inserting the numerical value for v into the differentiated equation; and (v) inverting the resulting numerical value for $\partial e_1/\partial v$ to obtain $\partial v/\partial e_1$. Although Mathematica is capable of solving the equation obtained in step (i) for v and of differentiating the resulting explicit equation with respect to e_1 , it understandably does so with relatively great difficulty owing to the equation being third-order in terms of v .

According to the above procedure, the condition for maximum v under a particular set of conditions of a , p , and t , is that $\partial v/\partial e_1 = \partial v/\partial e_2 = \partial v/\partial e_3 = v/t$. When this condition exists, $C_v^1 = e_1/t$, $C_v^2 = e_2/t$, and $C_v^3 = e_3/t$ (i.e. the flux control coefficients are proportioned among the individual enzymes exactly as the total concentration of enzyme is proportioned among the individual reactions, a result also obtained recently by Pettersson and Pettersson [14] using a different approach). In addition, v is directly proportional to t at any specific combination of values for a , p , and the kinetic parameters that result in a net velocity,

as would be the case if the overall reaction were catalysed by a single enzyme. A number of combinations of kinetic parameters and concentrations of a and p were employed in testing this procedure and invariably these results were obtained.

As is evident from eqn. (5) and the fact that the control coefficients for the individual components of a system always add up to unity, the flux control coefficient and relative value of $\partial v/\partial e$ for a specific individual enzyme of reaction (1) are equal when the concentrations of the enzymes among the individual reactions are equal. When, in addition, the rate equations and kinetic parameters of the individual reactions are the same, the concentrations of the enzymes are optimally proportioned among the individual reactions with respect to v and thus all of the enzymes have the same flux control coefficient and relative value of $\partial v/\partial e$. This is so regardless of the concentrations of A and P . On the other hand, when the concentrations of the enzymes are not equal, the flux control coefficient and relative value of $\partial v/\partial e$ for a specific enzyme differ, and changes in the concentrations of A and P result in changes in the magnitude of the difference, regardless of whether or not the rate equations and kinetic parameters are identical for the individual reactions.

Figure 1 shows the effects of varying a of the multienzyme reaction (1) occurring according to eqns. (3) and (4) under conditions of $p = 0$ and of a constant total concentration of enzyme t which is proportioned among the individual reactions such that v is maximal for the conditions of a and p indicated. As noted above, the $\partial v/\partial e$ values for the individual enzymes are identical under such conditions and are equal to v/t . Thus the relative values of $\partial v/\partial e$ are identical. If the rate equations and kinetic parameters for the individual reactions were identical, this would be true also of the flux control coefficients. Therefore, to make possible a comparison between the flux control coefficient and the relative value of $\partial v/\partial e$ as indicators of relative rate limitation or control by the individual enzymes, V_{11} , V_{12} , and V_{13} were chosen to differ and were assumed to be 1, 2, and 3 respectively in Figure 1(a) and 3, 2, and 1 respectively in Figure 1(b). All of the other kinetic parameters were assumed to be unity. Note that, according to the Haldane relationship, this choice of kinetic parameters says that the equilibrium constants of the individual reactions are identical with the V_1 values and that, since the equilibrium constant for the overall reaction (1) is given by the product of the equilibrium constants of the individual reactions, the equilibrium constants for the overall reactions of Figures 1(a) and 1(b) are assumed to be identical. Note also that the above choice of kinetic parameters says that the potential activities of the individual enzymes, with respect to the net forward velocities of the reactions the enzymes catalyse, are higher as the equilibrium constants (V_1 values) are higher, and thus that, for a specific individual reaction, the concentration of enzyme required to achieve a particular net forward velocity under a particular set of conditions of reactant and product concentration will be lower as the equilibrium constant (V_1 value) of the reaction is higher. As one might expect, the rate of the overall reaction and the relative degrees to which it is limited or controlled by the individual enzymes differ considerably depending on one's choice of kinetic parameters to account for the equilibrium constants of the individual reactions. This will be shown in detail below.

Figure 1 shows that when the total concentration of enzyme is distributed among the individual reactions such that the rate of reaction (1) is maximal, the flux control coefficients for the individual enzymes vary considerably depending on the values assumed for a and the equilibrium constants (V_1 values) of the individual reactions. Under such conditions and saturation of the system with A , $e_1 V_{11} = e_2 V_{12} = e_3 V_{13} = V_{\max}$ for the overall

Figure 1 Effects of varying a on the net velocity and flux control coefficients of the multienzyme reaction (1) for conditions of $p = 0$, of constant total concentration of enzyme t , and of $\partial v/\partial e_1 = \partial v/\partial e_2 = \partial v/\partial e_3 = v/t$.

The overall reaction was assumed to occur according to eqns. (3) and (4). $V_1:V_2:V_3$ was assumed to be 1:2:3 in (a) and 3:2:1 in (b). All of the other kinetic parameters were assumed to be unity. The condition $\partial v/\partial e_1 = \partial v/\partial e_2 = \partial v/\partial e_3 = v/t$ was achieved as described in the text and corresponds to the condition in which the total concentration of enzyme is proportioned among the individual reactions such that v is maximal at all of the indicated values of a . In consequence of this condition, $C_{e1}^v = e_1/t$, $C_{e2}^v = e_2/t$, $C_{e3}^v = e_3/t$ and v is directly proportional to t at any specific finite value of a .

reaction. From this relationship and the fact that the flux control coefficients are proportioned among the individual enzymes exactly as the total concentration of enzyme is proportioned among the individual reactions, it is clear that if two of the individual reactions had been assigned V_i values much greater than the V_1 value of the other, the flux control coefficients could indicate that the enzyme of this other reaction is virtually the only one limiting the rate of the overall reaction. Consequently, if one were to arrange for this condition and were to assume the flux control coefficient to be an appropriate indicator of relative rate control, one could expect a doubling of the concentration of the enzyme to have a near doubling effect on the rate of the overall reaction in the case of the enzyme having the extremely

large coefficient, and to have a very small effect on the rate in cases where the enzymes have extremely small coefficients. This is precisely what is observed. Therefore, the results of this comparison appear to be consistent with the conclusions: (i) that the above notion, that the individual enzymes of reaction (1) would be equally rate limiting if the available amount of enzyme were being used with maximum efficiency, is incorrect, and (ii) that the relative degrees to which the individual enzymes limit or control the rate of the overall reaction are given by the flux control coefficients for the enzymes. The success of the flux control coefficients here can be seen to result from their ability to account properly for differences in specific activity among the enzymes.

Direct calculation of flux control coefficients can be achieved in a number of ways without the use of sophisticated computer software and without having to eliminate all of the intermediate concentrations from the rate equation for the overall reaction. For example, in the case of reaction (1), $\partial v/\partial e_1$ may be obtained by determining first $\partial e_1/\partial v$ by partial differentiation of eqn. (3) and then inverting the result. This may be accomplished with an equation for the total differential of e_1 which has been converted into the derivative form by dividing both sides by dv :

$$de_1/dv = \partial e_1/\partial v + (\partial e_1/\partial b) db/dv + (\partial e_1/\partial c) dc/dv \quad (6)$$

The partial derivatives may be obtained either by differentiation of eqn. (3) in the implicit form or by solving eqn. (3) for e_1 and differentiating the resulting explicit equation. Of course all variables other than those being differentiated are considered to be constants during partial differentiation. On the other hand, db/dv must be determined with an equation that includes b and v but not e_1 and c , and dc/dv must be determined with an equation that includes c and v but not e_1 and b . A suitable equation for the determination of db/dv is the E_2E_3 equation obtained by eliminating c between the E_2 and E_3 components of eqn. (2), and a suitable equation for the determination of dc/dv is the E_3 component of eqn. (2). After de_1/dv of eqn. (6) has been evaluated with a set of numerical values that satisfies eqn. (3), it is inverted and designated as $\partial v/\partial e_1$ for determination of C_{e1}^v according to eqn. (5). Similar methods may be employed to obtain numerical values for C_{e2}^v and C_{e3}^v . Although this procedure is more difficult than solving eqn. (4) for each of the enzyme concentrations and differentiating the resulting explicit equations, it is useful in cases in which the individual rate equations are such as to make it difficult or impossible to eliminate all of the intermediate concentrations from the rate equation for the overall reaction. In any case, it is generally beneficial to eliminate as many of the intermediate concentrations as possible before differentiating.

In recent years a number of ingenious methods for the determination of flux control coefficients have been developed according to the principles or theorems of Metabolic Control Analysis (MCA) [6-9,15]. For the most part the MCA approach involves determination of elasticity coefficients, which gauge the velocity responses of the individual reactions to changes in the concentrations of the intermediates, and assembling these coefficients in accordance with summation and connectivity relationships into equations that yield the control coefficients. It has been tested here with sets of numerical values that satisfy the rate equations for a variety of model multienzyme reactions which differ as to structural arrangement of the individual reactions and in all cases has been found to yield the same results as the direct calculation methods described above. In the cases of reaction (1) and several other multienzyme reactions considered below, acquisition of flux control coefficients by this approach is achievable with the use of relatively simple connectivity relation-

ships of the type described in a recent review by Kacser and Porteous [15]. For convenience, the method described in this review shall be referred to below as the simple MCA approach.

MISCELLANEOUS APPLICATIONS

The direct calculation approach is applicable to a wide variety of multienzyme reactions which differ with regard to structural arrangement of the individual reactions. Whether or not a particular arrangement can be handled successfully depends on whether there are enough equations to eliminate or evaluate all of the dependent variables. Of course this is true of any approach that attempts to avoid loss of information concerning the kinetic and thermodynamic characteristics of the individual enzymes and reactions. In testing the feasibility of handling a particular arrangement, it is unnecessary to include the kinetic and thermodynamic parameters in the rate equations because they have no bearing on the feasibility of the approach. However, since most rate equations include terms consisting only of these parameters, they cannot be ignored completely and thus are best eliminated from consideration by setting them equal to unity. Another simplification that has no bearing on the feasibility of the approach may be achieved by assuming the concentrations of all final products to be zero.

The feasibility of handling the various multienzyme reactions considered below was tested using Haldane's generalized Michaelis-Menten equation in the case of Uni Uni individual reactions and the equations given by Cleland [3] in the cases of Uni Bi, Bi Uni, and Bi Bi individual reactions. Association or dissociation of reactants and products with or from the enzyme in the cases of bireactant reactions was assumed to be ordered. Since the equations for ordered bireactant reactions are not algebraically symmetrical with respect to the two reactants and/or two products, flux control coefficients differ depending on the order in which the reactants or products are considered to add to or leave the enzyme. One's choice in this regard determines to some extent also the approach that one might find best for handling multienzyme reactions that include bireactant individual reactions. In accordance with the terminology of Cleland [3], the order of addition or dissociation of multiple reactants or products is indicated here by the order of the letters of the alphabet used to represent them.

Simple sequential multienzyme reactions

The prototype for this structural arrangement is reaction (1). Since detailed rate equations are given, this reaction will be employed to illustrate further the effects of one's choice of kinetic parameters and of reactant and product concentrations on the net velocity of the overall reaction and on the flux control coefficients for the individual enzymes. As is evident from Figure 1, the concentration of the initial reactant and the thermodynamics of the individual reactions, as well as the concentrations of the enzymes, are important determinants of relative rate limitation or control among the individual enzymes. Of course this is true also of the properties of the enzymes. Such is readily evident from Table 1, which compares five multienzyme reactions which differ only with regard to the kinetic and thermodynamic properties of the E_2 reaction. A control multienzyme reaction is included in which it was assumed that the equilibrium constants, kinetic parameters, and enzyme concentrations are equal to unity in each of the three individual reactions. As noted above, the flux control coefficients are equal under such conditions regardless of

Table 1 Dependence of the flux control coefficients, net velocity, and intermediate concentrations of the model multienzyme reaction (1) on a and one's choice of kinetic parameters to account for a change in one's choice of equilibrium constant for the E_2 reaction

The overall reaction was assumed to occur according to eqns. (3) and (4) under the condition $p = 0$. All of the kinetic parameters and enzyme concentrations were initially assumed to be unity and thus the equilibrium constants of the individual reactions were assumed to be unity. Then the equilibrium constant of the E_2 reaction, K_{E_2} , was assumed to be 10 and the kinetic parameters of the E_2 reaction were altered one at a time by a factor of 10 according to the requirements of eqn. (7), the Haldane relationship for the E_2 reaction. Although the units employed are arbitrary, the size of the unit for the enzyme concentrations is assumed to be much smaller than that of the unit for the concentrations of the reactant and intermediates.

K_{E_2}	E_2 parameter changed	a	b	c	v	C_e^r		
						E_1	E_2	E_3
1	None	0.1	0.065	0.031	0.030	0.333	0.333	0.333
		10	3.30	0.879	0.468	0.333	0.333	0.333
		1000	78.9	5.80	0.853	0.333	0.333	0.333
10	V_{12}	0.1	0.016	0.081	0.075	0.814	0.087	0.099
		10	0.781	3.60	0.783	0.586	0.052	0.362
		1000	10.2	46.1	0.979	0.509	0.006	0.485
10	V_{22}	0.1	0.051	0.044	0.043	0.458	0.475	0.068
		10	2.70	1.14	0.533	0.350	0.406	0.244
		1000	64.6	7.18	0.878	0.334	0.351	0.315
10	K_{b_2}	0.1	0.017	0.080	0.074	0.794	0.103	0.103
		10	1.43	2.22	0.689	0.394	0.303	0.303
		1000	36.5	13.0	0.929	0.336	0.332	0.332
10	K_{c_2}	0.1	0.050	0.045	0.043	0.474	0.474	0.051
		10	2.11	1.51	0.601	0.433	0.433	0.134
		1000	40.4	11.7	0.921	0.366	0.366	0.268

the concentrations of A and P. The E_2 reactions of the other four multienzyme reactions were assumed to have equilibrium constants of 10 and were assumed to differ otherwise from the control only in that one of the kinetic parameters was higher or lower by a factor of 10 to satisfy the requirement of the Haldane relationship that:

$$K_{E_2} = \frac{V_{12} K_{c_2}}{V_{22} K_{b_2}} \quad (7)$$

In all cases p was assumed to be zero and a was varied over a wide range.

As may be seen (Table 1), the net velocity of the overall reaction and the flux control coefficients for the individual enzymes differ considerably depending on the value assumed for a and on which of the kinetic parameters of the E_2 reaction is adjusted to account for the increase in the equilibrium constant for this reaction. Note that when either V_{22} , K_{b_2} , or K_{c_2} is altered to account for the increase in the equilibrium constant, the flux control coefficients tend toward equality as a is increased. On the other hand, when V_{12} is the E_2 parameter that is changed, the flux control coefficients for E_1 and E_3 approach equality and that for E_2 approaches zero as a is increased. Since the lowest v/V_1 of the overall reaction is in all cases unity, the net velocity of the overall reaction in all cases approaches unity as a is increased.

Although obtained with the use of unrealistic assumptions, the findings of Table 1 serve to point out the importance of knowing and including in the rate equation for a multienzyme reaction such as reaction (1) the details of the kinetic and thermodynamic properties of the individual enzymes and reactions if meaningful flux control coefficients are to be obtained, or if the bases for

Table 2 Effects of product concentration on the net velocity, flux control coefficients, and intermediate concentrations of the multienzyme reaction (1) under conditions of $a = 10$ and $V_{11}:V_{12}:V_{13}$ as indicated

The overall reaction was assumed to occur according to eqns. (3) and (4). The concentrations of the enzymes and all kinetic parameters other than V_1 were assumed to be unity. Consequently, the equilibrium constants of the individual reactions are identical with their V_1 values and the equilibrium constants of the overall reactions are given by $V_{11} V_{12} V_{13}$.

$V_{11}:V_{12}:V_{13}$	p	v	b	c	C'_p		
					E_1	E_2	E_3
1:2:3	0	0.743	1.05	0.329	0.719	0.197	0.085
	2	0.592	2.19	1.57	0.600	0.245	0.155
	10	0.353	4.52	5.24	0.498	0.284	0.219
	30	0.139	7.43	12.0	0.445	0.302	0.253
	59.9	0.000	9.99	20.0	0.420	0.310	0.270
3:2:1	0	0.862	11.0	6.27	0.103	0.141	0.756
	2	0.645	13.9	11.1	0.184	0.233	0.584
	10	0.369	18.9	22.3	0.237	0.281	0.481
	30	0.144	24.8	40.2	0.265	0.301	0.433
	59.9	0.000	30.0	59.9	0.279	0.310	0.412

experimentally determined flux control coefficients are to be understood.

Table 2 shows the effects on the flux control coefficients and net velocity of reaction (1) of increasing p under the condition $a = 10$ to a point at which the overall reaction is near equilibrium. The concentrations of the enzymes and all of the kinetic parameters other than V_1 were assumed to be unity. As may be seen, the flux control coefficients are quite sensitive to the presence of product of the overall reaction under these conditions and tend to become less unequal as p is increased to a point where the overall reaction is very close to equilibrium.

Branched multienzyme reactions

The branched structural arrangement that is usually considered to be the simplest consists of three individual reactions which are linked by a single intermediate:

In considering this so-called branch-point multienzyme reaction, it will be assumed that $p = q = 0$ to assure that all of the individual reactions have a net forward velocity regardless of the finite value of a . When the overall reaction is in the steady state, the net velocity of the E_1 reaction is equal to the sum of the net velocities of the E_2 and E_3 reactions. In consequence, the rate expression for the E_1 reaction is equal to the sum of the rate expressions for the E_2 and E_3 reactions. Therefore, a rate equation for the overall reaction free of dependent variables other than v can be obtained simply by (i) adding the rate expressions for the E_2 and E_3 reactions, (ii) setting the resulting expression equal to the net velocity of the E_1 reaction, (iii) solving the E_1 rate equation for b , and (iv) substituting the resulting equivalent of b into the $E_2 E_3$ equation obtained in step (ii). With the $E_1 E_2 E_3$ equation thus obtained in hand, one can obtain numerical values for b , the net velocities, and the flux control coefficients by direct

calculation essentially as described for reaction (1). All of this can be done very easily within Mathematica.

Since there are three velocities in the case of reaction (8), there is a choice as to which of the three is used to obtain the flux control coefficients. The treatment described above permits acquisition of the coefficients only with respect to the velocity of the E_1 reaction. If coefficients with respect to one of the other velocities were desired, for example the velocity of the E_3 reaction, one could go about obtaining them by (i) subtracting the rate expression for the E_2 reaction from that of the E_1 reaction, (ii) setting the resulting expression equal to the net velocity of the E_3 reaction, and (iii) eliminating b between the resulting $E_1 E_2$ equation and the E_3 equation. As predicted by the observations of Kacser [16], the $E_1 E_2 E_3$ equation thus obtained yields a negative flux control coefficient for E_2 . This is one of a number of rather odd characteristics of reaction (8). Another is observed when the coefficients are obtained with respect to the velocity of the E_1 reaction, in which case all of the coefficients are positive. Thus, when the enzyme of one of the two reactions linked to the E_1 reaction is made more limiting by decreasing its concentration, the flux control coefficients indicate that the enzymes of both reactions become more rate limiting. In contrast, when the enzyme of one of the two reactions is made more rate limiting by decreasing V_1 , the coefficients indicate that the enzyme of the reaction with the decreased V_1 value becomes less rate limiting and that enzyme of the other becomes more rate limiting. These odd characteristics are due to the fact that the velocities of the individual reactions are not constrained to be equal, a consequence of which is that the flux control coefficients are largely meaningless when compared in the usual manner. The coefficients make sense only when they are determined with respect to the velocity of the E_1 reaction and the coefficients for E_2 and E_3 are added before comparing them with the coefficient for E_1 .

Flux control coefficients for the individual enzymes of overall reaction (8) cannot be obtained according to the simple MCA approach. However, Kacser [16] has presented connectivity relationships that do apply. These have been tested and were found to yield the same numerical results as the direct calculation approach described above.

Another type of branched structural arrangement may be exemplified by the reaction:

This multienzyme reaction consists of a Uni Bi reaction linked to two Uni Uni reactions. Since in the steady state the net velocities of the individual reactions are constrained to be equal, and since there are three rate equations which may be used to eliminate two of the three dependent variables, this structural arrangement can be handled by the direct calculation approach essentially as described for reaction (1). Of course to do so is slightly more difficult owing to the relatively large number of terms in the rate equations of Uni Bi reactions as compared with Uni Uni reactions. Flux control coefficients can be determined either by direct calculation or by the simple MCA approach.

A branched structural arrangement that is relatively difficult to handle may be exemplified by the reaction:

In this 'internally branched' case there are four dependent variables and three rate equations. To deal with it one must come up with an additional equation. For example, one could assume that the intermediates are very stable and that the only sources for them are A and P through the E_1 and E_3 reactions. If such were the case, one could expect c to equal $b + d$. This condition would allow elimination of one of the dependent variables. Doing so results in three rate equations, each of which is in terms of v and the two remaining intermediate concentrations. Mathematica readily evaluates these variables simultaneously after numerical values for the independent variables and kinetic parameters have been inserted into a list of the three equations.

Acquisition of an $E_1 E_2 E_3$ equation free of intermediate concentrations is virtually impossible in this case. However, flux control coefficients can be obtained fairly easily by partial differentiation in accordance with an abbreviated version of eqn. (6). This may be achieved by (i) eliminating d by assuming it to be equal to $c - b$; (ii) solving all of the individual rate equations for b and combining the E_2 and E_3 expressions for b by adding their numerators and denominators; (iii) eliminating b between the resulting $E_2 E_3$ equation and the E_1 equation; (iv) using the resulting $E_1 E_2 E_3$ equation to obtain the partial derivatives of the enzyme concentrations with respect to v and c ; and (v) eliminating b between the E_2 and E_3 equations, between the E_1 and E_3 equations, and between the E_1 and E_2 equations and using the resulting $E_2 E_3$, $E_1 E_3$, and $E_1 E_2$ equations to obtain dc/dv for determination of $\partial v/\partial e_1$, $\partial v/\partial e_2$, and $\partial v/\partial e_3$ respectively.

As noted by Fell and Sauro [17], the simple MCA approach for the determination of flux control coefficients is not applicable to multienzyme reactions in which an assumption has been made as to the total or relative concentrations of the intermediates. However, these workers have developed a simple method whereby appropriate connectivity relationships can be obtained for such reactions. In the case of reaction (10), handled as described above, three of these relationships may be obtained by assuming $\delta b = -\delta d$, $\delta c = \delta d$, and $\delta c = \delta b$, any two of which yields numerical values for the flux control coefficients identical with those obtained by direct calculation when solved simultaneously for $C_{e_1}^v$, $C_{e_2}^v$, and $C_{e_3}^v$ in conjunction with the summation relationship $C_{e_1}^v + C_{e_2}^v + C_{e_3}^v = 1$.

An interesting characteristic of reaction (10) is that within certain limits the concentration of any one of the intermediates can be fixed without precluding the achievement of a steady state. There is, in addition, a particular combination of intermediate concentrations that results in the net velocity of the overall reaction being maximal. This combination, for a particular set of values for the independent variables and kinetic parameters, may be determined with an $E_1 E_2 E_3$ equation obtained by eliminating d between the E_2 and E_3 equations and then either b or c between the resulting $E_2 E_3$ equation and the E_1 equation. For example, if the $E_1 E_2 E_3$ equation thus obtained is in terms of v and b , one can do this by (i) determining $\partial v/\partial b$ by implicit differentiation, (ii) imposing the condition maximum v by setting the expression that is equivalent to $\partial v/\partial b$ equal to zero, (iii) using this equation and the $E_1 E_2 E_3$ equation to determine v and b simultaneously, and (iv) using the values thus obtained in the individual rate equations to determine the corresponding values of c and d . Flux control coefficients can be determined by direct calculation very easily in this case with an $E_1 E_2 E_3$ equation obtained by eliminating b between the above $E_1 E_2 E_3$ equation and the equation obtained in step (ii). Since no assumptions as to the total or relative concentrations of the intermediates were made in this case, the simple MCA approach is applicable and yields the same numerical results as the direct calculation approach.

Cyclic multienzyme reactions

The prototype for this structural arrangement is:

Since here, as in reaction (10), there are four dependent variables and only three rate equations, successful treatment of this arrangement requires imposition of a conservation condition which permits elimination of one of the dependent variables other than v . Such a condition is that $b + c + d = s$, where s is the concentration of a 'sparker' which must be added initially to get the reaction going and which must consist of one or more of the intermediates. The need for such a sparker in cyclic systems that are initially devoid of intermediates is well known to investigators who have studied pyruvate oxidation via the tricarboxylic acid cycle with isolated plant mitochondria, which tend to lose their tricarboxylic acid cycle intermediates during isolation. Elimination of either b or c by assuming this condition results in a set of equations very similar to that obtained in the case of reaction (10). Consequently, the dependent variables and flux control coefficients of reaction (11) can be evaluated by direct calculation essentially as described for reaction (10). Because of the conservation condition imposed, acquisition of correct flux control coefficients by way of the MCA approach requires the use of connectivity relationships of the sort given by Fell and Sauro [17] and later also by Hofmeyr et al. [18]. These relationships have been tested and found to yield the same numerical results as the direct calculation approach. An unusual characteristic of reaction (11) is that the V_{max} value of the overall reaction is much less than the lowest eV_{i1} , regardless of sparker concentration.

A cyclic arrangement that is cyclic with respect to the intermediates linking the individual reactions rather than to the arrangement of the individual reactions may be exemplified by the reaction:

This multienzyme reaction consists of two Bi Bi reactions which are linked by a coenzyme-type intermediate that transfers something between the reactants of the two reactions. For example, if X were ADP and Y were ATP, a phosphoryl group would be transferred from A to B in the forward reaction and from Q to P in the backward reaction. Since the adenine nucleotide moiety of ADP and ATP would be conserved and cycled between two forms, the structural arrangement that results from this type of linkage is often referred to as a moiety-conserved cycle. Since the sum of the concentrations of the two forms does not change during the reaction, the concentration of one of the forms can be expressed as the difference between a constant concentration c and the concentration of the other. Doing this by letting $y = c - x$ results in both rate equations being second-order in terms of x , a consequence of which is that x is difficult, if not impossible, to eliminate. However, since there are two equations in terms of the two remaining dependent variables, v and x and then y can

be evaluated for particular combinations of the independent variables and kinetic parameters. Alternatively, v may be eliminated between the modified E_1 and E_2 equations and then evaluated with one of the individual rate equations after x and y have been evaluated. Flux control coefficients can be determined directly by partial differentiation of an $E_1 E_2$ equation obtained either by adding the numerators and denominators of the two modified rate expressions that are equivalent to v , or by eliminating x^2 between the two rate equations. dx/dv for determination of $\partial v/\partial e_1$ and $\partial v/\partial e_2$ according to an abbreviated version of eqn. (6) may be obtained with the modified forms of the E_2 and E_1 equations respectively.

Because of the need to impose a conservation condition, valid flux control coefficients for reaction (12) cannot be determined by the simple MCA approach. Appropriate connectivity relationships for this reaction have been given by Hofmeyr et al. [18]. These have been tested and found to yield the same numerical results as the direct calculation approach.

FEEDBACK REGULATION

The activities of enzymes in living systems must be closely regulated to prevent excessive accumulations of metabolites [19]. An important mechanism whereby this is achieved within multi-enzyme sequences such as reaction (1) is inhibition of the first reaction in the sequence through modification of the enzyme by a downstream product. Of course the key to success in modelling the kinetics of such sequences is knowing in quantitative detail how the downstream product decreases the activity of the upstream enzyme. One possibility is that the downstream product binds to the upstream enzyme and thereby inactivates it completely. If such were the case with respect to the action of C on E_1 of reaction (1) and the binding of C to E_1 were unaffected by changes in other variables of the system, the rate equation for the E_1 reaction could be altered to account for this action simply by multiplying all the terms in e_1 by $K_1/(K_1 + c)$, K_1 being the dissociation constant of the $E_1 C$ complex.

Another possibility is that the binding of the downstream product to the upstream enzyme does not inactivate the enzyme but decreases its activity through modification of its catalytic properties. Assuming this to be the case with respect to the binding of C to E_1 of reaction (1) as described above, the factor $K_1/(K_1 + c)$ must be used to modify the kinetic parameters of the E_1 reaction rather than the concentration of active E_1 . Since variation of c would not affect the equilibrium constant of the E_1 reaction, this must be done in such a way that the equilibrium constant is in fact a constant. In other words, this must be done in such a way that the Haldane equivalent of the equilibrium constant, $V_{11} K_{b1}/V_{21} K_a$, is constant regardless of the concentration of C. To illustrate application of the approach of this study to this sort of regulation of reaction (1), it shall be assumed that the binding of C to E_1 decreases the activity of E_1 by increasing the affinity of the enzyme for A, thereby decreasing K_a and decreasing V_{11} as a result of increased stability of the $E_1 A$ complex relative to the $E_1 B$ complex [4]. The likelihood that V_{21} and K_{b1} would also be affected shall be ignored. To account for these effects of C on the E_1 reaction, the E_1 component of eqn. (2) will be modified by substituting K_a^{app} for K_a and V_{11}^{app} for V_{11} , K_a^{app} and V_{11}^{app} being defined as follows:

$$K_a^{\text{app}} = K_a \left(\frac{K_1}{K_1 + c} \right) + K'_a \left(1 - \frac{K_1}{K_1 + c} \right) \quad (13)$$

$$V_{11}^{\text{app}} = \frac{V_{11} K_a^{\text{app}}}{K_a} = V_{11} \left[\frac{K_1}{K_1 + c} + \frac{K'_a}{K_a} \left(1 - \frac{K_1}{K_1 + c} \right) \right] \quad (14)$$

Figure 2 Effects of feedback regulation on the net velocity, flux control coefficients, and intermediate concentrations of the model multienzyme reaction (1) under the condition $p = 0$

Except as indicated below, the overall reaction was assumed to proceed in accordance with eqns. (3) and (4). All of the kinetic parameters and enzyme concentrations given in eqns. (3) and (4) were assumed to be unity. Part (a) was obtained without further assumptions and thus is the control. In the case of part (b), binding of C to E_1 was assumed to modify K_a and V_{11} according to eqns. (13) and (14), assuming $K'_a = 0.5$ and $K'_a = 0.1$. Parts (c) and (d) were obtained by assuming that the binding of C to E_2 and E_1 respectively inactivates the enzyme according to $K_1/(K_1 + c)$, used as a multiplier of the enzyme concentration; in both cases the dissociation constant of the EC complex, K_p , was assumed to be 0.5.

Here K'_a is the Michaelis constant for A when c is saturating with respect to E_1 . Please note that, as required, substitution of the above expressions for K_a^{app} and V_{11}^{app} for K_a and V_{11} in the Haldane relation for the E_1 reaction does not affect the magnitude of the equilibrium constant regardless of the values assumed for K_1 , K'_a , and c .

Although it appears from eqns. (13) and (14) that making the above substitutions in the E_1 equation would greatly increase the complexity of the equation, owing to cancellations it merely doubles the number of terms. The intermediate concentrations can be easily eliminated from the rate equation for the overall reaction by first eliminating c between the E_1 and E_3 equations and between the E_2 and E_3 equations, and then eliminating b between the resulting $E_1 E_3$ and $E_2 E_3$ equations. Flux control coefficients can then be determined either by direct calculation using the resulting $E_1 E_2 E_3$ equation or by the simple MCA approach.

Figure 2 compares multienzyme reactions (i) without feedback inhibition (Figure 2a), (ii) with feedback inhibition of the E_1 reaction by C according to eqns. (13) and (14) (Figure 2b), and (iii) with feedback inhibition of the E_2 (Figure 2c) and E_1 (Figure 2d) reactions by C through enzyme inactivation as described above. To facilitate observation of the effects of the regulation over a wide range of concentration of the initial reactant, p was assumed to be zero and the concentrations of the enzymes and all of the kinetic parameters given in the primary rate equations [eqns. (2), (3) and (4)] were assumed to be unity. As may be seen by comparing Figure 2(b) with Figure 2(d), the two different mechanisms by which the product of the E_2 reaction is assumed to inhibit the E_1 reaction do not differ greatly with regard to their qualitative effects. Comparison of Figure 2(c) with Figure 2(d)

shows that when E_2 rather than E_1 is assumed to be inactivated by C, only b and the flux control coefficients for E_1 and E_2 are affected. In general, the results show that, as expected, inhibition of the first reaction in the sequence by a downstream product effectively decreases the concentrations of the intermediates at the expense of a decrease in the maximum velocity of the overall reaction. Such a decrease in velocity of a multienzyme reaction *in vivo* is not likely to be important, because the concentration of the initial reactant would probably also be closely controlled and thereby maintained at a low level.

CONCLUSION

This study describes an approach whereby the steady-state kinetics of multienzyme reactions can be determined quantitatively from experimentally determined kinetic and thermodynamic characteristics of the individual enzymes and reactions. It involves the use of mathematical techniques which can be easily mastered by virtually all investigators. Since it permits retention of all the information concerning the kinetic and thermodynamic properties of the individual enzymes and reactions, the degree of its success for a manageable multienzyme reaction depends only on how well these properties are known for the conditions under which the overall reaction occurs. Although the approach requires the use of algebraic rate equations, the only requirement as to the nature of these equations is that they be of the steady-state or stationary-state variety.

In view of the great complexity of the metabolic machinery of living systems and of the fact that numerous interactions occur among the enzymes and metabolites, accurate modelling of the activities of whole cells or large parts thereof by the approach of this study seems unlikely to be feasible. However, much can and remains to be done with metabolic systems that are not much more complex than the simple models considered above. The

major challenge to further progress with these systems appears to be the acquisition of accurate and detailed information concerning the properties of the individual enzymes and reactions under the conditions in which the reactions occur.

I thank Nancy Sally for drawing the figures and Drs. Gregory Forest and David Terman of the Department of Mathematics for providing me with a very helpful session on the fundamentals of implicit and partial differentiation. This work was supported in part by the Stoner Research Fund of the Ohio State University Development Fund.

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