The kinetics of consecutive enzyme reactions

The design of coupled assays and the temporal response of pathways

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A regime is proposed for the design of coupled enzyme assays in which auxiliary enzymes are added at concentrations proportional to their K_m values. Under these conditions it is possible to calculate the complete time course of the assay including the time required for the system to approach its steady state. The consequence of increasing the number of coupling enzymes is shown to be a considerable decrease in time required to reach the steady state provided that the overall transient time remains the same. The method is extended to the general consideration of pathways and shows that pathways of the same length exhibit identical temporal responses provided that the units of concentration and time used are based on the steady-state concentration of intermediates and the transient time respectively. An unexpected finding is that increasing the number of intermediates in a pathway can decrease the time required to enter a steady state.

Previous analyses of the behaviour of consecutive enzyme reactions have shown that, in a coupled enzyme system, the steady-state formation of product is preceded by a transient period during which the intermediates of the pathway are accumulating, resulting in a lag in formation of end product. This lag or transient is a measure of the time it takes the sequence to switch on or to switch between steady states, and in the case of a pathway is indicative of its responsiveness to changing metabolic requirements within the cell. Its determination has usually been restricted to situations in which the primary, initiating, enzyme is severely rate-limiting and the auxiliary enzymes obey pseudo-first-order kinetics (McClure, 1969; Hess & Wurster, 1970; Easterby, 1973). In these circumstances the transient is exclusively determined by the kinetic properties of the auxiliary coupling enzymes and is independent of the primary enzyme. Usually the analysis has been restricted to systems with one or two auxiliary enzymes (McClure, 1969; Hess & Wurster, 1970), but a complete solution for pathways of unlimited length has been proposed (Easterby, 1973). A problem with such analyses has been the complexity of the relationship between the velocity of product formation and the transient times. This has precluded general statements concerning the time required to reach a specified fraction of the steady-state rate, and has caused difficulty in

predicting the accuracy of steady-state rate measurements in all but the simplest case of a single auxiliary enzyme. The solutions do, however, provide a complete time course for the reactions concerned given specific values of transient time.

More general treatments (Storer & Cornish-Bowden, 1974; Easterby, 1981) have lifted the restriction of pseudo-first-order kinetics for the auxiliary enzymes and have allowed the study of systems obeying Michaelis–Menten kinetics, but, owing to the complexity of the non-linear differential equations generated, do not allow a complete description of the time course of events except again in the simplest case. One of these treatments does, however, allow very simple determination of the transient time for even the most complex systems in terms of steady-state intermediate concentrations and pathway flux (Easterby, 1981).

Solutions of the first type, assuming first-order kinetic behaviour, are only applicable when the transient times ($K_m/V_{max.}$) are distinct. Singularities occur in the solution when two or more auxiliary enzymes have the same transient time (McClure, 1969; Easterby, 1973), and the general solution is not then applicable, as the functions are undefined.

The present paper provides a solution to the equations when all enzymes have the same transient time and suggests (a) an approach to the

design of coupled assays and (b) an approach to the understanding of pathway responses. This is achieved by considering the effect of extending the pathway by the addition of 'modular' enzyme units of fixed transient time.

Results and discussion

Solution for identical transient times

In a previous analysis (Easterby, 1973) it was shown that a system containing a primary enzyme

$$\frac{d}{dt} \begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_4 \\ \vdots \\ S_n \end{bmatrix} = \begin{bmatrix} -1/\tau & 0 & 0 & 0 & \dots & \dots & \dots \\ 1/\tau & -1/\tau & 0 & 0 & \dots & \dots & \dots \\ 0 & 1/\tau & -1/\tau & 0 & \dots & \dots & \dots \\ 0 & 0 & 1/\tau & -1/\tau & 0 & \dots & \dots \\ 0 & 0 & 0 & 0 & \dots & \dots & \dots \\ \vdots & \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & 0 & 0 & \dots & 1/\tau & -1/\tau \end{bmatrix} \begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_4 \\ \vdots \\ S_n \end{bmatrix} + \begin{bmatrix} v_0 \\ 0 \\ 0 \\ 0 \\ \vdots \\ 0 \end{bmatrix} \quad (4)$$

of constant velocity v_0 and succeeded by n irreversible enzymes with K_m and $V_{\max}$ values K_i and V_i ($i = 1$ to n) possesses n distinct transient times. These are equivalent to the reciprocals of the pseudo-first-order rate constants and are given by the ratios K_i/V_i . The transient time for the complete sequence is the sum of these individual transients. Product formation by the sequence is described by the equation:

$$[P] = v_0 \left(t + \sum_{i=1}^n \frac{\tau_i^n \cdot e^{-t/\tau_i}}{\prod_{\substack{j=1 \\ j \neq i}}^n (\tau_i - \tau_j)} \right) \quad (1)$$

If two or more of the transient times are the same, the solution fails.

In the following scheme all of the auxiliary enzymes are given the same first-order rate constant corresponding to the $K_m/V_{\max}$ ratio. The S_i represent the intermediates of the pathway, and P represents the measured end product:

In the steady state, the concentration of S_i is given by:

$$[S_i] = v_0/k \quad (2)$$

and according to a previous general analysis (Easterby, 1981) the transient time is:

$$\tau = [S_i]/v_0 = 1/k \quad (3)$$

All of the enzymes and intermediates of the pathway share a common transient time, and transient times are additive when considering the overall pathway transient. The previous, general, theory of pathway transience therefore establishes that the pathway is 'well-behaved', despite apparent singularities in previous complete solutions of the time course (McClure, 1969; Easterby, 1973). It is therefore reasonable to seek solutions to the differential equations.

The complete set of equations describing the pathway is:

The eigenvalues (λ) of the coefficient matrix are given by:

$$(-1)^n (1/\tau + \lambda)^n = 0 \quad (5)$$

There is therefore a single eigenvalue $-1/\tau$ of multiplicity n associated with the equivalent set of homogeneous differential equations. The solution for the i th intermediate is therefore of the form (see, e.g., Boyce & Di Prima, 1977):

$$[S_i] = \mu_i(t) \cdot e^{-t/\tau} \quad (6)$$

Where $\mu_i(t)$ is a continuous function of t . The general solution may be determined by the method of variation of parameters and yields:

$$[S_i] = v_0 \tau - v_0 \sum_{j=0}^{i-1} \frac{\tau}{j!} \cdot \left(\frac{t}{\tau} \right)^j \cdot e^{-t/\tau} \quad (7)$$

Whence:

$$[S_n] = v_0 \tau - v_0 \sum_{j=0}^{n-1} \frac{\tau}{j!} \cdot \left(\frac{t}{\tau} \right)^j \cdot e^{-t/\tau} \quad (8)$$

The velocity of formation of P is given by:

$$v = \frac{[S_n]}{\tau} \quad (9)$$

and the overall transient time for the sequence is simply the sum of the individual enzyme transients (Easterby, 1981). The normalized velocity may

therefore be written:

$$\frac{v}{v_0} = 1 - \sum_{j=0}^{n-1} \frac{n^j}{j!} \cdot \left(\frac{t}{\tau_0}\right)^j \cdot e^{-nt/\tau_0} \tag{10}$$

where:

$$\tau_0 = n\tau \tag{11}$$

Further integration of eqn. (10) gives the following normalized description of product concentration:

$$\frac{[P]}{[S]_{ss}} = \frac{t}{\tau_0} + \sum_{j=0}^{n-1} n^{(j-1)} \cdot \frac{(n-j)}{j!} \cdot \left(\frac{t}{\tau_0}\right)^j \cdot e^{-nt/\tau_0} - 1 \tag{12}$$

where:

$$[S]_{ss} = \sum_{i=1}^n [S_i]_{ss} = v_0 \tau_0 \tag{13}$$

[S]_{ss} is the sum of the steady-state concentrations of the *n* intermediates.

It is clear from the foregoing equations that, if τ_0 is used as the unit of time, v_0 as the unit of velocity and [S]_{ss} as the unit of concentration, all pathways of the same length have the same shape of response, but in time and concentration zones determined by τ and the steady-state flux. It is then possible to calculate the fractional attainment of a steady-state parameter such as velocity or concentration as a function of transient time, with the pathway length as the only variable. This in turn makes the design of coupled enzyme assays much simpler.

Fig. 1 contains curves for the attainment of steady-state velocity for several representative path lengths. As expected, increase in the pathway length delays the appearance of product and the establishment of a significant velocity. However, the longer the pathway the greater the acceleration into the steady state once end product starts to appear. This means, rather surprisingly, that the longer the pathway the quicker it approaches the steady state. Consequently, if two pathways have the same overall transient time, the longer pathway will approach the steady state first. However, this in turn means that individually the enzymes of the longer pathway must have shorter transient times.

The curves of Fig. 1 possess an inflexion at:

$$\frac{t}{\tau_0} = \frac{n-1}{n} \tag{14}$$

The inflexion progresses from the origin for $n = 1$ towards $t = \tau_0$ as n tends to infinity. In the case of an extremely long pathway the function is essentially discontinuous. No product is formed until the transient time is reached, and at this point the pathway jumps into its steady state (Fig. 1).

Fig. 1. Dependence of velocity on time for representative path lengths

The velocity, as a fraction of steady-state velocity of product formation, is shown as a function of time. The numbers of auxiliary enzymes are appended to the curves.

Table 1. Attainment of steady-state velocity in a coupled enzyme sequence

The half-time, the time required to reach 99% of the steady-state flux and the velocity at the transient time are shown respectively.

No. of auxiliary enzymes (<i>n</i>)	$\left(\frac{t}{\tau_0}\right)_{0.5}$	$\left(\frac{t}{\tau_0}\right)_{0.99}$	$\frac{v}{v_0}$ at $t = \tau_0$
1	0.693	4.605	0.632
2	0.839	3.319	0.594
3	0.891	2.802	0.577
4	0.918	2.511	0.567
5	0.934	2.321	0.560
6	0.945	2.185	0.554
7	0.952	2.082	0.550
8	0.959	2.000	0.547
9	0.963	1.934	0.544
10	0.967	1.879	0.542
15	0.978	1.696	0.534
20	0.983	1.592	0.532

The appearance of product in a long pathway therefore behaves very much like a switch mechanism. The velocity of end-product formation will rise abruptly rather than by a steady progression. These properties are demonstrated in Table 1 and Figs. 1 and 2.

Application to the design of coupled assays

Table 1 and Fig. 2 show that the complete time course of a coupled enzyme system is readily obtainable provided that all enzymes present possess the same transient time. Bearing in mind that τ is given by K_m/V_{max} , this can be achieved in two ways, as follows.

Fig. 2. Dependence of temporal response on path length. The time required to reach specified fractions of steady-state velocity is shown as a function of the number of auxiliary enzymes. The times required to reach 50% and 99% of the steady-state rate are shown, together with the (t/τ_0) value at the inflexion of the curves in Fig. 1.

(a) The K_m values of the coupling enzymes are determined under the conditions of assay. The enzyme of lowest K_m is added at a concentration to give at least 6 times the maximum rate to be measured (this ensures first-order behaviour; Easterby, 1981). The other coupling enzymes are then added at activities in proportion to their K_m values.

(b) Alternatively, the τ values may be determined experimentally by first adding the coupling enzymes at low concentration to generate a significant transient. Their concentrations would then be individually greatly increased and the decrease in transient time would be a measure of each enzyme's transient. The final concentrations would then be adjusted accordingly to give identical values of the required magnitude.

Many reaction measurements use the coupling of ADP to NADH oxidation through pyruvate kinase and lactate dehydrogenase. As an example of the calculation required in the first method, this system may be considered. With rabbit muscle enzymes the K_m of commercial preparations of pyruvate kinase (PK) for ADP is reportedly 2×10^{-4} M. That of lactate dehydrogenase (LDH) for pyruvate is 3.5×10^{-4} M.

Thus:

$$\tau_{PK} = \tau_{LDH} = \frac{2 \times 10^{-4}}{V_{PK}} = \frac{3.5 \times 10^{-4}}{V_{LDH}}$$

and:

$$\frac{V_{LDH}}{V_{PK}} = 1.75$$

This gives the required ratio of lactate dehydrogenase to pyruvate kinase units in the coupled assay. The actual units of pyruvate kinase must be at least 6 times the maximum rate of generation of ADP to be measured.

Previously it was known that in systems of a single auxiliary enzyme a time of at least $4-6\tau_0$ must elapse before the velocity reaches 99% of the steady-state value. However, it was not clear what the consequences of extending the sequence would be. Table 1 and Fig. 2 show that the extension of the pathway greatly decreases the time required to reach the steady state, and, if calculations of temporal response are based on the single-coupling-enzyme model, there will be a considerable safety margin in estimating the accuracy of steady-state measurements.

General considerations of pathway transience

The behaviour described for coupled assay systems is also applicable to pathways that enter a steady state. The dependence of pathway temporal responses on path length is directly analogous, and one can define the effects of extending a pathway by considering the addition of extra, 'modular', enzyme units of identical transient time. The validity of this approach depends on the assumption that the identical transient-time model is realistic. Consideration of previous analysis (Easterby, 1973) and the general equation (1) demonstrates this to be so. If any one enzyme possesses a markedly longer transient time than any other in a sequence, then it will dominate the temporal response and the others may be ignored. Similarly an enzyme possessing a markedly shorter transient time will not contribute to the response and may be ignored. Therefore only when all enzymes have similar transient times is there any problem in defining the temporal response. In view of the fact that the appropriate functions are continuous in the region of the transient identity, the model used here provides a reasonable basis for understanding pathways in this region.

The most striking conclusion one may draw is that, if two pathways have identical transient times, the longer enters the steady state more rapidly. This is achieved with no increase in the total matter bound-up as intermediate, as this will be the same in both cases. The improved response to flux changes is therefore achieved by breaking the interconversion of substrate and end product into a greater number of discrete steps, and is

analogous to the kinetic and energetic improvements seen in multistep single-enzyme mechanisms. Rapid pathway response is therefore achieved without the waste of 'metabolic energy' by accumulation of unwanted intermediates. As with all things, this improvement is not without penalty. It requires that the individual enzymes of the pathway should either be more active or have a higher affinity for their substrates than their short-pathway counterparts. This is required in order to achieve the same overall transient time.

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