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COUPLED ENZYME ASSAYS: A GENERAL EXPRESSION FOR THE TRANSIENT

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SUMMARY

The rate of formation of product in a coupled enzyme reaction is subject to a transient phase prior to the attainment of the steady-state. General expressions are presented to describe the transient in a multi-enzyme sequence in which the initial enzyme is rate-limiting. This reveals that the initial enzyme does not contribute to the transient but does determine the steady-state velocity. Each coupling enzyme has a characteristic transient time given by the ratio of its Michaelis constant to its maximum velocity. The transient for the complete sequence is a simple sum of the individual enzyme transients. In metabolic sequences for which the initial enzyme is rate-limiting the lag phase will be controlled by the enzymes of the sequence with greatest transient times and not by the initial rate-limiting enzyme.

INTRODUCTION

It is common practice to couple an enzyme reaction to one or more secondary enzymes in order to generate a measurable product. It is generally assumed that if the initial enzyme reaction of the sequence is rate-limiting it will determine the steady-state rate of product formation. However, the steady-state will be preceded by a transient period during which the intermediates in the sequence are accumulating and the rate of product formation is not a true reflection of the activity of the enzyme under study. In order to use a coupled enzyme reaction successfully and to optimise the conditions for the assay it is necessary to describe the lag phase and to estimate its duration. The approach to this problem has been outlined previously[1,2] and a number of solutions for systems containing one[1,3–5] or two[3,4,6] coupling enzymes have been reported. The present report extends the theory of coupled enzyme reactions to provide general expressions for the description of multi-enzyme sequences. It is shown that each enzyme in a sequence has a characteristic and simply defined transient time and that the transient for the complete sequence is the sum of the individual transients. The initial, rate-limiting enzyme does not contribute to the transient and therefore does not influence the lag period of the reaction.

The conclusions drawn are also of significance to simple metabolic sequences and some implications of the theory to metabolic regulation are discussed.

MATERIALS AND METHODS

ATP, NADP and glucose-6-phosphate dehydrogenase, grade II (EC 1.1.1.49) were obtained from C. F. Boehringer & Soehne (Mannheim, Germany). Trizma base was a product of Sigma Chemical Co. (London). All other reagents were AnalaR grade of British Drug Houses (Poole, Dorset, England). Hexokinase (EC 2.7.1.1) was purified from porcine heart (Easterby, J. S., unpublished).

Hexokinase assay

Hexokinase was coupled to glucose-6-phosphate dehydrogenase and the rate of generation of NADPH followed spectrophotometrically at 340 nm in a Perkin–Elmer Model 356 spectrophotometer. The assay contained glucose (0.02 M), $MgCl_2$ (0.02 M), Tris–HCl (0.02 M), NADP (10^{-4} M), ATP (10^{-2} M), and glucose-6-phosphate dehydrogenase in a final volume of 1.5 ml. The pH of the assay was 7.6 and the temperature 30 °C. An absorbance change of 4.14 corresponded to the generation of 1 μmole of NADPH. The reaction was initiated by the addition of hexokinase. For a normal hexokinase assay glucose-6-phosphate dehydrogenase would be present at a concentration of 0.2 unit/ml. This would produce a transient time of about 6 s. In the present assay the concentration was about 0.03 unit/ml producing a measurable transient of about 40 s.

RESULTS AND DISCUSSION

The simplest example of a coupled enzyme reaction is that in which the product of the enzyme under study is converted by a single coupling enzyme to a measurable product. This may be described by the following scheme.

$$S \xrightarrow{v_0} I_1 \xrightarrow{V_1/K_1} P$$

where S is the initial substrate, P the measurable product and I_1 the single intermediate (product of the reaction under study). v_0 is the velocity of the reaction under study and V_1 and K_1 are the maximum velocity (M/unit time) and Michaelis constant respectively of the coupling enzyme. For the rate of formation of product to reflect the activity of the initial enzyme, V_1 must necessarily be much greater than v_0 and consequently the steady-state concentration of the intermediate I_1 will be small and less than K_1 (refs 1 and 2). Providing this condition is met and the reactions are considered irreversible, the rate of formation of product is given by

$$\frac{d[P]}{dt} = \frac{V_1}{K_1} \cdot [I_1] \qquad (1)$$

where V_1/K_1 is a first-order rate constant. The time course of the reaction is described by the solution to the equation

$$\frac{d[I_1]}{dt} + \frac{V_1}{K_1}[I_1] = v_0 \qquad (2)$$

with boundary conditions $[I_1] = 0$, $[P] = 0$ at $t = 0$.

This results in

$$[I_1] = v_0 \tau_1 (1 - e^{-t/\tau_1}) \quad (3$$

$$[P] = v_0 (t + \tau_1 e^{-t/\tau_1} - \tau_1) \quad (4$$

where the transient time is given by

$$\tau_1 = K_1/V_1 \quad (5$$

and the steady-state concentration of the intermediate I_1 by

$$[I_1]_{ss} = v_0 \tau_1 \quad (6$$

Fig. 1 shows the time required to approach the steady state condition as a function of τ_1. Thus for the concentration of the intermediate to approach within 1% of its steady-state value (and hence for the velocity of product formation to approach v_0

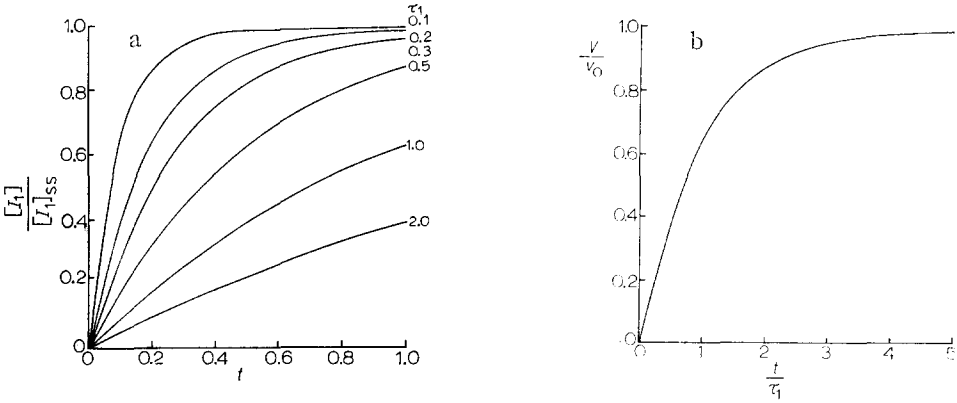
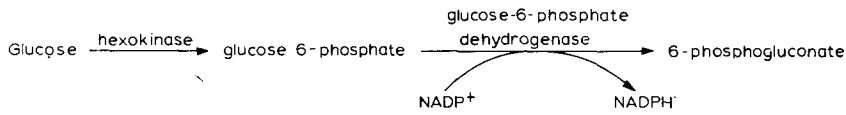


Fig. 1. The effect of the transient time on the approach to the steady-state: (a) the concentration of the intermediate (I_1) is shown as a function of time for various values of the single transient time (τ_1). Transient times in the range 0.1–2.0 are appended to the curves. Units of time are arbitrary. (b) normalised functions relating the measured velocity of product formation to time are shown. It will be seen that a time at least five times the transient time must elapse before the steady-state velocity can be determined to an accuracy of 1%.

to within 1%) a time equal to at least five times the transient time must elapse. The expression for product concentration contains an exponential term which disappears as the steady-state is approached ($t \to \infty$). Eqn 4 then reduces to

$$\lim_{t \to \infty} [P] = v_0(t - \tau_1) \quad (7$$

This describes a straight line intersecting the abscissa at τ_1 and the ordinate axis at $-[I_1]_{ss}$. Fig. 2 shows the correspondence between the theoretical expressions and experimental data on the coupling of heart hexokinase to glucose-6-phosphate dehydrogenase *via* the intermediate glucose 6-phosphate.



The change in absorbance at 340 nm due to the formation of the product NADPH was monitored. The figure also demonstrates the physical significance of the transient time. The steady-state concentration of glucose 6-phosphate can be estimated as 3.5 μM, which is an order of magnitude less than the K_i value of the enzyme with respect to this inhibitor (Easterby, J. S., unpublished). It is therefore possible to rule

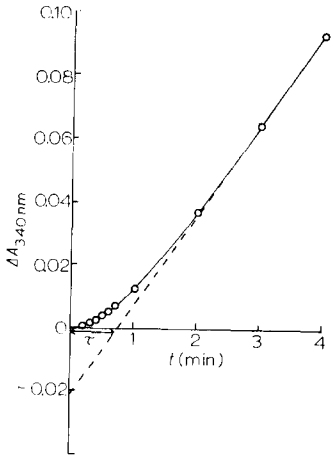


Fig. 2. The coupling of hexokinase to glucose-6-phosphate dehydrogenase. Conditions were as described under Materials and Methods. Experimental points and a theoretical curve based on a transient time of 0.725 min are shown. The ordinate intercept corresponds to a steady-state glucose 6-phosphate concentration of 3.5 μM. The steady-state velocity is 4.5 μM/min.

out inhibition by this product as a limiting factor in the assay. This would not be possible without reference to the description of the transient given by the above equations.

It is frequently necessary to make use of several coupling reactions before a measurable product is reached. For example, an alternative to the assay described for hexokinase is to couple this enzyme through its other reaction product (ADP) to pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) monitoring the fall in NADH concentration (this system is not applicable to animal hexokinases with glucose as substrate as the accumulation of glucose 6-phosphate results in inhibition). This system has been analysed previously by Barwell and Hess[6]. Such multienzyme systems may be described by the following scheme.

$$S \xrightarrow{v_0} I_1 \xrightarrow{V_1/K_1} I_2 \xrightarrow{V_2/K_2} I_3 \cdots\cdots I_n \xrightarrow{V_n/K_n} P$$

where $V_1, V_2 \ldots\ldots\ldots V_n$ are the maximum velocities of the n coupling enzymes and $K_1, K_2 \ldots\ldots\ldots K_n$ are their respective Michaelis constants. The derivation of a general expression to describe the time course of such an enzyme sequence

requires the solution of the series of differential equations for the intermediates

$$\frac{d[I_i]}{dt} + \frac{[I_i]}{\tau_i} = \frac{[I_{i-1}]}{\tau_{i-1}} \quad i = 2,3,\cdots n \tag{8}$$

with boundary conditions $[I_i] = 0$ at $t = 0$.

The trivial solution for I_1 is given above and the solution for successive intermediates may be obtained by progressive substitution into Eqn 8. This leads to the general solution

$$[I_i] = v_0 \tau_1 (1 - \sum_{j=1}^{i} A_i e^{-t/\tau_j}) \quad i = 2, 3\cdots n \tag{9}$$

where

$$A_j = \tau_j^{i-1} \prod_{k=1}^{i} \frac{1}{(\tau_j - \tau_k)} \quad k \neq j \tag{10}$$

The steady-state concentration is given by

$$[I_i]_{ss} = v_0 \tau_i \tag{11}$$

where the transient time is

$$\tau_i = K_i/V_i \tag{12}$$

The concentration of end product is described by the solution to the equation

$$\frac{d[P]}{dt} = \frac{V_n}{K_n} \cdot [I_n] \tag{13}$$

with boundary condition $[P] = 0$ at $t = 0$. The result is

$$[P] = v_0 (t + \sum_{i=1}^{n} C_i e^{-t/\tau_i} - \sum_{i=1}^{n} \tau_i) \tag{14}$$

where

$$C_i = \tau_i^n \prod_{j=1}^{n} \frac{1}{(\tau_i - \tau_j)} \quad j \neq i \tag{15}$$

As the steady-state is entered Eqn 14 reduces to

$$\lim_{t \to \infty} [P] = v_0 (t - \sum_{i=1}^{n} \tau_i) \tag{16}$$

This describes a line intersecting the time axis at $\sum_{i=1}^{n} \tau_i$. Thus the transient for the sequence is simply the sum of the transients for the individual enzymes. The ordinate is intersected at $-v_0 \sum_{i=1}^{n} \tau_i$, which corresponds to the sum of the steady-state concentrations of the n intermediates and the equation is therefore directly analogous to the simple case involving a single coupling enzyme. The specific form of equation 14 which applies to the commonly encountered systems involving two coupling enzymes (*e.g.* hexokinase–pyruvate kinase–lactate dehydrogenase) is

$$[P] = v_0 (t + \frac{\tau_1^2}{(\tau_1 - \tau_2)} e^{-t/\tau_1} + \frac{\tau_2^2}{(\tau_2 - \tau_1)} e^{-t/\tau_2} - \tau_1 - \tau_2) \tag{17}$$

and in the case of three coupling enzymes

$$[P] = v_0 \left(t + \frac{\tau_1^3 e^{-t/\tau_1}}{(\tau_1-\tau_2)(\tau_1-\tau_3)} + \frac{\tau_2^3 e^{-t/\tau_2}}{(\tau_2-\tau_1)(\tau_2-\tau_3)} + \frac{\tau_3^3 e^{-t/\tau_3}}{(\tau_3-\tau_1)(\tau_3-\tau_2)} - \tau_1 - \tau_2 - \tau_3\right) \quad (18)$$

These equations are symmetrical with respect to the transient times and interchanging values for the enzymes do not alter the time course of the reaction. As has been noted by McClure[3] a special case arises if the transient times of two enzymes are identical. The equations given above do not then apply and such cases must be solved individually. However, the transient times may approach each other very closely with no invalidation of the equations and the case of exact identity must be regarded as an extremely unlikely occurrence.

It is of considerable significance that the transient time of an enzyme sequence depends only on the coupling enzymes and is independent of the initial, rate-limiting enzyme. Modification of the activity of the initial enzyme will not affect the time required for the sequence to approach its maximum velocity of product formation although it will determine the magnitude of this velocity.

In choosing a coupled enzyme assay three conditions must be met. The initial enzyme must be rate-limiting ($v_0 \ll V_i$), the transient time must be minimised ($K_i \ll V_i$) and the steady-state concentrations of intermediates must be small relative to the Michaelis constants ($v_0 K_i / V_i \ll K_i$). These conditions may be summarised as a single requirement

$$V_i \gg v_0, K_i \quad (19)$$

Thus with a knowledge of the Michaelis constants and activities of all coupling enzymes, it is possible to optimise the assay and reduce the transient time to a practical value.

Particular care must be exercised when using coupled assays to study the effects of inhibitors (especially as the substrate for the coupling enzyme is likely to be structurally similar to the inhibitor of the enzyme under study). However, inhibition of the coupling enzyme is usually easily distinguished from inhibition of the initial enzyme. The former results in an increased transient time, the latter results in a reduction of the steady-state reaction velocity. Thus the assay system described for the coupling of hexokinase to glucose-6-phosphate dehydrogenase cannot be used safely at high Mg–ATP concentrations (above 10 mM) owing to inhibition of glucose-6-phosphate dehydrogenase at high ionic strength[7]. This results in a dramatic and impractical increase in the transient time.

Metabolic pathways are typically sequences of enzyme reactions, directed towards formation of an end product, in which the initial reaction is rate limiting. From the above theory, it is clear that the first enzyme of a pathway may determine the maximum flux, but later enzymes in the sequence will determine the time-lag before the pathway is fully operative. If one enzyme possesses a transient significantly longer than those of the other enzymes of the pathway, this enzyme will control the switching on process and may be termed the time-limiting enzyme. Moreover, the greater the number of separate reactions constituting a metabolic sequence, the greater the transient is likely to be. Much of the study of metabolic regulation has been directed towards the effects of modifiers on rate-limiting enzymes (usually

catalysing the initial reaction of a sequence). It is clear that activation or inhibition of these enzymes may determine the maximum rate which the pathway can achieve but cannot control the time required for the pathway to switch on. Thus a study of the later, time-limiting enzymes is necessary to decide whether a pathway will be initiated. This suggests that more attention should be directed to those enzymes which have large transient times. This does not necessarily mean that they will be enzymes of low activity, they may have low affinities for their substrates. It is also clear that, to be effective, modifiers of a metabolic sequence must have lives at least comparable to the transient time for the sequence. This could provide a homeostatic mechanism preventing changes in response to short-lived fluctuations in the concentrations of metabolites. Where intermediates in the sequence act as modifiers the simple equations given cannot be applied and in general a numerical solution to the differential equations will be necessary[8,9]. The transient times of individual enzymes will also regulate the steady-state concentration of intermediates. Thus enzymes with long transients might control the distribution of metabolites between branches of a pathway if they succeed the branch point.

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