A general expression for coupled enzyme assays of hysteretic enzymes

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A general description of a system consisting of a hysteretic enzyme and a series of coupling enzymes is presented. The chloroplast enzyme sedoheptulose-1,7-bisphosphatase and a sequence of three coupling enzymes is used as an example. An analysis using first-order rate equations for the coupling enzymes shows that the observed relaxation time of the system is the sum of the relaxation times of the coupling enzymes and that of the hysteretic enzyme. The behaviour of a system with relatively low concentrations of coupling enzymes, where the first-order assumption is not valid, is analysed by computer simulation. Several methods for the accurate determination of the relaxation time of the hysteretic enzyme are discussed.

Several studies have described the behaviour of coupled enzyme assays where the system approaches a steady state and the velocity of the primary enzyme reaction (which is constant) is reflected by the rate of product formation (Bergmeyer, 1953; McClure, 1969; Barwell & Hess, 1970; Easterby, 1973; Kuchel *et al.*, 1974; Storer & Cornish-Bowden, 1974). In earlier studies, the coupling enzyme kinetics were assumed to be first-order (Bergmeyer, 1953; McClure, 1969; Barwell & Hess, 1970; Easterby, 1973). This assumption is valid only when the concentration of a given intermediate is substantially less than the Michaelis constant and other substrates are saturating. The Storer & Cornish-Bowden (1974) treatment, however, used the more general assumption that the coupling enzyme reactions follow Michaelis-Menten kinetics. In contrast with the first-order treatments, this analysis was able to describe the behaviour of assay systems with relatively low concentrations of coupling enzymes.

Most of these studies of coupled enzyme assays describe a transient period during which intermediates accumulate and the rate of measurable product formation does not reflect the velocity of the primary enzyme reaction. These studies also outline methods designed to quantify and shorten this period. In contrast, the Kuchel *et al.* (1974) treatment accommodates systems with very long lags where the velocities of the primary and auxiliary enzymes are similar and a steady state is not attained.

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No treatment has been devised to accommodate enzymes the activity of which changes with time. When handling these enzymes it has been common practice to maximize the amount of coupling enzyme, thereby minimizing the contribution of the assay system to the observed transient. Apart from being wasteful and expensive, it is not always feasible to ignore the assay system in this way, unless the parameters of the coupling enzyme are extremely favourable.

In the present study, we present a general description of a multi-enzyme sequence with a primary enzyme (enzyme under study) that undergoes a velocity transition that can be described by a single rate constant. It should be noted that most hysteretic enzymes (Frieden, 1970) also undergo such a velocity transition and that the general description is equally valid for these enzymes. The chloroplast enzyme sedoheptulose-1,7-bisphosphatase is used to demonstrate the application of the analysis.

Materials and methods

Materials

Biochemicals and 6-phosphofructokinase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., Sephadex products from Pharmacia, North Ryde, N.S.W., Australia, and other auxiliary enzymes from Boehringer, North Ryde, N.S.W., Australia. Wheat (*Triticum aestivum* L. cultivar. Sappo) was grown in vermiculite under sunlight.

Methods

Stromal sedoheptulose-1,7-bisphosphatase was prepared by the procedure described by Woodrow & Walker (1982). Enzyme activity was measured by coupling sedoheptulose 7-phosphate formation to the oxidation of NADH:

The standard reaction mixture contained, in a final volume of 1 ml: 50mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/NaOH buffer, pH 8.2, 10mM-MgCl₂, 20mM-KCl, 0.1mM-ATP, 1mM-phosphoenolpyruvate, 0.15mM-NADH, 30mM-dithiothreitol, 0.1mM-sedoheptulose 1,7-bisphosphate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, 0.5 unit of fructose-6-phosphate kinase (rabbit muscle) and sedoheptulose-1,7-bisphosphatase solution. The last-named was normally used to initiate the reaction. The auxiliary enzymes were desalted on a Sephadex PD-10 column of Sephadex G-25 (medium grade) equilibrated with 20mM-Tricine/NaOH buffer, pH 8.2, before being used. The oxidation of NADH was monitored at 340nm. The continuous spectrophotometric assay and basic activation kinetics of sedoheptulose-1,7-bisphosphatase are described in detail elsewhere (Woodrow & Walker, 1982). Measurement of enzyme activity by using the assay of P_i liberated is described by Woodrow & Walker (1982). One unit of enzyme is defined as the amount that catalyses the formation of 1 μmol of product/min at 20°C under the above conditions.

Theory

Reaction-velocity equation for the primary enzyme

The activation of sedoheptulose-1,7-bisphosphatase in the presence of the reductant dithiothreitol is described by:

$$v_t = v_f + (v_0 - v_f)e^{-t/\tau_0} \quad (5)$$

where v_t is the reaction velocity at time t (which is relative to the change in conditions that initiates the slow activation), v_f the final reaction velocity at $t = \infty$, v_0 the initial reaction velocity, and τ_0 the relaxation time describing the transition to the new steady state (Woodrow & Walker, 1982). The rate constant is a function of the reductant and sub-

strate concentrations (Woodrow & Walker, 1983), which are held constant in the present experiments. Eqn. (5) describes the reaction-velocity transition of most of the hysteretic enzymes (Frieden, 1970) and is used in the following analysis.

Solution with first-order coupling reactions

The following scheme describes a multi-enzyme sequence of n coupling enzymes and a primary enzyme that uses the substrate (S):

where P is the measureable product, and I_n and E_n are the n th intermediate and coupling enzyme respectively. The rate of change of the I_1 concentration is given by:

$$\frac{d[\text{I}_1]}{dt} + \frac{[\text{I}_1]}{\tau_1} = v_f + (v_0 - v_f)e^{-t/\tau_0} \quad (7)$$

In this expression $\tau_1 = K_1/V_1$, K_1 is the Michaelis constant of E_1 for I_1 , V_1 is the maximum velocity of the reaction catalysed by E_1 , and τ_0 is the relaxation time for the primary enzyme. The following equations provide solutions for the other intermediates:

$$\frac{d[\text{I}_i]}{dt} + \frac{[\text{I}_i]}{\tau_i} = \frac{[\text{I}_{i-1}]}{\tau_{i-1}} \quad (8)$$

$$i = 2, 3 \dots n$$

where $[\text{I}_i] = 0$ at $t = 0$.

Certain assumptions underlie eqns. (7) and (8). Firstly, v_f must be independent of [S]. This requires that S is saturating or that only a small quantity of S is consumed. Secondly, the primary enzyme reaction must be essentially irreversible. This may be assumed under most circumstances because I_1 is continuously removed by E_1 . Finally, the coupling reactions must be approximately first-order and essentially irreversible. These assumptions will be valid if the concentration of I_j is substantially less than K_j , and if other substrates of the coupling reactions are saturating.

Solving eqns. (7) and (8):

$$[I_i] = v_f \tau_i \left(1 - \sum_{j=0}^i \tau_j A_j e^{-t/\tau_j} \right) + v_0 \tau_0 \tau_i \left(\sum_{j=0}^i A_j e^{-t/\tau_j} \right) \quad (9)$$

$i = 1, 2 \dots n$

where

$$A_j = \tau_j^{i-1} \prod_{\substack{k=0 \\ k \neq j}}^i \frac{1}{(\tau_j - \tau_k)}$$

This solution can be confirmed by substitution into eqns. (7) and (8).

The concentration of product is described by:

$$\frac{d[P]}{dt} = [I_n]/\tau_n \quad (10)$$

where $[P] = 0$ at $t = 0$. Solving eqns. (9) and (10):

$$[P] = v_f \left(t + \sum_{j=0}^n \tau_j^2 A_j e^{-t/\tau_j} \right) - v_0 \tau_0 \sum_{j=0}^n \tau_j A_j e^{-t/\tau_j} + v_0 \tau_0 \sum_{j=0}^n \tau_j A_j - v_f \sum_{j=0}^n \tau_j^2 A_j \quad (11)$$

As the steady state is approached ($t \rightarrow \infty$), eqn. (11) approaches the line:

$$[P] = v_f t + \tau_0 (v_0 + v_f) - v_f \sum_{j=1}^n \tau_j \quad (12)$$

which intersects the time axis ($[P] = 0$) at:

$$t = \sum_{j=1}^n \tau_j + \frac{(v_f - v_0)}{v_f} \tau_0 \quad (13)$$

Thus the contribution of the assay system to the measured induction period is the sum of the individual coupling enzyme transients, i.e. $\sum_{j=1}^n \tau_j$.

The steady-state concentration of intermediates can be derived from eqn. (9):

$$[I_i]_{ss} = v_f \tau_i \quad (14)$$

This expression involves both the K_m and V_{max} of the coupling enzyme together with the flux through the system.

The rate of measureable product formation will approach v_f as long as the first-order assumption is valid and the primary enzyme determines the flux through the system. Under these conditions the measured induction period will also be independent of v_f . Its length will depend on both the K_m and V_{max} of the coupling enzymes in addition to the relaxation time of the primary enzyme (τ_0).

Solutions with Michaelis-Menten coupling reactions

Circumstances sometimes arise when the activity of a coupling enzyme is comparable with that of

the primary enzyme. Under these conditions Storer & Cornish-Bowden (1974) showed that the first-order assumption is invalid, and a more general equation, such as the Michaelis-Menten equation, must be used to describe the coupling reactions. Their system contained one coupling enzyme and a primary enzyme that catalysed a reaction at an essentially constant rate. The Storer & Cornish-Bowden (1974) approach, however, cannot be readily extended to multi-enzyme systems such as that of the sedoheptulose-1,7-bisphosphatase assay.

In order to analyse the sedoheptulose-1,7-bisphosphatase assay system, eqns. (7) and (8) were rewritten with the use of Michaelis-Menten expressions:

$$\frac{d[I_i]}{dt} + \frac{V_i [I_i]}{K_i + [I_i]} = \frac{V_{i-1} [I_{i-1}]}{K_{i-1} + [I_{i-1}]} \quad (15)$$

$i = 2, 3 \dots n$

$$\frac{d[I_1]}{dt} + \frac{V_1 [I_1]}{K_1 + [I_1]} = v_f + (v_0 - v_f) e^{-t/\tau_0} \quad (16)$$

The equations were numerically solved by using a fifth-order Runge-Kutta method. A range of coupling enzyme activities was tested, the lowest being twice that of the primary enzyme. In each case, the rate of measurable product formation approached the velocity of the primary enzyme reaction (Fig. 1). However, at the lower concentrations of coup-

Fig. 1. Computer simulation of the sedoheptulose-1,7-bisphosphatase continuous spectrophotometric assay

The ordinate axis expresses the rate of NADH oxidation as a percentage of the velocity of the sedoheptulose-1,7-bisphosphatase-catalysed reaction. The quantity of each coupling enzyme is the same and is given next to each trace in units/ml. The K_m (sedoheptulose 7-phosphate), K_m (ADP) and K_m (pyruvate) are 0.6 mM, 0.3 mM and 0.17 mM respectively. The relaxation time of the primary enzyme (τ_0) is 10 min, and the final reaction velocity (v_f) is 0.01 unit/ml. The initial reaction velocity (v_0) is zero. In each case the rate of measurable product formation approaches the velocity of the primary enzyme reaction.

Fig. 2. Theoretical reaction progress curves for the sedoheptulose-1,7-bisphosphatase assay

It is assumed that the coupling reactions follow first-order (●) or Michaelis-Menten (○) kinetics. The concentration of each coupling enzyme is the same and is given next to the traces (units/ml). The 'first-order' curves are derived from eqn. (11). Numerical integration of eqns. (15) and (16) gave the 'Michaelis-Menten' curves. The coupling-enzyme kinetic parameters are the same as those outlined in the legend to Fig. 1. The relaxation time (τ_0) and final reaction velocity for the sedoheptulose-1,7-bisphosphatase-catalysed reaction are 10 min and 0.01 unit/ml respectively. The initial reaction velocity is zero.

ling enzymes there was a marked deviation from the behaviour predicted by the first-order treatment (Fig. 2). In the present case, when a significant deviation from first-order behaviour occurred, the induction period was too long to be measured by using the spectrophotometric assay. The computer simulation technique has the disadvantage that it does not provide a general solution and must be adapted to the individual systems.

Results and discussion

When a coupled enzyme assay is used to analyse hysteretic-enzyme kinetics, it should be ascertained whether the transient is an intrinsic property of the primary enzyme or an artifact of the system. Eqn. (12) approximates the contribution of the coupling-enzyme sequence to the measured induction period. This expression shows that, if the concentration of coupling enzyme is increased, the measured induction period approaches that of the primary enzyme. This can be graphically analysed by varying the concentrations of the coupling enzymes proportionally and plotting the measured induction period versus the inverse of the coupling-enzyme concentration. The

Fig. 3. Determination of the relaxation time of a hysteretic enzyme by varying the concentration of coupling enzymes

The sedoheptulose-1,7-bisphosphatase assay was used as a model system. The three coupling enzymes were prepared in a single solution and were therefore varied proportionally. The coupling-enzyme solution contained, in 300 μ l, 1 unit of sedoheptulose-7-phosphate kinase, 2 units of pyruvate kinase and 2 units of lactate dehydrogenase. τ represents the induction period measured by extrapolating the linear portion of the product-versus-time graph to the time axis. The ordinate intercept provides an estimate of τ_0 , the relaxation time of sedoheptulose-1,7-bisphosphatase. V is the volume of coupling-enzyme solution.

Fig. 4. Reaction progress curve obtained by using P_i production as a measure of sedoheptulose-1,7-bisphosphatase activity

The reaction mixture was essentially as described in the Materials and methods section except that 400 μ M-sedoheptulose 1,7-bisphosphate was used. Reactions were initiated by adding enzyme solution. Product formation did not significantly inhibit enzyme activity.

plot, according to eqn. (12), will be linear and intercepts the ordinate axis at the induction time of the primary enzyme. Such a plot was made for the sedoheptulose-1,7-bisphosphatase system, and a τ_0 value of 4.6 min was estimated (Fig. 3). This value

was verified by measuring the amount of P_i liberated with time (Fig. 4). A computer simulation of the system with the use of eqns. (15) and (16) indicated that, as the system deviates significantly from first-order behaviour, there is a deviation from linearity. This was observed at low concentrations of coupling enzyme (Fig. 3). However, the linear region was broad enough to permit accurate determination of the ordinate intercept.

A second means of calculating τ_0 involves the use of a semi-logarithmic plot of $\log(v_f - v_i)$ versus time. Consider a system of three coupling enzymes. The observed instantaneous velocity of product formation is given by:

$$v_{\text{obs.}} = \frac{d[P]}{dt} = v_f - \frac{\tau_0^3 e^{-t/\tau_0}(v_f - v_0)}{(\tau_0 - \tau_1)(\tau_0 - \tau_2)(\tau_0 - \tau_3)} - \frac{\tau_3^2 e^{-t/\tau_1}(v_f \tau_1 - v_0 \tau_0)}{(\tau_1 - \tau_0)(\tau_1 - \tau_3)(\tau_1 - \tau_2)} - \frac{\tau_2^2 e^{-t/\tau_2}(v_f \tau_2 - v_0 \tau_0)}{(\tau_2 - \tau_0)(\tau_2 - \tau_1)(\tau_2 - \tau_3)} - \frac{\tau_3^2 e^{-t/\tau_3}(v_f \tau_3 - v_0 \tau_0)}{(\tau_3 - \tau_0)(\tau_3 - \tau_1)(\tau_3 - \tau_2)} \quad (17)$$

When t and τ_0 are large compared with τ_1 , τ_2 and τ_3 , and provided that τ_1 , τ_2 and τ_3 do not approach each other too closely, the last three terms in eqn. (17) are negligible and:

$$\log(v_f - v_{\text{obs.}}) \simeq \log(v_f - v_0) + 3 \log \tau_0 -$$

$$\log[(\tau_0 - \tau_1)(\tau_0 - \tau_2)(\tau_0 - \tau_3)] - t/\tau_0 \quad (18)$$

Plotting $\log(v_f - v_{\text{obs.}})$ against time gives a line of slope $-\tau_0^{-1}$. Therefore τ_0 can be calculated directly by ensuring that the coupling-enzyme τ values are at least an order of magnitude smaller than τ_0 . Semi-logarithmic plots describing the observed kinetics of sedoheptulose-1,7-bisphosphatase activation at various concentrations of coupling enzyme are shown in Fig. 5. These data confirm that accurate determination of τ_0 by this method can only be achieved when τ_0 is significantly less than the coupling-enzyme τ values. It was noted in a previous treatment of coupled enzyme assays (Easterby, 1973) that a special case arises when two τ values are equal. These cases must be solved individually because eqn. (16) does not apply.

Conclusions

In preliminary studies of the reaction-velocity transition of hysteretic enzymes it is necessary to vary the quantity of coupling enzymes and compare plots of $\log(v_f - v_i)$ versus time. In this way, a multi-exponential decay is not confused with one that can be described by a single rate constant. Variations in the quantity of coupling enzymes can also be used to estimate τ_0 by extrapolating a plot of the measured induction period versus the

inverse of the coupling-enzyme concentration to the ordinate axis.

Once the nature of the transient has been determined, it is advisable to use sufficient coupling enzyme to allow direct measurement of τ_0 and v_f . This requires that the reaction proceeds to a steady state that is several times longer than the period of obvious acceleration. The first-order rate constants for the coupling-enzyme reactions should also be at least an order of magnitude larger than that for the reaction-velocity transition of the primary enzyme. v_0 can be calculated from eqn. (13).

Several difficulties in defining the parameters develop as the concentration of coupling enzyme is

Fig. 5. Determination of the relaxation time of sedoheptulose-1,7-bisphosphatase activation by a semi-logarithmic plot

v_f and v_i represent the final, steady-state, reaction velocity and the observed instantaneous reaction velocity respectively. The relaxation time is derived from the slope of the linear portion of the graph. Reaction mixtures contained 300 μl (●), 140 μl (○), and 80 μl (■) of coupling enzyme (see the legend to Fig. 3).

lowered to one comparable with (but not lower than) the v_f of the primary enzyme reaction. In the sedoheptulose-1,7-bisphosphatase experiments, for example, the time required to approach a steady state was so long that inactivation of the primary enzyme became a problem. Under these conditions, it is possible to fit τ_0 , v_0 and v_f values to the initial portion of the reaction progress curve, as long as the K_m and V_{max} values for the coupling enzymes are accurately calculated.

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