Kinetic study of an enzymic cycling system coupled to an enzymic step: determination of alkaline phosphatase activity

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A kinetic study is made of a system consisting of a specific enzymic cycling assay coupled to an enzymic reaction. A kinetic analysis of this system is presented, and the accumulation of chromophore involved in the cycle is seen to be parabolic, i.e. the rate of the reaction increases continuously with constant acceleration. The system is illustrated by the measurement of alkaline phosphatase activity using β -NADP⁺ as substrate. The enzymes alcohol dehydrogenase and diaphorase are used to cycle β -NAD⁺ in the presence of ethanol and *p*-Iodonitrotetrazolium

Violet. During each turn of the cycle, one molecule of the tetrazolium salt is reduced to an intensely coloured formazan. A simple procedure for evaluating the kinetic parameters involved in the system and for optimizing this cycling assay is described. The method is applicable to the measurement of any enzyme, and its amplification capacity as well as the simplicity of determining kinetic parameters enable it to be employed in enzyme immunoassays to increase the magnitude of the measured response.

INTRODUCTION

The technique of enzymic cycling arose from the need to increase sensitivity when determining very small quantities of a metabolite [1]. It is based on the regeneration *in situ* of the target metabolite by using a second reaction to allow it to re-enter the reaction cycle, so that its concentration remains constant. Thus the system constitutes a response-amplification mechanism similar to that of a futile cycle in metabolism [2]. The amount of product accumulated is directly proportional to the original amount of metabolite in the sample when determined conditions are verified [3]. The efficiency of such a recycling process is measured by the number of cycles that can be achieved before a metabolite molecule is destroyed, and is expressed as the total number of turnovers, which is the total number of mol of product formed per mol of metabolite during the course of a complete reaction.

Enzymic cycling is also used to measure low levels of activity of a given enzyme [4–7]. These methods are quite sensitive and useful for determining microamounts of an enzyme in a tissue extract. However, they are usually indirect assays which require several additional steps, including the preincubation of the sample with a substrate of the enzyme (sometimes involving the additional use of a coupled reaction). Furthermore, the concentration of the product thus formed needs to be evaluated by means of a cycling system, which means stopping the reaction by inactivating the enzyme by heat, pH extremes or detergent treatment, before measuring the accumulated amount of a chromophore involved in the cycle in a separate reaction step [8]. These are slow procedures that must be accurately monitored to ensure proportionality between product formation and amount of sample.

This problem does not arise when several enzymic reactions are linearly coupled in order to measure the activity of the first enzyme, because mathematical solutions have been obtained for these reaction schemes [9–11] that allow the establishment of the

conditions necessary to evaluate the velocity of the first enzyme [12].

In an attempt to overcome these problems, we describe the kinetic study of the direct coupling of the enzymic reaction to be measured to a specific enzymic cycle, with continuous monitoring of the time course of the reaction. This procedure was applied to the assay of alkaline phosphatase activity with β -NADP⁺ as substrate. This example was used in a discontinuous way by Self [13] and further characterized by Johannsson et al. [14], to improve sensitivity of enzyme immunoassays in which alkaline phosphatase is employed as the label. The first catalytic step is the dephosphorylation of β -NADP⁺ to produce β -NAD⁺, which catalytically activates a redox cycle involving the enzymes alcohol dehydrogenase and diaphorase (Scheme 1). In each turn of the cycle, one molecule of a tetrazolium salt [*p*-Iodonitrotetrazolium Violet (INT)] is reduced to an intensely coloured formazan; thus one molecule of β -NAD⁺ generated by alkaline phosphatase can

Scheme 1 Coupling of the dephosphorylation reaction of β -NADP⁺ by alkaline phosphatase to a β -NAD⁺-specific cycle involving the enzymes alcohol dehydrogenase and diaphorase

One molecule of coloured INT formazan is generated in each turn of the cycle.

catalyse the production of many molecules of formazan dye. The kinetic analysis of these systems is presented and a single procedure for determining the kinetic parameters involved in the model is described.

THEORY

The enzymic cycling system shown in Scheme 1 may be described as follows:

where A, B and C are β -NADP⁺, β -NAD⁺ and β -NADH respectively, S_1 and S_2 are ethanol and INT respectively, P_1 and P_2 are acetaldehyde and formazan respectively, and E_1 , E_2 and E_3 are alkaline phosphatase, alcohol dehydrogenase and diaphorase respectively.

Assuming that β -NADP⁺ concentration is saturating or its variation during the reaction time is very small, we can consider that the reaction rate of step (1) remains constant (V_1). In addition, the ethanol and INT concentrations (S_1 and S_2) in the reaction medium must also be high enough to be saturating or remain constant during the reaction time. Under these conditions and with relatively high activities of both enzymes E_2 and E_3 , a small quantity of B synthesized from A can 'catalyse' the formation of large quantities of the products P_1 and P_2 . When either P_1 or P_2 is measured, the system acts as a chemical amplifier in the determination of E_1 activity. During the cycling, the concentrations of B and C must clearly be lower than their respective Michaelis-Menten constants (K_{mB} and K_{mC} towards E_2 and E_3 respectively), so that the reaction rates of steps (2) and (3) are proportional to their respective concentrations.

Taking into account these assumptions, the differential equation system that describes the mechanism shown by eqns. (1)–(3) is:

$$d[B]/dt = V_1 - k_2[B] + k_3[C] \quad (4)$$

$$d[C]/dt = k_2[B] - k_3[C] \quad (5)$$

$$d[P_1]/dt = k_2[B] \quad (6)$$

$$d[P_2]/dt = k_3[C] \quad (7)$$

where k_2 and k_3 are apparent first-order rate constants, $k_2 = V_{m2}/K_{m2}$ [V_{m2} is the reaction rate of step (2) at saturating concentrations of B and S_1 ; K_{m2} is a function of Michaelis-Menten constants of B and S_1 towards E_2 and of the initial concentration of S_1 , with $K_{m2} \simeq K_{mB}$ if S_1 is saturating], $k_3 = V_{m3}/K_{m3}$ [V_{m3} is the reaction rate of step (3) at saturating concentrations of C and S_2 ; K_{m3} is a function of Michaelis-Menten constants of C and S_2 towards E_3 and of the initial concentration of S_2 , with $K_{m3} \simeq K_{mC}$ if S_2 is saturating]. The initial conditions of this differential equation system are at $t = 0$, $[B] = B_0$ (due to contamination of β -NADP⁺ by β -NAD⁺; see the Materials and methods section), $[C]$, $[P_1]$ and $[P_2] = 0$.

The differential eqns. (4) and (5) constitute a non-homogeneous linear system that may become homogeneous by further derivation and performing of the variable changes $d[B]/dt = X$, and $d[C]/dt = Y$, giving

$$dX/dt = -k_2X + k_3Y \quad (8)$$

$$dY/dt = k_2X - k_3Y \quad (9)$$

the initial conditions of which are at $t = 0$, $X_0 = V_1 - k_2B_0$ and $Y_0 = k_2B_0$. The solution to this system is given by:

$$X = \frac{k_2(V_1 - \lambda B_0)}{\lambda} e^{-\lambda t} + \frac{k_3V_1}{\lambda} \quad (10)$$

$$Y = -\frac{k_2(V_1 - \lambda B_0)}{\lambda} e^{-\lambda t} + \frac{k_2V_1}{\lambda} \quad (11)$$

where $\lambda = k_2 + k_3$. Integration of eqns. (10) and (11) to return to our original symbolism yields:

$$[B] = \frac{k_2(V_1 - \lambda B_0)}{\lambda^2} (1 - e^{-\lambda t}) + \left(\frac{k_3V_1}{\lambda} \right) t + B_0 \quad (12)$$

$$[C] = -\frac{k_2(V_1 - \lambda B_0)}{\lambda^2} (1 - e^{-\lambda t}) + \left(\frac{k_2V_1}{\lambda} \right) t \quad (13)$$

Inserting eqns. (12) and (13) into eqns. (6) and (7) respectively and integrating again we obtain:

$$[P_1] = k_2 \left\{ -\frac{k_2(V_1 - \lambda B_0)}{\lambda^3} (1 - e^{-\lambda t}) + \left(\frac{k_3V_1}{2\lambda} \right) t^2 + \left[\frac{k_2(V_1 - \lambda B_0)}{\lambda^2} + B_0 \right] t \right\} \quad (14)$$

$$[P_2] = k_3 \left\{ \frac{k_2(V_1 - \lambda B_0)}{\lambda^3} (1 - e^{-\lambda t}) + \left(\frac{k_2V_1}{2\lambda} \right) t^2 - \left[\frac{k_2(V_1 - \lambda B_0)}{\lambda^2} \right] t \right\} \quad (15)$$

In the steady state ($t \rightarrow \infty$), exponential terms in eqns. (14) and (15) are negligible and, reordering terms:

$$[P_1] = \left(\frac{k_2k_3V_1}{2\lambda} \right) t^2 + \left[\frac{k_2^2(V_1 - \lambda B_0)}{\lambda^2} + k_2B_0 \right] t - \frac{k_2^2(V_1 - \lambda B_0)}{\lambda^3} \quad (16)$$

$$[P_2] = \left(\frac{k_2k_3V_1}{2\lambda} \right) t^2 - \left[\frac{k_2k_3(V_1 - \lambda B_0)}{\lambda^2} \right] t + \frac{k_2k_3(V_1 - \lambda B_0)}{\lambda^3} \quad (17)$$

Eqns. (16) and (17) indicate that both products of the cycle, P_1 and P_2 , are accumulated in a parabolic shape when the steady state is reached, i.e. the rate of the reaction is continuously increasing as long as the conditions assumed are fulfilled. By analogy with the parabolic shape of uniformly accelerated motion in mechanical physics, whose integrated equation for covered space

$$S = S_0 + V_0t + \frac{1}{2} at^2$$

is similar to these latter equations, we can define a kinetic parameter that keeps constant during the reaction, the acceleration of the process:

$$a = \frac{k_2k_3}{k_2 + k_3} V_1 \quad (18)$$

with

$$\frac{a}{V_1} = \frac{k_2k_3}{k_2 + k_3} \quad (19)$$

the acceleration per unit of activity to be measured.

If λ is high enough to make the exponential terms in eqns. (14) and (15) negligible, then eqns. (16) and (17) are valid from the onset of the reaction. One way of ascertaining whether the steady state has been reached is to take the first derivative of the progress curve corresponding to P_1 or P_2 and to ensure that it is linear. These equations are second-order polynomials and so data from the progress curves of either P_1 or P_2 may be easily fitted to them by linear regression for the kinetic parameters involved in the system to be evaluated.

MATERIALS AND METHODS

Alkaline phosphatase (EC 3.1.3.1) from chicken intestine, β -NADP⁺, β -NAD⁺, INT, *o*-carboxyphenyl phosphate (*o*-CPP), glycine and BSA (fraction V) were obtained from Sigma. Alcohol dehydrogenase (EC 1.1.1.1) from yeast and diaphorase (NADH-dye oxidoreductase, EC 1.8.1.4) from micro-organisms were obtained from Boehringer-Mannheim. All other buffers and reagents were of analytical grade and were used without further purification. Spectrophotometric readings were obtained on a Uvikon 940 spectrophotometer from Kontron Instruments. All measurements were performed at 25 °C.

Determination of alkaline phosphatase activity with *o*-CPP as substrate

The activity of alkaline phosphatase was determined separately by following the assay method indicated in the Worthington Enzyme Manual [15]. The standard reaction mixture consisted of 1 mM *o*-CPP and 6.95 mM MgCl₂ in 0.2 M glycine buffer, pH 8.8. The reaction was started by the addition of 0.1 ml of stock enzyme solution (1 mg/ml in 0.1 M Tris/HCl, pH 8.5, containing 1 mg/ml BSA), the final volume being 3.6 ml. The reaction was followed at 25 °C by recording the increase in A_{300}/min from the initial linear portion of the curve (the molar absorption coefficient, ϵ , of salicylic acid at 300 nm is 3500 M⁻¹·cm⁻¹, [15]). One unit of alkaline phosphatase activity was defined as the amount of enzyme that hydrolyses 1 μmol of *o*-CPP/min under the specified conditions. The specific activity of the enzyme under these conditions was 2.2 units/mg of protein.

Cycling assay for alkaline phosphatase

The cycling reagent consisted of 0.1 M Tris/HCl buffer, pH 8.8, 0.5 mM INT (with 0.1 % Tween 80), 0.33 M ethanol, 8.33 mM MgCl₂, 20 $\mu\text{g}/\text{ml}$ alcohol dehydrogenase (in 0.01 M Tris/HCl, pH 7.5, with 5 mg/ml BSA), 66.4 $\mu\text{g}/\text{ml}$ diaphorase (in 0.1 M Tris/HCl, pH 8.5, with 5 mg/ml BSA) and different volumes of a stock solution of alkaline phosphatase of 0.5 $\mu\text{g}/\text{ml}$ (in 0.1 M Tris/HCl, pH 8.5, containing 1 mg/ml BSA). The reaction was started by the addition of β -NADP⁺ to give a final concentration of 0.25 mM, the final volume being 3 ml. The time course of the reaction was followed by measuring the appearance of the formazan product of the reaction at 492 nm ($\epsilon = 1.94 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [16]).

If the reaction is started by the addition of the enzyme (alkaline phosphatase) instead of its substrate (β -NADP⁺), spontaneous reduction of tetrazolium salt is observed during preincubation. This indicates the presence of β -NAD⁺ and/or β -NADH in the β -NADP⁺ solution. The presence of the reduced form was disregarded by spectrophotometrically scanning the sample, no absorbance being detected in its absorption maximum at 340 nm. The β -NAD⁺ present was titrated as indicated below.

Cycling assay for β -NAD⁺

The amount of β -NAD⁺ present in the β -NADP⁺ solution was evaluated by using the same enzymic cycling system but in the absence of alkaline phosphatase, under the above conditions. A calibration curve was made with β -NAD⁺ of known concentration. The reaction was started by the addition of β -NADP⁺ or β -NAD⁺. In this way, we ascertained that the concentration of β -NAD⁺ present at time zero (B_0) in a standard cuvette assay is 0.24 μM , which must be taken into account in the calculations.

Evaluation of kinetic parameters

We also used the β -NAD⁺-specific cycling assay system to evaluate the general rate of constant of the cycle, $k = k_2 k_3 / (k_2 + k_3)$ [3], allowing the activity of the enzyme E_1 (V_1) to be measured from acceleration of the process [eqn. (18)].

The apparent first-order rate constants k_2 and k_3 were similarly evaluated by performing several experiments, in which the concentration of the enzyme alcohol dehydrogenase was kept constant and the concentration of diaphorase was varied from 6.64 to 116.16 $\mu\text{g}/\text{ml}$, or the concentration of diaphorase was kept constant while the concentration of alcohol dehydrogenase was varied from 3.33 to 40 $\mu\text{g}/\text{ml}$. The concentration of β -NAD⁺ used in all these experiments was 0.44 μM .

Data were fitted by linear regression using the SigmaPlot Scientific Graphing System.

RESULTS AND DISCUSSION

Evaluation of optimum conditions

In a normal cycling system with only steps (2) and (3), the equation that describes product accumulation with time under the assumptions made here is of the form:

$$P = \gamma(1 - e^{-\lambda t}) + \alpha t$$

where $\lambda = k_2 + k_3$ and the steady-state rate $\alpha = k_2 k_3 B_0 / \lambda$. When the steady state is reached (it may be at the onset of the reaction if λ is high enough to make the exponential term negligible), the product is accumulated linearly with time.

Thus, working only with the β -NAD⁺-specific cycle with a known concentration of β -NAD⁺ (0.44 μM), we first check that the INT and ethanol concentrations were saturating, i.e. the rate of the reaction was independent of their concentration. The concentrations of the enzymes alcohol dehydrogenase and diaphorase were varied to obtain a straight line for chromophore accumulation at the very beginning of the reaction, indicating that exponential terms in the above equation are negligible and can therefore also be considered negligible in eqns. (14) and (15), when the reaction catalysed by alkaline phosphatase is coupled to the cycle.

Other authors [14] have included ZnCl₂ in the buffer used to dissolve alkaline phosphatase and β -NADP⁺ and so we tested the effect of this compound on the complete reaction. When its concentration was varied, we found that alkaline phosphatase activity was about a thousand times higher in the absence of Zn²⁺ than in its presence, which explains why we did not include ZnCl₂ in the reaction medium. This inhibitory effect of Zn²⁺ has been described in the literature for mammalian alkaline phosphatase [17]. Mg²⁺ ions are well-known activators of alkaline phosphatase from chicken intestine [18] and therefore the concentration of MgCl₂ used was such that the enzymes involved in the cycle were not affected.

Figure 1 (a) Chromophore accumulation with time at different concentrations of alkaline phosphatase and (b) acceleration of the process plotted against alkaline phosphatase concentration

In (a) the concentrations of alkaline phosphatase are 1.6, 3.3, 5.0, 7.5, 10.0, 12.5 and 15.0 ng/ml for curves 1–7 respectively. The points in (b) represent experimental data, and the straight line corresponds to regression analysis.

Time course of the reaction

The dephosphorylation of $\beta\text{-NADP}^+$ by alkaline phosphatase was followed by monitoring the increase in absorbance at 492 nm, the wavelength at which the chromophore formed in the $\beta\text{-NAD}^+$ -specific cycle by the diaphorase-catalysed reduction of the tetrazolium salt INT shows maximum absorbance. Figure 1(a) shows formazan (P_2) formation for different alkaline phosphatase concentrations under the conditions described in the Materials and methods section. It can be seen that the progress of the reaction was parabolic, according to the equations obtained. These data were therefore fitted by linear regression to second-order polynomial equations in accordance with eqn. (17), after a preliminary check, by taking the first derivative, that the exponential terms in eqn. (15) were negligible. The regression coefficients thus obtained varied from 0.999995 to 0.999997, indicating good concordance. Figure 1(b) shows the linear dependence of the acceleration (from the term in t^2) of the process on alkaline phosphatase concentration.

Evaluation of kinetic parameters

k_2 and k_3 are apparent first-order rate constants (V_{max}/K_m), as has been mentioned above, and they can therefore be evaluated by determining their corresponding kinetic parameters V_{max} and K_m in a classical way. However, a simpler method that ensures the same experimental conditions is to use only the $\beta\text{-NAD}^+$ -specific cycle shown in Figure 1. By this means, we evaluated the general rate constant of the cycle, $k = k_2k_3/(k_2 + k_3)$, obtaining a value of 8.8 min^{-1} under the present conditions (results not shown). Once k has been determined, it is very easy to find the activity of the enzyme alkaline phosphatase (V_1 in the above experiments from the acceleration of the process, according to eqn. 18). The activity of the primary enzyme, determined from the acceleration, was proportional to enzyme concentration over the specified range. The data obtained indicate that a much wider range of alkaline phosphatase activity can be detected than when using the classical assay of this enzyme with *o*-CPP as substrate. There is therefore a much greater degree of sensitivity in alkaline phosphatase measurement, as the concentrations used would not have been detectable by the second method. Specific activities thus determined were of the order of $5.8 \text{ pmol/min per ng}$.

Figure 2 Evaluation of the apparent first-order rate constants k_2 (a) and k_3 (b) corresponding to alcohol dehydrogenase and diaphorase respectively, by means of the $\beta\text{-NAD}^+$ -specific cycle

Points represent experimental data, and the straight line corresponds to regression analysis.

k_2 and k_3 (corresponding to alcohol dehydrogenase and diaphorase respectively) were determined individually to check the results obtained, by varying the concentration of one enzyme and keeping the concentration of the other fixed, as explained in the Materials and methods section. Taking into account that $1/k = 1/k_2 + 1/k_3$, a double-reciprocal plot of k versus diaphorase concentration will be a straight line with the ordinate value $1/k_2$, and by analogy, a double-reciprocal plot of k versus alcohol dehydrogenase concentration will be a straight line with ordinate value $1/k_3$. Figure 2 shows the results thus obtained, the values of k_2 and k_3 being $0.261 \text{ min}^{-1} \text{ per } \mu\text{g}$ of enzyme and $0.098 \text{ min}^{-1} \text{ per } \mu\text{g}$ of enzyme respectively. These values, together with that of B_0 ($0.24 \mu\text{M}$), evaluated by performing a calibration curve with $\beta\text{-NAD}^+$ of known concentration, were introduced into the linear regression of progress curves shown in Figure 1, and total coincidence was obtained.

Concluding remarks

The enzymic cycling assay procedure for kinetic amplification may be used for the continuous determination of low levels of an enzymic reaction. The kinetic equations obtained for the theoretical model described in the present paper allow the direct evaluation of the specific first-order rate constants involved in the cycle (k_2 and k_3) in the same reaction medium in which they will be used afterwards. In this way, we can ascertain experimentally the degree of acceleration attained in the measurement of initial enzyme per unit of activity to be detected.

Likewise, it is established that the kinetic parameter product-accumulation acceleration is linear with the concentration of initial enzyme. All these conclusions are illustrated by the measurement of alkaline phosphatase in the dephosphorylation of $\beta\text{-NADP}^+$ using the alcohol dehydrogenase/diaphorase cycling system. Alkaline phosphatase is a widely used enzyme label in enzyme immunoassay. The great sensitivity with which the enzyme may be determined, as well as the numerous advantages of using a continuous assay, make it possible to perform immunometric assays of high sensitivity and short duration using the technique of enzymic cycling described here.

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