Kinetic analysis of chemical reactions coupled to an enzymic step

Application to acid phosphatase assay with Fast Red

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1. Acid phosphatase assay with α -naphthyl phosphate as substrate and the use of diazonium salt (Fast Red TR) for chromophore formation was kinetically analysed as a system of two chemical reactions coupled to an enzymic reaction. This system follows a mechanism defined as enzymic–chemical–chemical (E_zCC). 2. The accumulation of chromophore with reaction time presented a marked lag period, which was only dependent on the rate constants of the chemical reactions and was independent of the enzymic step. 3. The specific rate constants of each chemical step were determined in 3.8–5.0 pH and 10–35°C temperature ranges. Thermodynamic parameters of the chemical steps were also obtained. 4. Measurement of acid phosphatase activity can be carried out in the pH range 3.8–5.0 (4.8 was optimal pH) without the need to eliminate the lag period.

Enzymic reactions where the products are unstable and undergo a chemical process of decomposition, deprotonation or, in general, stabilization by a first-order or second-order kinetics have previously been described (Childs & Bardsley, 1975; Claiborne & Fridovich, 1979; Boekeheide *et al.*, 1980; Frère *et al.*, 1982). On the other hand, chemical reactions have been coupled to many enzymic reactions in order to determine the time-dependent behaviour of the products (Ellman *et al.*, 1961; Jolley & Mason, 1965; Esterbauer *et al.*, 1977). Usually it has been considered that the velocity of an enzymic step, provided that it is the limiting one, can be measured by monitoring the formation of the chemical reaction product (Esterbauer *et al.*, 1977). A kinetic analysis has recently been made of the pathway of dopa (3,4-dihydroxy-phenylalanine) oxidation by tyrosinase to dopa-chrome, as a system of several chemical reactions coupled to an enzymic step (García-Carmona *et al.*, 1982), although with peculiar pathway characteristics such that it is not applicable for a study of a linear pathway of an enzymic reaction followed by various chemical reactions.

In the present work, kinetic analysis of a system

of two chemical reactions coupled to an enzymic reaction has been carried out, designating this type of mechanism as enzymic–chemical–chemical (E_zCC). This theoretical study was applied to assay for determination of acid phosphatase activity by the method of Hillman (1971), with α -naphthyl phosphate as substrate and the use of Fast Red TR for chromophore formation, as expressed in the mechanism shown in Scheme 1.

The formation of the chromophore shows a marked lag period, which strongly depended on pH. This method is not accepted by most clinical laboratories (Bais & Edwards, 1976) because of the existence of this lag period. Various modifications have been introduced to minimize the lag period, since according to several authors its existence means that the limiting step for this assay is the formation of the complex between the reaction product (α -naphthol) and Fast Red TR (Bais & Edwards, 1976), and therefore the reaction between α -naphthol and Fast Red TR must be rapid to reflect the enzyme activity accurately (Cooper *et al.*, 1982), suggesting that a pH range of 5.5–6.0 must be used to measure the enzyme activity (Bais & Edwards, 1976; Warren & Moss, 1977; von Gundlach & Mühlhausen, 1980; Cooper *et al.*, 1982).

The present paper shows that the need to eliminate the lag period, in order to measure

Abbreviations used: Fast Red TR salt, 5-chloro-2-toluidinediazonium chloride (hemizinc chloride salt).

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Scheme 1

accurately the enzyme activity in an E_2CC mechanism, is unfounded. Furthermore, analysis of this type of mechanism allows the specific rate constants of each chemical step to be easily evaluated. On the basis of the kinetic results obtained, some modifications to the assay of the acid phosphatase are proposed.

Theory

The hydrolysis of the α -naphthyl phosphate by acid phosphatase and the reaction of the product with Fast Red TR can be described as follows:

where A is α -naphthyl phosphate, B is α -naphthol, C is α -naphthyl and P is the chromophore obtained from the reaction between α -naphthyl and Fast Red TR; $k'_{-1} = k_{-1}[\text{H}^+]$ and $k'_{+2} = k_{+2}[\text{Fast Red}]$. Eqn. (1) shows the enzymic hydrolysis of α -naphthyl phosphate, eqn. (2) the deprotonation equilibrium of α -naphthol and eqn. (3) the chemical reaction between α -naphthyl and Fast Red TR.

On the assumption that the α -naphthyl phosphate concentration is saturating or its variation during reaction time is very small, we ought to consider that the enzymic reaction rate remains constant (V_0). On the other hand, the Fast Red TR concentration in the reaction medium should be high enough to remain constant during the reaction time.

Taking into account these assumptions, velocity equations for B and C accumulation are:

$$\frac{d[\text{B}]}{dt} = V_0 + k'_{-1}[\text{C}] - k_{+1}[\text{B}] \quad (4)$$

$$\frac{d[\text{C}]}{dt} = k_{+1}[\text{B}] - (k'_{-1} + k'_{+2})[\text{C}] \quad (5)$$

From eqns. (4) and (5) a second-order equation can be defined, the general solution of which is:

$$[\text{C}] = \frac{V_0}{k'_{+2}} \left(1 - \frac{1}{(\lambda_2 - \lambda_1)} (\lambda_2 \cdot e^{\lambda_1 t} + \lambda_1 \cdot e^{\lambda_2 t}) \right) \quad (6)$$

where λ_1 and λ_2 solutions are negative. From eqns. (5) and (6) it can be deduced that:

$$[\text{B}] = \frac{V_0}{k_{+1} \cdot k'_{+2}} \left((k'_{-1} + k'_{+2}) - \frac{1}{(\lambda_2 - \lambda_1)} [(\lambda_1 + k'_{-1} + k'_{+2}) \lambda_2 \cdot e^{\lambda_1 t} + (\lambda_2 + k'_{-1} + k'_{+2}) \lambda_1 \cdot e^{\lambda_2 t}] \right)$$

For $-\lambda_1 \cdot t \gg 1$ and $-\lambda_2 \cdot t \gg 1$, [B] and [C] in the steady state can be defined as:

$$[\text{C}]_{ss} = \frac{V_0}{k'_{+2}}$$

$$[\text{B}]_{ss} = \frac{(k'_{-1} + k'_{+2})}{k_{+1} \cdot k'_{+2}} \cdot V_0$$

On the other hand:

$$\frac{d[\text{P}]}{dt} = k'_{+2}[\text{C}] \quad (7)$$

From eqns. (6) and (7) and integrating, the following expression for [P] as a function of λ_1 and λ_2 can be established:

$$[\text{P}] = V_0 \left[t - \frac{1}{(\lambda_2 - \lambda_1)} \left(\frac{\lambda_2}{\lambda_1} \cdot e^{\lambda_1 t} + \frac{\lambda_1}{\lambda_2} \cdot e^{\lambda_2 t} + \frac{\lambda_2}{\lambda_1} - \frac{\lambda_1}{\lambda_2} \right) \right]$$

For $-\lambda_1 \cdot t \gg 1$ and $-\lambda_2 \cdot t \gg 1$, it is possible to define how P is accumulated when the system reaches its steady state:

$$[P]_{ss} = V_0 \left(t + \frac{\lambda_2 + \lambda_1}{\lambda_1 \cdot \lambda_2} \right)$$

Substituting λ_1 and λ_2 as a function of the rate constants, the following expression is obtained for the P accumulation in the steady state:

$$[P]_{ss} = V_0 \left[t - \left(\frac{k'_{-1}}{k_{+1} \cdot k'_{+2}} + \frac{1}{k_{+1}} + \frac{1}{k'_{+2}} \right) \right] \quad (8)$$

Therefore the lag period, L , is defined as:

$$L = \frac{[H^+]}{K_a \cdot k_{+2}[\text{Fast Red}]} + \frac{1}{k_{+1}} + \frac{1}{k_{+2}[\text{Fast Red}]} \quad (9)$$

where $K_a = k_{+1}/k_{-1}$ is the ionization constant of α -naphthol. A similar equation has been obtained by Easterby (1981), applying the mass-conservation balance.

A plot of lag period against proton concentration gives straight lines, the slopes of which allow us to evaluate k_{+2} , since K_a is known, and the intercept on the ordinate axis allows the k_{+1} values to be calculated.

With reference to eqn. (9), it is important to point out that the transition time depends on the rate constant of the chemical reactions, but it is independent of the rate of the enzymic step. However, concentrations of intermediates in the steady state depend on both enzymic and chemical reactions, and increase with enzymic reaction rate.

Materials and methods

Acid phosphatase (orthophosphoric-monoester phosphohydrolase, acid optimum, EC 3.1.3.2) (0.36 unit/mg), Fast Red TR salt and α -naphthyl phosphate were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other reagents commercially obtained were of analytical grade. One unit of enzyme is defined as the amount of enzyme that hydrolyses 1.0 μmol of p -nitrophenyl phosphate/min at pH 4.8 at 37°C.

Spectrophotometric measurements were carried out with an Aminco DW-2 spectrophotometer equipped with a Hewlett-Packard recorder with kinetic response, which allows the dead recording time to be minimized; when the rate constant of the chemical reaction was greater than 0.1 s^{-1} , an Aminco-Morrow Stopped Flow accessory equipped with a Dasar T.M. unit was also used. Temperature was controlled by a Gilson bath with a precision of $\pm 0.1^\circ\text{C}$. Acid phosphatase activity

was determined by the Hillman's (1971) method, where chromophore accumulation was monitored spectrophotometrically at $\lambda_{\text{max.}} = 405 \text{ nm}$. The reaction medium contained 0.1M-sodium citrate buffer (pH as indicated in each case), 3mM- α -naphthyl phosphate, 62.5 μg of acid phosphatase/ml and Fast Red TR concentration varied in the 1.3–13mM range. The reaction study was undertaken in the temperature range 10–35°C. The α -naphthyl phosphate and Fast Red TR solutions were prepared fresh every day.

Results

Variation of lag period and the steady-state rate with the enzyme concentration

The hydrolysis of α -naphthyl phosphate by acid phosphatase was followed by the absorbance increase at 405nm, where the chromophore formed by the reaction of α -naphthyl with Fast Red TR dye shows maximum absorbance. The reaction progress presented a marked lag period, which was strongly dependent on pH and temperature, but was independent of the enzyme concentration. Fig. 1 shows the chromophore formation

Fig. 1. Chromophore accumulation with time. This is followed by the absorbance increase at 405nm at different enzyme concentrations ($\mu\text{g/ml}$): 1, 77; 2, 59; 3, 40.3; 4, 30.6; 5, 20.6. The reaction medium contained 0.1M-citrate buffer, pH 4.5, 5.7mM-Fast Red TR and 1.5mM- α -naphthyl phosphate at 23°C. The inset shows a plot of chromophore accumulation rate in steady-state conditions against enzyme concentration. The same experimental conditions as indicated for the main Figure were used.

for different enzyme concentrations at 23°C and pH 4.5. The transient phase was defined by the lag period, the value of which was independent of the enzyme concentration, according to eqn. (9). However, the slope of the linear zone of the curve depended directly on the enzyme concentration, as shown by Fig. 1 (inset), and its value is V_0 , in accordance with eqn. (8).

Evaluation of the specific rate constants

The specific rate constants of each step were calculated from eqn. (9). The lag period was evaluated for each different concentration of Fast Red TR in the concentration range 1.3–13 mM, for the same enzyme concentration (62.5 µg/ml), at 23°C in 0.1 M-citrate buffer in the pH range 3.8–5.0. The plot of the lag period against the reciprocal of the Fast Red TR concentration, as shown in Fig. 2, gives straight lines for each pH value studied, the ordinate value of which is identical for all pH values, being the inverse of the rate constant k_{+1} . This value $k_{+1} = 3.1 \times 10^{-2} \text{ s}^{-1}$

Fig. 2. Variation of lag period with Fast Red TR concentration

The data are presented as plots of the lag period against the reciprocal of Fast Red TR concentration at the different pH values: ○, pH 3.8; ▲, pH 4.0; △, pH 4.5; □, pH 4.8; ●, pH 5.0. The reaction medium contained 0.1 M-citrate buffer, 1.5 mM- α -naphthyl phosphate and 62.5 µg of acid phosphatase/ml at 23°C. The inset shows a plot of each slope of straight lines obtained in the main Figure, according to eqn. (9), against proton concentration.

was independent of proton concentration. On the other hand, the plot of the value of each slope against proton concentration was a straight line, as shown in Fig. 2 inset. The second-order rate constant k_{+2} can be evaluated ($k_{+2} = 3.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) from its slope $m = 1/K_a \cdot k_{+2}$, by taking $K_a = 4.6 \times 10^{-10} \text{ M}$ (Weast, 1973).

Variation of lag period with pH and temperature

The rate constants for different values of pH and temperature were evaluated. The reaction medium

Fig. 3. Lag period variation at several pH values and temperatures

The reaction medium contained 0.1 M-citrate buffer, 1.5 mM- α -naphthyl phosphate, 5.7 mM-Fast Red TR and 62.5 µg of acid phosphatase/ml. Temperatures: ●, 10°C; ○, 15°C; ▲, 20°C; △, 25°C; ■, 30°C; □, 35°C.

Table 1. First-order rate constants for the deprotonation of α -naphthol and the reaction between α -naphthyl and Fast Red TR at several temperatures

The formation of the chromophore presents a lag period, which depended on temperature. From eqn. (9) the specific rate constants at several temperatures values can be evaluated.

Temperature (°C)	$10^{-2} \times k_{+1} \text{ (s}^{-1}\text{)}$	$10^3 \times k'_{+2} \text{ (s}^{-1}\text{)}$
10	1.73	0.99
15	2.53	1.07
20	2.86	1.54
25	3.41	2.39
30	5.95	2.88
35	6.84	3.69

Table 2. *Thermodynamic activation parameters for the deprotonation of α -naphthol and for the reaction between α -naphthyl and Fast Red TR*

	E_a (kJ·mol ⁻¹)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (kJ·mol ⁻¹ ·K ⁻¹)
Deprotonation (k_{+1})	48.5	46.0	81.1	0.12
Union to Fast Red (k'_{+2})	36.4	33.8	53.5	0.07

contained 0.1 M-citrate buffer in the pH range 3.8–5.0, 62.5 μ g of acid phosphatase/ml, 3 mM- α -naphthyl phosphate and 5.7 mM-Fast Red TR. The lag period increased when pH and/or temperature were decreased. From eqn. (9) the plot of the lag period against proton concentration for the same concentration of Fast Red TR gives linear plots, as shown in Fig. 3. From these linear-regression-fitted plots, k_{+1} can be evaluated from the intercepts on the ordinate axis and k'_{+2} from the slope of the straight lines. The different rate constants at several temperatures are given in Table 1.

For the evaluation of the activation energies for the deprotonation of α -naphthol and the reaction between α -naphthyl and Fast Red TR, Arrhenius plots were made with the k_{+1} and k'_{+2} values obtained. The derived activation parameters are summarized in Table 2.

Discussion

The theoretical analysis of an E_zCC mechanism and its application for the determination of acid phosphatase activity allows us to define a parameter, the lag period of the accumulation of the last product, that is only dependent on the rate constants of the chemical reactions (eqn. 9) and is independent of the enzymic step.

Thus, from a theoretical and experimental point of view, this mechanism shows that the accumulation rate of the chromophore in the steady state is independent of the existence of chemical steps and its rate constants, but depends on a linear behaviour of the enzyme concentration (eqn. 8 and Fig. 1 inset). Enzymic activity can be determined without the interference of the relatively slow chemical step. Therefore it is unnecessary to minimize the lag period (Bais & Edwards, 1976; Cooper *et al.*, 1982). Minimization of this lag period with a determination of acid phosphatase activity above pH 5.5 has introduced several further problems in the method of measurement. At these pH values the diazonium salt itself is unstable and may be precipitated from solution (Warren & Moss, 1977); a significant hydrolysis of Fast Red TR can also take place to yield 4-chloro-2-methylphenol, subsequently undergoing coupling with Fast Red TR to produce a chlorocresol-azodye complex (Cooper *et al.*, 1982); instability of a α -naphthol-azodye complex chromophore (War-

ren & Moss, 1977), probably due to the coupling of a second diazonium ion to the complex already formed between α -naphthol and diazonium ion (Sanders *et al.*, 1978), also occurs.

On the basis of the present paper, measurement of enzymic activity at a pH between 3.8 and 5.0 is recommended, since the molar absorption coefficient of chromophore is then highest (Bais & Edwards, 1976), at pH 4.8 the prostatic acid phosphatase activity is also at a maximum (Walter & Schütt, 1974), and furthermore in this pH range none of the problems present in the 5.5–6.0 range appear.

The theoretical and practical analysis of the transition phases of the E_zCC mechanism allows us to evaluate the specific rate constants of the chemical steps easily and to establish with greater accuracy the specific reactions that occur in the chromophore formation.

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