Kinetics of an enzyme reaction in which both the enzyme–substrate complex and the product are unstable or only the product is unstable

C. GARRIDO-DEL SOLO,* F. GARCÍA-CÁNOVAS,† B. H. HAVSTEEN,‡ E. VALERO* and R. VARÓN*§

*Departamento de Química-Física, Escuela Universitaria Politécnica, Universidad de Castilla-La Mancha, Avda. España, s/n. Campus Universitario, E-02071, Albacete, Spain, †Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Murcia, E-30071 Murcia, Spain, and ‡Biochemisches Institut der Medizinischen Fakultät, Christian-Albrechts Universität zu Kiel, Olshausenstr. 40, 24098 Kiel, Germany

A kinetic analysis of the Michaelis–Menten mechanism has been made for the case in which both the enzyme–substrate complex and the product are unstable or only the product is unstable, either spontaneously or as the result of the addition of a reagent. This analysis allows the derivation of equations which under conditions of limiting enzyme concentration relate the concentration of all of the species to the time. A kinetic data analysis

is suggested, which leads to the evaluation of the kinetic parameters involved in the reaction. The analysis is based on the equation which describes the formation of products with time and one's experimental progress curves. We demonstrate the method numerically by computer simulation of the reaction with added experimental errors and experimentally by the use of data from the kinetic study of the action of tyrosinase on dopamine.

INTRODUCTION

There are a few examples of enzymic systems in which the product instability significantly influences the evaluation of the kinetic parameters; two examples are peroxidase (Escribano et al., 1985; Vázquez et al., 1992) and tyrosinase (García-Cánovas et al., 1982; García-Carmona et al., 1982; Rodríguez-López et al., 1991). Enzymic reactions in which the products are unstable and undergo a chemical process of decomposition, deprotonation or other stabilization with first- or second-order kinetics have previously been described (Childs and Bardsley, 1975; Claiborne and Fridovich, 1979; Boekelheide et al., 1980; Frère et al., 1982; García-Carmona et al., 1982; Escribano et al., 1985; Rodríguez-López et al., 1991). Sometimes, both the enzyme and the enzyme–substrate complex are unstable (Duggleby, 1986; Pike and Duggleby, 1987; Garrido-del Solo et al., 1993). In some other cases, the enzyme is stable but the substrate induces instability in the complex; such is the case for the suicide substrates (García-Cánovas et al., 1987; Tudela et al., 1987a, 1987b, 1988; García Moreno et al., 1989). There are also enzymic reactions in which both the product and the enzyme–substrate complex are unstable (García-Carmona et al., 1982; García-Cánovas et al., 1987; Escribano et al., 1989). The influence of the instability of the product and of the enzyme–substrate complex on the Michaelis–Menten mechanism has, to our knowledge, not yet been studied.

In this paper, the kinetic analysis of a Michaelis–Menten mechanism in which both the product and the enzyme–substrate complex are unstable (see Scheme 1) has been carried out. We derive explicit equations relating the concentration of the product to the time and suggest a kinetic analysis for the evaluation of the kinetic parameters which is adequate if suitable experimental data are available. This has been illustrated by the kinetic study of the oxidation of 3,4-dihydroxyphenylethylamine (dopamine) to the corresponding *o*-quinone catalysed by the enzyme tyrosinase [monophenol, 3,4-dihydroxy-L-phenylalanine (L-dopa): oxygen oxidoreductase, EC 1.14.18.1]. It has been shown that the direct product of the enzymic catalysis is *o*-dopaminequinone- H^+ , which evolves non-enzymically to *o*-dopaminechrome by the cyclization of the molecule following a Michael intramolecular 1,4-addition (Jiménez et al., 1984).

In Scheme 1, E is the free enzyme catalysing the reaction, S and P are the substrate and the product respectively, ES is the enzyme–substrate complex and R_1 and R_2 are the species into which the species ES and P are transformed.

Scheme 1

KINETIC ANALYSIS

The following equations describe the kinetics of Scheme 1:

$$\frac{d[E]}{dt} = -k_{+1}[E][S] + (k_{-1} + k_{+2})[ES] \quad (1)$$

$$\frac{d[ES]}{dt} = k_{+1}[E][S] - (k_{-1} + k_{+2} + j_{+1})[ES] \quad (2)$$

$$\frac{d[S]}{dt} = -k_{+1}[E][S] + k_{-1}[ES] \quad (3)$$

$$\frac{d[P]}{dt} = k_{+2}[ES] - j_{+2}[P] \quad (4)$$

$$\frac{d[R_1]}{dt} = j_{+1}[ES] \quad (5)$$

$$\frac{d[R_2]}{dt} = j_{+2}[P] \quad (6)$$

Eqns. (1)–(6) do not admit any analytical solution, but approximate solutions can be derived if one assumes that

$$[S]_0 \gg [E]_0 \quad (7)$$

§ To whom correspondence should be addressed.

and

$$[P] + [R_2] \ll [S]_0 \quad (8)$$

$[S]_0$ and $[E]_0$ being the total concentrations of the enzyme and substrate respectively. In this case,

$$[S] \approx [S]_0 \quad (9)$$

Under these conditions, the only variables appearing on the right hand sides of eqns. (1)–(6) are E, ES and P. The three equations (1), (2) and (4) form a set of three independent linear equations in these variables that can be solved by standard techniques for a set of linear ordinary differential equations (Redheffer, 1991). This involves finding the eigenvalues and eigenvectors of the matrix of coefficients. The eigenvalues are the roots λ_i of a cubic equation:

$$\lambda_1 = \frac{-F_1 + \sqrt{F_1^2 - 4F_2}}{2} \quad (10)$$

$$\lambda_2 = \frac{-F_1 - \sqrt{F_1^2 - 4F_2}}{2} \quad (11)$$

$$\lambda_3 = -j_{+2} \quad (12)$$

The expressions F_1 and F_2 in eqns. (10) and (11) are:

$$F_1 = k_{+1}[S]_0 + k_{-1} + k_{+2} + j_{+1} \quad (13)$$

$$F_2 = k_{+1}j_{+1}[S]_0 \quad (14)$$

The complete solution has the form:

$$[P] = \sum_{h=1}^3 \gamma_h e^{\lambda_h t} \quad (15)$$

where the γ_h is determined to ensure that the initial conditions (in particular $[P]_0 = 0$, $[R_1]_0 = 0$, $[ES]_0 = 0$) at $t = 0$ are satisfied. Moreover:

$$\gamma_h = \frac{k_{+1}k_{+2}[S]_0[E]_0}{\prod_{\substack{q=1 \\ q \neq h}}^3 (\lambda_h - \lambda_q)} \quad (h = 1, 2, 3) \quad (16)$$

The following relations exist between λ_1 and λ_2 :

$$\lambda_1 + \lambda_2 = -F_1 \quad (17)$$

$$\lambda_1 \lambda_2 = F_2 \quad (18)$$

From the eqns. (10), (13) and (14), we obtain:

$$|\lambda_1| < j_{+1} \quad (19)$$

and from eqns. (11), (13) and (14):

$$|\lambda_2| > (k_{+1}[S]_0 + k_{-1} + k_{+2} + j_{+1})/2 \quad (20)$$

Since normally $(k_{+1}[S]_0 + k_{-1} + k_{+2}) \gg j_{+1}$, eqns. (19) and (20) yield the realistic assumption:

$$|\lambda_2| \gg |\lambda_1| \quad (21)$$

Insertion of eqn. (21) into eqn. (17) gives:

$$\lambda_2 \approx -F_1 \quad (22)$$

whereas eqns. (18) and (22) predict:

$$\lambda_1 \approx -\frac{F_2}{F_1} \quad (23)$$

Eqn. (21) allows the neglect of the terms containing the factor $e^{\lambda_2 t}$ in eqns. (15) and eqns. (A1)–(A4) of the Appendix. If,

in eqn. (16), we consider the relationship given by eqn. (21), we obtain:

$$\lambda_1 - \lambda_2 \approx -\lambda_2 \quad (24)$$

Then eqn. (15) yields:

$$[P] = \gamma(e^{\lambda_1 t} - e^{-j_{+2} t}) \quad (25)$$

and

$$\lambda_1 \approx -\frac{j_{+1}[S]_0}{[S]_0 + K_m} \quad (26)$$

$$\gamma = \frac{k_{+2}[S]_0[E]_0}{j_{+2}(K_m + [S]_0) - j_{+1}[S]_0} \quad (27)$$

In eqns. (26) and (27), K_m is the Michaelis constant, i.e. $(k_{-1} + k_{+2})/k_{+1}$. The time-course equations for the other species are given in the Appendix.

MATERIALS AND METHODS

Mushroom tyrosinase (2400 units/mg) and dopamine hydrochloride were purchased from Sigma. All other chemicals were of analytical grade and supplied by Merck.

The time course of the reaction was followed by measuring the appearance of dopaminochrome as A_{480} ($\epsilon = 3300 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The reaction media contained dopamine at the indicated concentration, 50 mM sodium phosphate buffer, pH 6.0, and 2.2 $\mu\text{g/ml}$ tyrosinase. Assay temperature was 20 °C to diminish the possible conversion of aminechrome into indole. The reaction was started by the addition of the enzyme.

The simulated progress curves were obtained by the same methods as in a previous contribution (Garrido-del Solo et al., 1993), i.e. by numerical solution of the non-linear set of differential eqns. (1)–(6) using arbitrary sets of rate constants and initial concentrations. The solutions of eqn. (28) were obtained using the Newton–Raphson method (De La Fuente-O'Connor, 1993). The solutions of the set of differential eqns. (1)–(6) were obtained by the Laplace transformation (Darvey, 1977; Jacquez, 1985).

Figure 1 Simulated progress curves obtained for the species S, P, R₁ and R₂ in Scheme 1 and for plots of the eqns. (15) and (A3)–(A4)

The values of the rate constants used in the simulation were those in the legend of Table 1. The initial concentrations used were $[E]_0 = 0.3 \mu\text{M}$ and $[S]_0 = 1 \text{ mM}$.

Figure 2 Curves showing the dependence of $[P]_{\max}$ and $t_{\max}$ upon $[S]_0$ and $[E]_0$ using Scheme 1 and eqns. (28) and (29)

(a) Hyperbolic dependence of $[P]_{\max}$ upon $[S]_0$ at fixed $[E]_0$. (b) Linear dependence of $[P]_{\max}$ upon $[E]_0$ at fixed $[S]_0$. (c) Dependence of $t_{\max}$ upon $[S]_0$ at fixed j_{+2} value. (d) Dependence of $t_{\max}$ upon $[E]_0$ at fixed $[S]_0$ value. The arrow indicates the increase of $[E]_0$, $[S]_0$, j_{+2} and $[S]_0$ in (a), (b), (c) and (d) respectively.

RESULTS AND DISCUSSION

We have obtained the general time-course eqn. 15 and the Appendix eqns. (A1)–(A4) for the species in the reaction described in Scheme 1 under the sole assumption that the initial concentration of the substrate, $[S]_0$, is much higher than that of the initial concentration of the free enzyme, $[E]_0$. In Figure 1, we show, using a chosen set of rate constants, the simulated progress curves of the species S, P, R_1 and R_2 in Scheme 1 for initial conditions which fulfil the condition expressed in eqn. (9). In the same figure, the time-course curves calculated from eqns. (11), (15), (A1) and (A4) are shown. The two sets of curves overlap in the reaction period considered. The progress curve of P has a maximal value, $[P]_{\max}$. The corresponding value of the time, $t_{\max}$, can easily be determined experimentally and is of great value to the analysis of the kinetic data. At high t values, the concentration of P slowly decreases until it vanishes. The progress curves of R_1 and R_2 continuously increase and their formation rates steadily diminish until they reach constant values. The values of $t_{\max}$ and the corresponding $[P]_{\max}$ may be obtained from eqn. (25):

$$t_{\max} = -\frac{1}{\lambda_1 + j_{+2}} \ln \frac{-\lambda_1}{j_{+2}} \quad (28)$$

$$[P]_{\max} = \frac{k_{+2}[E]_0}{j_{+1}} e^{-j_{+2}t_{\max}} \quad (29)$$

When $t = 2t_{\max}$, the curve of P exhibits an inflexion point. The hyperbolic dependence of $[P]_{\max}$ on $[S]_0$ at fixed $[E]_0$ is shown in Figure 2(a), and its linear dependence upon $[E]_0$ at fixed $[S]_0$ is illustrated in Figure 2(b). Figure 2(c) shows the dependence of $t_{\max}$ upon $[S]_0$ at fixed j_{+2} value. At fixed $[S]_0$ values, $t_{\max}$ is independent of $[E]_0$ (see Figure 2(d)).

Particular cases

The particular cases of the Scheme 1 are easily obtained by setting $j_{+1} = 0$ or $j_{+2} = 0$ (see the Schemes 2 and 3 respectively). The corresponding time-course equations may easily be obtained by insertion of j_{+1} or $j_{+2} = 0$ into those of Scheme 1. Scheme 2 represents the case in which the substrate induces instability of the complex ES ($j_{+1} \neq 0$) but stabilizes after formation of a stable product ($j_{+2} = 0$). Scheme 3 corresponds to the case in which only the product is unstable ($j_{+1} = 0$ and $j_{+2} \neq 0$).

Scheme 2

Scheme 3

Scheme 2 has already been dealt with by Garrido-del Solo et al. (1993).

In Scheme 3, if eqn. (9) is fulfilled, the time-course equations of P and R_2 are:

$$[P] = \gamma(1 - e^{-j_{+2}t}) \quad (30)$$

Figure 3 Simulated progress curves for the species P and R_2 in Scheme 3

The values of the initial concentrations and the rate constants used in the simulations were: $[E]_0 = 0.1 \mu\text{M}$, $[S]_0 = 5 \text{ mM}$, $k_{+1} = 5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-1} = 500 \text{ s}^{-1}$, $k_{+2} = 5 \text{ s}^{-1}$, $j_{+1} = 0 \text{ s}^{-1}$ and $j_{+2} = 0.05 \text{ s}^{-1}$.

$$[R_2] = -\gamma + \alpha t + \gamma e^{-j_{+2}t} \quad (31)$$

Where $\alpha = \gamma j_{+2}$ and:

$$\gamma = \frac{k_{+2}[S]_0[E]_0}{j_{+2}([S]_0 + K_m)} \quad (32)$$

Eqn. (32) can also be written as:

$$\frac{1}{\alpha} = \frac{1}{V_{\max.}} + \frac{K_m}{V_{\max.}} \cdot \frac{1}{[S]_0} \quad (33)$$

where $V_{\max.} = k_{+2}[E]_0$.

Figure 3 shows the simulated progress curves of P and R_2 under the condition expressed in eqn. (9). In the steady state ($t \rightarrow \infty$), $[P] \approx \gamma$ if the condition of eqn. (9) is fulfilled. When the concentration of [S] substantially decreases, the condition of eqn. (9) is no longer fulfilled. Then, the concentration of P decreases and the time-course eqns. (15), (30), (31) and (A1)–(A4) are rendered invalid.

Kinetic data analysis

We suggest that the kinetic data analysis reported above allows discrimination of the Schemes 1–3 and the evaluation of all of the kinetically important parameters of the mechanism in Scheme 1, i.e. the inactivation rate constants j_{+1} and j_{+2} , the catalytic rate constant k_{+2} and K_m . It is based upon the time progress curves of the product obtained under the condition expressed in eqn. (9).

Discrimination between Scheme 1 and Scheme 2

The following steps are suggested to obtain experimental progress curves for P at different $[E]_0$ and $[S]_0$ values.

If the progress curves of P show a maximum (see Figure 1) which immediately is followed by a decrease in the concentration of P, then the curve fits the bi-exponential eqn. (25) and Scheme 1 is suitable. If the progress curve of P has no maximum (see Figure 3), then the mechanism is compatible with those in Schemes 2 and 3 and the time-course equation of P has only one exponential term.

According to eqn. (27), a plot of $[E]_0/\gamma$ versus $1/[S]_0$ yields a straight line with the slope $j_{+2}K_m/k_{+2}$ and the ordinate intercept $(j_{+2} - j_{+1})/k_{+2}$. If the straight line obtained has the slope zero, then $j_{+2} = 0$. If the straight line obtained has a slope different from zero, then Scheme 3 is suitable.

Evaluation of k_{+2} , j_{+1} , j_{+2} and K_m

In the case in which the mechanism is compatible with Scheme 1, the kinetic parameters k_{+2} , j_{+1} , j_{+2} and K_m may be evaluated as follows.

According to eqn. (29), a plot of $\ln([P]_{\max.}/[E]_0)$ versus $t_{\max.}$ yields a straight line of the slope $-j_{+2}$ and the ordinate intercept $\ln(k_{+2}/j_{+1})$. For each curve, the corresponding λ_1 value is obtained using eqn. (28). According to eqn. (26), a plot of $1/\lambda_1$ versus $1/[S]_0$ yields a straight line of the slope $-K_m/j_{+1}$ and the ordinate intercept $-1/j_{+1}$. If the mechanism is compatible with Scheme 1, then the k_{+2}/j_{+1} value obtained from the previous plot of $\ln([P]_{\max.}/[E]_0)$ versus $t_{\max.}$ permits the evaluation of the rate constant k_{+2} . If the mechanism is compatible with Scheme 3, then the j_{+2}/k_{+2} and $j_{+2}K_m/k_{+2}$ values obtained from the plot of $[E]_0/\gamma$ versus $1/[S]_0$ permit the evaluation of all of the kinetic parameters.

An alternative procedure which allows the evaluation of the kinetic parameters in Scheme 1 is as follows. The parameter j_{+2} is obtained in an independent assay. The λ_1 value is obtained from each progress curve of P, using the j_{+2} and $t_{\max.}$ values and eqn. (28). The j_{+2} and λ_1 values are used as initial estimates to fit each progress curve of P to eqn. (25), by non-linear regression, to obtain the value of γ . The K_m and j_{+1} values are obtained from the plot of $1/\lambda_1$ versus $1/[S]_0$. According to eqn. (27), the plot of $[E]_0/\gamma$ versus $1/[S]_0$ yields a straight line with the slope $j_{+2}K_m/k_{+2}$ and the ordinate intercept $(j_{+2} - j_{+1})/k_{+2}$. These parameters permit the evaluation of k_{+2} . If the evaluation of the $[R_2]_{\infty}$ value is possible, then k_{+2} can be obtained from eqn. (A9).

The evaluation of the kinetic parameters in Scheme 3 can be carried out according to eqn. (33). A plot of $1/\alpha$ versus $1/[S]_0$ yields a straight line of ordinate intercept $1/V_{\max.}$ and slope $K_m/V_{\max.}$. In the cases in which the species R_2 can be monitored, according to eqn. (31) and at high t values, $[R_2]$ is plotted against

Figure 4 Simulated progress curves of P obtained using the same rate constants as those in Figure 3 but different values of $[E]_0$ and $[S]_0$

The rate constants used as well as the values of $[S]_0$ and $[E]_0$ are shown in Table 1.

Table 1 Values of $[S]_0$, $[E]_0$, $[P]_{\max}$, $t_{\max}$, λ_1 and λ_2/λ_1 for the progress curves of P in Figure 4

The quotients λ_2/λ_1 were calculated from eqns. (10) and (11). The parameters used in the simulations were: $k_{+1} = 5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-1} = 500 \text{ s}^{-1}$, $k_{+2} = 5 \text{ s}^{-1}$, $j_{+1} = 0.07 \text{ s}^{-1}$ and $j_{+2} = 0.03 \text{ s}^{-1}$.

Curve	$[S]_0$ (μM)	$[E]_0$ (μM)	$[P]_{\max}$ (μM)	$t_{\max}$ (s)	$10^2 \times \lambda_1$ (s^{-1})	λ_2/λ_1
1	150	0.08	2.5	28.1	-4.290	30002
2	200	0.10	3.2	26.6	-4.752	32359
3	300	0.15	5.1	24.9	-5.363	38287
4	400	0.20	7.0	24.0	-5.732	44822
5	1000	0.30	11.0	22.4	-6.483	86586

Figure 5 Linear plot for the evaluation of the progress curves of P from the numerical example

(a) Plot of $\ln([P]_{\max}/[E]_0)$ versus $t_{\max}$. The straight line has the slope $-j_{+2}$ and the ordinate intercept $\ln(k_{+2}/j_{+1})$. (b) Plot of $1/\lambda_1$ versus $1/[S]_0$. The straight line obtained has the slope $-K_m/j_{+1}$ and the ordinate intercept $-1/j_{+1}$.

t to yield a straight line of slope α and ordinate intercept $-\gamma$. The point in which this straight line intercepts the abscissa axis corresponds to the $1/j_{+2}$ value.

Numerical example

We illustrate the procedure for the evaluation of the rate

Figure 6 Experimental data corresponding to the oxidation of dopamine catalysed by tyrosinase

(a) Progress curves of dopaminochrome accumulation. (b) Plot of $1/\alpha$ versus $1/[S]_0$ corresponding to curves 1–6; the points fitted by linear regression yields a straight line of the slope $K_m/V_{\max}$ and the ordinate intercept $1/V_{\max}$.

constants and K_m by simulating some curves for Scheme 1 using the same set of rate constants, but different values of $[E]_0$ and $[S]_0$. The condition given in eqn. (9) must be satisfied for each curve. Simulated experimental errors with an S.D. of 1% were incorporated into the data as described in the Materials and methods section. These simulated curves are here considered as equivalent to experimental curves. They are shown in Figure 4. In Table 1, the $[E]_0$ and $[S]_0$ values used for each curve are listed.

In Figure 4, the progress curves of P show a maximum which immediately is followed by a decrease in the concentration of P. Hence, the mechanism is compatible with Scheme 1. The values of $[P]_{\max}$ and $t_{\max}$ obtained from the progress curve 1 of P are $2.5 \mu\text{M}$ and 28.1 s respectively.

A plot of $\ln([P]_{\max}/[E]_0)$ versus $t_{\max}$ yields a straight line of the slope $(-j_{+2}) - 0.0292 \text{ s}^{-1}$ and an ordinate intercept $\ln(k_{+2}/j_{+1})$ of 4.253 (see Figure 5a). From eqn. (28), we obtained the value $\lambda_1 = -4.29 \times 10^{-2} \text{ s}^{-1}$. The values obtained for the other simulated curves in Figure 4 are listed in Table 1.

The plot of $1/\lambda_1$ versus $1/[S]_0$ yielded a straight line of the ordinate intercept $(-1/j_{+1}) - 14 \text{ s}$ and the slope $(-K_m/j_{+1}) - 1.4 \times 10^{-3} \text{ M} \cdot \text{s}$ (see Fig. 5b).

The values obtained for j_{+1} , j_{+2} , k_{+2} and K_m are $(7.14 \pm 0.02) \times 10^{-2} \text{ s}^{-1}$, $(2.9 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$, $5.0 \pm 0.3 \text{ s}^{-1}$ and $(1.01 \pm 0.01) \times 10^{-4} \text{ M}$ respectively.

Table 2 Values of $[S]_0$ corresponding to the progress curves of R_2 in Figure 6(a) as well as α and j_{+2} values obtained by fitting of the experimental $[R_2]$ values at high t values to the equation $[R_2] = -\gamma + \alpha t$

Curve	$[S]_0$ (M)	$10^7 \times \alpha$ (M·s ⁻¹)	$10^2 \times j_{+2}$ (s ⁻¹)
1	1.667×10^{-4}	1.317	5.69
2	3.333×10^{-4}	2.370	4.83
3	6.667×10^{-4}	3.804	5.69
4	1.000×10^{-3}	4.629	4.60
5	1.667×10^{-3}	5.461	4.83
6	2.000×10^{-3}	5.753	4.60

Experimental example

We have performed some experiments on the oxidation of dopamine by the enzyme tyrosinase yielding *o*-dopamine-quinone as an experimental example of the system described here (Jiménez et al., 1984). The *o*-quinone product is unstable and evolves non-enzymically along two branches consisting of cyclization or hydroxylation reactions. The hydroxylation branch only operates significantly at pH < 6, whereas the cyclization branch is important over the entire pH range. It is the only significant branch at pH ≥ 6 (García-Moreno et al., 1991). This route leads to the formation of dopaminechrome by the cyclization of the *o*-quinone following an intramolecular 1,4-Michael addition. Moreover, dopamine has been described as a suicide substrate of tyrosinase (Tudela et al., 1987b), although this effect may be considered as negligible in the range time of several minutes.

Figure 6(a) shows the progress curves obtained when the appearance of *o*-dopaminechrome, which is equivalent to R_2 in Scheme 3, is measured. In Table 2, the values of $[S]_0$ corresponding to the progress curves of R_2 in Figure 6(a), as well as the α and j_{+2} values obtained by linear fitting of the $[R_2]$ values at high t values to the equation $[R_2] = -\gamma + \alpha t$ are shown. The plot of $1/\alpha$ versus $1/[S]_0$ yields a straight line of the slope 1.07×10^3 s and the abscissa intercept 1.12×10^6 M⁻¹·s (Figure 6b). The values obtained for j_{+2} , $V_{\max}$, and K_m are $(5.0 \pm 0.5) \times 10^{-2}$ s⁻¹, $(8.93 \pm 0.46) \times 10^{-7}$ M·s⁻¹ and $(9.56 \pm 0.67) \times 10^{-4}$ M respectively. It should be noted that j_{+2} , in this case, must be interpreted as the apparent rate constant of the process (Jiménez et al., 1984).

APPENDIX

Time-course equations for the enzyme species in Scheme 1

$$[E] = \sum_{h=1}^2 \mu_h e^{\lambda_h t} \quad (A1)$$

$$[ES] = \sigma(e^{\lambda_1 t} - e^{\lambda_2 t}) \quad (A2)$$

$$[R_1] = [E]_0 + \sum_{h=1}^2 a_h e^{\lambda_h t} \quad (A3)$$

$$[R_2] = [R_2]_\infty + \sum_{h=1}^3 r_h e^{\lambda_h t} \quad (A4)$$

where λ_1 , λ_2 and λ_3 are given by eqns. (10)–(12). Moreover,

$$\mu_h = \frac{(\lambda_h + k_{-1} + k_{+2} + j_{+1})[E]_0}{(\lambda_h - \lambda_q)} \quad (h, q = 1, 2; q \neq h) \quad (A5)$$

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$$\sigma = \frac{k_{+1}[S]_0[E]_0}{\lambda_1 - \lambda_2} \quad (A6)$$

$$a_h = \frac{k_{+1}j_{+1}[S]_0[E]_0}{\lambda_h(\lambda_h - \lambda_q)} \quad (h, q = 1, 2; q \neq h) \quad (A7)$$

$$r_h = \frac{k_{+1}k_{+2}j_{+2}[S]_0[E]_0}{\lambda_h \prod_{\substack{q=1 \\ q \neq h}}^3 (\lambda_h - \lambda_q)} \quad (h = 1, 2, 3) \quad (A8)$$

$$[R_2]_\infty = \frac{k_{+2}[E]_0}{j_{+1}} \quad (A9)$$