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The kinetics of substrate-induced inactivation

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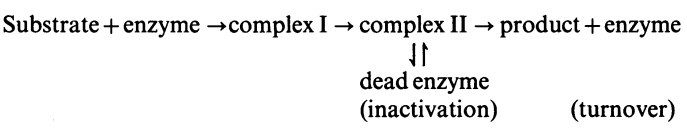
The kinetics of a branched-pathway mechanism for a simple enzymic reaction were studied. In this mechanism there is reversible formation of an inactive form of the second complex along the pathway. This substrate-induced inactivation typically results in the progress curve showing a burst. Three parameters can be obtained from the progress curve: the initial rate, the final rate and the rate constant characterizing the transient. The rate constant for the conversion of the inactive form of the complex into the active form can be obtained either from these parameters or by measuring the regain of enzymic activity. The partition ratio can also be obtained from the three parameters; this is the ratio of the rate of conversion of complex into product to the rate of conversion of complex into inactive form. Simulations give guidance to the conditions required for accurate determinations of the rate constants.

INTRODUCTION

In some enzyme-catalysed reactions a substrate brings about inactivation of the enzyme: there is inactivation during turnover. The inactivation may be effectively irreversible; the kinetics of the action of such mechanism-based inhibitors (or suicide substrates) has already been described (Waley, 1980, 1985; Tatsunami *et al.*, 1981; Tudela *et al.*, 1987; Burke *et al.*, 1990). When the inactivation is reversible, however, the kinetics are quite different. After the transient phase is over, normal Michaelis–Menten kinetics are evinced. Experimentally, progress curves typically show a burst, i.e. a rapid exponential phase preceding a linear phase (Fig. 1). The concentration of product formed in the rapid exponential phase is greater (typically, much greater) than the concentration of enzyme. This signals the likelihood of a branched pathway. Even when a burst is not detected a branched pathway may be inferred by comparing the rates of re-activation and turnover, as discussed below. The sole use of initial rates would miss the signs of the operation of this mechanism.

The kinetics of the branched-pathway mechanism:



have not hitherto been elucidated in a practically useful form, although the mechanism has been put forward on several occasions. Thus this kinetic scheme in which a second intermediate (here an acyl-enzyme) is reversibly converted into a dead-end complex has been postulated for different β-lactamases (Fisher *et al.*, 1978; Kiener *et al.*, 1980; Frère *et al.*, 1982*a*,*b*; Fink *et al.*, 1987; Persaud *et al.*, 1986), and the burst has been clearly demonstrated (Citri *et al.*, 1976). Related mechanisms have been put forward to account for the kinetics of sulphatase A (O'Fagain *et al.*, 1982; Roy & Mantle, 1989).

Unless simplifying assumptions are made, the equations are so complex and the rate constants so numerous that there is a wide gap between theory and practice. With the advent of site-directed mutagenesis of enzymes that evince substrate-induced inactivation it has become especially important to obtain values for rate constants so that the consequences of the mutagenesis can be described quantitatively. The present paper shows how the introduction of two assumptions enables us to obtain analytical solutions that are simple enough to be useful.

These two assumptions are (*a*) that the change in the concentration of substrate may be neglected and (*b*) that the steady-state approximation can be applied to the two 'ordinary' intermediates, i.e. those on the unbranched pathway. The range of validity of the first assumption is examined later below; the basis of the second assumption is that the burst is due to the branch, and that when the data fit a curve with only one exponential the time variation of only one species needs to be considered.

The reaction is written as above with the branch at the second intermediate (complex II) partly because this is the counterpart to the scheme considered earlier (Waley, 1980), and partly because this seems to be more commonly put forward; the alternatives are, however, discussed briefly at the end.

I start by showing how the kinetic mechanism leads to an equation for the progress curve. Then the problem of evaluating rate constants is considered, and simulations are used to see how well certain rate constants can be determined.

SIGNIFICANCE OF PARAMETERS

The burst equation

When the velocity v at time t changes exponentially from an initial value v_i to a steady-state value v_s then we may write:

$$v = v_s - (v_s - v_i)\cdot e^{-kt}$$

where k is the rate constant characterizing the change, here referred to as the burst rate constant. It is clear that when $t = 0$ then $v = v_i$, and when t is much larger than $1/k$ then $v = v_s$. This equation, which was put forward by Frieden (1970) in connection with the hysteretic enzyme concept, may also be written as:

$$v = v_s(1 - e^{-kt}) + v_i\cdot e^{-kt}$$

which makes it clear that the velocity is being regarded as a weighted mean of v_s and v_i, the weights being $1 - e^{-kt}$ and e^{-kt}.

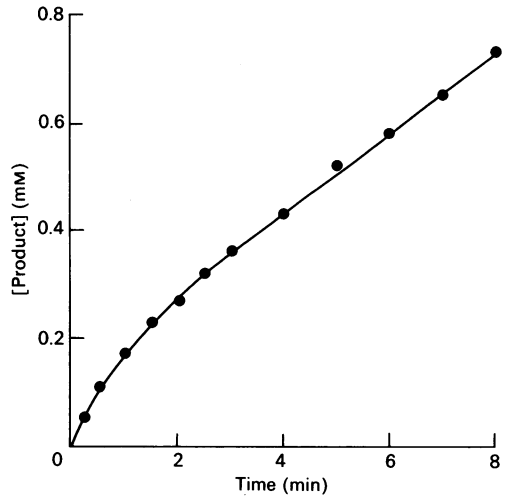
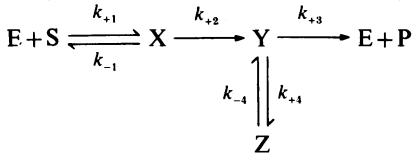


Fig. 1. Progress curve showing burst

The hydrolysis of 5 mM-methicillin by 15 nM-β-lactamase I in 0.5 M-NaCl/1 mM-EDTA at 30 °C was measured in a pH-stat by proton production. The experimental points were fitted to eqn. (1) in the text by the Marquardt–Nash procedure, giving $v_i = 0.25$ mM/min, $v_s = 0.075$ mM/min and $k = 1.28$ min^{-1}.



Scheme 1.

Integration then gives eqn. (1) (here called the burst equation) for the progress curve:

$$p = v_s t - (v_s - v_i)(1 - e^{-kt})/k \qquad (1)$$

where the concentration (p) of product at time t is given as a function of v_i, v_s and k. The progress curve (e.g. Fig. 1) then consists of an initial exponential phase followed by a steady-state phase, which is linear until substrate depletion intervenes. Fitting progress curves (see, e.g., Neet & Ainslie, 1980) then provides values for v_i, v_s and k at a given initial concentration (s) of substrate. Our task now is to show how the kinetic mechanism of Scheme 1 can lead to progress curves obeying eqn. (1). Moreover, we wish to see which of the rate constants in Scheme 1 can be determined experimentally from progress curves.

Parameters of the burst equation

Table 1 shows the expressions for the parameters in terms of the rate constants for the mechanism in Scheme 1. There are five rows in Table 1, corresponding to five measurable entities. The first two rows give the expressions for the initial and steady-state rates, v_i and v_s of eqn. (1). The next row gives the expression for the burst rate constant, k of eqn. (1). Then the fourth row gives the expression for the burst; this may be expressed in the same units of concentration as that of the enzyme; it is characteristic of these reactions that the burst is much greater than the concentration (e_0) of enzyme. Finally, there are instances (referred to later below) where it is useful to measure the extent of inactivation of the enzyme, and this is given in the last row of Table 1.

Table 1. Significance of parameters for branched pathway

Initial rate	$\dfrac{k_{+2}k_{+3}e_0 s}{s(k_{+2}+k_{+3}+k_{+4})+K(k_{+3}+k_{+4})}$
Final rate	$\dfrac{k_{+2}k_{+3}k_{-4}e_0 s}{s[k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}]+k_{+3}k_{-4}K}$
Burst rate constant	$\dfrac{s[k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}]+k_{+3}k_{-4}K}{s(k_{+2}+k_{+3}+k_{+4})+K(k_{+3}+k_{+4})}$
Burst	$\dfrac{k_{+2}k_{+3}k_{+4}e_0 s[s(k_{+2}-k_{-4})-k_{-4}K]}{\{s[k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}]+k_{+3}k_{-4}K\}^2}$
Fraction of enzyme activated	$\dfrac{k_{+2}k_{+4}s}{s[k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}]+k_{+3}k_{-4}K}$

Table 2. Michaelis–Menten parameters for the initial and steady-state phases

The expressions for the rates are given in Table 1; K is defined as $(k_{-1}+k_{+2})/k_{+1}$.

Initial phase

$$k_{cat.} = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}+k_{+4}}$$

$$K_m = \frac{K(k_{+3}+k_{+4})}{k_{+2}+k_{+3}+k_{+4}}$$

$$\frac{k_{cat.}}{K_m} = \frac{k_{+2}}{K(1+k_{+4}/k_{+3})}$$

Steady-state phase

$$k_{cat.} = \frac{k_{+2}k_{+3}k_{-4}}{k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}}$$

$$K_m = \frac{Kk_{+3}k_{-4}}{k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}}$$

$$\frac{k_{cat.}}{K_m} = \frac{k_{+2}}{K}$$

Initial and steady-state rates

The initial and steady-state rates are hyperbolic functions of the initial substrate concentration s. This distinguishes the present mechanism from the more general hysteretic mechanisms where the rate constant is a 2:2 function of s (i.e. a function containing terms in s and s^2 in the numerator and denominator) (Ainslie *et al.*, 1972; Cheron *et al.*, 1990).

The variation of the initial and steady-state rates with initial concentration of substrate gives the expressions for the Michaelis parameters in Table 2. The extent of the difference between the initial and steady-state phases depends on the initial concentration of substrate. At low concentrations the relevant parameter is $k_{cat.}/K_m$, and this is decreased by a factor of $1/(1+k_{+4}/k_{+3})$. When $k_{+3} \gg k_{+4}$, as is expected for these reactions [see (*b*) below], then this factor will be approximately 1. In other words, if the initial concentration of substrate is too low, then the initial and steady-state phases will have similar rates and a burst will not be observed.

At high concentrations of substrate the relevant parameter is

$k_{cat.}$, and the equations are simpler when it is the reciprocals of $k_{cat.}$ for the initial and steady-state phases that are compared:

$$\left(\frac{1}{k_{cat.}}\right)_s - \left(\frac{1}{k_{cat.}}\right)_i = \frac{k_{+4}}{k_{+3}}\left(\frac{1}{k_{-4}} - \frac{1}{k_{+2}}\right)$$

This equation shows that the difference between the initial and steady-state phases will be large when $k_{-4}/k_{+2} \ll k_{+4}/k_{+3}$; this inequality is mentioned again below in (*c*).

I have compared the initial and steady-state phases, first at low and then at high concentrations of substrate. The general expression is:

$$\frac{v_i}{v_s} = \frac{s[k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}]+k_{+3}k_{-4}K}{s[k_{-4}(k_{+2}+k_{+3})+k_{-4}k_{+4}]+k_{+3}k_{-4}K+k_{+4}k_{-4}K}$$

A well-marked burst requires that $v_i \gg v_s$, and so this expression is now used to see the conditions required.

The three conditions:

(*a*) $k_{+2} \gg k_{-4}$

(*b*) $k_{+3} \gg k_{+4}$

(*c*) $(k_{+4}/k_{-4}) \gg (k_{+3}/k_{+2})$

are clearly sufficient for $v_i \gg v_s$, because (*b*) implies that the last term in the denominator is much smaller than the preceding term; when this last term is dropped, the only difference between the numerator and the denominator is that k_{+2} in the former is replaced by k_{-4} in the latter; (*c*) entails this differing term not being negligible. It is also clear that the limiting case, as *s* tends to zero, is that, when (*b*) holds, v_i/v_s tends to 1. This emphasizes the point that the substrate concentration should not be too low, as was noted above. The three conditions (*a*) to (*c*) are now used to give approximate forms of the equations for several of the parameters.

Burst rate constant

The rate constant characterizing the transient burst is given in the third row of Table 1; the value changes with the initial substrate concentration *s* from a limiting low value of:

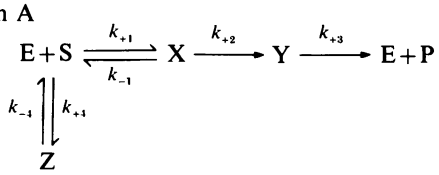
$$\frac{k_{+3}k_{-4}}{k_{+3}+k_{+4}} \simeq k_{-4}$$

to a limiting high value:

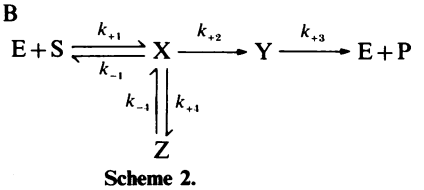
$$\frac{k_{+2}k_{+4}+k_{-4}(k_{+2}+k_{+3})}{k_{+2}+k_{+3}+k_{+4}} \simeq \frac{k_{+2}k_{+4}}{k_{+2}+k_{+3}}$$

The increase of the burst rate constant with *s* distinguishes this mechanism from mechanism A in Scheme 2, in which it is the free enzyme that is converted into an alternative form. In this mechanism the burst rate constant decreases with increasing *s* (see below).

Mechanism A



Mechanism B



Scheme 2.

Size of the burst

The size of the burst is given in the fourth row of Table 1. The value of the burst increases with *s*. An approximate value is obtained when, as well as the three conditions (*a*) to (*c*) above, the concentration of substrate is large enough, as judged by the conditions: $s \gg K \cdot k_{-4}/k_{+2}$ and $s \gg K \cdot (k_{-4}/k_{+2})(k_{+3}/k_{+4})$. This approximate value for the burst is $(k_{+3}/k_{+4})e_0$. Condition (*b*) above may thus be correlated with the burst being much larger than the concentration of enzyme. The partition ratio, k_{+3}/k_{+4}, measures the relative tendency of the second intermediate to form the dead-end complex Z.

Inactivation of the enzyme

Hitherto the information from progress curves has been discussed. This may be supplemented by the information obtained by withdrawing samples from the reaction mixture of enzyme and inactivating substrate and diluting many-fold into assay mixture containing non-inactivating substrate. The fraction of enzyme that is present as dead-end complex Z when the equilibrium concentration has been attained is given in Table 1; this value increases with *s* to a plateau:

$$\frac{k_{+2}k_{+4}}{k_{-4}(k_{+2}+k_{+3})+k_{+2}k_{+4}}$$

The approximate value of this fraction is 1. The maximum extent of inactivation is only attained after virtually all the substrate has been consumed, and so the present analytical treatment does not apply. Curves relating the maximum extent of inactivation to the concentration of substrate have to be interpreted on the basis of numerical solutions of the differential equations [e.g. KINSIM (Barshop *et al.*, 1983) or the methods indicated in the Appendix]. The initial rate of inactivation, however, will be first-order, and the rate constant will be the same as that characterizing the transient. These experimental features were noted by Charnas & Knowles (1981).

The five parameters given in Table 1 have now been discussed. Some further consideration of the relative magnitudes of the rate constants is now given, before turning to see which can be evaluated experimentally.

Relative magnitudes of rate constants

The reciprocal forms of $k_{cat.}$ from Table 2 for the initial phase:

$$1/k_{cat.} = 1/k_{+2} + 1/k_{+3} + k_{+4}/(k_{+2}k_{+3})$$

and for the steady-state phase:

$$1/k_{cat.} = 1/k_{+2} + 1/k_{+3} + k_{+4}/(k_{+3}k_{-4})$$

show that both k_{+2} and k_{+3} must be greater than $k_{cat.}$, and, since $k_{cat.} \geqslant v_i/e_0$, where e_0 is the concentration of enzyme, lower limits for k_{+2} and k_{+3} are readily obtained from the measured v_i. Experimentally, k_{-4} is determined as described below. Then the quotient $k_{-4}/k_{+2} \ll 1$, a reflection of the differing time scales for the 'horizontal' and 'vertical' parts of Scheme 1. In the steady state, the branch step $Y \rightleftharpoons Z$ has attained equilibrium, and, for the burst to be significant, $k_{+4} > k_{-4}$.

The evaluation of rate constants, individually or as quotients, is now considered.

EVALUATION OF RATE CONSTANTS

The rate constant k_{-4}

The rate constant k_{-4} is the most readily evaluated; from fitting progress curves to eqn. (1) v_i, v_s and k are obtained, and it may be shown that $k_{-4} = (v_s/v_i) \cdot k$. When v_s is very low it is

hard to measure precisely, and then it may be better to use a separate method for k_{-4}. The regain of activity after most (or all) of the enzyme has been converted into the dead-end complex Z is a more sensitive method for determining k_{-4} because the enzyme rather than the substrate is being monitored. The rate constant for regain of enzymic activity can be found from the limiting value of the burst rate constant (Table 1) when s is very small, since the inactivating substrate is being highly diluted in the assay mixture; this limiting value is:

$$\frac{k_{+3}k_{-4}}{k_{+3}+k_{+4}}$$

In most cases $k_{+3} \gg k_{+4}$, as discussed below, and so the limiting value becomes k_{-4}. In practice, a small portion of the solution containing inactivated enzyme is diluted into an assay mixture and the progress curve is recorded. The lag can conveniently be used as described previously (Monks & Waley, 1988); it is essential to use a high enough concentration of substrate for there to be no significant depletion of substrate in the assay during the time of the lag, as emphasized by Fink *et al.* (1987).

Comparison with suicide substrate kinetics

The rate constant k_{-4} has significance in the following context. If k_{-4} were zero, the mechanism would become that associated with suicide substrates (mechanism-based inhibitors); the most easily observed practical distinction is that linear progress curves are not observed if k_{-4} is zero, because the concentration of active enzyme is declining throughout the reaction. A convenient criterion for distinguishing k_{-4} from zero is the magnitude of $1/k_{-4}$ compared with the time of the experiment. For instance, if $1/k_{-4}$ is, say, 100 times the time of the experiment then k_{-4} may be regarded as zero. On the other hand, if $1/k_{-4}$ is of the same order of magnitude as the time of the experiment then the present treatment holds until the concentration of substrate declines too much; subsequently, regain of enzyme activity may be observed. As an example, when k_{-4} was about 0.01 min^{-1} and thus $1/k_{-4}$ was 100 min regain of enzyme activity (as measured in an assay with non-inactivating substrate) was observed in an experiment carried out over about 2 h (Monks & Waley, 1988).

The partition ratio k_{+3}/k_{+4}

The values of v_i and v_s can be utilized to determine the quotient k_{+3}/k_{+4} as follows. The expression:

$$e_0\left(\frac{1}{v_s}-\frac{1}{v_i}\right)=\frac{k_{+4}(1-k_{-4}K/k_{+2}s)}{k_{+3}k_{-4}} \simeq \frac{k_{+4}}{k_{+3}k_{-4}}$$

Since k_{-4} has been determined, the k_{+3}/k_{+4} ratio can be found.

The quotient k_{+2}/K

The variation of v_s with substrate concentration gives k_{+2}/K; the individual values can only be determined if the quotient k_{+2}/k_{+3} can be evaluated.

Limits for values of rate constants

From the lower limit for k_{+3} and the value of k_{+3}/k_{+4} a lower limit for k_{+4} can be found. The absolute values of k_{+2}, k_{+3} and k_{+4} cannot be determined from steady-state measurements. If, however, measurements of the fraction of enzyme present as Y (Scheme 1) can be made on a time scale short compared with $1/k_{+4}$ then the quotient k_{+2}/k_{+3} can be found, and this enables all the rate constants to be determined. Thus knowledge of k_{+3}/k_{+4} and the $k_{cat.}$ for the steady-state phase and k_{+2}/k_{+3} suffices to obtain k_{+2}, k_{+3} and k_{+4}, and hence K from k_{+2}/K. Some (but less) information can be obtained if k_{+2} and k_{+3} are very different in magnitude. If $k_{+2} \gg k_{+3}$ and k_{+4}, then k_{+3} is approximately equal to $k_{cat.}$ for the initial phase, and so k_{+3} and hence k_{+4} could be found. Conversely, if there were evidence that $k_{+3} \gg k_{+2}$ and k_{+4}, then k_{+2} could similarly be found, and hence K.

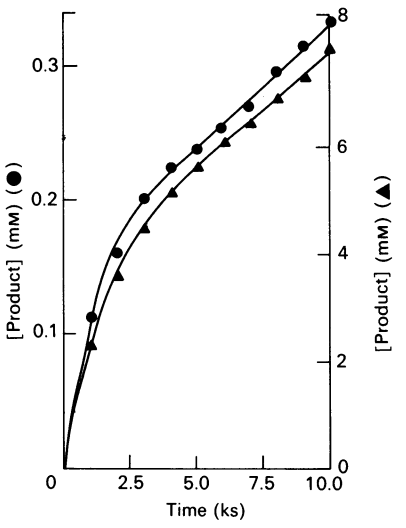


Fig. 2. Simulated progress curves for branched pathway

The points were obtained by numerical solution of the differential equations, with errors added, and the curves were fits to the progress-curve equation. Upper curve (●): [enzyme] = 8 nM, [substrate] = 1 mM, K = 50 mM, k_{+2} = 1250 s^{-1}, k_{+3} = 250 s^{-1}, k_{+4} = 0.01 s^{-1} and k_{-4} = 0.0001 s^{-1}, noise with standard deviation 0.004 added; lower curve (▲): [enzyme] = 40 nM, [substrate] = 25 mM, K = 50 mM, k_{+2} = 250 s^{-1}, k_{+3} = 1250 s^{-1}, k_{+4} = 0.01 s^{-1}, k_{-4} = 0.0001 s^{-1}, noise with standard deviation 0.05 added.

The significance of k_{+2}/k_{+3}

A burst had been thought to imply that $k_{+2} \gg k_{+3}$, but this is not so; a burst is observed even when $k_{+2} \ll k_{+3}$ (Fig. 2). The quotient k_{+2}/k_{+3}, however, markedly affects the concentration of substrate required to observe the burst.

Testing the validity of the assumptions

The assumption that the steady-state approximation may be applied to Y (Scheme 1) is tested by the adherence to first-order kinetics during the transient. An important practical point is that (as in all cases where progress curves are utilized) product inhibition should be tested for; product inhibition, when $K_i \ll K_m$, gives apparent bursts, although the later portion is not accurately linear. The assumption that the concentration of substrate has not changed significantly during the transient should first be tested by looking for a trend in the residuals when the progress curves are fitted to eqn. (1). The calculated concentration of product may be too high if substrate depletion is significant. However, this test is necessary, but not sufficient. A satisfactory fit of an experimental progress curve turns out to be inadequate as a guarantee of reliable values of the parameters. Some guidelines are now given; it is assumed that the final (steady-state) parameters have been measured.

NUMERICAL SIMULATIONS

Accuracy of values of the parameters

Simulations (carried out as described in the Appendix) showed that the effects of depletion of substrate varied. When the initial concentration of substrate was 10 times the final (steady-state) K_m, then as long as the depletion of substrate at the end of the burst was less than approx. 25% the theoretical treatment given

above held adequately (Fig. 3). The points, obtained by numerical solution of the differential equations, lie on the curve obtained by the use of eqn. (1). Moreover, when these points are used in a non-linear regression (curve-fitting) program to find the parameters v_i, v_s and k by the use of eqn. (1), the values are reasonably close (within 10%) to those calculated, and the inferred values of k_{-4} and k_{+3}/k_{+4} are within 2% [Table 3, series (*a*)]. When the initial concentration of substrate was only 2.5 times the final K_m, however, the procedure was less tolerant; at 10% depletion of substrate, k_{-4} was about 5% low and k_{+3}/k_{+4} was about 20% low [Table 3, series (*b*)]. The last row in Table 3 gave an excellent fit to the progress curve (Fig. 3), but only poor values of the parameters (both k_{-4} and k_{+3}/k_{+4} about 30% low); this illustrates the point that a satisfactory fit does not preclude unsatisfactory values of the parameters.

These tests show the extent of systematic errors, arising from the neglect of substrate depletion, on noise-free data. The effects of added errors were greater for the lower concentration of substrate. When random errors, with zero mean and standard deviation 0.004 (a reasonable value for accurate spectrophotometric data) were introduced into a simulated experiment in which the absorbance change was 0.05 to 0.3) [5 nM enzyme, Table 4], the standard deviations of the values for k_{-4} and k_{+3}/k_{+4} were about 35% and 20%. The errors were considerably lower for the higher concentration of substrate [Table 4, series (*a*), top two rows]. Thus these simulations, with added noise, also serve to show that experiments at a substrate concentration considerably greater than (the final) K_m are to be preferred.

The same conclusion may be drawn from the simulations shown in Table 4, series (*b*), where $k_{+2} \gg k_{+3}$, and in Table 4,

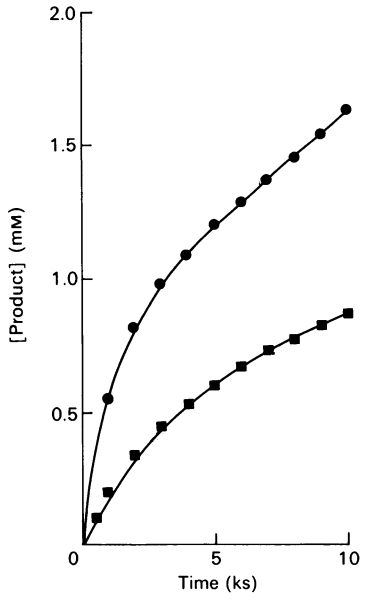


Fig. 3. Effects of substrate depletion on simulated and fitted progress curves

The points were obtained by numerical solution of the differential equations and the curves were fits to the progress-curve equation. Upper curve (●): [enzyme] = 20 nM, [substrate] = 5 mM, K = 50 mM, k_{+2} = 500 s^{-1}, k_{+3} = 500 s^{-1}, k_{+4} = 0.01 s^{-1} and k_{-4} = 0.0001 s^{-1}; lower curve (■): as upper curve, except that [substrate] = 1.25 mM.

Table 4. Parameters from fitted progress curves with errors added

The rate constants were as in Table 3 except that in series (*b*) k_{+2} = 1250 s^{-1} and k_{+3} = 250 s^{-1}, and in series (*c*) k_{+2} = 250 s^{-1} and k_{+3} = 1250 s^{-1}; the simulated progress curves, obtained by numerical solution of the differential equations, were then modified by adding random errors from a normal distribution with zero mean to the concentration of product. The fitting of eqn. (1) was then carried out by the Nash–Marquardt algorithm (Nash & Walker-Smith, 1987) for ten curves. The dispersion in the parameters is the standard deviation derived from these ten replicates.

[Enzyme] (nM)	[Substrate] (mM)	Added standard deviation	$10^5 \times k_{-4}$ (s^{-1})	$10^{-4} \times k_{+3}/k_{+4}$
Series (*a*)				
20	5	0.004	9.84±0.18	4.88±0.04
20	5	0.02	9.88±0.70	4.83±0.15
5	1.25	0.004	11.8±3.45	4.45±1.0
	Theoretical values		10.0	5.0
Series (*b*)				
8	1	0.004	9.91±1.0	2.45±1.3
	Theoretical values		10.0	2.5
Series (*c*)				
40	25	0.05	10.1±0.5	12.1±0.25
	Theoretical values		10.0	12.5

Table 3. Results of fitting simulated progress curves

The concentration of substrate was 5 mM in series (*a*) and 1.25 mM in series (*b*); K = 50 mM, k_{+2} = 500 s^{-1}, k_{+3} = 500 s^{-1}, k_{+4} = 0.01 s^{-1} and k_{-4} = 0.0001 s^{-1}. The values for depletion of substrate (second column), which are approximate, refer to a time of $5/k$ s, i.e. after the exponential term has become small. The values for the initial rate, the final rate and the transient rate constant were obtained by fitting eqn. (1) to the simulated progress curves, and k_{-4} and k_{+3}/k_{+4} were derived from the initial and final rates and the transient rate constant as described in the text.

[Enzyme] (nM)	Depletion of substrate (%)	$10^4 \times v_i$ (mM·s^{-1})	$10^5 \times v_s$ (mM·s^{-1})	$10^5 \times k$ (s^{-1})	$10^5 \times k_{-4}$ (s^{-1})	$10^{-4} \times k_{+3}/k_{+4}$
Series (*a*)						
2	2.5	0.829	0.890	9.30	9.98	4.99
5	6	2.05	2.21	9.23	9.95	4.98
10	12	4.06	4.38	9.21	9.94	4.93
20	24	7.92	8.54	9.10	9.81	4.88
Series (*b*)						
5	6	0.586	1.60	3.54	9.67	4.55
10	10	1.165	2.91	3.80	9.49	4.09
20	18	2.26	4.00	3.88	6.87	3.54

series (*c*), where $k_{+3} \gg k_{+2}$; in both series the initial substrate concentration was 10 times the (final) K_m and the parameters k_{-4} and k_{+3}/k_{+4} were obtained satisfactorily, except that the standard deviation of the latter in series (*b*) was high.

CONCLUSIONS

Detection of branched-pathway mechanism from progress curves

The initial and steady-state rates have to be sufficiently different to detect a burst. Conditions for the initial rate being much larger than the final rate have been given above.

A second requirement for the observation of a burst concerns k_{-4}, in particular the value of $1/k_{-4}$ relative to the time of the experiment; this was discussed above. The enzyme concentration is another factor, and it may be shown that the ratio of the enzyme concentration to the substrate concentration must not be too different from $k_{-4}/k_{cat.}$, so that the time scales for the transient and the steady state are not too different. At times much longer than $1/k$ normal steady-state kinetics are observed (see, e.g., Kiener *et al.*, 1980); the time range can be extended if stopped-flow measurements are made, or if low temperatures are used. This point (see, e.g., Crompton *et al.*, 1988) also applies to the kinetics of slow-binding inhibition (Morrison & Walsh, 1988), which are somewhat similar to the kinetics considered here.

Detection of branched-pathway mechanism from regain of activity

The branched-pathway mechanism may operate even when no burst is observed, perhaps owing to failure of one or more of the conditions just mentioned. When the rate constant for the regain of enzymic activity is less than $k_{cat.}$ then a branched pathway is indicated. This is a useful and widely applicable criterion. Finally, when the kinetics give an unexpectedly low $k_{cat.}$ and K_m, perhaps with a high $k_{cat.}/K_m$ ratio (as with some proteinase inhibitors), then the possibility of a branched pathway should be kept in mind.

Progress curves with two exponentials

Progress curves that show two exponentials can be accounted for by a more complex treatment in which dy/dt (see the Appendix) is not put equal to zero. This can be shown to lead to:

$$p = k'_{+2}k_{+3}e_0\left(\frac{k_{-4}}{a_1a_2}+\frac{a_1-k_{-4}}{a_1^2(a_2-a_1)}(1-e^{-a_1t})+\frac{a_2-k_{-4}}{a_2^2(a_1-a_2)}(1-e^{-a_2t})\right)$$

where k'_{+2} is $k_{+2}(1+K/s)$, and:

$$a_1a_2 = k'_{+2}(k_{+4}+k_{-4})+k_{-4}(k_{+3}+k_{+4})-k_{+3}k_{+4}$$
$$a_1+a_2 = k'_{+2}+k_{+3}+k_{+4}+k_{-4}$$

In general this expression seems too cumbersome to be useful.

Comparison of schemes

The three branched pathways for a kinetic mechanism in which there are two intermediates (e.g. a non-covalent enzyme–substrate complex and a covalent acyl-enzyme) differ according to whether the branch is at the free enzyme (Scheme 2, mechanism A) or at the first intermediate (Scheme 2, mechanism B) or at the second intermediate (Scheme 1). Hitherto it is the last that has been discussed. The kinetics of the other two schemes have also been elucidated, and the expressions for the initial rate, the final rate, the transient rate constant and the burst are given in Table 5. Expressions for $k_{cat.}$ and K_m, and approximate expressions, may readily be calculated from the expressions in Table 5. In practice, it would be hard to distinguish by kinetics

Table 5. Expressions for branched-pathway mechanisms A and B

Mechanisms A and B of Scheme 2 have parameters with the significance shown.

Mechanism A

v_i: $\dfrac{k_{+2}k_{+3}e_0s}{s(k_{+2}+k_{+3})+k_{+3}K}$

v_s: $\dfrac{k_{+2}k_{+3}k_{-4}e_0s}{k_{-4}s(k_{+2}+k_{+3})+k_{+3}K(k_{+3}+k_{+4})}$

Rate constant: $\dfrac{k_{-4}s(k_{+2}+k_{+3})+k_{+3}K(k_{+4}+k_{-4})}{s(k_{+2}+k_{+3})+k_{+3}K}$

Burst: $\dfrac{k_{+2}k_{+3}^2k_{+4}Ke_0s}{[k_{-4}s(k_{+2}+k_{+3})+k_{+3}K(k_{+4}+k_{-4})]^2}$

Fraction of enzyme as Z: $\dfrac{k_{+3}k_{+4}K}{k_{-4}s(k_{+2}+k_{+3})+k_{+3}K(k_{+4}+k_{-4})}$

Mechanism B

v_i: $\dfrac{k_{+2}k_{+3}e_0s}{s(k_{+2}+k_{+3})+k_{+3}K}$

v_s: $\dfrac{k_{+2}k_{+3}k_{-4}e_0s}{s[k_{-4}(k_{+2}+k_{+3})+k_{+3}k_{+4}]+k_{+3}k_{-4}K}$

Rate constant: $\dfrac{[k_{-4}(k_{+2}+k_{+3})+k_{+3}k_{+4}]+k_{+3}k_{-4}K}{s(k_{+2}+k_{+3})+k_{+3}K}$

Burst: $\dfrac{k_{+2}k_{+3}^2k_{+4}e_0s^2}{\{s[k_{-4}(k_{+2}+k_{+3})+k_{+3}k_{+4}]+k_{+3}k_{-4}K\}^2}$

Fraction of enzyme as Z: $\dfrac{k_{+3}k_{+4}s}{s[k_{-4}(k_{+2}+k_{+3})+k_{+3}k_{+4}]+k_{+3}k_{-4}K}$

alone mechanisms in which partition is at the first or the second intermediate. Similarly, mechanisms in which Z is formed irreversibly from X or Y and converted into E (free enzyme) and P (product) are similar kinetically to the schemes discussed above (e.g. Bicknell & Waley, 1985). If the complex Z can be isolated, e.g. by rapid centrifugal Minigel chromatography (Fink *et al.*, 1987), then it may be possible to decide whether it regenerates free enzyme directly or via X or Y. The distinction will rest on whether or not Z is deemed to be active.

The mechanism in which the free enzyme isomerizes (Scheme 2, mechanism A) is rather different, in that the substrate does not induce inactivation but merely reveals it. Preincubation of the enzyme on its own eliminates the burst in this mechanism. Moreover, there may be a burst when the reaction is started by adding enzyme and a lag when the reaction is started by adding substrate (e.g. Cheron *et al.*, 1990). This mechanism is also distinguished by the decrease of transient rate constant with increasing concentration of substrate.

Mechanisms in which there are branches at more than one species yield more complex equations (e.g. Frère, 1981). The difficulty of distinguishing between such mechanisms have been noted (Persaud *et al.*, 1986). There is much to be said for interpreting experimental findings by the simplest mechanism that fits, and this has been the motivation for the present paper.

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APPENDIX

Derivation of progress-curve equation

The stoicheiometry for the enzyme (total concentration e_0) is:

$$e_0 = e+x+y+z \tag{A1}$$

The steady-state concentration of intermediate X (Scheme 1 of the main paper) is:

$$x = e\cdot s/K$$

where $K = (k_{-1}+k_{+2})/k_{+1}$, and this is used to eliminate e from eqn. (A1):

$$e_0 = x(1+K/s)+y+z \tag{A2}$$

For intermediate Y we have:

$$dy/dt = k_{+2}x+k_{-4}z-(k_{+3}+k_{+4})y = 0$$

whence:

$$x = [(k_{+3}+k_{+4})y-k_{-4}z]/k_{+2} \tag{A3}$$

Now this value for x is substituted in eqn. (2) to get:

$$e_0 = (1+K/s)[(k_{+3}+k_{+4})y-k_{-4}z]/k_{+2}+y+z$$

whence:

$$y = \frac{e_0-z[1-k_{-4}(1+K/s)/k_{+2}]}{1+(k_{+3}+k_{+4})(1+K/s)/k_{+2}} \tag{A4}$$

Now substitute for y in the equation for the rate of change of z (concentration of dead enzyme):

$$dz/dt = k_{+4}y-k_{-4}z \tag{A5}$$

hence the form of dz/dt is:

$$dz/dt = a-b\cdot z \tag{A6}$$

$$z = a(1-e^{-bt})/b \tag{A7}$$

where a and b are $k_{+4}e_0/N$ and L/N respectively,

$$M = [1-k_{-4}(1+K/s)/k_{+2}]$$
$$N = 1+(k_{+3}+k_{+4})(1+K/s)/k_{+2}$$

and

$$L = k_{-4}N+k_{+4}M$$

Now this value for z may be substituted in eqn. (4), and used in:

$$dp/dt = k_{+3}y \tag{A8}$$

to obtain:

$$dp/dt = k_{+3}e_0(k_{-4}N+k_{+4}M\cdot e^{-bt})/NL \tag{A9}$$

Hence:

$$p = \frac{k_{+3}e_0}{L}\left(k_{-4}t+\frac{k_{+4}M}{L}(1-e^{-Lt/N})\right) \tag{A10}$$

This equation is of the same form as that put forward by Frieden (1970):

$$p = v_s t-(v_s-v_i)(1-e^{-kt})/k \tag{A11}$$

where v_s and v_i are the final and initial rates respectively and k is the parameter (time constant) characterizing the burst.

Simulation of progress curves

The differential equations for the kinetic mechanism of Scheme 1 of the main paper for dy/dt, dz/dt and dp/dt have been given above. Their solution by the trapezoidal method (see, e.g., Fox & Mayers, 1987) is now outlined. The advantage of this procedure is that the method is so transparent that it can readily be understood, and hence applied to other mechanisms. The binding of substrate is assumed to be an equilibrium step, i.e. E, S and X are present at their equilibrium proportions so that the initial value for x is given by:

$$x_0 = 0.5[w - \sqrt{(w^2 - 4e_0s_0)}]$$

where $w = e_0 + s_0 + K_s$, K_s being the dissociation constant for the first complex, X. In the procedure used there was an equilibrium-updating subroutine given by:

$$s_1 = s_0 - p_1 - y_1 - z_1 - x_1 \quad \text{(A12)}$$
$$x_1 = (e_0 - y_1 - z_1)/(1 + K_s/s_1) \quad \text{(A13)}$$

iterated until convergence; here the suffix 1 in s_1, x_1 etc. refers to the value at the end of a step (i.e. time interval). In the trapezoidal method, with a step size h, the approximations to:

$$dy/dt = k_{+2}x + k_{-4}z - (k_{+3} + k_{+4})y$$
$$dz/dt = k_{+4}y - k_{-4}z$$

are:

$$(y_1 - y_0)/h = k_{+2}(x_0 + x_1)/2 + k_{-4}(z_0 + z_1)/2 - (k_{+3} + k_{+4})(y_0 + y_1)/2$$
$$(z_1 - z_0)/h = k_{+4}(y_0 + y_1)/2 - k_{-4}(z_0 + z_1)/2$$

The values of y_1 and z_1 were obtained from:

$$y_1 = \{y_0 + H[k_{+2}(x_0 + x_1) - (k_{+3} + k_{+4})y_0 + k_{-4}(z_0 + z_1)]\}/Y \quad \text{(A14)}$$
$$z_1 = \{z_0 + H[k_{+4}(y_0 + y_1) - k_{-4}z_0]\}/Z \quad \text{(A15)}$$

where $Y = 1 + H(k_{+3} + k_{+4})$ and $Z = 1 + Hk_{-4}$, and $H = h/2$, h being the step size. Eqn. (A12) to eqn. (A16) gave the next value in the iteration for y, z and p, and were followed in the subroutine by the 'updating' assignment statements:

$$y_0 \Leftarrow y_1; z_0 \Leftarrow z_1; p_0 \Leftarrow p_1; x_0 \Leftarrow x_1 \quad \text{(A16)}$$

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The essence of the program, which has been implemented both in FORTRAN and BASIC, is that a large number, usually 10^4, small time steps are taken first, followed by the same number of steps with a much larger step size. The size of the small steps was usually $1/10^4$ of the later steps. Thus, in eqns. (A14) to (A16), the step size h was set to $(10^{-4}h)$ for the first call of the subroutine, and to h for the second. This simple manoeuvre sufficed for the simulations carried out above, and has been useful in other instances; it depends on the fact that very small steps are required for the early stages of enzymic reactions while steady states are being set up; subsequently much larger steps may be taken. The simplest check on the accuracy of the values obtained for p was to compare the results from two simulations, the second having step sizes half the first. The results were considered acceptable when the two sets agreed to three significant figures.

The trapezoidal method with the equilibrium assumption for substrate binding outlined above was compared with a program based on a more elaborate backwards differentiation method of solution of the differential equations (Scraton, 1987); in this program the equilibrium assumption was no longer made. The results were identical, to three significant figures, in examples where the constant K [defined as $(k_{-1} + k_{+2})/k_{+1}$] was 50 mM or 0.05 mM, with k_{-1}/k_{+2} 2000 or 1 respectively; the substrate concentration was 50 mM in these two examples, and the same held when K was 0.05 mM, $k_{-1}/k_{+2} = 1$, and the substrate concentration was 5 mM. That the assumption of equilibrium in substrate binding should make so little difference when $k_{-1} = k_{+2}$ was not expected.

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