Kinetic parameters from progress curves of competing substrates

Application to β -lactamases

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The use of two substrates, one of them a reporter substrate, is often convenient. The time course of an enzymic reaction with competing substrates is given explicitly in the present paper. Kinetic parameters can be readily obtained. The method is applied to the hydrolysis of benzylpenicillin, with cephalosporin C as the reporter substrate, catalysed by a *Pseudomonas* β -lactamase.

It is possible to follow the enzymic reaction of one substrate (B) by its effect on the reaction of a monitoring substrate (A), the latter being chosen because of its favourable spectroscopic properties (or sometimes because during reaction it releases a proton). The two substrates are assumed to compete for the same active site. This method is also helpful if the K_m for substrate B (K_m^B) is very low; direct determination of K_m^B is difficult because it is usually not practicable to follow the reaction at a low enough substrate concentration. The purpose of the present paper is to show how to obtain steady-state kinetic parameters by the use of competing substrates. One-substrate reactions are considered, as the kinetics of reactions of two substrates in the presence of an alternative substrate (Wong, 1975; Huang, 1979) do not readily lend themselves to the analysis of progress curves. This method has been used in studying the hydrolysis of benzylpenicillin by a β -lactamase from *Pseudomonas aeruginosa* (Knott-Hunziker *et al.*, 1982; R. Bicknell, V. Knott-Hunziker & S. G. Waley, unpublished work). The monitoring substrate was cephalosporin C; there is a change in A_{260} of the cephalosporin when it is hydrolysed (Sabath *et al.*, 1965), but the change in absorbance of the penicillin is at a lower wavelength (Waley, 1974), and so it is easy to follow the hydrolysis of the former selectively.

When an enzyme is acting on a mixture of two substrates, the rate of reaction of each is given, when the reactions are effectively irreversible and simple Michaelis–Menten kinetics apply, by eqn. (1) (Dixon & Webb, 1979):

$$-\frac{da}{dt} = \frac{aV_A/K_m^A}{1 + \frac{a}{K_m^A} + \frac{b}{K_m^B}} \quad \text{and} \quad -\frac{db}{dt} = \frac{bV_B/K_m^B}{1 + \frac{a}{K_m^A} + \frac{b}{K_m^B}} \quad (1)$$

The concentrations of A and B are a and b (initially, a_0 and b_0); K_m^A and K_m^B are the Michaelis constants for A and B, and V_A and V_B are the maximum velocities for A and B, when the enzyme acts on either A or B alone. When both substrates are present, each substrate acts as a competitive inhibitor of the other.

Put

$$\frac{V_B/K_m^B}{V_A/K_m^A} = R$$

Then

$$\frac{d(\ln b)}{d(\ln a)} = R \quad (2)$$

Integration of eqn. (2) gives:

$$\frac{b}{b_0} = \left(\frac{a}{a_0} \right)^R$$

The dimensionless constant R , which may be called the relative specificity constant (Nomenclature Committee of the International Union of Biochemistry, 1982), determines the relative amount of each substrate converted into product. The progress curve for A, obtained by substituting for b , is thus:

$$t = \frac{a_0 - a}{V_A} + \frac{K_m^A}{V_A} \cdot \ln \left(\frac{a_0}{a} \right) + \frac{b_0}{V_B} \cdot \left[1 - \left(\frac{a}{a_0} \right)^R \right] \quad (3)$$

This curve shows a point of inflexion (see, e.g., Fig. 1) when $R > 1$. The shape of the progress curve thus gives information directly about the specificity of the enzyme. Many different substrates can be quickly screened like this.

The first two terms on the right-hand side of eqn. (3) are the usual ones for the reaction of A on its own. The presence of B results in extra time taken (Δt) to reach a given extent of reaction, given by:

$$\Delta t = \frac{b_0}{V_B} \cdot \left[1 - \left(\frac{a}{a_0} \right)^R \right] \quad (4)$$

How R and V_B may be obtained is described below.

Materials and methods

The β -lactamase was from a mutant of *Pseudomonas aeruginosa* that produces the enzyme constitutively (Flett *et al.*, 1976), and was purified as previously described (Berks *et al.*, 1982). Progress curves (Fig. 1) for the hydrolysis of cephalosporin C alone, or in the presence of $30 \mu\text{M}$ -benzylpenicillin, were compared directly. Measurements of the time difference (Δt) at the same extent of reaction were used to fit eqn. (4) by the procedure now outlined. The sum (Q) of the squares of the residuals, for N points, is:

$$Q = \sum_{i=1}^N [\Delta t_i - B'(1 - x_i^R)]^2$$

where $B' = b_0/V_B$ and $x_i = a_i/a_0$. Differentiating with respect to the parameters and equating the differentials to zero gives:

$$\sum_i \Delta t_i (1 - x_i^R) - B' \cdot \sum_i (1 - x_i^R)^2 = 0 \quad (5)$$

$$\sum_i \Delta t_i \cdot x_i^R \cdot \ln x_i - B' \cdot \sum_i x_i^R \cdot \ln x_i + B' \cdot \sum_i x_i^{2R} \cdot \ln x_i = 0 \quad (6)$$

When the value of B' from eqn. (5) is substituted into eqn. (6), there results an equation for R that was solved numerically by the bisection method (Conte & de Boor, 1980); a PDP11 computer was used.

Results and discussion

The presence of benzylpenicillin caused the progress curve for the hydrolysis of cephalosporin C by the *Pseudomonas* β -lactamase to show a point of inflexion (Fig. 1). Values of $R = 6.28$ and $k_{\text{cat}}^B = 4150 \text{ min}^{-1}$ were obtained by fitting the curve. A direct measure of k_{cat} for benzylpenicillin (from the initial rate with $200 \mu\text{M}$ substrate) gave a value of 4030 min^{-1} . The agreement confirms that the en-

Fig. 1. Progress curve for competing substrates. The hydrolysis of cephalosporin C ($180 \mu\text{M}$) alone (O), or in the presence of benzylpenicillin ($30 \mu\text{M}$) (Δ), by the *Pseudomonas* β -lactamase (about 3 nM) was measured in $0.1 \text{ M-NaCl}/20 \text{ mM-Mes}$ (4-morpholine-ethanesulphonic acid)/NaOH buffer, pH 6, at 30°C . The time difference (Δt) was measured at the same extent of reaction: e.g. $\Delta t = 64 \text{ s}$ at $A = 1.2$ (9% reaction). The continuous curve is theoretical, calculated from eqn. (4) in the text with $R = 6.28$ and $V_B = 13.5 \mu\text{M} \cdot \text{min}^{-1}$. These values, together with $k_{\text{cat}}/K_m = 236 \mu\text{M}^{-1} \cdot \text{min}^{-1}$ for cephalosporin C, give $K_m^B = 2 \mu\text{M}$ for benzylpenicillin.

zyme was stable. Repeated experiments were undertaken to test the accuracy of the method. When the variation within fourfold replicates was measured, the coefficient of variation (from seven such sets) was 13% for R and 7% for V_B .

When R is low (< 0.1 , approximately) the extent of hydrolysis of B is too low to measure accurately. Then B behaves as a competitive inhibitor, and the measured K_i is equal to K_m^B . The K_i may conveniently be obtained from the direct comparison of progress curves (Waley, 1982). On the other hand, when R is much greater than 1, there is little reaction of A until virtually all B has been consumed; for much of the course of the reaction the term $(a/a_0)^R$ in eqn. (4) is small compared with 1. For instance, if $R > 20$, after more than 11% reaction, $\Delta t = b_0/V_B$ (to within 1%). The progress curve soon becomes parallel to that of the control (lacking B), and the separation (Δt) gives V_B . The ease of obtaining V_B is balanced by the difficulty of obtaining R . Values of R must be found from the early stages of reaction of A. This requires measurement of low concentrations of product in the presence of high concentrations of substrate. This is usually feasible if A is a proton-releasing reporter, and sometimes feasible if it is a spectrophotometric reporter.

If the product from substrate A is a competitive inhibitor eqn. (3) is altered, but eqn. (4) is not. [Similarly, the time differences in competitive inhibition are unaltered; they have mistakenly been stated to be altered (Waley, 1982).] If the product from substrate B is a competitive inhibitor

(inhibition constant K_Q), and is initially present at concentration q_0 , eqn. (4) is replaced by eqn. (7):

$$\Delta t = - \left(\frac{K_m^A}{V_A} \right) \cdot \frac{(b_0 + q_0)}{K_Q} \cdot \ln x + \left(\frac{b_0}{V_B} \right) \cdot \left(1 - \frac{K_m^B}{K_Q} \right) \cdot (1 - x^R) \quad (7)$$

Experiments with a range of initial concentrations of B, and its product, will be necessary to obtain the parameters.

The feature of a sudden increase in the rate of reaction of one substrate ('lag-burst') as a competing substrate is consumed has been known for more than 50 years (Willstätter *et al.*, 1927). Although the usefulness of competing substrates was stressed recently (Hollaway & Ticho, 1979), the general solution (eqn. 3) was not given.

The 'lag-burst' feature has been noted for transport (Hackette *et al.*, 1970), and perhaps could feature in metabolic control as follows. The rate of an enzymic reaction of substrate A will be low as long as a second substrate B for the same enzyme is present, in the case that both K_m^B/K_m^A and V_B/V_A are low (say less than 0.01). The low rate accounts for the lag. When substrate B has been consumed, the rate increases sharply. Hence the burst.

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