A quick method for the determination of inhibition constants

Stephen G. WALEY

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

(Received 20 May 1982/Accepted 12 July 1982)

The inhibition constant K_i in the common case of competitive inhibition can be obtained by simple comparison of progress curves in the presence and in the absence of inhibitor. The difference between the times taken for the concentration of substrate to fall to the same value is used to obtain K_i . The procedure to use when the product inhibits is described. When there is mixed inhibition, reactions at different substrate concentrations are used to obtain both inhibition constants.

The measurement of an inhibition constant (K_i) is one of the commonest tasks of those working with enzymes. There is an easy procedure that, although not new in principle, is, I believe, not widely practised. The method makes use of progress curves. This is seldom a disadvantage; most measurements nowadays are made in a recording spectrophotometer, and, if necessary, no more of the progress curve need be utilized than would otherwise be used to measure the initial rate (see, e.g., Waley, 1981). Successive time differences are exploited as indicated in the following sections.

Competitive inhibition

Consider competitive inhibition, where the integrated Michaelis–Menten equation has the form:

$$t = \frac{s_0 - s}{V} + \frac{K}{V} \cdot \left(1 + \frac{i}{K_i}\right) \cdot \ln\left(\frac{s_0}{s}\right) \quad (1)$$

where s_0 and i stand for the initial concentrations of substrate and inhibitor, and s is the concentration of substrate at time t . V and K stand for the maximum velocity and Michaelis constant respectively of the uninhibited reaction. Now compare this with the time (t_c) taken for the concentration of substrate in the control reaction lacking inhibitor to fall to the same value:

$$t_c = \frac{s_0 - s}{V} + \frac{K}{V} \cdot \ln\left(\frac{s_0}{s}\right) \quad (2)$$

By subtraction:

$$t - t_c = \frac{K}{V} \cdot \frac{i}{K_i} \cdot \ln\left(\frac{s_0}{s}\right) \quad (3)$$

The reactions with and without an inhibitor are compared, and several values of the time difference ($t - t_c$) for equal values of s are obtained (Fig. 1a).

Then $t - t_c$ is plotted against $\ln(s_0/s)$ and the slope measured (Fig. 1b). (The foregoing assumes that the inhibited and control experiments were performed with the same concentration of enzyme. If, however, the concentration of enzyme in the inhibited experiment was M -fold that in the control experiment, then $t - t_c$ in eqn. (3) should be replaced by $M \cdot t - t_c$.) From the slope, given in eqn. (4), K_i is obtained from eqn. (5):

$$\text{Slope} = \frac{K}{V} \cdot \frac{i}{K_i} \quad (4)$$

$$K_i = i \cdot \frac{K}{V} \cdot \frac{1}{\text{Slope}} \quad (5)$$

The dimension of the slope is time, and K/V must be expressed in the same units of time for K_i to be obtained in the same units as i . The extent of reaction (percentage conversion of substrate into product) should normally be in the range 15–40%. In the experiment shown in Fig. 1, the extent of reaction was 25%. The concentration of inhibitor should be large enough for $t - t_c$ to be at least as large as t_c , i.e. t should be at least twice as large as t_c . As a guide, if an approximate value of K_i is known, choose the concentration of inhibitor to be at least as large as $K_i(1 + s_0/K)$. The magnitude of the error in K_i (ΔK_i) brought about by errors in s (Δs) and T (ΔT) is obtained from eqn. (6):

$$\frac{\Delta K_i}{K_i} = -\frac{\Delta s}{s} \cdot \frac{1}{\ln\left(\frac{s_0}{s}\right)} \quad (6)$$

and eqn. (7):

$$\frac{\Delta K_i}{K_i} = \frac{\Delta T}{T} \quad (7)$$

Fig. 1. Use of time differences to obtain K_i .

(a) Progress curves for a reaction with inhibitor [▲, eqn. (1)] and without inhibitor [●, eqn. (2)] are shown. The differences between the times taken for the concentration of substrate (or the absorbance) to reach the same value are shown by the broken horizontal lines. These differences, referred to as $t - t_c$ in the text, are given by eqn. (3). In (b), these differences are plotted against $\ln(s_0/s)$, where s_0 is the initial concentration of substrate. The effect of boric acid (12.8 mM) as inhibitor on the hydrolysis of benzylpenicillin (0.85 mM) by β -lactamase I (0.66 nM) in 0.5 M-NaCl/50 mM-sodium phosphate buffer, pH 7, at 30°C is shown, the reaction being monitored by the absorbance (path length 10 mm). The first 25% of reaction is shown. The value of K_i calculated from the slope in (b) was 1.2 mM, in agreement with the value found previously from initial rates (Kiener & Waley, 1978).

where T is written for $t - t_c$. At 25% reaction, for example, a 2% error in s brings about a 7% error in K_i . Or, a 1% error in s makes a 2–6% error in K_i for 40–15% reaction.

It is not uncommon for the product to be a competitive inhibitor, and it is important to allow for this. Test by comparing progress curves in the presence and in the absence of product added initially. If they differ, then find K_p from eqn. (8), a parallel to eqn. (3), in which p_0 is the initial concentration of product and K_p its inhibition constant:

$$t - t_c = \frac{K}{V} \cdot \frac{p_0}{K_p} \cdot \ln \left(\frac{s_0}{s} \right) \quad (8)$$

The difference in time for the progress curves for the reactions with and without added inhibitor is now:

$$t - t_c = \frac{K}{V} \cdot \frac{i}{K_i} \cdot \left[\frac{s_0 - s}{K_p} + \left(1 + \frac{s_0}{K_p} \right) \cdot \ln \left(\frac{s_0}{s} \right) \right] \quad (9)$$

and here $t - t_c$ is plotted against the term in square brackets. The slope is again given by eqn. (4).

The effect of reversibility is now considered. If the equilibrium constant is K_{eq} , and the concentration of product at equilibrium is p_{eq} , it may be shown that eqn. (10) replaces eqn. (3):

$$t - t_c = -\frac{K}{V} \cdot \frac{i}{K_i} \cdot \ln \left(1 - \frac{s_0 - s}{p_{eq}} \right) \cdot \left(\frac{1}{1 + \frac{1}{K_{eq}}} \right) \quad (10)$$

If the equilibrium constant is 10, the effect of reversibility causes K_i to be underestimated by about

1% at 25% reaction; the difference is correspondingly less if the equilibrium constant is larger.

The effect of the enzyme's being unstable was examined with the equation given by Cornish-Bowden (1975); if there were 5% inactivation when 25% of the substrate had been converted into product in the control reaction lacking inhibitor, in an example with $s_0 = 4K$, $i = 5K_i$, $V = 1$, the value of K_i obtained would be in error by about 5%. This calculation is based on the assumption that all forms of enzyme are inactivated at the same rate. If, as is common, the enzyme-inhibitor complex is less unstable than the free enzyme, the longer time in the reaction containing inhibitor will be partly compensated for, and the errors will be less than that given above.

Mixed inhibition

If the inhibition is purely competitive, then the slope in the plot of $t - t_c$ against $\ln(s_0/s)$ is independent of the initial concentration of substrate, as shown by eqn. (4). This provides an adequate test for purely competitive inhibition. Mixed inhibition (Cornish-Bowden, 1979) is not uncommon. If the inhibitor combines with the enzyme-substrate complex (dissociation constant K'_i) as well as with the free enzyme (dissociation constant K_i), then instead of eqn. (3) we have:

$$t - t_c = \frac{1}{V} \cdot \frac{i}{K'_i} \cdot (s_0 - s) + \frac{K}{V} \cdot \frac{i}{K_i} \cdot \ln \left(\frac{s_0}{s} \right) \quad (11)$$

and the slope of the plot of $t - t_c$ against $\ln(s_0/s)$ is given by:

$$\text{Slope} = \frac{1}{V} \cdot \frac{i}{K'_i} \cdot s + \frac{K}{V} \cdot \frac{i}{K_i} \quad (12)$$

As the concentration of substrate changes relatively little during the early stages of reaction, the approximation is made that the value of s in eqn. (12) may be replaced by $(s_0 + s')/2$, where s' is the concentration at the end of the period taken. Thus, if the extent of reaction is 25%, $s' = 0.875 \times s_0$. Now $t - t_c$ is plotted against $\ln(s_0/s)$ for several values of s_0 , and the slopes are then plotted against s' . The slope and the intercept of this replot are used to find K_i and K'_i respectively. When this procedure was tested with the following values, $V = 1$, $K_i = 1$, $K'_i = 5$, $K = 1$, $s_0 = 1, 2, 4, 6$ and 8 , the first 25% of reaction being taken in each case, the values of K_i and K'_i returned by the approximate procedure were 1.02 and 5.02 respectively. The approximation described above was partly based on the work of Lee & Wilson (1971) and Morgan (1972). Since eqn. (11) is of the same form as eqn. (2), K_i and K'_i may also be found from the replots described by Orsi & Tipton (1979).

Conclusions

The proposed method is reliable and seems likely to be useful. The integrated Michaelis-Menten equation (eqn. 2) is widely applicable in practice (Orsi & Tipton, 1979; Wharton & Szawelski, 1982), and was used by Schwert (1969) in an extended

treatment that includes some of the present examples. However, the usefulness of the method for the commonest case of competitive inhibition was not stressed by Schwert (1969), nor was the plot for non-competitive inhibition correctly described. It is an advantage of the present method that the effectiveness of an inhibitor is gauged by a direct comparison with the control. This may be particularly helpful when the enzyme is unstable. Perhaps the main advantage is speed: it is often possible to perform some six reactions and their controls, at various initial concentrations of substrate and inhibitor, in an hour. Experience suggests that the value of K_i may be expected to be reliable to about 20%.

The support of the Medical Research Council is gratefully acknowledged. This paper is a contribution from the Oxford Enzyme Group.

References

- Cornish-Bowden, A. (1975) *Biochem. J.* **149**, 305–312
- Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp. 76–78, Butterworths, London
- Kiener, P. A. & Waley, S. G. (1978) *Biochem. J.* **169**, 197–204
- Lee, H. J. & Wilson, I. B. (1971) *Biochim. Biophys. Acta* **242**, 519–522
- Morgan, M. R. J. (1972) *Enzymologia* **42**, 219–233
- Orsi, B. A. & Tipton, K. F. (1979) *Methods Enzymol.* **63**, 159–183
- Schwert, G. W. (1969) *J. Biol. Chem.* **244**, 1278–1284
- Waley, S. G. (1981) *Biochem. J.* **193**, 1009–1012
- Wharton, C. W. & Szawelski, R. J. (1982) *Biochem. J.* **103**, 351–360