Biochem. J. (1990) 265, 647–653 (Printed in Great Britain)

Progress-curve equations for reversible enzyme-catalysed reactions inhibited by tight-binding inhibitors

Stefan E. SZEDLACSEK,*|| Vasile OSTAFE,* Ronald G. DUGGLEBY,† Mihai SERBAN‡ and Marcel O. VLAD§

*Department of Biochemistry, Institute of Biological Sciences, Spl. Independenţei 296, 77748 Bucharest, Romania, †Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia, ‡Department of Biochemistry, Institute of Agronomy, B-dul Marasti 59, Bucharest, Romania, and §I. Med. Bucharest, Casuta Postala 77-49, Bucharest, Romania

The rate equation for a tight-binding inhibitor of an enzyme-catalysed first-order reversible reaction was used to derive two integrated equations. One of them covers the situations in which competitive, uncompetitive or non-competitive inhibition occurs and the other refers to the special non-competitive case where the two inhibition constants are equal. For these equations, graphical and non-linear regression methods are proposed for distinguishing between types of inhibition and for calculating inhibition constants from progress-curve data. The application of the non-linear regression to the analysis of simulated progress curves in the presence of a tight-binding inhibitor is also presented. The results obtained are valid for any type of 'dead-end'-complex-forming inhibitor and can be used to characterize an unknown inhibitor on the basis of progress curves.

INTRODUCTION

The analysis of progress-curve data has many well-known advantages over initial-velocity measurements: more information from fewer experiments, more reliable data, the possibility of simultaneous analysis of product effect and so on. Although it has not been used very often, this is probably a temporary problem (Duggleby, 1985), which will be overcome as methods for data analysis are improved and simplified (Duggleby & Wood, 1989).

There have been several attempts to use progress curves for the determination of inhibition parameters. Thus Waley (1982) reported a method to determine inhibition constants by the comparison of progress curves recorded in the presence and in the absence of an inhibitor. This treatment assumes the validity of rate equations for classical inhibitors, as does the work of Kellershohn & Laurent (1985). This latter paper analyses the influence of product inhibition on progress curves at high concentrations of enzyme. For low enzyme concentrations the product influence is described by the integrated equations deduced by Boeker (1984) for reversible mono- and bi-molecular reactions.

Though there is not a clear demarcation between the classical type of inhibition and that caused by tight-binding inhibitors, classical inhibition is produced only at inhibitor concentrations considerably higher than the enzyme concentration, whereas tight-binding inhibition occurs at inhibitor concentrations comparable with that of the enzyme (Morrison, 1969). Although a limited number of studies have been made on the kinetics of tight-binding inhibitors, interest in the subject is increasing constantly, mainly because of their importance as chemotherapeutic agents (Williams & Morrison, 1979; Williams *et al.*, 1979).

The kinetic analysis of tight-binding inhibition is complex because Michaelis–Menten-type equations are not valid. As a result, double-reciprocal plots become non-linear in the presence of tight-binding inhibitors, as demonstrated by Morrison (1969). He gave a general initial steady-state rate equation for any enzyme-catalysed reaction in the presence of a tight-binding reversible inhibitor, but he has underlined the difficulties related to the determination of inhibition constants.

A linear form of the Morrison equation was derived by Henderson (1972). It allows a graphical determination of the mechanism of inhibition and the enzyme concentration. It was also shown and illustrated for the Michaelis–Menten-type irreversible reaction that secondary plots give the inhibition constants for competitive and uncompetitive cases. However, for non-competitive inhibition the two inhibition constants can be evaluated only by extrapolating a non-linear curve, a procedure that is unlikely to yield accurate values. The experimental part of this work (Henderson, 1972) confirms that, at least in the case of the mitochondrial ATPase inhibition by rutamycin, the scatter of the data points is too large to allow accurate evaluation of the equation parameters.

It should be noted here that in this paper we use the term 'non-competitive' in the broad sense where inhibition depends on two inhibition constants (Duggleby, 1988). In the special case where the two constants are coincident, which, for want of a better term, we here call 'pure non-competitive' inhibition, Henderson's equation yields a straight line that can be used to calculate the inhibition constant.

Greco & Hakala (1979), by using a Monte Carlo simulation, evaluated the strong and weak points for 11 of the existing initial-rate methods used in calculating dissociation constants. They concluded that computer methods that utilized non-linear regression based on the equations of Ackermann & Potter (1949) and Morrison (1969) are significantly more precise.

More recently, Sculley & Morrison (1986) developed a new method for the determination of kinetic constants governing slow tight-binding inhibition, by analysing progress curve data. However, this method, as well as

|| To whom correspondence should be addressed.

other methods for analysing slow-binding inhibition (Williams & Morrison, 1979; Williams *et al.*, 1979, 1980), consider only the initial part of product–time curves where there is a transition from the initial velocity to the steady-state velocity, and they do not allow for depletion of the substrates or inhibition by accumulated product as the reaction proceeds.

The aim of the present paper is to try to overcome the difficulties in the determination of the inhibition constants by using the advantages of progress-curve analysis. Starting from Morrison's general equation and from a widely used form of the reversible-type Michaelis–Menten equation, we derived a general form of the progress-curve equation for competitive, uncompetitive and non-competitive inhibition. Thus these equations, which have not been reported previously, cover the main types of tight-binding inhibition.

Our principal aim is to show the origin and interpretation of these equations, rather than to fit them to progress-curve data. However, some selected examples are given to illustrate possible approaches to non-linear-regression analysis of experimental measurements.

THEORY

The initial-velocity equation for any enzyme-catalysed reaction may be represented in a general form (Morrison, 1969) as:

$$v = \frac{N[\mathrm{E}]_t}{D} \tag{1}$$

where N contains the rate constants and substrate concentrations that determine the maximum velocity of the reaction, D is a sum of several terms related to the distribution of the enzyme in all its complexed forms (Cleland, 1963; Morrison, 1969) and $[\mathrm{E}]_t$ represents the total enzyme concentration. Morrison (1969) also gives the general rate equation (2) when there are multiple 'dead-end' enzyme–inhibitor complexes ($\mathrm{E_i I}$) having dissociation constants of K_i:

$$v_i = \frac{N[\mathrm{E}]_t}{D + [\mathrm{I}]_f \Sigma(N_i/K_i)} \tag{2}$$

Here N_i is part of D representing the distribution of that form of enzyme which combines with the inhibitor to form $\mathrm{E_i I}$, and $[\mathrm{I}]_f$ represents the concentration of the free inhibitor. The distinctive feature of tight-binding inhibitors is that $[\mathrm{I}]_f$ is not equal to the total inhibitor concentration $[\mathrm{I}]_t$; in this case v_i is the solution of Morrison's equation (Morrison, 1969):

$$v_i^2 + N\left(\frac{1}{\Sigma(N_i/K_i)} + \frac{[\mathrm{I}]_t - [\mathrm{E}]_t}{D}\right)v_i - \frac{N^2[\mathrm{E}]_t}{D\Sigma(N_i/K_i)} = 0 \tag{3}$$

Consider a reversible one-substrate–one-product enzyme-catalysed reaction ($\mathrm{S} \rightleftharpoons \mathrm{P}$) in the presence of a non-competitive inhibitor. We have to derive the particular form for N, D and $\Sigma(N_i/K_i)$.

For the sake of simplicity we begin by considering the scheme containing only one enzyme complex:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{E} + \mathrm{P} \tag{4}$$

The rate equation for this mechanism is:

$$v = \frac{V_f[\mathrm{S}]/K_s - V_r[\mathrm{P}]/K_p}{1 + [\mathrm{S}]/K_s + [\mathrm{P}]/K_p} \tag{5}$$

where the maximum velocities in the forward and reverse direction (V_f and V_r) and the Michaelis constants for S and P (K_s and K_p) are given by:

$$\left.\begin{aligned} V_f &= k_{+2}[\mathrm{E}]_t \\ V_r &= k_{-1}[\mathrm{E}]_t \\ K_s &= \frac{k_{-1} + k_{+2}}{k_{+1}} \\ K_p &= \frac{k_{-1} + k_{+2}}{k_{-2}} \end{aligned}\right\} \tag{6}$$

Note that [S] and [P] are related by stoichiometry embodied in the conservation equation:

$$[\mathrm{S}]_o + [\mathrm{P}]_o = [\mathrm{S}] + [\mathrm{P}] = [\mathrm{S}]_e + [\mathrm{P}]_e$$

where the subscript 'o' means initial and subscript 'e' equilibrium value.

The progress-curve equation generally is expressed as a function of $z = [\mathrm{P}] - [\mathrm{P}]_o$, that is z represents the amount of product formed during the enzyme-catalysed reaction. It is usual to replace [S] and [P] with functions of z in the kinetic equations, but in the following we find it more useful to express these equations in terms of a new variable ζ, defined as:

$$\zeta = z_\infty - z = [\mathrm{P}]_e - [\mathrm{P}] = [\mathrm{S}] - [\mathrm{S}]_e \tag{7}$$

where z_∞ is the value of z at equilibrium, i.e.:

$$z_\infty = [\mathrm{P}]_e - [\mathrm{P}]_o = (V_f K_p[\mathrm{S}]_o - V_r K_s[\mathrm{P}]_o)/(V_f K_p + V_r K_s).$$

We now rewrite the rate equation for the uninhibited reaction (eqn. 1) in the form $v = \rho\zeta/(\zeta + \delta)$, i.e.:

$$\left.\begin{aligned} N &= \frac{\rho\zeta}{[\mathrm{E}]_t} \\ D &= \zeta + \delta \end{aligned}\right\} \tag{8}$$

Now consider the equilibria involving the inhibitor:

$$\left.\begin{aligned} \mathrm{E} + \mathrm{I} &\overset{K_I}{\rightleftharpoons} \mathrm{EI} \\ \mathrm{ES} + \mathrm{I} &\overset{K_I'}{\rightleftharpoons} \mathrm{ESI} \end{aligned}\right\} \tag{9}$$

where K_I and K_I' are dissociation constants for EI and ESI complexes respectively.

The distribution equations (Cleland, 1963; Morrison, 1969) for the free enzyme and for the ES complex are:

$$\left.\begin{aligned} \frac{[\mathrm{E}]}{[\mathrm{E}]_t} &= \frac{\Delta_E}{\Delta} \\ \frac{[\mathrm{ES}]}{[\mathrm{E}]_t} &= \frac{\Delta_{ES}}{\Delta} \end{aligned}\right\} \tag{10}$$

where

$$\left.\begin{aligned} \Delta_E &= k_{-1} + k_{+2} \\ \Delta_{ES} &= k_{+1}[\mathrm{S}] + k_{-2}[\mathrm{P}] \\ \Delta &= \Delta_E + \Delta_{ES} \end{aligned}\right\} \tag{11}$$

In the absence of the inhibitor, the steady-state velocity equation is:

$$v = k_{+2}[ES] - k_{-2}[P]\cdot[E] = \frac{k_{+2}\Delta_{ES} - k_{-2}[P]\cdot\Delta_E}{\Delta_E + \Delta_{ES}}[E] \quad (12)$$

Allowing for the possibility that the inhibitor reacts with both E and ES, then, using eqn. (2), we obtain:

$$v_i = \frac{k_{+2}\Delta_{ES} - k_{-2}[P]\cdot\Delta_E}{\Delta_E + \Delta_{ES} + [I]_f[(\Delta_E/K_I) + (\Delta_{ES}/K_I')]} \quad (13)$$

which may be rewritten as:

$$v_i = \frac{\rho\zeta}{\zeta + \delta + [I]_f \Sigma(N_i/K_i)} \quad (14)$$

with

$$\rho = \frac{V_r K_s + V_f K_p}{K_p - K_s} \quad (15)$$

$$\delta = \frac{K_s K_p}{K_p - K_s}\left(1 + \frac{([S]_o + [P]_o)(V_r + V_f)}{V_f K_p + V_r K_s}\right) \quad (16)$$

$$\Sigma(N_i/K_i) = \frac{K_s K_p}{K_p - K_s}\left(\frac{1}{K_I} + \frac{([S]_o + [P]_o)(V_r + V_f)}{V_f K_p + V_r K_s}\cdot\frac{1}{K_I'}\right) + \frac{\zeta}{K_I'} \quad (17)$$

Provided that $K_s < K_p$ then $\rho > 0$ and $\delta > 0$. It is of interest that $K_s = K_p$ implies that the progress curve follows a simple exponential because v depends linearly on ζ, i.e. $v = \nu\zeta$ with $\nu = (V_f + V_r)/(K_s + [S]_o + [P]_o)$. This case can be treated by the same procedure putting $N = (\nu/[E]_t)\zeta$, $D = 1$ and then deriving the proper form for $\Sigma(N_i/K_i)$. We consider that this situation would be so rare that it does not warrant further analysis in a separate section.

It is evident that $\Sigma(N_i/K_i)$ has the form:

$$\Sigma(N_i/K_i) = a + b\zeta \quad (18)$$

The particular forms of a and b for competitive, pure non-competitive and uncompetitive inhibition can be derived by setting $K_I' = \infty$, $K_I = K_I'$ and $K_I = \infty$ respectively (see Table 1).

Setting $K_p = \infty$ and $[P]_o = 0$, a and b from Table 1 then become identical with the expressions given by Henderson (1972) for the case of irreversibility.

Introducing now eqns. (8) and (18) into eqn. (3) and solving it for $v_i = -d\zeta/dt$, we obtain:

$$-\frac{dt}{d\zeta} = p_1\frac{\sqrt{(p_2\zeta^2 + 2p_3\zeta + p_4)} + p_5\zeta + p_6}{\zeta} \quad (19)$$

In eqn. (19) the following notations were used:

$$\left.\begin{aligned} p_1 &= 1/(2\rho) \\ p_2 &= [1 + b([I]_t - [E]_t)]^2 + 4b[E]_t \\ p_3 &= ab([I]_t - [E]_t)^2 + (a + b\delta)([I]_t + [E]_t) + \delta \\ p_4 &= [\delta + a([I]_t - [E]_t)]^2 + 4a\delta[E]_t \\ p_5 &= 1 + b([I]_t - [E]_t) \\ p_6 &= \delta + a([I]_t - [E]_t) \end{aligned}\right\} \quad (20)$$

Table 1. Expressions for a and b

Type of inhibition	a	b
Non-competitive	$\frac{K_s K_p}{K_p - K_s}\left(\frac{1}{K_I} + \frac{([S]_o + [P]_o)(V_r + V_f)}{V_f K_p + V_r K_s}\cdot\frac{1}{K_I'}\right)$	$\frac{1}{K_I'}$
Competitive	$\frac{K_s K_p}{K_p - K_s}\cdot\frac{1}{K_I}$	0
Pure non-competitive	$\frac{K_s K_p}{K_p - K_s}\left(1 + \frac{([S]_o + [P]_o)(V_r + V_f)}{V_f K_p + V_r K_s}\right)\cdot\frac{1}{K_I'}$	$\frac{1}{K_I'}$
Uncompetitive	$\frac{K_s K_p}{K_p - K_s}\cdot\frac{([S]_o + [P]_o)(V_r + V_f)}{V_f K_p + V_r K_s}\cdot\frac{1}{K_I'}$	$\frac{1}{K_I'}$

We emphasize that p_2, p_3 and p_4 are all strictly positive.

Examine now the roots of the quadratic equation:

$$p_2\zeta^2 + 2p_3\zeta + p_4 = 0 \quad (21)$$

The roots can be obtained from the general solution:

$$\left.\begin{aligned} s_1 &= (-p_3 + \sqrt{D})/p_2 \\ s_2 &= (-p_3 - \sqrt{D})/p_2 \end{aligned}\right\} \quad (22)$$

where $$D = p_3^2 - p_2 p_4 = 4[I]_t[E]_t(a - b\delta)^2 \quad (23)$$

Clearly, two situations appear here:

(i) $\delta \neq a/b$ implying s_1 and s_2 are real and distinct;

(ii) $\delta = a/b$ when the real roots s_1 and s_2 are identical.

From Table 1 we can see that the first situation corresponds to competitive, uncompetitive and non-competitive inhibition and the second one to pure non-competitive inhibition. The integration of eqn. (19), presented in detail in the Appendix, gives, in the case of competitive, uncompetitive and non-competitive inhibition, the following equation:

$$t = \frac{1}{2\rho}\left[\sum_{i=1}^{4} A_i\cdot\ln\left(\frac{x - x_i}{x_0 - x_i}\right) + \left(\frac{B_1}{x_0 - 1}\right)\left(\frac{x - x_0}{x - 1}\right) + \left(\frac{B_2}{x_0 + 1}\right)\left(\frac{x - x_0}{x + 1}\right)\right] \quad (24)$$

where

$$\left.\begin{aligned} x &= \sqrt{\left(\frac{z_\infty - s_2 - z}{z_\infty - s_1 - z}\right)} \\ x_0 &= \sqrt{\left(\frac{z_\infty - s_2}{z_\infty - s_1}\right)} \\ x_1 &= 1 \\ x_2 &= -1 \\ x_3 &= \sqrt{(s_2/s_1)} \\ x_4 &= -\sqrt{(s_2/s_1)} \end{aligned}\right\} \quad (25)$$

and A_1 to A_4, B_1 and B_2 are defined in the Appendix.

For pure non-competitive inhibition a simpler equation results:

$$t = \frac{1}{\rho^*}\left[z - \delta \cdot \ln\left(1 - \frac{z}{z_\infty}\right)\right] \quad (26)$$

with

$$\rho^* = \frac{\rho}{2[E]_t}\left(\sqrt{\{(K_I' + [I]_t - [E]_t)^2 + 4[E]_t K_I'\}} - (K_I' + [I]_t - [E]_t)\right) \quad (27)$$

While eqn. (26) characterizes the progress curves for tight-binding pure non-competitive inhibition where K_I is exactly equal to K_I', there are also several other situations when formally similar types of equations result. Thus, for $[I]_t = 0$, by using successively eqns. (23) and (20) and finally integrating eqn. (19), we obtain eqn. (28), which is the integrated form of the uninhibited reversible reaction:

$$t = (1/\rho)[z - \delta \cdot \ln(1 - z/z_\infty)] \quad (28)$$

The same equation (28) results when K_I and K_I' tend to ∞, that is for the absence of any inhibitory effects on the enzyme.

A similar type of equation is derived in the case of a classical inhibitor. Indeed, taking into account eqn. (8) and eqn. (18) and setting $[I]_f = [I]_t$, eqn. (2) integrates to:

$$t = (1/\rho^{app.})[z - \delta^{app.} \cdot \ln(1 - z/z_\infty)] \quad (29)$$

where

$$\left.\begin{aligned} \rho^{app.} &= \frac{\rho}{1 + b[I]_t} \\ \delta^{app.} &= \frac{\delta + a[I]_t}{1 + b[I]_t} \end{aligned}\right\} \quad (30)$$

Consider now the significance of ρ^* from eqn. (26) for a tight-binding pure non-competitive inhibitor. Eqn. (A11) from the Appendix gives the initial rate for this type of inhibition and shows that ρ^* is the maximum velocity for a given inhibitor concentration, that is $\rho^* = v_i(\zeta \to \infty)$. In other words, accounting for $\zeta(t = 0) = z_\infty = ([S]_o - [P]_o/K_e)/(1 + 1/K_e)$, ρ^* represents the initial velocity of the reaction at saturating values of substrate concentration in the presence of the inhibitor. Thus we can derive from eqn. (27):

$$\frac{[I]_t}{1 - (\rho^*/\rho)} = [E]_t + K_I' \frac{\rho}{\rho^*} \quad (31)$$

It is worth mentioning that the above equation can be equally well derived from the equation given by Henderson (1972) (a linearized form of eqn. 3), by substituting appropriately $\Sigma(N_i/K_i)$ and setting $[S]_o \to \infty$.

METHODS

Simulation of experimental progress curves

By using particular values for the various enzyme kinetic parameters, the initial concentrations of substrate and product and the total concentrations of enzyme and inhibitor, eqn. (24) or eqn. (26) may be used to calculate the time required to accumulate any amount of product which is less than z_∞. These times were taken as exact values, and the effect of experimental error was mimicked by adding to each product concentration a pseudo-random number taken from a normally distributed population with a mean of zero and a standard deviation proportional to the product concentration, followed by rounding the resultant value to four significant digits.

Non-competitive tight-binding inhibition simulated by using the following parameters and conditions: $V_f = 100$ mM/h, $V_r = 50$ mM/h, $K_s = 1$ mM, $K_p = 4.5$ mM, $K_I = 10$ nM and $K_I' = 40$ nM. $[S]_o$, $[P]_o$ and $[E]_t$ were fixed at 5 mM, 0 mM and 20 nM respectively, and three progress curves were simulated at inhibitor concentrations of 10, 30 and 50 nM. From each curve eight points were taken corresponding to expected product concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM, i.e. they are equally spaced between zero and the equilibrium concentration of 4.5 mM.

The competitive inhibition of bovine chymotrypsin A by aprotinin was simulated at 25 °C and pH 7.8 in Tris/HCl buffer containing 30% (v/v) methanol. Under these conditions, with benzoyl-L-tyrosine ethyl ester as substrate, the enzyme has the following characteristics: $K_s = 2.6$ mM, $V_f = 51.6$ μM/min at $[E]_t = 20$ nM, $V_r = 0$, $K_p \gg K_s$ and $K_I = 10$ nM (Folk & Schirmer, 1965; Lazdunski *et al.*, 1974). Progress curves were simulated with 2% error at $[S]_o = 100$ μM and 200 μM at $[I]_t$ values of 10, 30 and 50 nM. In addition, four curves without inhibitor were calculated at $[S]_o$ values of 1, 2, 3 and 4 mM. From each curve, nine points were collected, equally spaced from 10 to 90% substrate utilization.

Analysis of data

Eqn. (24) was fitted to the simulated experimental data for non-competitive inhibition by non-linear regression by using the BASIC program DNRP53 (Duggleby, 1984). K_I, K_I' and $[E]_t$ were considered as unknown parameters. Eqn. (24) expresses the time (t) as function of product concentration ([P]) (i.e. the independent variable versus the dependent one), but the correct way of regression analysis requires the expression of [P] as a function of t in order to minimize the sum of squares (SSQ) on the [P] axis. This was achieved by solving eqn. (24) by using a bracketing method (Duggleby & Ward, 1988), knowing that [P] must be between $[P]_o$ and $[P]_e$. The program gives the best-fit values and standard errors of the unknown parameters.

A similar procedure may be used to calculate the inhibition constant and the enzyme concentration for competitive inhibition and for pure non-competitive inhibition, except that in the latter case the fit is based on a solution of eqn. (26).

RESULTS AND DISCUSSION

The purpose in analysing the progress curves described either by eqn. (24) or by eqn. (26) is to determine the values of kinetic parameters. One of the major goals would be to calculate the inhibition constants K_I and/or K_I'. These parameters together with $[E]_t$ are directly related to the inhibition process, but eqns. (24) and (26) also contain several parameters characterizing the basic enzyme-catalysed reaction. We suggest that the calculation of parameters should be carried out in two main steps. The first one would involve determining the kinetic

parameters of the uninhibited reaction. In the second stage those parameters that are related to the inhibition would be determined.

Determination of kinetic parameters of the uninhibited reaction

By integrating the eqn. (1) in which N and D from eqn. (8) are substituted, we get eqn. (28), which is the well-known form of the integrated Michaelis–Menten-type equation (Duggleby, 1986). There are many reports related to the calculation of parameters of this equation. Although we would not recommend this approach, it could be done by linear regression on different linearized forms of eqn. (28). This method cannot be applied when z_∞ is unknown, so it is preferable to use non-linear regression to obtain the unknown parameters (e.g. Atkins & Nimmo, 1973; Fernley, 1974; Duggleby, 1984, 1986; Szedlacsek & Ostafe, 1987).

In the case of pure non-competitive inhibition the parameters ρ, δ and z_∞ thus obtained are sufficient for the subsequent use in the calculation of the inhibition constant (see eqns. 26 and 27). However, in the case of competitive, uncompetitive and non-competitive inhibition, an additional parameter of the uninhibited reaction is necessary in order to express a and b (see Table 1) in terms of these parameters. In fact, there are four parameters of the uninhibited reaction: V_f, V_r, K_s and K_p. These could be calculated as described by Duggleby & Wood (1989), and used to calculate ρ, δ and z_∞ if required. With this information at hand the inhibited progress-curve equations would be expressed as functions of parameters evaluated from the uninhibited reaction together with $[E]_t$ and the inhibition constant(s) K_I and/or K_I'. We consider first the problem of establishing the type of inhibition, then go on to look at the problem of calculating inhibition constants. Their determination is examined both for the general case and for the special case of pure non-competitive inhibition.

Distinction between different types of inhibition

The first problem in characterizing a tight-binding inhibitor is to ascertain the type of inhibition. We here accept that ρ, δ, z_∞ and V_f have been previously determined by processing the progress-curve data obtained for the uninhibited reaction. Thus the following procedure can be used.

For the inhibited reaction the progress curve is monitored and the resulting set of data is analysed by linear regression of t/z versus $\ln(1-z/z_\infty)/z$ (see eqn. 26). If this transformation of the progress-curve data is well described by a straight line, this means that we have to deal with a pure non-competitive tight-binding inhibition or a classical type of inhibition. In order to discriminate between them, we should make an additional study: $\rho^{app.}$ and $\delta^{app.}$ are calculated from the linear regression mentioned above and the dependence of $1/\rho^{app.}$ and of $\delta^{app.}/\rho^{app.}$ on $[I]_t$ is examined. The linearity of both representation proves (see eqns. 29 and 30) the existence of a classical-type inhibition. If these plots are non-linear, the inhibitor is of the pure non-competitive tight-binding type and K_I' and $[E]_t$ can be calculated as described below. It can be demonstrated that in the case of this type of inhibition both $1/\rho^{app.}$ and $\delta^{app.}/\rho^{app.}$ as functions of $[I]_t$ are constantly increasing, the curves being always concave-up.

If the plot of the transformed progress-curve data is not linear, it means that the inhibited reaction obeys the general eqn. (24). In this case, in order to decide which of the three possible types of inhibition occurs, it is possible to fit eqn. (24) to the set of data by non-linear regression.

If the absolute value of K_I' is very high relative to both K_I and the largest $[I]_t$, this suggests that we have a competitive inhibitor. The set of data should be fitted again to eqn. (24) but setting $b = 0$ and taking for a the corresponding expression from Table 1. An example of such a result was obtained by simulating the inhibition of bovine chymotrypsin A by aprotinin. Fitting eqn. (24) to the data gave $K_I = 9.86 \pm 0.26$ nM and $K_I' = 210 \pm 1061$ μM. Clearly the inhibition is dominated by the K_I component, and re-analysis as competitive inhibition gave an equally good fit.

If the absolute value of K_I is very high relative to both K_I' and the largest $[I]_t$, this suggests that the inhibition is uncompetitive. It is useful to refit the data to eqn. (24) but using for a and b the appropriate forms taken from Table 1. Finally, if K_I and K_I' are of comparable magnitudes, the inhibitor is non-competitive and no re-analysis is necessary.

Determination of inhibition constants for a competitive, uncompetitive or non-competitive inhibitor

At first sight, eqn. (24) seems to be quite complicated and the evaluation of parameters would be rather difficult. However, taking into account that many of the parameters can be determined from the uninhibited reaction, the fitting of progress-curve data by a non-linear-regression technique becomes feasible. Many attempts have been made to apply non-linear regression to progress-curve analysis (e.g. Atkins & Nimmo, 1973; Fernley, 1974; Duggleby, 1984, 1986; Kellershohn & Laurent, 1985). The majority of the authors have concluded that these non-linear procedures are reliable provided that some precautions have been taken [see, e.g., Atkins & Nimmo (1973) and Matyska & Kovar (1985)].

Despite of the complexity of eqn. (24), we have been successful in applying non-linear regression to the analysis of simulated progress curves referring to non-competitive tight-binding inhibitor. A typical example of the fit is shown in Table 2. Note that K_I and K_I' are of

Table 2. Analysis of simulated progress curves for a non-competitive tight-binding inhibitor

The progress curves with 1% error were simulated as described in the Methods section. Eqn. (24) was then fitted to these simulated data by fixing V_f, V_r, K_s and K_p equal to their 'correct' values while treating K_I, K_I' and $[E]_t$ as parameters to be estimated by regression.

Iteration	K_I (nM)	K_I' (nM)	$[E]_t$ (nM)	$10^3 \times$ SSQ
Initial	20.000	80.000	32.000	1190.603
1	5.777	37.898	2.426	1118.473
2	8.064	68.297	4.924	68.858
3	9.639	42.679	13.999	21.807
4	11.037	40.291	16.633	1.475
5	11.375	39.392	17.188	1.147
Final	11.366	39.361	17.274	1.146
Standard error	1.092	3.027	1.364	

comparable magnitudes because the inhibitor is non-competitive and no re-analysis as competitive or uncompetitive inhibition would be needed.

The final fitted values given in Table 2 were found to depend to some extent on the values chosen for the parameters associated with the uninhibited reaction: V_f, V_r, K_s and K_p. For example, when V_r and K_p were both decreased from their correct values by 10% (thereby maintaining an unchanged equilibrium constant for the reaction), the final fitted values of K_I, K_I' and $[E]_t$ were altered by +13.0%, −11.0% and +8.5% respectively.

We have also investigated the robustness of the calculation procedure to poor initial estimates of the parameters and found that fitting was successful over the following ranges: K_I, 4–60 nM; K_I', 9–150 nM; $[E]_t$, 1–70 nM. Table 2 illustrates one such trial, in which the initial estimates were each approximately double the final values. Thus initial estimates do not need to be particularly accurate, and simple guesswork will usually provide suitable starting values for the regression.

In some instances a starting value for $[E]_t$ could be obtained by active-site titration, and estimates of K_I, K_I' and $[E]_t$ may be determined from initial-velocity measurements as described by Henderson (1972). According to this method, when $[E]_t$ and $[S]_0$ are kept constant, a plot of $[I]_t/(1-v_i/v_0)$ against v_0/v_i is linear with a slope of $([S]_0+K_s)/(K_s/K_I+[S]_0/K_I')$ and an intercept (at $v_0/v_i=0$) equal to $[E]_t$. If the slope is evaluated at two concentrations of $[S]_0$ and knowing K_s, K_I and K_I' can be determined by numerical solution of a pair of simultaneous equations.

When eqn. (24) was fitted to error-free progress curves, the original parameters were recovered almost exactly. As the error level increases, the fitted parameters tend to get further from their true values and the estimated standard errors increase. To get a better idea of the effect of experimental error, 40 sets of data were simulated at 1% error. The overall mean of these 40 values of K_I was 10.577 nM with a standard deviation of 1.502 nM. The corresponding values for K_I' and $[E]_t$ are 40.204 ± 4.905 nM and 19.173 ± 2.071 nM respectively. The 'correct' values for K_I, K_I' and $[E]_t$ are 10 nM, 40 nM and 20 nM respectively.

Determination of inhibition constant for a pure non-competitive inhibitor

As mentioned above, eqn. (26) characterizes the progress curves for tight-binding pure non-competitive inhibition, where K_I is exactly equal to K_I'. The theory outlined above suggests a possible way to determine graphically approximate values for K_I' and $[E]_t$. Thus:

(i) For the inhibited reaction, t/z is plotted against $\ln(1-z/z_\infty)/z$. A straight line is obtained, because the following linearized form of eqn. (26) is valid:

$$\frac{t}{z}=\frac{1}{\rho^*}-\frac{\delta}{\rho^*}\cdot\frac{\ln(1-z/z_\infty)}{z} \qquad (32)$$

The progress curves plotted for several different values of $[I]_t$ will give thus the corresponding values of ρ^*.

(ii) Plotting $[I]_t/[1-(\rho^*/\rho)]$ against (ρ/ρ^*), the slope and the intercept on the $[I]_t/[1-(\rho^*/\rho)]$ axis, for the straight line obtained in accordance with eqn. (31), will give K_I' and $[E]_t$ respectively.

We would recommend that values for K_I' and $[E]_t$ are determined by fitting eqns. (26) and (27) to the progress-curve data by non-linear regression, perhaps using the approximate values determined graphically as initial estimates for the regression. We expect pure non-competitive inhibition would be quite rare and have not attempted fitting the relevant equations to simulated data, but the principle would be the same as that outlined for eqn. (24).

Conclusions

The general approach described here can be applied to both classical and tight-binding inhibition. Therefore, if we are not sure about the strength of inhibitor binding, we can assume it to be a tight-binding inhibitor and proceed as described above. Indeed, Morrison (1969) mentioned that the initial-velocity equation that characterizes the tight-binding inhibition (eqn. 3) reduces to the form of the classical-type inhibition if

$$[E]_t \ll D/\Sigma(N_i/K_i).$$

Thus, if we decide to use progress-curve analysis in characterizing an unknown inhibitor, it is useful to begin with the more general treatment described in the present paper. Clearly, if we conclude that our inhibitor is of a classical type, we can then re-analyse our experimental data by using simpler techniques (e.g. Waley, 1982).

In the present paper we have considered only the case of 'dead-end'-complex-forming inhibitors, often called 'linear' inhibitors. For the more complex case of 'hyperbolic' tight-binding inhibition, in which the enzyme–inhibitor complexes are still able to yield the reaction product, the equations given here are not valid. However, a method for determining inhibition constants from initial-velocity data in this situation has been reported (Szedlacsek *et al.*, 1988). For 'linear' tight-binding inhibitors, we prefer to use the progress-curve analysis instead of the initial-velocity measurements, because of the richness of data offered by the former.

This work was supported in part by C.I.M.C.–Bucharest and by the Australian Research Council.

REFERENCES

Ackermann, W. W. & Potter, V. R. (1949) Proc. Soc. Exp. Biol. Med. **72**, 1–9
Atkins, G. L. & Nimmo, I. A. (1973) Biochem. J. **135**, 779–784
Boeker, E. A. (1984) Biochem. J. **223**, 15–22
Cleland, W. W. (1963) Biochim. Biophys. Acta **67**, 104–137
Duggleby, R. G. (1984) Comput. Biol. Med. **14**, 447–455
Duggleby, R. G. (1985) Biochem. J. **228**, 55–60
Duggleby, R. G. (1986) Biochem. J. **235**, 613–615
Duggleby, R. G. (1988) Biochem. Med. Metab. Biol. **40**, 204–212
Duggleby, R. G. & Ward, L. C. (1988) Comput. Biol. Med. **18**, 245–251
Duggleby, R. G. & Wood, C. (1989) Biochem. J. **258**, 397–402
Fernley, H. N. (1974) Eur. J. Biochem. **43**, 377–378
Folk, J. E. & Schirmer, E. W. (1965) J. Biol. Chem. **240**, 181–192
Greco, W. R. & Hakala, M. T. (1979) J. Biol. Chem. **254**, 12104–12109
Henderson, P. J. F. (1972) Biochem. J. **127**, 321–333
Kellershohn, N. & Laurent, M. (1985) Biochem. J. **231**, 65–74
Lazdunski, M., Vincent, J.-P., Schweitz, H., Peron-Renner, M. & Pudles, J. (1974) Bayer Symp. **5**, 420–431
Matyska, L. & Kovar, J. (1985) Biochem. J. **231**, 171–177
Morrison, J. F. (1969) Biochim. Biophys. Acta **185**, 269–286

Sculley, M. J. & Morrison, J. F. (1986) Biochim. Biophys. Acta **874**, 44–53
Szedlacsek, S. E. & Ostafe, V. (1987) Rev. Roum. Biochim. **24**, 347–351
Szedlacsek, S. E., Ostafe, V., Serban, M. & Vlad, M. O. (1988) Biochem. J. **254**, 311–312
Waley, S. G. (1982) Biochem. J. **205**, 631–633
Williams, J. W. & Morrison, J. F. (1979) Methods Enzymol. **63**, 437–467
Williams, J. W., Morrison, J. F. & Duggleby, R. G. (1979) Biochemistry **18**, 2567–2573
Williams, J. W., Duggleby, R. G., Cutler, R. & Morrison, J. F. (1980) Biochem. Pharmacol. **29**, 589–595

APPENDIX

Integration of eqn. (17)

Competitive, uncompetitive and non-competitive inhibition. As mentioned, we have here $s_1 \neq s_2$, both having real and negative values (as $p_4/p_2 > 0$) and $-p_3/p_2 < 0$).

Let us define a new dependent variable x by:

$$\sqrt{(p_2\zeta^2+2p_3\zeta+p_4)} = \sqrt{(p_2)}(\zeta-s_1)x \qquad \text{(A1)}$$

i.e.

$$x = \sqrt{\left(\frac{\zeta-s_2}{\zeta-s_1}\right)} \quad \text{and} \quad d\zeta = \frac{2x(s_2-s_1)}{(x^2-1)^2}dx \qquad \text{(A2)}$$

Substituting ζ and $d\zeta$ from eqns. (A2) into eqn. (19) we get:

$$\frac{dt}{dx} = q_0\frac{x(q_1x^2+q_2x+q_3)}{(x-x_1)^2(x-x_2)^2(x-x_3)(x-x_4)} \qquad \text{(A3)}$$

where

$$\left.\begin{aligned} x_1 &= 1\\ x_2 &= -1\\ x_3 &= \sqrt{(s_2/s_1)} = r\\ x_4 &= -r \end{aligned}\right\} \qquad \text{(A4)}$$

and

$$\left.\begin{aligned} q_0 &= (1-r^2)/\rho\\ q_1 &= p_5s_1+p_6\\ q_2 &= \sqrt{(p_2)}(s_1-s_2)\\ q_3 &= -(p_5s_2+p_6) \end{aligned}\right\} \qquad \text{(A5)}$$

Eqn. (A3) can be written as:

$$\frac{dt}{dx} = \frac{1}{2\rho}\left(\sum_{i=1}^{4}\frac{A_i}{x-x_i}+\frac{B_1}{(x-x_1)^2}+\frac{B_2}{(x-x_2)^2}\right) \qquad \text{(A6)}$$

The expressions of coefficients A_i, B_1 and B_2 are:

$$\left.\begin{aligned} A_1 &= \frac{r^2(2q_1+q_2)+q_2+q_3}{2(r^2-1)}\\ A_2 &= \frac{r^2(2q_1-q_2)-q_2+2q_3}{2(r^2-1)}\\ A_3 &= \frac{q_1r^2+q_2r+q_3}{r^2-1}\\ A_4 &= \frac{q_1r^2-q_2r+q_3}{r^2-1}\\ B_1 &= \frac{q_1+q_2+q_3}{2}\\ B_2 &= \frac{q_1-q_2+q_3}{2} \end{aligned}\right\} \qquad \text{(A7)}$$

Now, integrating eqn. (A6) over time from 0 to t we obtain:

$$t = \frac{1}{2\rho}\left[\sum_{i=1}^{4}A_i\cdot\ln\left(\frac{x-x_i}{x_0-x_i}\right)+\left(\frac{B_1}{x_0-1}\right)\left(\frac{x-x_0}{x-1}\right)+\left(\frac{B_2}{x_0+1}\right)\left(\frac{x-x_0}{x+1}\right)\right] \qquad \text{(A8)}$$

where x_0 is the value of x when $t = 0$:

$$x_0 = \sqrt{\left(\frac{z_\infty-s_2}{z_\infty-s_1}\right)} \qquad \text{(A9)}$$

Pure non-competitive inhibition. We observe from Table 1 that in this case a and b have particular forms, i.e.:

$$\left.\begin{aligned} a &= \delta/K_I'\\ b &= 1/K_I' \end{aligned}\right\} \qquad \text{(A10)}$$

Introducing these values in eqn. (20) and using the values of p_1 to p_6 so obtained, eqn. (19) becomes:

$$\frac{dt}{d\zeta} = -\frac{1}{\rho^*}\cdot\frac{\zeta+\delta}{\zeta} \qquad \text{(A11)}$$

where

$$\rho^* = \frac{\rho}{2[E]_t}\left(\sqrt{\{(K_I'+[I]_t-[E]_t)^2+4[E]_tK_I'\}}-(K_I'+[I]_t-[E]_t)\right) \qquad \text{(A12)}$$

Eqn. (A11) can be integrated directly, giving:

$$t = \frac{1}{\rho^*}\left[z_\infty-\zeta+\delta\cdot\ln\left(\frac{z_\infty}{\zeta}\right)\right] \qquad \text{(A13)}$$

Substituting ζ for $(z_\infty - z)$ yields eqn. (26).

Received 20 March 1989/10 August 1989; accepted 16 August 1989