Graphical and statistical analysis of hyperbolic tight-binding inhibition

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Equations are described for the analysis of enzyme-catalysed reactions in the presence of hyperbolic tight-binding inhibitors using both graphical and non-linear-regression methods. The general character of the equations allows the interpretation of several other kinetic mechanisms. As an example, the tight-binding hyperbolic non-competitive inhibition of human leucocyte elastase by a polysulphated glycosaminoglycan is analysed.

INTRODUCTION

An enzyme inhibitor, *I*, is classified as linear or hyperbolic depending on the shape of a reciprocal-velocity-versus-*[I]* plot. The inhibitor is called 'linear' or 'hyperbolic' depending on this plot (or similar) being a straight line or a hyperbola respectively. A linear inhibitor will drive the velocity of the enzyme-catalysed reaction to zero under saturating conditions, whereas in a hyperbolic system an infinitely high *[I]* will convert the enzyme into a modified, but still functional, complex, ESI, with a decreased rate of product formation. A graphical procedure, the specific-velocity plot, has been developed to facilitate the analysis of hyperbolic-inhibition mechanisms (Baici, 1981). This method can not be applied when the inhibitors are tightly bound to the enzyme, a feature in common with other equations used in the analysis of steady-state kinetics. In fact, if the association constant for the formation of the EI complex is high, a significant fraction of *I* may become bound to the enzyme. The application of the steady-state equations is then invalid, because the assumption that the concentration of inhibitor that is free in solution is the same as the total inhibitor concentration is no longer true (Morrison, 1969; Williams & Morrison, 1979).

The kinetic properties of tight-binding inhibitors have been discussed by several authors (Straus & Goldstein, 1943; Ackermann & Potter, 1949; Goldstein, 1944; Reiner, 1959). The interest of these reports was essentially qualitative. A profound treatment of this subject was undertaken by Morrison (1969), who derived a general steady-state rate equation that allows for the combination of a tight-binding inhibitor with one or more enzyme forms. Henderson (1972) rearranged Morrison's (1969) equation to facilitate the calculation of the kinetic parameters using graphical procedures. In principle, Morrison's (1969) and Henderson's (1972) equations can be applied to any kinetic mechanism, including multi-substrate reactions. The particular case of hyperbolic inhibition was not considered explicitly by these authors, in which case the equations contain additional terms.

During the investigation of the inhibition mechanism of human leucocyte elastase by polysulphated glycos-

aminoglycans it was found that some of the compounds tested behaved as hyperbolic tight-binding inhibitors (Baici *et al.*, 1980). Owing to the lack of adequate methods, *K_i* was expressed as an approximate order of magnitude. The present paper describes the derivation of equations, based on the general modifier mechanism (Botts & Morales, 1953), which allow the analysis of linear and hyperbolic tight-binding inhibitors by graphical means and by non-linear regression.

MATERIALS AND METHODS

Enzyme and assays

Human leucocyte elastase (EC 3.4.21.37) was isolated from polymorphonuclear leucocytes as detailed elsewhere (Baici *et al.*, 1982) and the enzyme active sites were determined by titration with azapeptides as described by Powers & Gupton (1977). The enzyme was assayed with Suc-Ala-Ala-Pro-Val-NPhNO₂ as substrate (Bachem, Bubendorf, Switzerland) in 50 mM-sodium/potassium phosphate buffer, *I* 0.15, pH 7.40 at 25 ± 1 °C. Steady-state velocities were determined by monitoring the release of *p*-nitroaniline at 410 nm ($\epsilon = 8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) with a Uvikon 810 spectrophotometer programmed for calculating initial slopes by means of linear regression of absorption data. GAGPS (Arteparon) was obtained from Luitpold-Werk, Munich, Germany, and its molecular size was determined by analytical ultracentrifugation. The properties of this compound were discussed previously (Baici *et al.*, 1980).

Analysis of kinetic data

Weighted non-linear regression analysis of initial-velocity data was performed by using the program described by Duggleby (1984) (see also the Theory section). According to Mannervik *et al.* (1986), the observed experimental variance in spectrophotometric assays of enzyme activity is primarily due to errors in determination of concentrations of substrate and inhibitor and not to error in measuring the velocity. Therefore the weighting factors were based on the experimental variance determined from replicate measurements

Abbreviations used: Suc-Ala-Ala-Pro-Val-NPhNO₂, succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine 4-nitroanilide; GAGPS, glycosaminoglycan polysulphate.

($n = 9$). This method was preferred to the assumptions of constant absolute or constant relative error.

THEORY

Reversible linear tight-binding inhibition

The treatment shown below is based on the assumption that the rate of establishment of equilibrium between E, I and EI is fast, i.e. 'tight-binding inhibition' is considered, not 'slow, tight-binding inhibition', in which case other equations apply (Cha, 1980; Morrison, 1982). The rate equation can be expressed as follows (Morrison, 1969):

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

where N is a term containing the rate constants that determine the maximum velocity of the reaction, together with the concentrations of substrates, N_i is a term(s) in the denominator of the rate equation that represent the form(s) of the enzyme with which the inhibitor reacts, K_i is the dissociation constant(s) of the EI complex(es), $[E]_t$ is the total concentration of the enzyme, $[I]_t$ is the total concentration of the inhibitor, D is the denominator of the rate equation in the absence of inhibitor and v terms are initial velocities.

Eqn. (1) was rearranged by Henderson (1972) into the following linear form:

$$\frac{[I]_t}{\left(1 - \frac{v_i}{v_0}\right)} = [E]_t + \frac{D}{\Sigma(N_i/K_i)} \cdot \frac{v_0}{v_i} \quad (2)$$

According to eqn. (2), a plot of $[I]_t/[1 - (v_i/v_0)]$ versus v_0/v_i is linear, with the intercept on the ordinate equal to $[E]_t$ and slope varying according to the mechanism. The major objection to eqn. (2) is that the dependent variable v_i appears on both sides of the equation, thus precluding the use of regression analysis (Williams & Morrison, 1979). In fact, the errors in the dependent and independent variables are related, and hence a correct weighting factor cannot be assigned (Johansen & Lumry, 1961). Therefore it is inappropriate to make a least-squares fit of a set of experimental data to eqn. (2). This problem has been considered by Henderson (1973) himself, but the computer program did not actually minimize the residuals of squares, as already pointed out by Williams & Morrison (1979). Nevertheless, Henderson's (1972) plot has a powerful diagnostic value and is an indispensable tool for a preliminary study of the kinetics of tight-binding inhibitors and to obtain initial estimates of the kinetic parameters to be used in non-linear regression analysis.

Reversible hyperbolic tight-binding inhibition

For the derivation of a general equation it is convenient to focus attention on the general modifier mechanism (Botts & Morales, 1953) shown in Scheme 1:

Scheme 1.

This mechanism adequately describes the inhibition patterns of a single substrate reaction, but the equations derived can be extended to multi-substrate reactions.

The derivation is started on the basis of the following equation [reported as eqn. (4) by Baici, 1981] and rearranging with simple algebra ($\sigma = [S]/K_m$):

$$\frac{v_i}{v_0} = \frac{(1 + \sigma) \left(1 + \beta \frac{[I]}{\alpha K_1}\right)}{\sigma \left(1 + \frac{[I]}{\alpha K_1}\right) + 1 + \frac{[I]}{K_1}} \quad (3)$$

$$[I] = \frac{(1 + \sigma)(1 - v_i/v_0)}{\frac{v_i}{v_0} \left[\frac{\sigma}{\alpha K_1} + \frac{1}{K_1} \right] - \frac{\beta}{\alpha K_1}(1 + \sigma)} \quad (4)$$

$[I]$ in eqn. (4) represents the concentration of free inhibitor, i.e. the fraction of the total inhibitor which is not bound to the enzyme. The conservation equation for the inhibitor (Henderson, 1972) is given by:

$$[I]_t = \Sigma[E_i I] + [I]_f = [E]_t (1 - v_i/v_0) + [I]_f \quad (5)$$

where the subscripts t and f mean total and free respectively and $E_i I$ are the enzyme-inhibitor complexes. Substituting the expression of $[I]_f$ given by eqn. (4) into eqn. (5) and rearranging in a way similar to eqn. (2) we obtain:

$$\frac{[I]_t}{1 - \frac{v_i}{v_0}} = [E]_t + \frac{(1 + \sigma) \frac{v_0}{v_i}}{\frac{1}{K_1} + \frac{\sigma}{\alpha K_1} - \frac{\beta(1 + \sigma)}{\alpha K_1} \frac{v_0}{v_i}} \quad (6)$$

The novel aspect brought by eqn. (6) is the fact that a Henderson plot of the data is no longer linear, since the ratio v_0/v_i is present both in the numerator and in the denominator on the right side of the equation, which is a hyperbola. Direct graphical analysis is precluded in this case. A convenient transformation of eqn. (6) into a linear form can be achieved by shifting $[E]_t$ to the left side of the equation and taking the double-reciprocal form:

$$\frac{1}{\left(\frac{[I]_t}{1 - v_i/v_0} - [E]_t \right)} = \frac{\alpha + \sigma}{\alpha K_1 (1 + \sigma)} \cdot \frac{v_i}{v_0} - \frac{\beta}{\alpha K_1} \quad (7)$$

β and αK_1 can be obtained graphically. This method has obviously the same statistical limitations discussed above for eqn. (2). Therefore the graphical method can be used as a source of preliminary information and for obtaining initial estimates of the parameters, which will be refined by least-squares fit (see below). The correct use of eqn. (7) supposes a precise knowledge of $[E]_t$. If this is not the case, the plots will be curved, as shown in Fig. 1.

Statistical treatment of hyperbolic tight-binding inhibition

Eqn. (6) can be converted into a quadratic expression in v_i , thus producing an equation analogous to eqn. (1), which is more suitable for computer fitting. The transformation is easy and requires only algebraic manipulation of eqn. (6): the terms containing v_i and v_0

are grouped and the collected terms are equated to zero. The final expression is given by:

$$v_i^2 - v_i \left\{ v_0 \left[\frac{1+\sigma}{\alpha+\sigma} \left(\beta - \frac{\alpha K_1}{[E]_t} \right) + 1 - \frac{[I]_t}{[E]_t} \right] \right\} - v_0^2 \left\{ \frac{1+\sigma}{\alpha+\sigma} \left[\frac{\beta([I]_t - [E]_t) + \alpha K_1}{[E]_t} \right] \right\} = 0 \quad (8)$$

The solution of the quadratic eqn. (8) is:

$$v_i = \frac{v_0}{2} \left\{ \left[\frac{1+\sigma}{\alpha+\sigma} \left(\beta - \frac{\alpha K_1}{[E]_t} \right) + 1 - \frac{[I]_t}{[E]_t} \right] \pm \sqrt{\left(\left[\frac{1+\sigma}{\alpha+\sigma} \left(\beta - \frac{\alpha K_1}{[E]_t} \right) + 1 - \frac{[I]_t}{[E]_t} \right)^2 - 4 \left\{ \frac{1+\sigma}{\alpha+\sigma} \left[\frac{\beta([I]_t - [E]_t) + \alpha K_1}{[E]_t} \right] \right\}} \right\} \quad (9)$$

Notice that in eqn. (9) only the + sign gives a solution with physical meaning. Eqns. (8) and (9) are general expressions, not restricted to hyperbolic inhibition; according to Scheme 1, with appropriate values of α and β , the equations can describe several mechanisms, either linear or hyperbolic.

Experimental data can be fitted to eqn. (9) by means of weighted non-linear regression. A versatile computer program for this kind of analysis was developed by Duggleby (1984). The program, named DNRP53, uses the matrix solution subroutine popularized by Cleland (1967) and was modified to use the Marquardt's (1963) algorithm. The program also provides an approximation of the partial derivatives of the fitted function to be evaluated by numerical differentiation. The program listing was given in full by Duggleby (1984) and the expression of eqn. (9) in BASIC language, to be inserted into program lines 5100–5130, is as follows:

```
5100 G1 = (1 + X(2))/(B(5) + X(2))
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5110 G2 = G1*(B(3) - B(5)*B(2)/B(1)) + 1 - X(1)/B(1)
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5120 G3 = 4*(G1*(B(3)*(X(1) - B(1)) + B(5)*B(2))/B(1))
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5130 G = .5*B(4)*(G2 + SQR(G2^2 + G3))
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where $B(1) = [E]_t$, $B(2) = K_1$, $B(3) = \beta$, $B(4) = v_0$, $B(5) = \alpha$, $X(1) = [I]_t$, and $X(2) = \sigma$. The kinetic parameters to be fitted are α , β and K_1 . Strictly speaking, $[E]_t$ and v_0 are constants. However, they are also considered as parameters for two reasons: (1) one may wish to know the extent to which the parameter estimates are dependent on the values of $[E]_t$ and v_0 ; (2) this procedure simplifies the use of the program. In fact, one very useful feature of DNRP53 is the option of treating one or more parameters as fixed constants, rather than as parameters to be estimated, by 'masking' them. This method avoids the need of entering each time the specific values of the constants into program lines 5100–5130 and saves time when re-running the program with modified parameters to check a fit with particular mechanisms. As an example, a set of experimental data can be fitted to eqn. (9), first assuming a non-competitive mechanism [this is done by setting $B(5) = \alpha = 1$ and masking this value] and then assuming a competitive mechanism [done by entering for $B(5)$ a large value, say 10^8 , and masking it]. The weighting factors can be supplied to the program as experimentally determined standard deviations (option S).

RESULTS AND DISCUSSION

As a support for the theoretical treatment shown above, a practical example will now be analysed. The inhibition of human leucocyte elastase by GAGPS has been investigated from a qualitative point of view (Baici *et al.*, 1980). It was established that the interaction between GAGPS and the enzyme is electrostatic, with multiple binding, and that the mechanism is of the tight-binding type. This is due to the particular structure of GAGPS, which possesses, on an average, three to four negatively charged sulphate groups per disaccharide unit. Owing to the high content of arginine residues, leucocyte elastase is a highly basic protein, with pI near 10 (Janoff & Basch, 1971). Thus an electrostatic interaction occurring between the sulphate groups of GAGPS and the positively charged guanidinium groups located on the surface of the enzyme was hypothesized (see, in particular, Fig. 3 in Baici *et al.*, 1980). Recently the three-dimensional structure of leucocyte elastase was published (Bode *et al.*, 1986) and the arginine residues were shown to be located at the enzyme surface (W. Bode, personal communication).

Fig. 1. Inhibition of human leucocyte elastase by GAGPS

The M_r of GAGPS was 6500 and the concentration used for calculations was twice the molar concentration (stoichiometry 1:2 with the enzyme). Triplicate steady-state-velocity measurements were performed with three concentrations of the substrate (0.25, 0.7 and 2.0 mM). The titrated enzyme-active-site concentration was 29 ± 2 nM. Initial-velocity data were plotted according to eqn. (7a), using the titrated enzyme concentration ($\bullet$). The straight line, drawn by hand, has abscissa and ordinate intercepts equal to β and $-\beta/K_1$ respectively, from which $\beta \cong 0.32$ and $K \cong 12$ nM. If the enzyme concentration used for calculations does not coincide with the effective concentration, curved plots are obtained: $\square$, $[E]_t = 40$ nM; $\circ$, $[E]_t = 15$ nM.

In the present study an oligodisperse GAGPS fraction, $M_r = 6500$, was used. This fraction has a stoichiometry of 1:2 with leucocyte elastase, meaning that one inhibitor molecule binds two enzyme molecules (Baici *et al.*, 1980). Owing to this fact the effective concentration of the inhibitor corresponds to twice its concentration in mol/litre. Steady-state velocities at various substrate and inhibitor concentrations were measured and analysed, as a preliminary approach, by means of eqn. (6) (not shown). The curve was hyperbolic, and it was established that the v_0/v_i ratio at any inhibitor concentration was independent of the substrate concentration. These features are typical of hyperbolic non-competitive inhibition, which means $\alpha = 1$ and $0 < \beta < 1$ in Scheme 1. Preliminary estimates of β and K_i were obtained by plotting the data according to eqn. (7) as shown in Fig. 1. In the particular case considered, eqns. (6), (7) and (9) simplify to eqns. (6a), (7a) and (9a) respectively:

$$\frac{[I]_t}{1 - v_i/v_0} = [E]_t + \frac{K_i v_0/v_i}{1 - \beta v_0/v_i} \quad (6a)$$

$$\left(\frac{[I]_t}{1 - v_i/v_0} - [E]_t \right)^{-1} = \frac{1}{K_i} \frac{v_i}{v_0} - \frac{\beta}{K_i} \quad (7a)$$

$$v_i = \frac{v_0}{2} \left(\left[1 + \beta - \frac{[I]_t + K_i}{[E]_t} \right] \pm \sqrt{\left[1 + \beta - \frac{[I]_t + K_i}{[E]_t} \right]^2 + 4 \left[\frac{\beta([I]_t - [E]_t) + K_i}{[E]_t} \right]} \right) \quad (9a)$$

Fig. 1 also illustrates that a straight line can be obtained only if $[E]_t$ corresponds exactly to the effective concentration of enzyme which combines with the inhibitor. If a higher enzyme concentration is used for calculations the plot is curved upwards, whereas a lower enzyme concentration produces plots which are curved downwards. Thus, if $[E]_t$ is not accurately known, a trial-and-error procedure can be applied until a straight

Fig. 2. Inhibition of human leucocyte elastase by GAGPS

The data are the same as in Fig. 1 but are plotted as dose-response curve. The bars represent ± 1 s.d. and the curve was fitted by weighted non-linear regression analysis by using eqn. 9a and the preliminary values of β and K_i obtained from Fig. 1. Each point represents the mean of replicate measurements ($n = 9$).

line is obtained during graphical analysis of the data. The preliminary estimates of β and K_i were further used for a statistical refinement by non-linear regression analysis (see the Theory section) using eqn. (9a), as shown in Fig. 2. The expression of eqn. (9a) to be used in the computer program is in this case:

$$5100 \text{ G1} = 1 + \text{B}(3) - (\text{X}(1) + \text{B}(2))/\text{B}(1)$$

$$5110 \text{ G2} = 4 * (\text{B}(3) * (\text{X}(1)/\text{B}(1) - 1) + \text{B}(2)/\text{B}(1))$$

$$5120 \text{ G} = .5 * \text{B}(4) * (\text{G1} + \text{SQR}(\text{G1}^2 + \text{G2}))$$

Considering $[E]_t$ and v_0 as constants (equal to 29 nM and 33.5 nM/s respectively) and β and K_i as parameters, the best-fit values $\pm$ s.d. were $\beta = 0.31 \pm 0.01$ and $K_i = 13.2 \pm 0.8$ nM (Fig. 2). This value of K_i was calculated considering the stoichiometry of 1:2 for the inhibitor-enzyme complex. If the molar concentration of GAGPS is considered, then $K_i = 6.6$ nM. Considering also $[E]_t$ and v_0 as parameters to be fitted, the following results were obtained: $[E]_t = 22.2 \pm 8.9$ nM, $v_0 = 34.5 \pm 0.6$ nM/s, $\beta = 0.30 \pm 0.02$ and $K_i = 13.8 \pm 3.0$ nM. Despite relatively large s.d. for the fitted values of $[E]_t$ and v_0 , the values of the kinetic parameters β and K_i are very similar with both procedures used.

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