A graphical method for determining inhibition parameters for partial and complete inhibitors

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A new simple graphical method is described for the determination of inhibition type and kinetic parameters of an enzyme reaction without any replot. The method consists of plotting experimental data as $v/(v_0 - v)$ versus the reciprocal of the inhibitor concentration at different substrate concentrations, where v and v_0 represent the velocity in the presence and in the absence of the inhibitor respectively with a given concentration of the substrate. Partial inhibition gives straight lines that converge on the abscissa at a point away from the origin, whereas complete inhibition gives lines that go through the origin. The inhibition constants of enzymes and the reaction rate constant of the enzyme–substrate–inhibitor complex can be calculated from the abscissa and ordinate intercepts of the plot. The relationship between the slope of the plot and the substrate concentration shows characteristic features depending on the inhibition type: for partial competitive inhibition, the straight line converging on the abscissa at $-K_s$, the dissociation constant of the enzyme–substrate complex; for non-competitive inhibition, a constant slope independent of the substrate concentration; for uncompetitive inhibition, a hyperbola decreasing with the increase in the substrate concentration; for mixed-type inhibition, a hyperbola increasing with the increase in the substrate concentration. The properties of the replot are useful in confirmation of the inhibition mechanism.

INTRODUCTION

Enzyme-catalysed reactions are usually characterized by the kinetic parameters $V_{\max}$ and K_m , which can be related mathematically to the steady-state concentration of the enzyme–substrate (ES) complex and its rate of breakdown. The aim of enzyme kinetics is to find K_m and $V_{\max}$ for substrate, and to determine the inhibition constants K_i . Enzyme inhibition is divided into two types: complete and partial inhibition are also known as linear and hyperbolic inhibition, because plots of reciprocal velocity versus the inhibitor concentration give a straight line and a hyperbola respectively [1]. Various graphical methods have been developed to enable experimental workers to analyse complete enzyme inhibitors, which decrease the enzymic velocity to zero when in excess [1]. On the other hand, there are few analytical methods for investigation of the action of partial inhibitors, which will convert the enzyme into a modified, but still functional, enzyme–substrate–inhibitor complex with a decreased rate of product formation at an infinitely high concentration. Simple diagnostic methods distinguishing partial inhibition from complete inhibition type have hitherto not been presented. Partial inhibition is usually analysed by the replot of the primary reciprocal plot [2]. However, small errors in the determination of v are magnified, particularly at low substrate concentrations, when reciprocals are taken. Slope and intercept replots further magnify errors in the determination of kinetic constants. The present paper describes a new graphical procedure for analysis of enzyme inhibition, which is applicable to partial inhibition and complete inhibition, including competitive, non-competitive, uncompetitive and mixed-type inhibition. By simple inspection of the fractional velocity plot it is possible to see whether the inhibition is partial or complete, and one can calculate K_i values and

the reaction rate constants of the partial and complete inhibition from the slope and intercepts of the plot.

THEORY AND MODEL

We now derive a general equation for inhibition and activation effects. The modifier I is used in a general sense and takes into account both activators and inhibitors. Partial or hyperbolic mixed inhibition includes all of the common types of inhibition as asymptotic or special cases, and it is therefore taken as a general case. We may write the following reaction scheme:

Here it is assumed that the effect is not only on affinity but also on the rate of the breakdown of the ES complex; thus the ligand would influence the enzyme activity in two ways, affecting $V_{\max}$ as well as K_m . It is formally convenient to regard K_s , K'_s , K_i and K'_i as the dissociation constants of the enzyme–ligand complexes as described in eqn. (1). The ligand also affects the $V_{\max}$, that is to say the EIS complex is assumed to break down at a different velocity from the ES complex, and the rate constants k and k' apply to the breakdown of the ES complex and EIS complex respectively. The overall velocity will be given as follows:

$$v = k[\text{ES}] + k'[\text{EIS}] \quad (2)$$

A rate equation for the reaction scheme presented in eqn. (1) is derived according to the rapid-equilibrium method:

$$\frac{v}{V_{\max.}} = \frac{[1 + ([I]/K_1') \cdot (k'/k)] \cdot ([S]/K_s)}{(1 + [I]/K_1') \cdot \left([S]/K_s + \frac{1 + ([I]/K_1') \cdot (K_s'/K_s)}{1 + [I]/K_1'} \right)} \quad (3)$$

where $[I]$ and $[S]$ are the concentrations of modifier and substrate respectively. The relationship among the four dissociation constants of the enzyme-ligand complexes is $K_1'/K_1 = K_s'/K_s$.

Eqn. (3) is represented in the reciprocal form thus:

$$\frac{V_{\max.}}{v} = \frac{(1 + [I]/K_1') \cdot \left([S]/K_s + \frac{1 + ([I]/K_1') \cdot (K_s'/K_s)}{1 + [I]/K_1'} \right)}{([S]/K_s) \cdot [1 + ([I]/K_1') \cdot (k'/k)]} \quad (4)$$

Eqn. (4) can be rearranged writing v_0 for $(V_{\max.} \cdot [S]/K_s) / (1 + [S]/K_s)$, where v_0 is the velocity that can be attained in the presence of a given concentration of the substrate without the inhibitor:

$$\frac{v}{v_0 - v} = \frac{(1 + [S]/K_s)(K_1'/[I] + k'/k)}{(1 - k'/k) \cdot ([S]/K_s) + (K_s'/K_s - k'/k)} \quad (5)$$

Eqn. (5) gives a straight-line relationship between $v/(v_0 - v)$ and the reciprocal of the inhibitor concentration: the straight lines converge on the abscissa at $-(1/K_1') \cdot (k'/k)$. I term this plot the fractional-velocity plot. Partial inhibition yields straight lines that do not converge on the origin because $k' \neq 0$; however, complete inhibition gives straight lines that go through the origin since the EIS complex cannot break down ($k' = 0$).

TYPES OF THE INHIBITION AND REPLOTS OF SLOPES VERSUS THE SUBSTRATE CONCENTRATIONS

Partial and complete competitive inhibition

In competitive inhibition, the substrate binds to free enzyme with a greater affinity than to the EI complex ($K_s' > K_s$), and the ES and EIS complexes both yield product with equal facility ($k = k'$). This situation is generally called partial competitive inhibition or hyperbolic competitive inhibition [1]. Eqn. (5) can be reduced as follows:

$$\frac{v}{v_0 - v} = \frac{1 + [S]/K_s}{1/K_1 - 1/K_1'} \cdot \left(\frac{1}{[I]} + \frac{1}{K_1'} \right) \quad (6)$$

The relationship between $v/(v_0 - v)$ and $1/[I]$ gives straight lines converging on the abscissa at $-1/K_1'$ at different concentrations of the substrate (Fig. 1a). In complete competitive inhibition, binding of the substrate and the inhibitor to the enzyme is mutually exclusive; $K_1' = \infty$ and $K_s' = 0$. Thus we have the following equation for the pure competitive inhibition:

$$\frac{v}{v_0 - v} = (1 + [S]/K_s) \cdot K_1 \cdot \frac{1}{[I]} \quad (7)$$

Eqn. (7) gives straight lines converging on the origin regardless of the substrate concentration (Fig. 1b).

When the inhibition is competitive type ($k' = k$), one can calculate the K_1' value from the abscissa intercept of the fractional-velocity plot. K_1 value is determined from the slope by using the K_1' value.

Fig. 1. Fractional-velocity plots for partial (a) and complete (b) competitive inhibition

The lines were drawn in accordance with eqn. (6). The following values of parameters were used: $K_s = 1$, $K_1 = 0.005$, $K_1' = 0.05$ and $K_s' = 10$ for the partial competitive inhibition or $K_s' = \infty$ for the complete competitive inhibition. The substrate concentration is indicated by each line.

The slope of eqn. (6) is given as the following form:

$$\text{Slope} = \frac{1 + [S]/K_s}{1/K_1 - 1/K_1'} \quad (8)$$

Eqn. (8) is a linear function of the substrate concentration, and provides a straight line converging on the abscissa at $-K_s$ (Fig. 2). The replot may be useful in diagnosis or confirmation of competitive inhibition including the partial and the complete types.

Partial and complete non-competitive inhibition

In non-competitive inhibition, both the substrate and inhibitor bind to the enzyme at completely independent sites, and the affinity of the substrate (inhibitor) is unaffected by the inhibitor (substrate); $K_s = K_s'$ and $K_1 = K_1'$. The EIS complex is inactive in complete non-competitive inhibition ($k' = 0$), but when the inhibitor is of the partial non-competitive type the complex does break down, though more slowly than does ES ($0 < k'/k < 1$). We have the following equation for the non-competitive inhibition:

$$\frac{v}{v_0 - v} = \frac{1}{1 - k'/k} \cdot (K_1/[I] + k'/k) \quad (9)$$

Eqn. (9) converts a series of inhibition curves obtained with different substrate concentrations into a single straight line converging on the abscissa at $-(1/K_i) \cdot (k'/k)$, and the k'/k values can be calculated from the ordinate intercept of $(k'/k)/(1-k'/k)$. The K_i value is readily calculated from the slope by using the k'/k values. If the inhibition is of the complete non-competitive type, where $k' = 0$, the line goes through the origin (Fig. 3). Non-competitive inhibition is confirmed by constant slope of the plot irrespective of the change in substrate concentration.

Fig. 2. Replot of the slope of the fractional-velocity plot for competitive inhibition

The relationship between the slope and the substrate concentration is calculated with eqn. (8) by using the values of kinetic parameters indicated in the legend to Fig. 1.

Fig. 3. Fractional-velocity plots for partial and complete non-competitive inhibition

Lines A and B indicate the partial and complete non-competitive inhibitors respectively. The lines were drawn in accordance with eqn. (9). The following values of parameters were used: $K_s = K_s' = 1$, $K_i = K_i' = 0.05$ and $k'/k = 0.5$ for the partial non-competitive inhibition or $k'/k = 0$ for the complete non-competitive inhibition.

Partial and complete uncompetitive inhibition

Uncompetitive inhibitor does not combine with the free enzyme, but only with the ES complex; $K_i = \infty$ and then $K_s' = 0$. Eqn. (5) can be rearranged into the following form:

$$\frac{v}{v_0 - v} = \frac{1 + [S]/K_s}{(1 - k'/k) \cdot ([S]/K_s) - k'/k} \cdot (K_i'/[I] + k'/k) \quad (10)$$

A relationship of $v/(v_0 - v)$ with $1/[I]$ shows a straight line converging at $-(k'/k) \cdot (1/K_i')$ (Fig. 4a). When the K_s value is determined by certain graphical methods [3], one can obtain the k'/k value from the ordinate intercept. The K_i' value can be calculated from the abscissa intercept by using the k'/k value determined. Complete uncompetitive inhibition gives straight lines that go through the origin (Fig. 4b).

The slope of the fractional-velocity plot is a hyperbolic function with respect to the substrate concentration as follows:

$$\text{Slope} = \frac{(1 + [S]/K_s) \cdot K_i'}{(1 - k'/k) \cdot [S]/K_s - k'/k} \quad (11)$$

This hyperbola has asymptotes at $K_s \cdot (k'/k)/(1 - k'/k)$ and $K_i'/(1 - k'/k)$ (Fig. 5). If the inhibition is of the complete uncompetitive type ($k' = 0$), the asymptotes are the ordinate and the K_i' value.

Fig. 4. Fractional-velocity plots for partial (a) and complete (b) uncompetitive inhibition

The lines were drawn in accordance with eqn. (10). The following values of parameters were used: $K_i = \infty$, $K_i' = 0.05$, $K_s' = 0$, $K_s = 1$ and $k'/k = 0.6$ for partial uncompetitive inhibition or $k'/k = 0$ for complete uncompetitive inhibition. The substrate concentration is indicated by each line.

Fig. 5. Replot of the slope of the fractional-velocity plot for uncompetitive inhibition

The relationship of the slope with the substrate concentration is calculated by using eqn. (11) with the values of the kinetic parameters indicated in the legend to Fig. 4.

Partial and complete mixed-type inhibition

Partial mixed-type inhibition consists of a mixture of partial competitive plus complete non-competitive inhibition. General eqn. (5) can be applied to the mixed-type inhibition (Fig. 6). In order to determine the k'/k , K_s' , K_i and K_i' values, one needs to solve four simultaneous equations. When the K_s value is determined by certain graphical methods [3], one can obtain the relation between k'/k and K_s' from the ordinate intercept, and further the relation between k'/k and K_i' is obtained from the abscissa intercept. The slope of the plot also gives the relation among K_i' , k'/k and K_s' . One can calculate the values of K_i , K_i' , K_s' and k'/k from these relations and the equation $K_i'/K_i = K_s'/K_s$ in the mixed-type inhibition.

The slope of the plot is a hyperbolic function of the substrate concentration:

$$\text{Slope} = \frac{(1 + [S]/K_s) \cdot K_i'}{(1 - k'/k) \cdot ([S]/K_s) + (K_s'/K_s - k'/k)} \quad (12)$$

The relationship of the slope with the substrate concentration is shown in Fig. 7. The maximum value of the slope reaches $K_i'/(1 - k'/k)$. Table 1 summarizes the values of slope, abscissa intercept and ordinate intercept of complete and partial inhibition.

APPLICATION OF THE FRACTIONAL-VELOCITY PLOT TO PRACTICAL CASES

Inosine nucleosidase (EC 3.2.2.2) from *Azotobacter vinelandii* catalyses the hydrolysis of inosine to form hypoxanthine and ribose, and shows some regulatory properties in relation to purine nucleotide metabolism [4,5]. Here I attempt to analyse the inhibition of inosine nucleosidase by bases and ATP by using the fractional-velocity plot. Purification of the enzyme and assay of the activity were described previously [4].

Inosine nucleosidase obeys typical Michaelis-Menten kinetics with a K_m value of 0.65 mM [4]. The enzyme is inhibited by ATP [4], and all the inhibition curves with

Fig. 6. Fractional-velocity plots for partial (a) and complete (b) mixed-type inhibition

The lines were drawn in accordance with eqn. (5). The following values of parameters were used: $K_s = 1$, $K_s' = 10$, $K_i = 0.005$, $K_i' = 0.05$ and $k'/k = 0.6$ for partial mixed-type inhibition or $k'/k = 0$ for complete inhibition. The substrate concentration is indicated by each line.

Fig. 7. Replot of the slope of the fractional-velocity plot for mixed-type inhibition

The relationship of the slope with the substrate concentration is calculated by using eqn. (12) with the values of the kinetic parameters indicated in the legend to Fig. 6.

respect to ATP were converted into straight lines converging on the origin (Fig. 8), implying that ATP acts as a complete-type inhibitor, i.e. k' is equal to zero.

Table 1. Values of slopes and intercepts of the fractional-velocity plots

Inhibition type		Slope	Abscissa intercept	Ordinate intercept	Replot of slope versus [S]
Competitive: $K_s = K_s'$, $k = k'$	Complete	$(1 + [S]/K_s)K_i$	0	0	Linear $y = ax + b$
	Partial	$\frac{1 + [S]/K_s}{1/K_i - 1/K_i'}$	$-1/K_i'$	$\frac{(1 + [S]/K_s)K_i'}{1/K_i - 1/K_i'}$	
Non-competitive: $K_s = K_s'$, $K_i = K_i'$	Complete	K_i	0	0	Constant $y = a$
	Partial	$K_i/(1 - k'/k)$	$-(k'/k) \cdot (1/K_i)$	$(k'/k)/(1 - k'/k)$	
Uncompetitive: $K_i = \infty$, $K_s' = 0$	Complete	$\frac{(1 + [S]/K_s)K_i'}{[S]/K_s}$	0	0	Hyperbolic $y = (cx + d)/(ax - b)$
	Partial	$\frac{(1 + [S]/K_s)K_i'}{(1 - k'/k) \cdot [S]/K_s - k'/k}$	$-(k'/k) \cdot (1/K_i')$	$\frac{(1 + [S]/K_s)k'/k}{(1 - k'/k) \cdot [S]/K_s - k'/k}$	
Mixed type	Complete	$\frac{(1 + [S]/K_s) \cdot K_i'}{[S]/K_s + K_s'/K_s}$	0	0	Hyperbolic $y = (cx + d)/(ax - b)$
	Partial	$\frac{(1 + [S]/K_s) \cdot K_i'}{(1 - k'/k) \cdot [S]/K_s + K_s'/K_s - k'/k}$	$-(k'/k) \cdot (1/K_i')$	$\frac{(1 + [S]/K_s) \cdot k'/k}{(1 - k'/k) \cdot [S]/K_s + K_s'/K_s - k'/k}$

Fig. 8. Fractional velocity plot of the inhibition by ATP of inosine nucleosidase

The reaction mixture (0.2 ml) contained 20 mM-Tris/HCl buffer, pH 7.1, various concentrations of ATP and the purified enzyme with a specific activity of about 2 $\mu\text{mol/min}$ per mg of protein [4] in the presence of 0.6 mM-, 1.5 mM- and 5 mM-inosine. Reaction was initiated by addition of the enzyme, and carried out at 37 °C for 5 min. Free ribose liberated was determined as described previously [4]. Data are plotted according to eqn. (5). Δ , 0.6 mM-Inosine; $\circ$, 1.5 mM-inosine; $\bullet$, 5 mM-inosine.

However, the slope of the plot is not a straight line but apparently a hyperbola, suggesting a characteristic of mixed inhibition (Figure not shown). Inhibition of the enzyme by nucleotides was shown to be essentially of the non-competitive type with respect to inosine, but addition of ATP induced a positive co-operativity with the substrate [4]. A more detailed model of the mixed inhibition consisting of non-competitive type plus allosteric mechanism will be required for explanation of the ATP inhibition of the enzyme, although the inhibition undoubtedly belongs to the complete type.

All the inhibition curves with respect to adenine were converted by the fractional-velocity plot into a single straight line converging on the abscissa at $-(k'/k) \cdot (1/K_i')$: the change in the substrate concentration did not affect the slope of the plot (Fig. 9a), implying that the inhibition is of the partial non-competitive type. The fractional-velocity method also showed that hypoxanthine acted as a partial non-competitive inhibitor (Fig. 9b). The values of k'/k were calculated to be 0.286 and 0.583 for the inhibition by adenine and hypoxanthine respectively from each ordinate intercept. The K_i values of the enzyme for adenine and hypoxanthine were also determined to be 4.4 and 3.65 mM respectively from the abscissa intercepts by using the k'/k values determined.

DISCUSSION

Kinetic analysis of enzyme inhibition forms an important and principal part of enzyme research. Enzyme inhibition is usually analysed in terms of the inhibition type and K_i value, the dissociation constant of

Fig. 9. Fractional-velocity plots of the inhibition by adenine (a) and hypoxanthine (b) of inosine nucleosidase

Reaction conditions were similar to those of Fig. 8 except that ATP was replaced by adenine or hypoxanthine. Inosine concentrations are: Δ , 0.6 mM; $\circ$, 1.5 mM; $\bullet$, 5 mM.

the enzyme-inhibitor complexes. An enzyme inhibitor is classified as being of complete or partial type, also being called a linear or hyperbolic inhibitor depending on the replot of the primary double-reciprocal plots being a straight line or a hyperbola respectively [2]. Complete or full inhibition has a dead-end EI complex or non-productive EIS complex or both; the increase in complete inhibitor can decrease the enzyme activity to zero. On the other hand, a partial inhibitor, by combining with the enzyme, does not give rise to a dead-end complex, but to an EIS complex that breaks down to form products with a decreased rate. Although some graphical methods, including Dixon [6] and Cornish-Bowden plots [7], are utilized for kinetic analysis of the inhibitor action, these methods are applicable to complete or linear inhibition only, but not to partial inhibition. In some instances, partial inhibition has been overlooked because a sufficient inhibitor concentration range was not examined. Partial

inhibition is usually analysed by the replot of the primary double-reciprocal plot [1,2]. However, of the various transformations of the Michaelis-Menten equation, the double-reciprocal plot provides the worst estimates of the kinetic constants: this feature arises because the double-reciprocal relationship emphasizes the separation of the low-substrate-concentration points so that visually the high-substrate-concentration points tend to coalesce to a single point [8,9]. Slope and intercept replots cause a further magnification of errors in determining kinetic parameters.

An example of the graphical method for partial inhibition is the specific velocity plot developed by Baici [10], but this method also requires secondary plots for the determination of the kinetic parameters. On the other hand, the fractional-velocity plot described in the present paper can graphically distinguish partial inhibition from the complete type, including competitive, non-competitive, uncompetitive and mixed-type inhibition. Simple inspection of the fractional-velocity plot is always sufficient to decide whether the inhibition is of the complete or the partial type. The K_i and K_i' values of the enzyme for the inhibitor and the reaction rate constants of the ES and EIS complexes can be readily calculated from the abscissa and ordinate intercepts of the plot. Simple transformation of the inhibition curves without secondary plots causes a smaller error in the estimates. Partial and complete inhibition of inosine nucleosidase was analysed successfully by this plot. As pointed out by Cornish-Bowden [9], partial inhibition should be regarded as the norm and complete inhibition a deviation from it for all types of inhibitor, whether physiologically significant or not. The fractional-velocity plot should be useful in the kinetic analysis of the partial inhibition of enzyme-catalysed reactions.

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