Determination of the Rate Constant of Enzyme Modification by Measuring the Substrate Reaction in the Presence of the Modifier†

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ABSTRACT: On the basis of the equations derived previously [Tsou, C. L. (1965) *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 5*, 398–408, 409–417] for the substrate reaction during the course of enzyme modification, the kinetic behavior of the system chymotrypsin–substrate–modifier has been studied. The kinetics of benzoyltyrosine ester hydrolysis during the course of irreversible inhibition of the enzyme has been found to be in satisfactory agreement with equations obtained previously. The apparent rate constant between the enzyme and an irreversible inhibitor can be easily obtained in one single experiment by following the course of substrate hydrolysis in the presence of the inhibitor. The results are also in accord with the assumption that diisopropyl fluorophosphate can be classified as an irreversible competitive inhibitor. For both phenylmethanesulfonyl fluoride and L-1-[(*p*-toluenesulfonyl)amino]-2-phenylethyl chloromethyl ketone, the inhibition has been found to be in agreement with the kinetics of the complexing type; i.e., a noncovalent enzyme–inhibitor complex is formed before irreversible enzyme modification. Both the equilibrium constants for the complex formation and the first-order rate constants for the irreversible modification step have been determined also by following the course of substrate hydrolysis in the presence of the irreversible inhibitor.

The study of enzyme inhibition has received much attention, owing not only to its importance in providing useful information on the nature of enzyme catalysis but also to its implications in pharmacology and toxicology. In most text books on enzyme kinetics, attention has always been focused on reversible inhibition, whereas the kinetics of irreversible inhibition usually receives either not much more than a passing mention (Cornish-Bowden, 1979; Segal, 1975) or no mention at all (Wong, 1975). Recent developments have, however, shown that in mechanistic as well as applied studies, irreversible inhibitors are as important as, if not more important than, reversible inhibitors. As affinity probes of the active sites of enzymes, irreversible inhibitors can sometimes provide definitive information which is not possible to obtain with reversible inhibitors (Singer, 1967; Shaw, 1970; Wold, 1977). Moreover, as alkylating, phosphorylating, and acylating agents, many irreversible inhibitors are being used as chemotherapeutic agents (Connors, 1975) or insecticides (O'Brien, 1963). Some years ago, one of the present authors (Tsou, 1965a,b) made a systematic study on the kinetics of irreversible modification of enzyme activity. It was shown that the concept for competitive, noncompetitive, and uncompetitive inhibitions can also be applied, and it was proposed that the effect of substrate concentration on the rate constants for enzyme modification can be used to distinguish these types in the case of irreversible inhibition. It was also shown that the kinetics of the substrate reaction in the presence of the modifier can be used conveniently for the determination of the apparent rate constants of enzyme modification in one single experiment. Furthermore, the kinetics of systems in which the irreversible modification step followed a reversible association between the enzyme and the modifier was shown to be also amenable to such an analysis.[1] More recently, Laidler, in the new edition of his well-known textbook (Laidler & Bunting, 1973), treated the kinetics of substrate reaction during the course of enzyme denaturation with similar results.

It has now been shown in the present paper that the course of BTEE[2] and ATEE hydrolysis by α-chymotrypsin in the presence of the irreversible inhibitors DFP, TPCK, and PMSF is in satisfactory agreement with the equations previously derived (Tsou, 1965a,b) and the kinetic constants conveniently determined by the methods proposed.

Materials and Methods

Crystalline α-chymotrypsin with a specific activity of 68.5 μmol of BTEE hydrolyzed at 25 °C (pH 7) (mg of enzyme)$^{-1}$ min^{-1} was kindly provided by the Department of Biology, Beijing University. ATEE, BTEE, DFP, TPCK, and PMSF were from Dungfung Biochemicals, Shanghai. All other reagents were local products of analytical grade. Solutions were routinely prepared in double-distilled water.

The inhibitors TPCK and PMSF were initially dissolved in methanol and DFP in 2-propanol before addition to the reaction mixture. Suitable control experiments showed that the amount of organic solvent introduced had no appreciable effect on the enzyme activity. For the assay of chymotrypsin activity, absorbance changes were continuously recorded at 256 nm for BTEE (Walsh & Wilcox, 1970) and at 237 nm for ATEE (Wilcox, 1970) with a Specord UV–vis spectrophotometer thermostated at 25 °C. The concentrations of products formed were then calculated with the appropriate absorption coefficients.

The reaction mixture for the DFP or TPCK inhibition of BTEE hydrolysis contained 0.1 M Tris buffer, pH 7.7, 0.1 M $CaCl_2$, and the substrate, inhibitor, and enzyme concentrations as required. Both the inhibitor and the substrate were incubated together with the buffer system to reach temperature equilibrium before the addition of enzyme to start the reaction. When ATEE was the substrate, instead of Tris buffer, phosphate buffers, pH 7.7 for the inhibition by TPCK and pH 7.0 for the inhibition by PMSF, were used.

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[1] Since these two papers were published in Chinese and are not easily available, a brief summary of the salient points will be presented in the Appendix.

[2] Abbreviations: ATEE, acetyl-L-tyrosine ethyl ester; BTEE, benzoyl-L-tyrosine ethyl ester; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; TPCK, L-1-[(*p*-toluenesulfonyl)amino]-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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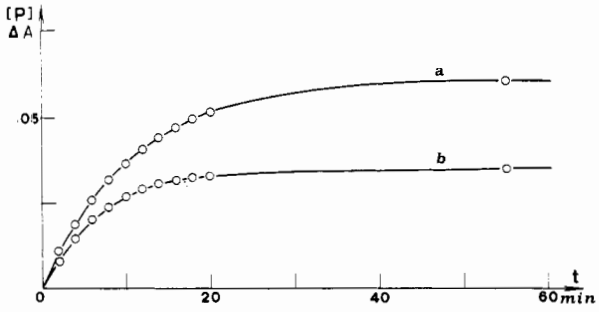


FIGURE 1: Course of BTEE hydrolysis in the presence of DFP. The reaction mixture contained the following: enzyme, 2.8 nM; DFP, 50 μM; BTEE, (a) 250 μM and (b) 150 μM (in 0.1 M Tris buffer, pH 7.7).

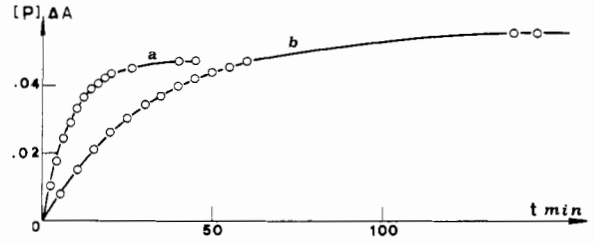


FIGURE 2: Course of BTEE hydrolysis in the presence of PMSF and TPCK. (a) PMSF inhibition, mixture contained the following: enzyme, 4.9 nM; PMSF, 33 μM; BTEE, 150 μM (in 0.1 M phosphate buffer, pH 7.0). (b) TPCK inhibition, mixture contained the following: enzyme, 1.4 nM; TPCK, 100 μM; BTEE, 150 μM (in 0.1 M Tris buffer, pH 7.7).

The integrated equations (see Appendix) on which the present work is based are derived on the assumption that during the whole reaction course, the change in [S] is not large enough so as to affect significantly the degree of substrate saturation on the native enzyme or, experimentally, the change in [S] would not affect significantly the steady-state rate had there been no inhibitor present. This was always checked in each series of experiments to ensure that the change in steady-state rate was generally less than 10% with the range of changes in [S] studied. Only in a few exceptional cases, owing to practical considerations, the change in [S] may produce steady-state rate changes up to 30%.

Results

Product Formation in the Presence of a Modifier. It is predicted from equations derived previously (Tsou, 1965b) that the product formation in the presence of an irreversible inhibitor should approach an asymptote. If $[P_\infty]$ represents the concentration of product formed at time approaching infinity, then

$$[P_\infty] = \frac{V[S]\bar{K}}{(1 + [S]\bar{K})A[Y]} \quad (1)$$

in which $\bar{K}$ is the inverted Michaelis Constant, A is the apparent inhibition rate constant in the presence of the substrate, and [Y] is the concentration of the inhibitor. That the product formation indeed approached an asymptote was shown to be the case with DFP (Figure 1), TPCK, or PMSF (Figure 2) as the inhibitor. It can also be seen that since [S] and [Y] are known and V and $\bar{K}$ can be determined in separate experiments, A can be calculated from $[P_\infty]$ immediately.

It has also been shown that

$$\log ([P_\infty] - [P]) = \log [P_\infty] - 0.43A[Y]t \quad (2)$$

where [P] is the product concentration at time t. Data in Figures 1 and 2 can be replotted as $\log ([P_\infty] - [P])$ against

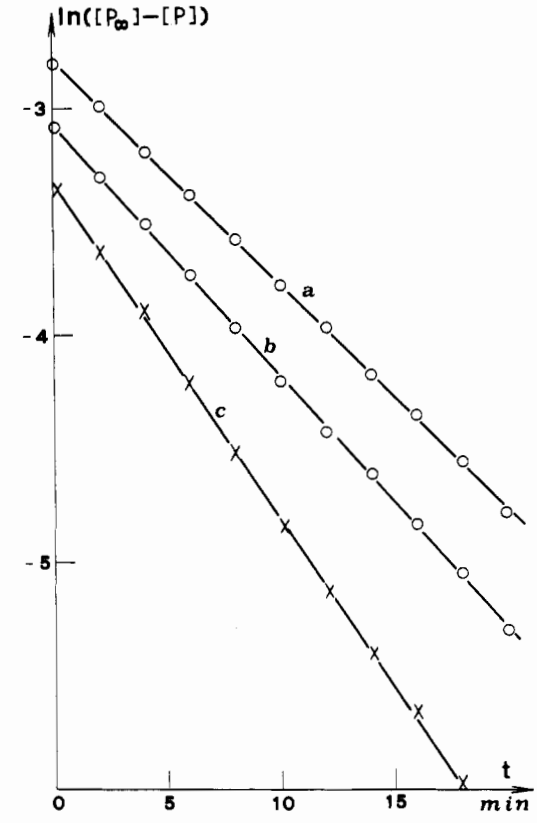


FIGURE 3: Plot of the DFP inhibition data by eq 2. Conditions as in Figure 1 except the substrate concentrations were (a) 250, (b) 200, and (c) 150 μM.

t, and Figure 3 shows the results for the DFP inhibition at three substrate (BTEE) concentrations. It can be seen that satisfactory straight lines were obtained. Equally satisfactory results were also obtained with TPCK and PMSF as the inhibitors. As [Y] is known, the apparent inhibition rate constant can be directly read off from the slope of the straight line thus obtained. Similar results were obtained with ATEE as substrate.

Effect of [S] on A. It has been suggested (Tsou, 1965a) that, like reversible inhibitors, irreversible inhibitors can also be classified as competitive, noncompetitive, and uncompetitive on the basis of similar considerations as used for reversible inhibitors. Experimentally, the type of inhibition can be ascertained by studying the effect of [S] on the apparent rate constant, A. As given previously (Tsou, 1965a)

competitive $$A = \frac{k_{+0}}{1 + \bar{K}[S]} \quad (3)$$

noncompetitive $$A = k_{+0} \quad (4)$$

uncompetitive $$A = \frac{k_{+0}'K[S]}{1 + \bar{K}[S]} \quad (5)$$

where k_{+0} and k_{+0}' are the rate constants between the inhibitor and the enzyme and ES complex, respectively. It is easily seen that these can be distinguished by suitable A and [S] plots. For noncompetitive inhibition, A is independent of [S]. For competitive or uncompetitive inhibition, the plot of $1/A$ against [S] or $1/[S]$ will be a straight line, respectively. From these plots, the respective rate constants can be obtained. Figure 4 shows the results for the effect of BTEE concentration on A between DFP and chymotrypsin. Similar results were obtained with TPCK and PMSF as inhibitors. It seems clear that all these inhibitors can be classified as irreversible competitive inhibitors.

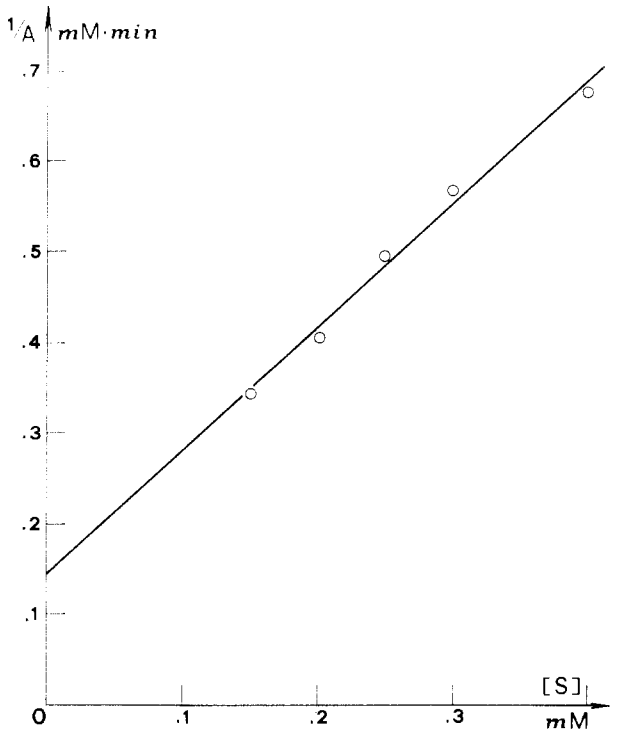


FIGURE 4: Effect of [S] on the apparent rate constant for the DFP inhibition of chymotrypsin. The apparent rate constants, A, were obtained from $[P_\infty]$ values under experimental conditions as in Figure 1.

Table I: Second-Order Rate Constant for the Irreversible Inhibition of Chymotrypsin

inhibitor	conditions	second-order rate constant (M^{-1} min^{-1}) from eq 3	by conventional method[a]	lit. value
DFP	25 °C, pH 7.7	7100		20 000[b]
PMSF	25 °C, pH 7.0	11500		12 000,[b] 14 900[c]
TPCK	25 °C, pH 7.7	1130	1000	590[d]

[a] This was carried out by incubating 72 μM TPCK with the enzyme at pH 7.7 and 0.1 M Tris buffer. Aliquots were taken at time intervals to determine the activity remaining with BTEE as substrate. [b] Fahrney & Gold (1963). [c] Gold (1967). [d] Kezdy et al. (1967).

Alternatively, the type of inhibition can be ascertained from the effect of [S] on $[P_\infty]$. When eq 3–5 are combined with eq 1

$$\text{competitive} \qquad [P_\infty] = \frac{V[S]\bar{K}}{k_{+0}[Y]} \tag{6}$$

$$\text{noncompetitive} \qquad [P_\infty] = \frac{k_{+0}V[S]\bar{K}}{(1 + \bar{K}[S])[Y]} \tag{7}$$

$$\text{uncompetitive} \qquad [P_\infty] = \frac{V}{k_{+0}'} \tag{8}$$

For competitive inhibition, a plot of $[P_\infty]$ against [S] will give a straight line passing through the origin, as indeed is the case for DFP as shown in Figure 5. Similar results were obtained for TPCK and PMSF. For noncompetitive inhibition, the plot of $1/[P_\infty]$ against $1/[S]$ will be a straight line whereas for uncompetitive inhibition $[P_\infty]$ will be independent of [S].

The kinetic constants thus obtained are listed in Table I.

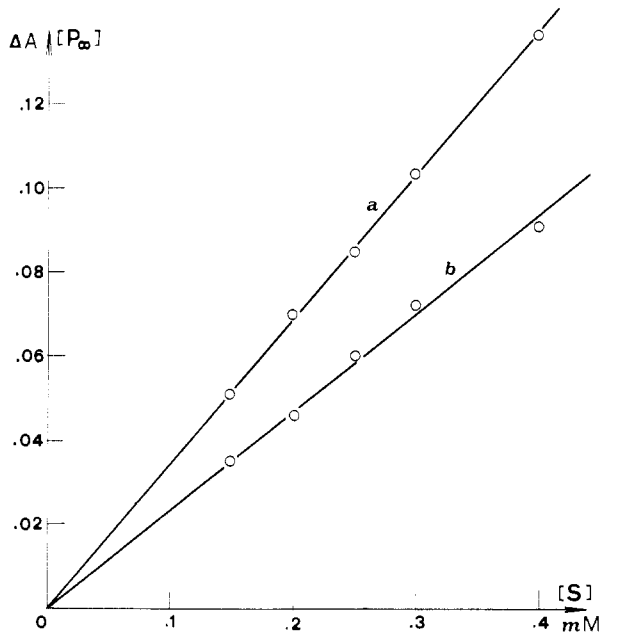


FIGURE 5: Effect of [S] on $[P_\infty]$. Experimental conditions as in Figure 1 except for the concentrations of DFP which were (a) 33 and (b) 50 μM.

Complexing Type of Irreversible Inhibition. Some inhibitors form a complex with the enzyme prior to the irreversible modification step:

$$E + Y \underset{k_{-0}}{\overset{k_{+0}}{\rightleftharpoons}} EY \xrightarrow{k_{+3}} EY' \tag{9}$$

It has been shown by similar considerations (Tsou, 1965b) that the apparent rate constant A and the final concentration of product formed $[P_\infty]$ in the presence of the inhibitor are given by

$$\frac{1}{A} = \frac{1 + \bar{K}[S] + \bar{K}_0[Y]}{k_{+3}\bar{K}_0} \tag{10}$$

$$[P_\infty] = \frac{V\bar{K}[S]}{k_{+3}K_0[Y]} \tag{11}$$

An inspection of eq 10 and 11 shows that the $[P_\infty]$ vs. [S] as well as the $1/A$ vs. [S] plots will have the same form as in the case of simple competitive irreversible inhibition, as indicated in the previous section. It can, however, be distinguished from the noncomplexing type of inhibition by a plot of $1/A$ vs. [Y]. Whereas for the simple competitive type of inhibition $1/A$ will be independent of [Y], as has indeed been found to be essentially the case for DFP in the present study, a straight line with a slope of $1/k_{+3}$ should be obtained for the complexing type of inhibition. Such a plot for the PMSF inhibition of chymotrypsin hydrolysis of BTEE is shown in Figure 6, and similar results were also obtained for TPCK.

For the complexing type of irreversible inhibition, the inhibitory power is determined by both the equilibrium constant $\bar{K}_0$ and the first-order rate constant k_{+3}. The values for k_{+3} can be easily obtained from the slope of the $1/A$ vs. [Y] plots whereas $\bar{K}_0$ can be calculated either from eq 11 or from the intercept of the x axis of the $1/A$ vs. [Y] plot, provided that V and $\bar{K}$ are known. The $\bar{K}_0$ and k_{+3} values found for PMSF and TPCK are listed in Table II.

Discussion

Although the concept of inhibitor competition with substrates can equally apply to both reversible and irreversible inhibitors, detailed kinetic criteria have hitherto been given only to reversible inhibitors. Experimentally, reversible com-

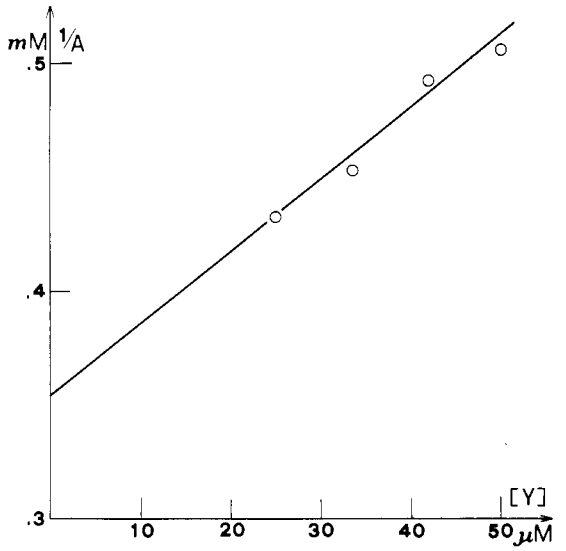


FIGURE 6: Effect of [Y] on the apparent rate constant in PMSF inhibition of chymotrypsin. The reaction mixture contained the following: enzyme, 4.9 nM; BTEE, 300 μM; PMSF as indicated (in 0.1 M phosphate buffer, pH 7.0).

Table II: Binding and Rate Constants for PMSF and TPCK Inhibition of Chymotrypsin

inhibitor	conditions	K_0 (M^{-1})	k_{+3} (min^{-1})	$\bar{K}_0 k_{+3}$ (M^{-1} min^{-1})	k_{+0} (M^{-1} min^{-1})[a]
PMSF	25 °C, pH 7.0	36000	0.32	11500	11500
TPCK	25 °C, pH 7.0	5290	0.22	1160	1130

[a] From Table 1.

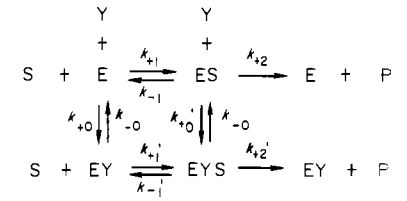
petitive, noncompetitive, and uncompetitive inhibitors are distinguished by their effects on the apparent Michaelis constants determined in the presence of the inhibitor (e.g., Cornish-Bowden, 1979). For irreversible inhibitors, it has been shown (Tsou, 1965a) that similar criteria can be derived to distinguish these three types of inhibitors on the basis of their effects on the apparent rate constant of enzyme inhibition also determined in the presence of both the substrate and the inhibitor, as in the usual practice in kinetic studies of reversible inhibition. Table III gives a summary of kinetic criteria of the different types of inhibition for both reversible and irreversible inhibitors. It should by pointed out that for reversible noncompetitive inhibitors it is required that $\bar{K}_0 = \bar{K}_0'$. Although, strictly speaking, $k_{+0} = k_{+0}'$ is not necessarily required for the two equilibrium constants to be equal, however, for an operational definition, it would be more convenient to define only those cases where $k_{+0} = k_{+0}'$ as irreversible noncompetitive inhibition and to treat cases where $k_{+0} \neq k_{+0}'$ as mixed type inhibition.

Experimentally, the first advantage in the method proposed here for the determination of the apparent rate constant of enzyme modification is its simplicity. By either eq 1 or 2, the apparent rate constant between the enzyme and an irreversible inhibitor can be determined in one single experiment as compared to taking aliquots and making activity determinations at time intervals from an enzyme–inhibitor incubation mixture. It would be particularly useful when the apparent rate constants of an inhibitor under a number of different conditions, or a number of similar inhibitors under the same condition, have to be compared. Second, the present method is applicable to fast reactions between the enzyme and the modifier. Reactions with a half-time about 10 s would be difficult to study by the usual practice but can be easily studied by the present method with manual mixing and a recording instrument. With a stopped-flow apparatus, reactions with half-times in the millisecond range can also be studied. Last, it should be noted that in the present approach, the apparent rate constants are determined under conditions which resemble more closely the in vivo situation where the substrate is constantly being turned over while the enzyme is being modified. This would be particularly important when the inhibitory powers of toxic substances or potentially useful drugs toward certain enzymes are being assessed.

Appendix

Kinetics of the Substrate Reaction during the Course of Enzyme Modification. The kinetic equations used in the present study are based on papers published in Chinese (Tsou, 1965a,b). The salient points of these papers will be presented here.

On the basis of the scheme



(A1)

the rate of decrease of the total unmodified forms of the enzyme E_T which includes both E and ES can be given by

$$-\frac{d[E_T]}{dt} = \left[\frac{[Y](k_{+0} + k_{+0}'\bar{K}[S])}{1 + \bar{K}[S]} + \frac{(k_{-0} + k_{-0}'\bar{K}'[S])}{1 + K'[S]}\right][E_T] - \frac{[E_0](k_{-0} + k_{-0}'\bar{K}'[S])}{1 + \bar{K}'[S]} \quad (A2)$$

or for irreversible modifications when both k_{-0} and k_{-0}' equal zero

$$-\frac{d[E_T]}{dt} = \frac{[E_T][Y](k_{+0} + k_{+0}'\bar{K}[S])}{1 + \bar{K}[S]} \quad (A3)$$

Table III: Kinetic Criteria for Different Types of Inhibition

		reversible		irreversible	
inhibition type	definition	condition	fractional act. at steady state	condition	A
competitive	Y does not bind to ES	$\bar{K}_0' = 0$	$\frac{1 + \bar{K}[S]}{1 + \bar{K}[S] + \bar{K}_0[Y]}$	$k_{+0}' = 0$	$\frac{k_{+0}}{1 + \bar{K}[S]}$
noncompetitive	Y does not affect ES binding	$\bar{K}_0 = \bar{K}_0'$	$\frac{1}{1 + \bar{K}_0[Y]}$	$k_{+0} = k_{+0}'$	k_{+0}
uncompetitive	Y binds to ES only	$\bar{K}_0 = 0$	$\frac{1 + \bar{K}[S]}{1 + \bar{K}[S] + \bar{K}_0'\bar{K}[S][Y]}$	$k_{+0} = 0$	$\frac{k_{+0}'\bar{K}[S]}{1 + \bar{K}[S]}$

in which $\bar{K}$ and $\bar{K}'$ are the inverted Michaelis constants $k_{+1}/(k_{+2} + k_{-1})$ and $k_{+1}'/(k_{+2}' + k_{-1}')$, respectively. Equation A2 can be written in the form

$$-\frac{d[E_T]}{dt} = (A[Y] + B)[E_T] - B[E_0] \quad (A4)$$

where A and B are the apparent rate constants for the modification of the enzyme and the dissociation of the EY complex, respectively. Integration of eq A4, provided that [S] and [Y] do not change appreciably, gives

$$\ln\left[\left(1 + \frac{B}{A[Y]}\right)\frac{[E_T]}{[E_0]} - \frac{B}{A[Y]}\right] = -(A[Y] + B)t \quad (A5)$$

The fractional activity, a, at any time t is

$$a = \frac{[E_T]}{[E_0]} = \frac{e^{-(A[Y]+B)t} + \frac{B}{A[Y]}}{1 + \frac{B}{A[Y]}} \quad (A6)$$

in which $[E_0]$ is the total enzyme concentration. The fractional activity when the system approaches equilibrium, i.e., when t approaches infinity, is

$$a_\infty = \frac{B}{A[Y] + B} \quad (A7)$$

The rate of product, P, formation while the enzyme is being modified can now be obtained from (A6), if $k_{+2}' = 0$, as

$$\frac{d[P]}{dt} = \frac{k_{+2}[E_0]\bar{K}[S]}{(1 + \bar{K}[S])(A[Y] + B)}[B + A[Y]e^{-(A[Y]+B)t}] \quad (A8)$$

and [P] at any time t as

$$[P] = \frac{v}{A[Y] + B}\left[Bt + \frac{A[Y]}{A[Y] + B}(1 - e^{-(A[Y]+B)t})\right] \quad (A9)$$

where v is the rate of the substrate reaction at a given [S] and in the absence of [Y]. When the inhibition is irreversible, the above equation is simplified to

$$[P] = \frac{v}{A[Y]}(1 - e^{-A[Y]t}) \quad (A10)$$

The concentration of the product formed when t approaches infinity is

$$[P_\infty] = \frac{v}{A[Y]} \quad (A11)$$

From eq A10 and A11, it can be easily shown that

$$\log([P_\infty] - [P]) = \log[P_\infty] - 0.43A[Y]t \quad (A12)$$

The apparent rate constant, A, for the enzyme modification can then be easily obtained from either eq A11 or A12 in one single experiment. As A is given by definition from eq A3 and A4

$$A = \frac{k_{+0} + k_{+0}'\bar{K}[S]}{1 + K[S]} \quad (A13)$$

and by combining eq A11 and A13

$$[P_\infty] = \frac{V\bar{K}[S]}{(k_{+0} + k_{+0}'\bar{K}[S])[Y]} \quad (A14)$$

If by analogy with reversible inhibitions we define for competitive, noncompetitive, and uncompetitive inhibitions $k_{+0}' = 0$, $k_{+0} = k_{+0}'$, and $k_{+0} = 0$, respectively, it can be easily shown that the different types of inhibition can be distinguished and the appropriate rate constants obtained by suitable plots of $1/A$ or $1/[P_\infty]$ against [S] or $1/[S]$.

For those inhibitors which form a noncovalent, dissociable complex with the enzyme before the irreversible modification step, it can be easily shown by similar considerations that the apparent rate constant is given by

$$\frac{1}{A} = \frac{1 + \bar{K}[S] + K_0[Y]}{k_{+3}K_0} \quad (A15)$$

where K_0 is the equilibrium constant for complex formation and k_{+3} the rate constant for the irreversible modification step. By $1/A$ against [Y] plots, this type of inhibition can be distinguished from the noncomplexing type, and both K_0 and k_{+3} can be easily obtained.

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