Kinetic differentiation between enzyme inactivation involving complex-formation with the inactivator and that involving a conformation-change step

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INTRODUCTION
Irreversible inhibitions in which an inhibitor, I, binds first with the enzyme reversibly before the irreversible inactivation step are of considerable importance as such reactions can be used to label the active-site groups specifically. It has been shown kinetically that for such complexing types of inactivation the dependence of the inactivation rate constant of the concentration of the inactivator exhibits saturation kinetics (Kritz & Wilson, 1962; Tsou, 1965; Tsou 1988). A plot of the reciprocal of the apparent inactivation rate constant, 1/k, against 1/[II] gives a straight line with positive values for both the y-axis intercept and the slope. On the other hand, a number of enzymes (Ainslie et al., 1972; Sanner & Tron, 1975; Gontero et al., 1984) are now known to exist in more than one conformational form, only one of which is susceptible to the attack by inactivators. Rakitzis (1986) pointed out that for enzymes whose inactivation step involves a slow conformational change, the corresponding equation derived for the dependence of the rate constant on inactivator concentration also shows saturation kinetics and gives a double-reciprocal plot similar to that for the complexing types of inactivation.

Some years ago, a systematic study on the kinetics of irreversible modification of enzyme activity was presented (Tsou, 1965, 1988). On the basis of the substrate reaction in the presence of an irreversible inhibitor, it has also been shown that the product formed at time infinity, [P] oo, approaches a constant value from which the apparent rate constant for the irreversible modification of enzyme activity can be obtained in a single experiment. The present paper compares the kinetics of substrate reaction during enzyme inactivation preceded either by complex-formation or by a relatively slow conformational change. It is shown that these two cases can be distinguished by plotting [P] oo against 1/[II] as has been illustrated experimentally with a number of enzymes.

THEORY
The complexing and the conformational-change types of inactivation can be represented by eqns. (1) and (2) respectively:

\[ E + I \xrightarrow{k_{s1}} EI \xrightarrow{k_{s2}} E_i \]  
\[ E \xrightarrow{k_{s1}} E_\times + I \xrightarrow{k_{s2}} E_i \]

where E and I are enzyme and inhibitor respectively. \( E_\times \) is a conformationally different form of E which binds with I to form \( E_i \) as the inactivated enzyme. Rakitzis (1986) showed that the equations for the decrease in the native form of the enzyme, E, with time have the same form for both cases:

\[ \frac{[E]}{[E]_0} = C e^{-m_1t} + (1-C) e^{-m_2t} \]

where \([E]_0\) is the total enzyme concentration. Although:

\[ m_1, m_2 = \frac{-(k_{s2}[I]+k_{-3}+k_{-4}) \pm \sqrt{(k_{s2}[I]+k_{-3}+k_{-4})^2-4k_{s3}k_{s4}[I]}}{2} \]  
for case (1) and:

\[ m_1, m_2 = \frac{-(k_{s2}+k_{-3}+k_{s4}[I]) \pm \sqrt{(k_{s2}+k_{-3}+k_{s4}[I])^2-4k_{s3}k_{s4}[I]}}{2} \]  
for case (2), where \( m_1 \) and \( m_2 \) are for the sum and difference respectively of the terms in the numerator of eqns. (4) and (5), kinetic discrimination of the two cases is impossible when constants \( k_{s3}, k_{-3} \) and \( k_{s4} \) are unknown.

Substrate reaction method can differentiate the two cases. Thus, for one-substrate reactions:

\[ S + E \xrightarrow{k_{s1}} ES \xrightarrow{k_{s2}} E + P \]

\[ [E] = \frac{k_m([E]+[ES])}{k_m+[S]} \]  
\[ [E] = \frac{[S][E]}{k_m} \]

Abbreviations used: MNP, 2-chloromercuri-4-nitrophenol; PMSF, phenylmethanesulfonyl fluoride; DFP, di-isopropyl fluorophosphate.

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For reactions involving two substrates obeying a random mechanism:

\[
\begin{align*}
  \text{E} & \xrightarrow{k_1} \text{EA} \\
  \text{E} & \xrightarrow{k_2} \text{E} + \text{P}
\end{align*}
\]

\[
[E] = \frac{[E] + [EA] + [EB] + [EAB]}{1 + K_1[A] + K_2[B] + K_1K_2[A][B]}
\]

\[
\text{EAB} = K_1K_2[A][B][E]
\]

where A and B are the two substrates and \( K_1 = K_2 \) are equilibrium binding constants. We may write \([E] = \frac{[E]_0}{\alpha} \) if we denote \((K_m + [S])/K_m \) or \(1 + K_1 + K_2 + K_1K_2[A]B \) as \( \alpha \).

As previously (Tsou 1988; Wang et al., 1988), it is assumed that the steady state of the substrate reaction is rapidly established and both \([S] \) and \([I] \) are \( \gg [E] \). Upon integration of the differential equations derived from eqns. (2) and (6), or (2) and (7), with the boundary conditions:

\[
[E]_0 = \alpha[E] + [E]_0, \quad \text{and} \quad ([E]_0)_{t=0} = k_{-3}(k_{-2} + k_2)[E]_0,
\]

we obtain the rate of disappearance of free enzyme as given by eqn. (3), with \( m_1 \) and \( m_3 \) as shown in eqn. (9), from the relationship:

\[
\frac{d[E]}{dt} = k_{-3}[EAB] = k_{-3} K_1[E][A][B]
\]

By integration, we have the expression for reactions with two substrates as follows:

\[
[P] = \frac{k_{-3} K_1 K_2[E][A][B] k_{-3}}{\alpha(k_{-3} + k_3)} \times \frac{m_2 - \left( \frac{1}{\alpha} \right) k_{-3}}{(m_2 - m_1) m_1 (e^{\alpha t - 1}) + \frac{m_1 - \left( \frac{1}{\alpha} \right) k_{-3}}{(m_1 - m_2) m_2} (1 - e^{\alpha t})}
\]

and

\[
m_1, m_2 = \frac{-(k_{-3} + k_{-3} + k_{-4}[I]) \pm \sqrt{(k_{-3} + k_{-3} + k_{-4}[I])^2 - 4(k_{-3} + k_{-3})[I]}}{2}
\]

Similarly, for complexing inactivations, the corresponding equations are:

\[
[P] = \frac{k_{-3} K_1 K_2[E][A][B]}{\alpha} \times \frac{m_2 + k_3[I]/\alpha}{(m_2 - m_1) m_1 (e^{\alpha t - 1}) + \frac{m_1 + k_3[I]/\alpha}{(m_1 - m_2) m_2} (1 - e^{\alpha t})}
\]

and

\[
m_1, m_2 = \frac{-(k_{-3}[I]/\alpha + k_{-3} + k_{-4}) \pm \sqrt{(k_{-3}[I]/\alpha + k_{-3})^2 - 4(k_{-3} + k_{-3})[I]/\alpha}}{2}
\]

In eqns. (9) and (11), \( m_1 \) and \( m_2 \) are, as previously, the sum and difference respectively of the terms in the numerator. It can be seen by a comparison of eqns. (8) and (10) that they contain \([I] \) in different terms. When the reaction time is sufficiently large, the exponential terms approaches zero. Rearranging the respective equations gives \([P]_x \) for the conformational-change type of inactivation as:

\[
[P]_x = k_{-3} K_1 K_2[E][A][B] \frac{k_{-3}}{(k_{-3} + k_{-3})[I] + (k_{-3} + k_{-3})[I]} + \frac{k_{-3}}{(k_{-3} + k_{-3})[I] + (k_{-3} + k_{-3})[I]}
\]

and for the complexing type of inactivation:

\[
[P]_x = k_{-3} K_1 K_2[E][A][B] \frac{k_{-3} + k_{-3}}{k_{-3} + k_{-3} + k_{-3} + k_{-3}} + \frac{k_{-3} + k_{-3}}{k_{-3} + k_{-3} + k_{-3} + k_{-3}}[I]
\]

From the above it can be easily seen that a plot of \([P]_x \) against \(1/[I] \) gives a straight line with a positive slope which passes through the origin for the complexing type and cuts at the ordinate with a positive intercept for the conformational-change type of inactivation. The same conclusion holds for one-substrate reactions, with \( K_1K_2[A][B] \) in eqns. (12) and (13) replaced by \([S]/K_m \).

### MATERIALS AND METHODS

Hexokinase and glucose-6-phosphate dehydrogenase were Sigma products. ATP was from Boehringer, 2-chloromercuri-4-nitrophenol (MNP) from Chemical Dynamics Corp., South Plainfield, N.J., U.S.A., and NADP* from Dongfeng Biochemicals, Shanghai, China. All other reagents used were local products of Analytical Grade.

Measuring the inactivation rate constants of hexokinase by monitoring the substrate reaction in the presence of the inactivator MNP, the method was essentially that described previously [Tian & Tsou, 1982; Tsou, 1988], except that the activity of hexokinase was monitored by a coupled assay in which the glucose-6-phosphate generated was coupled to the reduction of NADP* in the presence of glucose-6-phosphate dehydrogenase and the absorbance change at 340 nm was monitored. In a theoretical study, Wang & Zhao [1991] have recently shown that the substrate-reaction method using a coupled enzyme assay can be used for the study of enzyme-inactivation kinetics. In control experiments, MNP did not affect the activity of glucose-6-phosphate dehydrogenase under identical conditions.

### RESULTS

#### Inactivation of the complexing type

Inactivators which have some resemblance in structure to the substrate usually bind with the enzyme at the active site reversibly before the irreversible modification step (Stone & Hofsteenge, 1985; Silverberg et al., 1986; Liu & Tsou, 1986; Teruel et al., 1987) and are sometimes known as ‘affinity’ inactivators. The inactivation of chymotrypsin by either phenylmethanesulphonyl fluoride (PMSF) or di-isopropyl fluorophosphate (DFP) is of the complexing type as shown in this laboratory. A plot of \([P]_x \) against the reciprocal of inactivator concentration from the experimental data of Tian & Tsou (1982) is given in Fig. 1. A similar plot, shown in Fig. 2, was based on the data given by Silverberg et al. (1986). In both cases straight lines passing through the origin, as required for complexing type of inactivation, were obtained.

#### Conformation change before binding with inactivator

There are a number of reports suggesting that conformation changes may take place before binding with the inactivators (Rowe et al., 1970; Hatfield et al., 1970; Redkar & Kukare, 1975; Ellis et al., 1990). However, in most of the cases the kinetic data given were not sufficient for one to distinguish analytically
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change before its thiol becomes reactive to mercurial reagents. Fig. 3 shows the course of inactivation of hexokinase by MNP as monitored by the substrate reaction in a coupled enzyme assay with glucose-6-phosphate dehydrogenase. In the absence of MNP, the reaction followed a linear course, showing the saturation of hexokinase with the substrates throughout the entire course of the reaction under observation. Curve 5 shows that after the concentration of product formed had reached a constant value, $[P]_e$, indicating complete inactivation of hexokinase, the addition of glucose-6-phosphate resulted in the rapid formation of NADPH. It is therefore clear that, at this stage of reaction, the activity of glucose-6-phosphate dehydrogenase was not significantly affected. The inactivation was entirely due to hexokinase alone.

In general, the present approach of following the kinetics of the substrate reaction in the presence of the inactivator requires that the enzyme is stable during the entire course of study and that the accumulation of products does not produce significant inhibition. In the present study, hexokinase is stable under the experimental conditions, and complete linearity of the control reaction (curve 0, Fig. 3) indicates that there is no significant inhibition by the reaction products generated.

A similar plot of $[P]_e$ against the reciprocal of inactivator concentration from the data of Fig. 3 gives a straight line with a positive intercept at the ordinate, as shown in Fig. 4. This is to be expected, according to eqn. (12) above, if the enzyme undergoes a conformational change before binding with the inactivator.

**DISCUSSION**

Reports on the complexing type of enzyme inactivation are abundant in the literature. Inactivators which resemble the substrate in structure usually bind with the enzyme before the inactivation step and this has been extensively explored for affinity labelling and characterization of enzyme active sites (Stone & Hofsteenge, 1985; Silverberg et al., 1986; Teruel et al. 1987). Relatively little is known on the situation where inactivators bind only with one of the conformation states of the enzyme, leading to inactivation (Rowe et al., 1970; Hatfield et al. 1970; Redkar & Kenkare, 1975; Ellis et al., 1990). The method presented here provides a kinetic criterion for the characterization of a conformation change of the enzyme before binding with the inactivator and could be useful in revealing further examples of enzyme conformational changes. It is known that enzymes can exist in different conformation states, and it is generally accepted that substrate binding induces conformational changes in enzymes (Koshland & Neet, 1968; Sinev et al., 1989; Kostrewa et
REFERENCES


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