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

Chun LIU and Chen Lu TSOU*

National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing, People's Republic of China

It has been suggested that the complexing type of inactivation in which the inactivator binds reversibly with the enzyme before inactivation cannot be differentiated kinetically from that a slow enzyme conformation change is involved as a first step [Rakitzis (1986) *J. Theor. Biol.* **122**, 247–249]. The kinetics of the substrate reaction during modification of enzyme activity previously described [Tsou (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 381–436] have now been applied to this problem and equations derived to show that the slow-conformational-change type can be differentiated from the complexing type by plotting the final concentration of product formed, $[P]_{\infty}$, against the reciprocal of inactivator concentration. The reaction of hexokinase with 2-chloromercuri-4-nitrophenol has been shown to involve a conformational change of the enzyme before inactivation.

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 (Kitz & Wilson, 1962; Tsou, 1965; Tsou 1988). A plot of the reciprocal of the apparent inactivation rate constant, $1/k$, against $1/[I]$ 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 prior 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, $[P]_{\infty}$, 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 $[P]_{\infty}$ against $1/[I]$ 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:

where E and I are enzyme and inactivator respectively. E_x 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:

$$[E]/[E]_0 = C e^{m_1 t} + (1 - C) e^{m_2 t} \quad (3)$$

where $[E]_0$ is the total enzyme concentration. Although:

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

for case (1) and:

$$m_1, m_2 = \frac{-(k_{+3} + k_{-3} + k_{+4}[I]) \pm \sqrt{\{(k_{+3} + k_{-3} + k_{+4}[I])^2 - 4k_{+3}k_{+4}[I]\}}}{2} \quad (5)$$

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_{+3} , k_{-3} and k_{+4} are unknown.

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

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

*To whom correspondence should be sent.

For reactions involving two substrates obeying a random mechanism;

$$[\text{E}] = \frac{[\text{E}] + [\text{EA}] + [\text{EB}] + [\text{EAB}]}{1 + K_1[\text{A}] + K_3[\text{B}] + K_1 K_2[\text{A}][\text{B}]} [\text{EAB}] = K_1 K_2[\text{A}][\text{B}][\text{E}]$$

where A and B are the two substrates and K_1 – K_4 are equilibrium binding constants. We may write $[\text{E}] = [\text{E}]_0/\alpha$ if we denote $(K_m + [\text{S}])/K_m$ or $1 + K_1[\text{A}] + K_3[\text{B}] + K_1 K_2[\text{A}][\text{B}]$ as α .

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

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

we obtain the rate of disappearance of free enzyme as given by eqn. (3), with m_1 and m_2 as shown in eqn. (9), from the relationship:

$$d[\text{P}]/dt = k_{+2}[\text{EAB}] = k_{+2} K_1 K_2[\text{E}][\text{A}][\text{B}]$$

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

$$[\text{P}] = \frac{k_{+2} K_1 K_2 [\text{E}]_0 [\text{A}][\text{B}] k_{-3}}{\alpha(k_{+3} + k_{-3})} \times \left[\frac{m_2 - \left(1 - \frac{1}{\alpha}\right)k_{+3}}{(m_2 - m_1)m_1} (e^{m_1 t} - 1) + \frac{m_1 - \left(1 - \frac{1}{\alpha}\right)k_{+3}}{(m_1 - m_2)m_2} (1 - e^{m_2 t}) \right] \quad (8)$$

and

$$m_1, m_2 = \frac{-\left(\frac{k_{+3}}{\alpha} + k_{-3} + k_{+4}[\text{I}]\right) \pm \sqrt{\left(\left(\frac{k_{+3}}{\alpha} + k_{-3} + k_{+4}[\text{I}]\right)^2 - 4k_{+3}k_{+4}[\text{I}]\frac{1}{\alpha}\right)}}{2} \quad (9)$$

Similarly, for complexing inactivations, the corresponding equations are:

$$[\text{P}] = \frac{k_{+2} K_1 K_2 [\text{E}]_0 [\text{A}][\text{B}]}{\alpha} \times \left\{ \frac{m_2 + k_{+3}[\text{I}]/\alpha}{(m_2 - m_1)m_1} (e^{m_1 t} - 1) + \frac{m_1 + k_{+3}[\text{I}]/\alpha}{(m_1 - m_2)m_2} (1 - e^{m_2 t}) \right\} \quad (10)$$

and,

$$m_1, m_2 = \frac{-\left(\frac{k_{+3}[\text{I}]}{\alpha} + k_{-3} + k_{+4}\right) \pm \sqrt{\left(\left(\frac{k_{+3}[\text{I}]}{\alpha} + k_{-3} + k_{+4}\right)^2 - 4k_{+3}k_{+4}[\text{I}]\frac{1}{\alpha}\right)}}{2} \quad (11)$$

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 $[\text{I}]$ in different terms. When the reaction time is sufficiently large, the exponential terms approaches zero. Rearranging the respective

equations gives $[\text{P}]_\infty$ for the conformational-change type of inactivation as:

$$[\text{P}]_\infty = k_{+2} K_1 K_2 [\text{E}]_0 [\text{A}][\text{B}] \left\{ \frac{k_{-3}}{(k_{+3}k_{+4}[\text{I}] + (k_{+3} + k_{-3})k_{+3})} + \frac{k_{-3}}{(k_{+3} + k_{-3})k_{+3}} \right\} \quad (12)$$

and for the complexing type of inactivation:

$$[\text{P}]_\infty = k_{+2} K_1 K_2 [\text{E}]_0 [\text{A}][\text{B}] \frac{k_{-3} + k_{+4}}{k_{+3}k_{+4}[\text{I}]} \quad (13)$$

From the above it can be easily seen that a plot of $[\text{P}]_\infty$ against $1/[\text{I}]$ 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 $K_1 K_2[\text{A}][\text{B}]$ in eqns. (12) and (13) replaced by $[\text{S}]/K_m$.

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, NJ, 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 $[\text{P}]_\infty$ 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

Fig. 1. Plot of $[P]_{\infty}$ against $1/[I]$ for the inactivation of chymotrypsin

Data are from Tian & Tsou (1982).

Fig. 2. Plot of $[P]_{\infty}$ against $1/[I]$ for the inactivation of kallikrein by C1 inhibitor

Data are from Silverberg (1986). Arbitrary units are used for replotting.

Fig. 3. Course of the substrate reaction of hexokinase in the presence of different concentrations of MNP

Final concentrations were: 0.1 M-Tris/HCl buffer, pH 7.4, 7.5 mM-glucose, 3 mM-ATP, 5 mM-MgCl₂, 0.4 unit of glucose-6-phosphate dehydrogenase and 0.01 μ M-hexokinase. Concentrations of MNP were 0, 0 (control); 1, 6.75; 2, 9.0; 3, 13.5; 4, 27 μ M. The enzyme was added to the reaction mixture to start the reaction at 25 °C. Addition of glucose 6-phosphate at the end of the reaction of curve 4 results in curve 5 (see the text for details).

Fig. 4. Plot of $[P]_{\infty}$ against $1/[I]$ for the inactivation of hexokinase by MNP

Data are from Fig. 3.

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, $[P]_{\infty}$, 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 $[P]_{\infty}$ 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 conformational 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 conformational 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 conformational states, and it is generally accepted that substrate binding induces conformational changes in enzymes (Koshland & Neet, 1968; Sinev *et al.*, 1989; Kostrewa *et*

the two types of inactivation. It has been suggested (Colowick, 1973; Kosow & Rose, 1971; Shill & Neet, 1975; Ainslie *et al.*, 1972) that hexokinase, which is in its monomeric form under most of the experimental conditions, undergoes a conformational

al., 1989; Hiratsuka, 1990). However, it is equally possible that the substrate only stabilizes a conformation state which exists originally, although only as a minor component. The conformation-change type inactivators could act in a way similar to the substrate in covalently binding and stabilizing a minor conformational form of the enzyme, resulting in its inactivation.

The equations derived in the present study have been based on the irreversibility of the inhibition reagents as are the activations of chymotrypsin by PMSF or DFP and of hexokinase by 5,5'-dithiobis-(2-nitrobenzoic acid). However, the present approach can be easily applied to slow reversible inhibitions, as shown for the inhibition of trypsin by its specific inhibitors (Zhou *et al.*, 1989).

Conformational changes in enzymes can be an important feature of catalysis, since it has been suggested that enzymes pass through many different conformational forms during catalytic action (Clarke *et al.*, 1985; Schultz *et al.*, 1990). The inactivator may bind either with a conformational state which is on the catalytic pathway or with one on a side path. In either case, the irreversible binding with the inactivator would eventually lead to complete inactivation. The present kinetic criterion does not differentiate between the above two possibilities, which would require further investigation.

The present investigation was supported in part by a grant no. 38970207) from the China National Natural Science Foundation.

REFERENCES

- Ainslie, G. R., Jr., Shill, J. P. & Neet, K. E. (1972) *J. Biol. Chem.* **247**, 7088–7096
- Clarke, A. R., Waldman, A. D. B., Hart, K. W. & Holbrook, J. J. (1985) *Biochim. Biophys. Acta* **829**, 397–407
- Colowick, S. P. (1973) *Enzymes* 3rd Ed. **9**, 1–28
- Ellis, V., Wun, T. C., Behrendt, N., Ronne, E. & Dano, K. (1990) *J. Biol. Chem.* **265**, 9904–9908
- Gontero, B., Meunier, J. C., Buc, J. & Ricard, J. (1984) *Eur. J. Biochem.* **145**, 485–488
- Hatfield, W. G., Ray, W. J., Jr. & Umbarger, H. E. (1970) *J. Biol. Chem.* **245**, 1748–1754
- Hiratsuka, T. (1990) *J. Biol. Chem.* **265**, 18786–18790
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Koshland, D. E., Jr. & Neet, K. E. (1968) *Annu. Rev. Biochem.* **37**, 359–410
- Kosow, D. P. & Rose, I. A. (1971) *J. Biol. Chem.* **246**, 2618–2625
- Kostrewa, D., Choe, H. W., Heinemann, U. & Saenger, W. (1989) *Biochemistry* **28**, 7592–7600
- Liu, W. & Tsou, C. L. (1986) *Biochim. Biophys. Acta* **870**, 185–190
- Rakitzis, E. T. (1980) *J. Math. Biol.* **10**, 79–87
- Rakitzis, E. T. (1986) *J. Theor. Biol.* **122**, 247–249
- Redkar, V. D. & Kenkare, U. W. (1975) *Biochemistry* **14**, 4704–4712
- Rowe, P. B., Coleman, M. D. & Wyngaarden, J. B. (1970) *Biochemistry* **9**, 1498–1050
- Sanner, T. & Tron, L. (1975) *Biochemistry* **14**, 230–234
- Schultz, G. E., Muller, C. W. & Diederichs, K. (1990) *J. Mol. Biol.* **213**, 627–630
- Shill, J. P. & Neet, K. E. (1975) *J. Biol. Chem.* **250**, 2259–2268
- Silverberg, M., Longo, J. & Kaplan, A. P. (1986) *J. Biol. Chem.* **261**, 14965–14968
- Sinev, M. A., Razgulyaev, O. I., Vas, M., Timchenko, A. A. & Ptitsyn, O. S. (1989) *Eur. J. Biochem.* **180**, 61–66
- Stone, S. R. & Hofsteenge, J. (1985) *Biochem. J.* **230**, 497–502
- Teruel, J. A., Tudela, J., Fernandez Belda, F., Garcia Carmona, F., Gomez Fernandez, J. C. & Garcia Canovas, F. (1987) *Biochim. Biophys. Acta* **911**, 256–260
- Tian, W. X. & Tsou, C. L. (1982) *Biochemistry* **21**, 1028–1032
- Tsou, C. L. (1965) *Acta Biochim. Biophys. Sin.* **5**, 398–417
- Tsou, C. L. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 381–436
- Wang, Z. X. & Zhao, K. Y. (1991) *Sci. Sin. (Engl. Ed.)* **34B**, 162–173
- Wang, Z. X., Preiss, B. & Tsou, C. L. (1988) *Biochemistry* **27**, 5095–5100
- Zhou, J. M., Liu, L. & Tsou, C. L. (1989) *Biochemistry* **28**, 1070–1076

Received 9 July 1991/5 September 1991; accepted 11 September 1991