Kinetics of the course of inactivation of aminoacylase by 1,10-phenanthroline

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The kinetic theory of the substrate reaction during modification of enzyme activity previously described [Tsou (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 381–436] has been applied to a study on the kinetics of the course of inactivation of aminoacylase by 1,10-phenanthroline. Upon dilution of the enzyme that had been incubated with 1,10-phenanthroline into the reaction mixture, the activity of the inhibited enzyme gradually increased, indicating dissociation of a reversible enzyme–1,10-phenanthroline complex. The kinetics of the substrate reaction with different concentrations of the substrate chloroacetyl-L-alanine and the inactivator suggest a complexing mechanism for inactivation by, and substrate competition with, 1,10-phenanthroline at the active site. The inactivation kinetics are single phasic, showing that the initial formation of an enzyme–Zn²⁺–1,10-phenanthroline complex is a relatively rapid reaction, followed by a slow inactivation step that probably involves a conformational change of the enzyme. The presence of Zn²⁺ apparently stabilizes an active-site conformation required for enzyme activity.

INTRODUCTION

It is well known that metal ions play important roles in the catalysis of a large number of different enzymes, and zinc enzymes are particularly interesting in that they encompass all the six main classes of enzymes (Vallee & Galles, 1984; Vallee, 1988). It has been suggested by Vallee & Williams (1968) that the presence of Zn²⁺ helps to keep the conformation of the active site in a strained state, named the entatic state, required for the catalysis of the enzyme. Although the importance of Zn²⁺ in the catalytic processes is well established by inhibition with metal-ion chelators, inactivation by its removal from the enzymes and re-activation by its restoration (Vallee & Williams, 1968; Kidani & Hirose, 1977; Billo, 1979), the kinetics of the course of inactivation during metal chelation or removal have been but little explored.

Some years ago, a systematic study on the kinetics of the substrate reaction during irreversible modification of enzyme activity in the presence of a modifier was presented (Tsou, 1965, 1988). It has been shown not only that the apparent rate constant for the irreversible modification of enzyme activity can often be obtained in a single experiment, but that the effect of substrate complexing and competition with the inactivator can also be ascertained (Tian & Tsou, 1982). This kinetic approach has now been applied to the kinetics of irreversible inactivation (Liu & Tsou, 1986; Wang *et al.*, 1988), to slow binding reversible inhibition (Zhou *et al.*, 1989) and to activation (Liu *et al.*, 1985). It appears that the inactivation kinetics of zinc enzymes are particularly suitable for such studies, as the metal ion can be readily removed by chelators with inactivation and restored with complete re-activation.

Aminoacylase I (EC 3.5.1.14) is a dimeric enzyme (Kördel & Schneider, 1976, 1977a,b) containing one Zn²⁺ ion per each subunit. The kinetics of inactivation of aminoacylase I by chelating agents were studied by Kumpe *et al.* (1981) and Szajani *et al.* (1980). However, the method employed of monitoring the course of activity loss accompanying Zn²⁺ removal during dialysis

can hardly detect the initial phase of the inactivation process. The present paper reports a study on the complete kinetic course of 1,10-phenanthroline (OP) inactivation in which the hydrolysis of chloroacetyl-L-alanine was monitored in the presence of OP. Kinetic analysis of the results suggests a triphasic reaction involving a rapid formation of a reversible enzyme–OP complex, then a relatively slow conformational change at the active site, this leading to the inactivation of the enzyme before the removal of Zn²⁺.

MATERIALS AND METHODS

Aminoacylase I was prepared from pig kidney first according to the procedure of Birnbaum (1955) to the step of acetone fractionation, and the crude preparation was then chromatographed first by gel filtration through Sephadex G-150 followed by ion-exchange with DEAE-cellulose as described by Kördel & Schneider (1976). The final preparation was homogeneous on PAGE in the presence and the absence of SDS. Chloroacetyl-DL-alanine was from Kasei Industries (Tokyo, Japan); Sephadex G-150 was a Pharmacia product, and other chemicals were local products of analytical grade.

Enzyme concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient $A_{280,1\text{ cm}}^{1\%} = 13.5$ (Kördel & Schneider, 1976). Enzyme activity was determined at 25 °C by measuring the change of absorbance at 238 nm accompanying the hydrolysis of the substrate and using the molar absorption coefficient $\epsilon_{238} = 185 \text{ M}^{-1} \cdot \text{cm}^{-1}$ as reported by Kördel & Schneider (1977a) except that chloroacetyl-DL-alanine was used instead of the pure L-enantiomorph.

Both the conventional method and the progress-of-substrate-reaction method (Tsou, 1988) were used for the study of the inactivation kinetics of aminoacylase. In the conventional method the enzyme was incubated with OP in 0.3 M-sodium phosphate buffer, pH 7.3, and at different time intervals 40 μl portions of the incubation mixture were taken and each was diluted into 3 ml of the reaction mixture containing 6.25 mM

Abbreviation used: OP, 1,10-phenanthroline.

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substrate for assay of activity at 25 °C. In the progress-of-substrate-reaction method 10 μ l of 17.2 μ M-aminoacylase was added to 500 μ l of a reaction mixture containing 6.25 mM substrate in 0.3 M-sodium phosphate buffer, pH 7.3, containing different concentrations of OP and the progress curve for the substrate reaction was analysed (Tian & Tsou, 1982; Tsou, 1988) to obtain the rate constants as detailed below.

RESULTS

Kinetics of aminoacylase

The kinetic behaviour of aminoacylase I in the hydrolysis of acyl-L-amino acids has been well recorded in the literature (Szajani *et al.*, 1980; Galaev & Svedas, 1982; Henseling & Röhm, 1988). Under the conditions employed in the present study of the inactivation kinetics, the hydrolysis of chloroacetyl-DL-alanine follows Michaelis kinetics, with the K_m value in agreement with that obtained by Kördel & Schneider (1975) with the pure L-enantiomorph. The presence of the D-isomer had apparently little effect on the kinetics of hydrolysis of the substrate.

Kinetics of inactivation by removal of Zn^{2+} with OP

The inactivation of aminoacylase during removal of Zn^{2+} by OP was first studied conventionally by taking portions at different time intervals of an incubation mixture of the enzyme with the chelator and diluting each into the assay mixture for activity determination. The results, as presented in Fig. 1, show a first-order process with a rate constant $k = 0.25 \times 10^{-3} \text{ s}^{-1}$ at an OP concentration of 15 mM. The initial velocity at each OP concentration was taken as far as possible for the measurements of OP inhibition. However, the reaction rates increase gradually with the lengthening of time during assay. The initial-rate decrease and the time required for partial activity recovery increase with the increase in the incubation time with OP (Fig. 2). The activity recovery is due, at least in part, to the dissociation of an inactive enzyme-OP complex. The final activity recovered also decreases with incubation time, indicating the dissociation of Zn^{2+} from the enzyme. It appears that inactivation by removal

Fig. 1. Course of inactivation of aminoacylase by OP

A mixture of the enzyme (2.4 μ M) and OP (15 mM) in 0.3 M-sodium phosphate buffer, pH 7.3, was incubated at 25 °C. At different time intervals as indicated, 40 μ l portions were taken for activity determination in 3 ml of reaction mixture containing 6.25 mM substrate in the same buffer. The inset shows a semi-logarithmic plot of the results.

Fig. 2. Re-activation of aminoacylase after dilution of OP-incubated enzyme

Experimental conditions were as described for Fig. 1. Curves 1-4 are the course of reaction after dilution at incubation times of 0 s, 41 s, 978 s and 1620 s respectively.

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

Final concentrations were 6.25 mM substrate and 0.36 μ M enzyme in 0.3 M-sodium phosphate buffer, pH 7.3. Concentrations of OP were 0 mM (control) (curve 0), 0.10 mM (curve 1), 0.15 mM (curve 2), 0.20 mM (curve 3) and 0.25 mM (curve 4). The enzyme (10 μ l) was added to the reaction mixture (0.5 ml) to start the reaction.

Fig. 4. Semi-logarithmic plots of $\ln([P]_{\text{calc.}} - [P]_{\text{obs.}})$ against time for the substrate reaction in the presence of different concentrations of OP

Data are taken from Fig. 3.

Table 1. Dissociation and microscopic rate constants for the inactivation of aminoacylase by OP

Constant	From plot of $1/A$ versus $[S]$	From plot of $1/A$ versus $[OP]$
K_i (μM)	119	122
k_{+0} (s^{-1})	16×10^{-3}	15×10^{-3}
k_{-0} (s^{-1})	0.8×10^{-3}	1.0×10^{-3}

Fig. 5. Plot of $1/A$ against OP concentration

The values of the apparent rate constant, A , were calculated from data in Figs. 3 and 4. For details see the text.

of Zn^{2+} from the active site of the enzyme is preceded by the reversible formation of an inactive complex.

Kinetics of the substrate reaction in the presence of different concentrations of OP

The time courses of the hydrolysis of the substrate in the presence of different OP concentrations are shown in Fig. 3. At

each concentration of OP the rate decreases with increasing time until a straight line is approached, the slope of which decreases with increasing OP concentrations. As discussed by Tsou (1965, 1988), the above suggests the slow formation of a reversible inactive enzyme-OP complex, as in the cases of the formation of complexes of trypsin with ovomucoid and soya-bean trypsin inhibitor (Zhou *et al.*, 1989). The reaction scheme and the derivation of the kinetic equations have been given before (Tsou, 1988), and are briefly outlined in the Appendix. When the reaction between the inhibitor and the enzyme is reversible, the product formation is given by:

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

where $[P]_t$ is the concentration of the product formed at time t , v is the initial rate of the substrate reaction in the presence of OP, and A and B are the apparent rate constants for the forward and reverse reactions between OP and the enzyme respectively. When t is sufficiently large, the curves become straight lines:

$$[P] = \frac{vBt}{A[OP] + B} + \frac{vA[OP]}{(A[OP] + B)^2} \quad (2)$$

The slope, s , and x -axis intercept, i , are:

$$s = vB/(A[OP] + B)$$

$$i = A[OP]/B(A[OP] + B)$$

The apparent rate constants for the forward reaction, A , and reverse reaction, B , can be obtained from the s and i values at different OP concentrations.

Alternatively, subtraction of eqn. (1) from eqn. (2) gives:

$$[P]_{\text{calc.}} - [P]_{\text{obs.}} = \frac{vA[OP]}{(A[OP] + B)^2} e^{-(A[OP] + B)t} \quad (3)$$

where $[P]_{\text{calc.}}$ and $[P]_{\text{obs.}}$ are the product concentrations to be expected from the straight-line portions of the curves as can be calculated from eqn. (2) and the value actually observed respectively. Plots of $\log([P]_{\text{calc.}} - [P]_{\text{obs.}})$ against t give a series of straight lines at different concentrations of OP with slopes of $-(A[OP] + B)$, as shown in Fig. 4. The apparent forward and reverse rate constants A and B can then be obtained. The value of B directly gives the microscopic rate constant, k_{-0} , for the reverse reaction, and the value of k_{+0} can be obtained by suitable plots of A against $[S]$, as is discussed in another section below.

It has been pointed out that plots of $1/A$ against $[OP]$ can differentiate complexing and non-complexing types of inactivation (Tsou, 1965, 1988). In the non-complexing type OP reacts directly with the enzyme leading to inactivation (reaction 4), whereas in the complexing type OP first reacts with the enzyme reversibly before the inactivation step (reaction 5):

In cases where the complexing step is fast relative to the subsequent inactivation reaction, it has been shown that the apparent rate constant A is:

$$A = \frac{(k_{+0}/K_i)K_m}{K_m(1 + [OP]/K_i) + [S]} \quad (6)$$

where $[S]$ is the substrate concentration, K_m is the Michaelis

Fig. 6. Course of reaction at different substrate concentrations in the presence of OP

Final concentrations were 0.1 mM-OP and 0.36 μ M enzyme in 0.3 M-sodium phosphate buffer, pH 7.3. Concentrations of the substrate were: 6.25 mM (curve 1), 3.125 mM (curve 2), 2.08 mM (curve 3) and 1.55 mM (curve 4). The enzyme was added to the reaction mixture to start the reaction.

Fig. 7. Semi-logarithmic plot of $[P]_{\text{calc.}} - [P]_{\text{obs.}}$ against time for reactions at different substrate concentrations in the presence of OP

Data are taken from Fig. 6.

constant, K_i is the dissociation constant of the enzyme-OP complex and k_{+0} and k_{-0} are the first-order rate constants for the forward and reverse reactions respectively. The dissociation and the microscopic rate constants are summarized in Table 1.

The expressions for the apparent rate constants are different for complexing and non-complexing inhibitions in that eqn. (6) contains the term $[OP]$ whereas the expression for the non-complexing inhibition does not, thus providing the basis for the

Fig. 8. Plots of $1/A$ against substrate concentration

The values of the apparent rate constant, A , were calculated from data in Figs. 6 and 7. For details see the text.

differentiation of these two types of inhibition. A is independent of $[OP]$ for non-complexing inhibitions, but, as can be seen from eqn. (6), a plot of $1/A$ against $[OP]$ gives a straight line with a positive slope intersecting the ordinate for complexing inhibitions. This has been found to be the case for the inactivation by OP, as shown in Fig. 5.

Kinetics of the reaction at different substrate concentrations in the presence of OP

Substrate competition types can be ascertained by measurements of the rates of inactivation in the presence of different substrate concentrations (Tsou, 1965, 1988). For the substrate reaction in the presence of OP, when t is sufficiently large both the initial rate and the slope of the asymptote increase with increasing substrate concentration, as shown in Fig. 6. Similarly plots of $\log([P]_{\text{calc.}} - [P]_{\text{obs.}})$ against t give a series of straight lines at different concentrations of the substrate with slopes of $-(A[OP] + B)$, as shown in Fig. 7, whereas Fig. 8 shows that the apparent forward rate constant A obtained is dependent of substrate concentration and a plot of $1/A$ against $[S]$ gives a straight line with a positive intercept at the y -axis, indicating competition between OP and the substrate. The dissociation and microscopic rate constants can also be obtained by plotting $1/A$ against $[S]$, and the results obtained are in satisfactory agreement with those obtained by the plot of $1/A$ against $[OP]$, as shown in Table 1.

DISCUSSION

The action of OP on zinc enzymes has been extensively studied, and it is generally accepted that OP inactivates by chelating with or removal of Zn^{2+} and that the activity can usually be restored by the addition of Zn^{2+} (Kidani & Hirose, 1977; Billo, 1979; Vallee & Galdes, 1984; Gilles *et al.*, 1984). For aminoacylase I it has been shown that dialysis in presence of OP leads to the removal of Zn^{2+} and inactivation of the enzyme (Kördel & Schneider, 1977a,b; Gilles *et al.*, 1984). Kinetic studies now show that the dissociation is preceded by a relatively rapid step of reversible binding at the active site, presumably by complexing with Zn^{2+} , followed by a slow inactivation step. The

second slow step very probably involves changes from a strained, entactic and active state to a conformationally more stable and inactive state. It is likely that this precedes the removal of Zn^{2+} from the active site, as dilution at this stage results in a gradual recovery of activity and it is most unlikely that a re-association between Zn^{2+} and the enzyme could occur after a 25-fold dilution of the inactive enzyme-OP complex.

The formation of the reversible enzyme-OP complex appears to be a fast reaction, as the semi-logarithmic plots for the inactivation reaction (Figs. 4 and 7) are monophasic. If the two steps shown in reaction (4) had comparable rates, biphasic semi-logarithmic plots should have been obtained, as in the case shown previously for the inhibition of trypsin by soya-bean trypsin inhibitor (Zhou *et al.*, 1989).

Although the detailed structure of aminoacylase I is still unknown, results of structural analysis of carboxypeptidase A show that the Zn^{2+} is quinquevalently co-ordinated and the presence of substrate replaces a water molecule, leading to sexicovalent co-ordination (Rees *et al.*, 1981). It appears likely that the active site of aminoacylase has a similar structure and that OP binds by occupying the co-ordinate taken by the substrate and thus leading to a conformational change destroying the entactic state. Prolonged reaction in the presence of OP results in the removal of Zn^{2+} from the enzyme, probably in the form $Zn^{2+}(OP)_3$.

The competition between OP and the substrate is of interest, although substrate protection against OP inactivation has been previously reported for other zinc enzymes. It seems that the substrate binds at the active site by co-ordinating with Zn^{2+} and replacing a water molecule, as suggested by Henseling & Röhm (1988), and that OP competes with the substrate in co-ordinating with Zn^{2+} . It is possible that OP binding, like that of the substrate, results in a change releasing the strained conformational state that is required for the catalysis of the enzyme.

APPENDIX

The equations for irreversible inactivation with complex-formation before the inactivation step have been given before (Tsou, 1965, 1988). The inactivation of aminoacylase by OP appears to be a reversible reaction before the removal of Zn^{2+} by OP. This can be written as:

$$[E]_T = [E^*] + [E(OP)'] = [E] + [ES] + [E(OP)] + [E(OP)']$$

Z.-X.W. and C.-L.T. are grateful for support in part by Grant no. 38970207 from China Natural Science Foundation.

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As before it is assumed that the steady state of the substrate reaction is rapidly established and that both $[S]$ and $[OP]$ are $\gg [E]$. In addition, it is also assumed that the formation of the $E(OP)$ complex is a fast reaction relative to the inactivation step, which assumption is justified as the inactivation is apparently monophasic. The following relations hold at any time:

$$\begin{aligned}
 [E] &= \frac{[E^*]K_m}{K_m \left(1 + \frac{[OP]}{K_i}\right) + [S]} \\
 [ES] &= \frac{[E^*][S]}{K_m \left(1 + \frac{[OP]}{K_i}\right) + [S]} \\
 [E(OP)] &= \frac{[E^*][OP]K_m/K_i}{K_m \left(1 + \frac{[OP]}{K_i}\right) + [S]}
 \end{aligned}$$

For reversible reactions, the rate of inactivation is:

$$\begin{aligned}
 -\frac{d[E^*]}{dt} &= k_{+0}[E(OP)] - k_{-0}[E(OP)'] \\
 -\frac{d[E^*]}{dt} &= \left(\frac{k_{+0}[OP]K_m/K_i}{K_m \left(1 + \frac{[OP]}{K_i}\right) + [S]} + k_{-0} \right) [E^*] - k_{-0}[E]_T
 \end{aligned}$$

The above can be written as:

$$-\frac{d[E^*]}{dt} = (A[OP] + B)[E^*] - B[E(OP)]$$

where A and B are the apparent rate constants for the forward and reverse reactions respectively. The substrate reaction in the presence of OP is:

$$\frac{d[P]}{dt} = \frac{k_{+2}[E^*][S]}{K_m \left(1 + \frac{[OP]}{K_1}\right) + [S]}$$

It can be easily shown that the equation for product concentration at time t is:

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

where $[P]_t$ is the concentration of the product formed at time t and v is the initial rate of the substrate reaction in the presence of OP.

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Received 2 April 1991/12 June 1991; accepted 20 June 1991