Kinetics of Inactivation of Aminoacylase by 2-Chloromercuri-4-Nitrophenol: A New Type of Complexing Inhibitor

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ABSTRACT: The kinetic theory of the substrate reaction during modification of enzyme activity previously described [Tsou (1988) *Adv. Enzymol. 61,* 381−436] has been applied to a study of the inactivation kinetics of aminoacylase by 2-chloromercuri-4-nitrophenol (MNP). The results indicate that the mechanism of reaction between MNP and aminoacylase is a special type of irreversible inhibition. The main features of this type of inhibitor are as follows: (i) the reaction kinetics of inhibitor with enzyme is a single exponential process; (ii) inhibition shows a noncompetitive, complexing behavior; (iii) the first inhibitor−enzyme complex, EI, still has some enzyme activity, and hence the plot of $[P]_\infty$ versus the reciprocal of inhibitor concentration gives a straight line with a positive intercept at the ordinate. On the basis of the kinetic equation of substrate reaction in the presence of the inhibitor, a plotting method has been developed for determining the inhibition kinetic constants. As an example, all reaction kinetic constants of aminoacylase with 2-chloromercuri-4-nitrophenol have been determined. The results of the present study suggest that the essential thiol group at the active site of aminoacylase may have a significant effect on the catalytic step but is not involved in substrate binding.

According to their reversibility, strength, and reaction rates, reversible inhibitors of enzymes can be classified into four categories: (i) classical, (ii) tight-binding, (iii) slow-binding, and (iv) slow, tight-binding (Morrison, 1982; Morrison & Walsh, 1988). The first two classes of inhibitors have been recognized for a very long time, and their kinetics have been dealt with in many textbooks on enzymology (Segel, 1975; Laidler & Bunting, 1973; Fersht, 1985; Hammes, 1982; Williams & Morrison, 1979). During the last two decades, the behavior and kinetics of slow-binding and slow, tight-binding inhibitors have also been systematically studied (Cha, 1975, 1976; Williams *et al.*, 1979; Sculley & Morrison, 1986; Wang & Tsou, 1987; Tsou, 1988; Wang, 1990a, 1993). A common feature for these two classes of inhibitors is that the inhibition of enzyme activity is time dependent, and the steady-state rate of an enzyme-catalyzed reaction is attained within a second−minute time scale.

There are two basic mechanisms, noncomplexing and complexing type of inhibitions, for the slow-binding and slow, tight-binding inhibitions (Morrison, 1982; Morrison & Walsh, 1988; Tsou, 1988). In noncomplexing inhibition, an assumption is that the interaction of inhibitor with the enzyme is slow due to low inhibitor concentration, and/or it encounters barriers to its binding at the active site of the enzyme. For complexing inhibition, it is assumed there is an initial rapid interaction between an enzyme and a competitive inhibitor (inactive substrate analog) to form EI, which undergoes a slow isomerization reaction to a second complex (EI*). The irreversible modification can be regarded as a special case of reversible inhibition in which the off-rate constants of enzyme−inhibitor complexes in some steps are practically equal to zero. In this regard, the site-directed inhibitor or affinity labeling reagent has attracted particular attention because of its usefulness in elucidating the nature of the functional groups located at the active site, essential for enzyme activity, and likely to be involved in the catalytic process. The kinetics of enzyme modification and denaturation in the presence of substrate have been treated in two different ways depending upon whether a significant proportion of substrate is consumed in the enzyme-catalyzed reaction (Laidler & Bunting, 1973; Duggleby, 1986; Pike & Duggleby, 1987; Wang & Tsou, 1990), or whether the substrate concentration can be considered to be essentially constant during the period of observation and, hence, set equal to its initial value in the derivation of integrated rate expressions describing the time dependence of product formation (Tsou, 1988; Tian & Tsou, 1982). The latter approach has been extended beyond the treatment of relatively simple cases of irreversible modification of an enzyme catalyzing a single intermediate, one substrate reaction to include more complicated models: reactivation of an enzyme obeying a three-step acylenzyme mechanism (Liu *et al.*, 1985), product inhibition of a reactivated enzyme (Liu & Tsou, 1987), enzyme inactivation by a mechanism-based inhibitor (suicide substrate) (Tudela *et al.*, 1987; Escribano et al., 1989; Wang, 1990b), the use of a coupled-enzyme system to continuously monitor product formation (Teruel *et al.*, 1986, 1987; Wang & Zhao, 1992a,b), irreversible inhibition for several two-substrate kinetic mechanisms (Wang & Tsou, 1987; Wang *et al.*, 1988), the two slow step inhibition mechanism (Zhou *et al.*, 1989), enzyme inactivation by an unstable irreversible modifier (Topham, 1990), inactivation of metalloenzyme by

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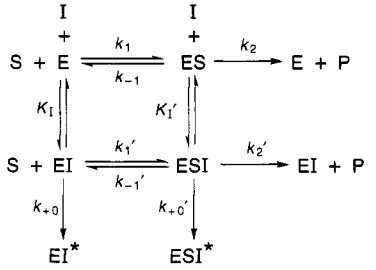
Scheme 1



a metal chelating agent (Wang *et al.*, 1992), reconstitution of the apo-metalloenzyme (Wu & Tsou, 1993), and the differentiation between enzyme involving complex formation with inactivator and that involving a conformation-change step (Liu & Tsou, 1992).

In this paper, we report a new type of irreversible complexing inhibitor. The main features of this type of inhibitor are as follows: (i) the reaction kinetics of inhibitor with enzyme is a single exponential process; (ii) the inhibition shows a noncompetitive, complexing behavior; (iii) the first inhibitor−enzyme complex, EI, still has some enzyme activity, and hence the plot of $[P]_\infty$ versus the reciprocal of inhibitor concentration gives a straight line with a positive intercept at the ordinate. The kinetic equation of substrate reaction in the presence of inhibitor has been derived, and as an example, the kinetics of inactivation of aminoacylase by 2-chloromercuri-4-nitrophenol has been studied.

MATERIALS AND METHODS

Aminoacylase (EC 3.5.1.14) was purchased from Sigma Chemical Co. Chloroacetyl-DL-alanine was from Kasei Industries (Tokyo, Japan); 2-chloromercuri-4-nitrophenol (MNP) was from Aldrich Chemical Co. All other chemicals were local products of analytical grade.

The aminoacylase concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient $E_{280}^{1\%} = 13.5$ (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 $\epsilon_{238} = 185\ M^{-1}\ cm^{-1}$ as reported by Kördel and Schneider (1976) except that chloroacetyl-DL-alanine was used instead of the pure L-enantiomorph.

Modification of thiol groups in the aminoacylase was carried out in 0.1 M phosphate buffer (pH 7.2) at 25 °C. The enzyme was incubated with different amounts of MNP, and the modification reaction was followed by the spectral changes between 300 and 500 nm. After the reaction was finished, the enzyme activity remaining was measured by adding 10 μL of the reaction mixture to 1 mL of an assay system containing 12.5 mM substrate (pH 7.2, 25 °C). Since the reaction of MNP with different SH groups in a protein molecule may have different spectral characteristics (Wu *et al.*, 1989), the extent of SH group modification was calculated directly from the molar ratio of MNP/enzyme in the reaction solution after the modification reaction was finished.

The kinetics of the inactivation reaction was followed by the substrate reaction in the presence of the modifier as described before (Tian & Tsou, 1982). All measurements were carried out in 0.1 M sodium phosphate buffer (pH 7.0) at 25 °C.

KINETIC ANALYSIS

Let us consider the reaction mechanism in Scheme 1 where E, S, and I represent enzyme, substrate, and inhibitor, respectively. As will be seen later, the experimental data obtained from the kinetic study of MNP inhibition of aminoacylase are in accordance with this theoretical model. The concentration of product formed at time t is given by (see Appendix)

$$[P] = [P]_\infty\{1 - \exp(-k_{obs}t)\} \quad (1)$$

where $[P]_\infty$ is the product concentration at time infinity, and k_{obs} is the apparent inactivation rate constant:

$$[P]_\infty = \frac{\{k_2 + (k_2'[I]/K_I')\}[E]_0[S]}{\{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')\}[I]} = \frac{k_2[E]_0[S]}{\{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')\}[I]} + \frac{(k_2'/K_I')[E]_0[S]}{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')} \quad (2)$$

and

$$k_{obs} = \frac{\{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')\}[I]}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]} \quad (3)$$

where [S] and [I] are respectively the concentrations of substrate and inhibitor, and K_m is the Michaelis constant. From eq 1, it can be seen that when reaction time t approaches infinity, [P] approaches constant values, and plots of $\ln([P]_\infty - [P])$ against t give straight lines with slopes of $-k_{obs}$. When keeping [S] constant, a plot of $[P]_\infty$ against 1/[I] should give a straight line with a slope s and an ordinate intercept i of

$$1/s = \frac{k_{+0}K_m}{K_Ik_2[E]_0[S]} + \frac{k_{+0}'}{K_I'k_2[E]_0} \quad (4)$$

$$1/i = \frac{k_{+0}K_I'K_m}{K_Ik_2'[E]_0[S]} + \frac{k_{+0}'}{k_2'[E]_0} \quad (5)$$

Both the slope and the intercept are functions of the substrate concentration. Secondary plots of $1/s$ against 1/[S] also give straight lines, and the values of k_{+0}/K_I and k_{+0}'/K_I' can be determined from the slope and intercept of the straight line when k_2 and K_m are known. Similarly, the value of K_I'/k_2' can then be calculated from the slope of the secondary plot of $1/i$ against 1/[S].

Equation 3 can be rewritten as

$$1/k_{obs} = \frac{K_m + [S]}{\{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')\}[I]} + \frac{(K_m/K_I) + ([S]/K_I')}{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')} \quad (6)$$

It can be seen from eq 6 that while keeping [S] constant, a plot of $1/k_{obs}$ against 1/[I] should also give a straight line,

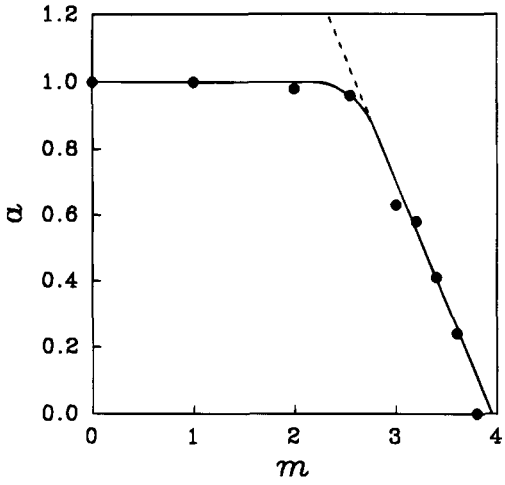


FIGURE 1: Relationship between fraction of activity remaining and the number of modified thiol groups for each subunit.

and the ratio of the intercept to the slope of this straight line is given by

$$\gamma = \text{intercept/slope} = \frac{(K_m/K_I) + ([S]/K_I')}{K_m + [S]}$$

$$= \frac{1}{K_I} + \left\{\frac{1}{K_I'} - \frac{1}{K_I}\right\} \frac{[S]}{K_m + [S]} \quad (7)$$

Thus, the values of K_I and K_I' can be determined from the secondary plot of γ against $[S]/(K_m + [S])$, and k_{+0}, k_{+0}', and k_2' can then be calculated.

RESULTS

Modification of the Cys Residues. Aminoacylase is a dimeric enzyme composed of identical subunits with 10 cysteine residues (Mitta *et al.*, 1992; Wang *et al.*, 1994). The native enzyme was treated with different amounts of MNP, and the enzyme activity remaining was determined as described previously. Figure 1 shows the relationship between the fractional activity remaining and the extent of modification of the reactive thiol groups. It can be seen from Figure 1 that out of five Cys residues in a subunit, four of them can be modified by MNP under the nondenaturation condition, and among the four reactive thiol groups, three nonessential groups were rapidly modified followed by one slow reacting essential thiol group (Tsou, 1962; Wang, 1991). Since the modification of the first three fast reacting SH groups has no effect on enzyme activity, the modification of the slower reacting essential thiol group can then be treated as the case of specific irreversible modification.

Kinetics of Aminoacylase. The kinetic behavior of aminoacylase in the hydrolysis of acyl-L-amino has been well recorded in the literature (Galaev & Svedas, 1982; Henseling & Rohm, 1988). Under the conditions employed in the present study of the inactivation kinetics (pH 7.0, 25 °C), the hydrolysis of chloroacetyl-DL-alanine follows Michaelis kinetics, with $K_m = 6.7 \pm 0.6$ mM, $k_2 = 50 \pm 4$ s^{-1}, which is in agreement with that obtained by Kördel and Schneider (1975) using the pure L-enantiomorph. The presence of the D-isomer had apparently little effect on the kinetics of hydrolysis of the substrate.

Kinetics of Substrate Reaction in the Presence of MNP. The time courses of the hydrolysis of the substrate in the presence of different concentrations of MNP are shown in Figure 2. It can be seen that [P] approach constant final values, $[P]_\infty$, which decrease with increasing concentrations of MNP. Semilogarithmic plots according to eq 1 are given in the inset of Figure 2, and the apparent rate constant k_{obs} can then be calculated from the slopes of the straight lines obtained. Results presented in the inset of Figure 2 also show that the inactivation is a monophasic pseudo-first-order reaction. Figure 3 shows the time course of enzyme inactivation in the different substrate concentrations. Plots of $\ln([P]_\infty - [P])$ against t also give a series of straight lines at different concentrations of substrate. A plot of $[P]_\infty$ against the reciprocal of MNP concentration gives a straight line with a positive intercept at the ordinate as shown in Figure 4. As predicted from eq 2, the values of both intercept and slope increase with increasing substrate concentrations. A secondary plot of the reciprocal of the slopes, $1/s$, versus $1/[S]$ is shown in the insert of Figure 4. From the intercept and the slope of the secondary plot, $k_{+0}/K_I = 0.96$ and $k_{+0}'/K_I' = 0.63$ mM^{-1} s^{-1} can then be obtained according to eq

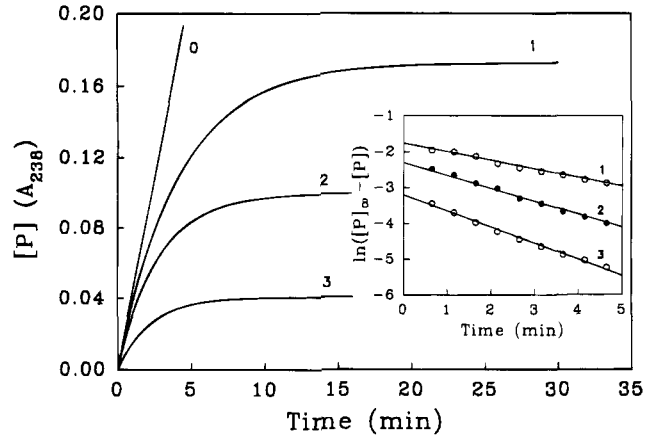


FIGURE 2: Course of substrate reaction in the presence of different concentrations of MNP. Final concentrations were 12 mM substrate and 0.143 μM enzyme in 0.1 M sodium phosphate buffer (pH 7.0) at 25 °C. Concentrations of MNP were 0 (control, curve 0), 8 (curve 1), 13.3 (curve 2), and 40 μM (curve 3). The enzyme (10 μL) was added to the reaction mixture (1 mL) to start the reaction. The inset shows semilogarithmic plots of curves 1−3 according to eq 1.

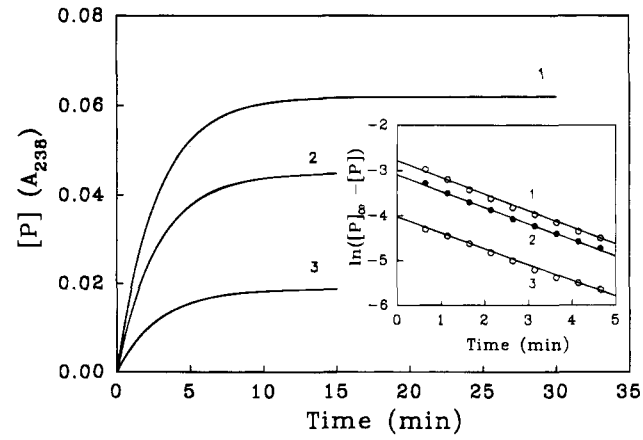


FIGURE 3: Course of substrate reaction at different substrate concentrations in the presence of MNP. Final concentrations were 20 μM MNP and 0.143 μM enzyme in 0.1 M sodium phosphate buffer (pH 7.0) at 25 °C. Concentrations of the substrate were 12 (curve 1), 6 (curve 2), and 2 mM (curve 3), respectively. The enzyme (10 μL) was added to the reaction mixture (1 mL) to start the reaction. The inset shows semilogarithmic plots of curves 1−3 according to eq 1.

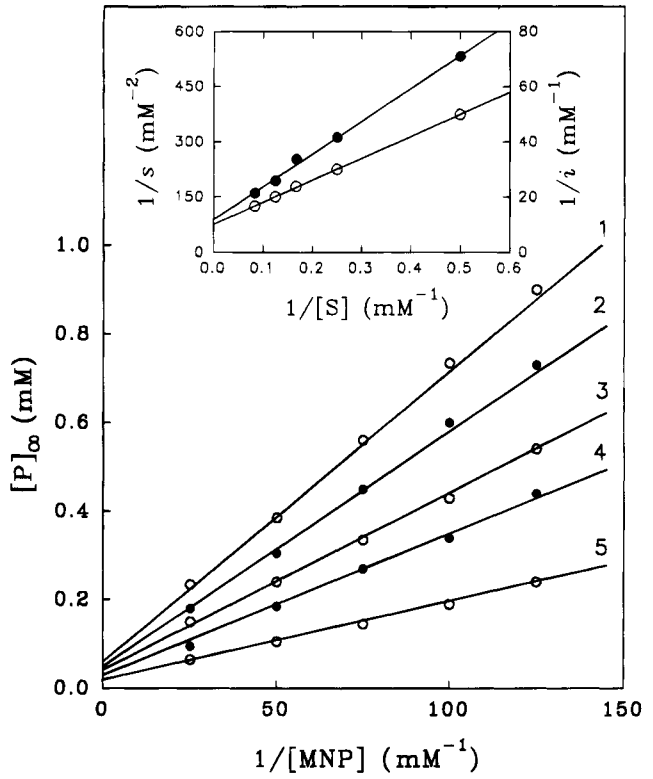


FIGURE 4: Plot of $[P]_\infty$ against 1/[MNP] for the inactivation of aminoacylase by MNP. The values of $[P]_\infty$ were calculated from the time course of aminoacylase inactivation shown in Figures 2 and 3. The insert shows the secondary plots of the reciprocals of the slopes (full circles and left-hand scale) and the ordinate intercepts (open circles and right-hand scale) against 1/[S].

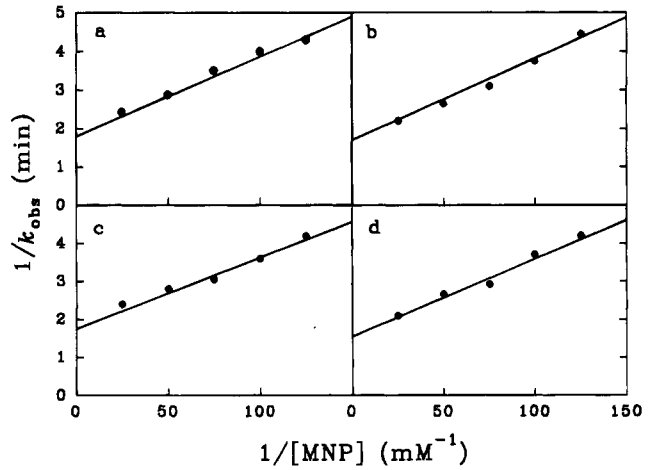


FIGURE 5: Plot of $1/k_{obs}$ against 1/[MNP] at different fixed substrate concentrations. The substrate concentrations are (a) 2, (b) 4, (c) 6, and (d) 8 mM, respectively. The values of the apparent rate constant, k_{obs}, were calculated from the time courses of substrate reaction in the presence of MNP shown in Figures 2 and 3.

4. Similarly, Figure 4 also shows a secondary plot of the reciprocal of intercept $1/i$ against 1/[S], and $K_I'/k_2' = 1.55$ μM s^{-1} can be obtained from the slope of this plot. Figure 5 shows plots of the reciprocal of apparent rate constant, $1/k_{obs}$, against 1/[I] at different concentrations of the substrate. It can be seen that at a fixed substrate concentration, the plot of $1/k_{obs}$ against 1/[I] gives a straight line with a positive intercept, indicating that a rapid formation of a noncovalent enzyme–MNP complex precedes the slow irreversible step. It can be seen from Figure 6 that a secondary plot of

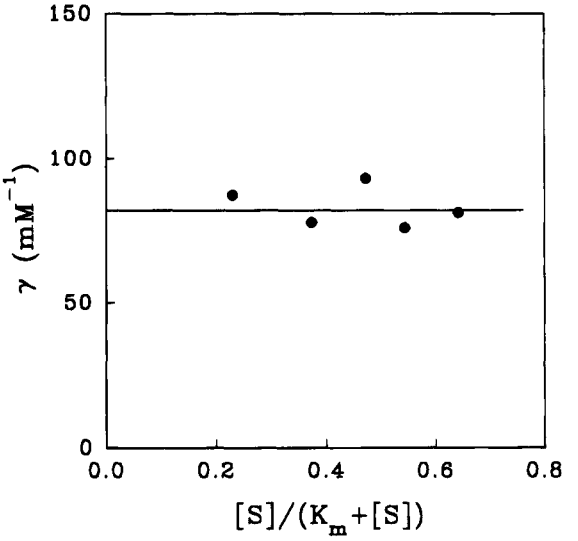


FIGURE 6: Secondary plot of the ratio of intercept to slope, γ, against $[S]/(K_m + [S])$.

Table 1: Rate Constants of the Reaction of Aminoacylase with MNP

K_m (mM)	6.7 ± 0.6	K_I (μM)	12.1 ± 1.5
K_m' (mM)	6.7 ± 0.6	K_I' (μM)	12.1 ± 1.5
k_{+2} (s^{-1})	50 ± 4	k_{+0} (min^{-1})	0.70 ± 0.13
k_{+2}' (s^{-1})	7.8 ± 1.2	k_{+0}' (min^{-1})	0.46 ± 0.11

intercept/slope, γ, against 1/[S] gives a horizontal line. According to eq 7, this result indicates that $K_I = K_I'$. The value of K_I (=12.1 μM) can be obtained from the intercept of the secondary plot. The kinetic constants determined are listed in Table 1. It can be seen from Table 1 that MNP is a pure noncompetitive modifier with respect to the substrate. The binding of substrate at active site has no effect on the binding of MNP to enzyme ($K_I = K_I'$). This result suggests that the essential thiol group in aminoacylase may be involved in the catalytic rather than substrate binding step.

DISCUSSION

The conventional method for determining rate constant of the irreversible modification of enzyme activity is to take aliquots from an enzyme–modifier incubation mixture at definite time interval and assay for the enzyme activity. This method is laborious and not easily applied to fast reactions with a half-life of less than 1 min. In comparison to the conventional method, the advantage of the progress curve method is not only its usefulness in study of fast modification reactions but its convenience in study of substrate effects on the interaction between the modifier and enzyme. In this respect, the study of modification of aminoacylase thiol groups by MNP is a good example. It is very difficult to obtain all information on the reaction of MNP with the thiol group of aminoacylase by the conventional method.

During the last decade, a systematic study on the kinetics of irreversible modification of enzyme activity has been presented (Tsou, 1988). It can be seen from the equations derived that, in the presence of an irreversible inhibitor, the time course of substrate reaction follows a single exponential process. As the reaction time increases, the concentration of product formed approaches a constant value, $[P]_\infty$. For the two types of basic reaction mechanisms mentioned above, a plot of the final product concentration, $[P]_\infty$, against the reciprocal of inhibitor concentration should give a straight

line passing through the origin. This result indicates that when the inhibitor concentration approaches infinity, all the enzyme molecules in the system are either modified by inhibitors immediately or combined with inhibitors to form noncovalent inactive enzyme−inhibitor complexes at the instant of reaction initiation. Thus, the concentration of active enzyme species is always equal to zero, and no product can be formed during the whole process of the reaction.

In the study of thiol group modification of hexokinase by MNP, Liu and Tsou (1992) have recently found that the plot of $[P]_\infty$ against 1/[MNP] gives a straight line with a positive intercept on the $[P]_\infty$ axis. They proposed a slow conformation-change model to explain this phenomenon. In this model, the enzyme can exist in two interconvertible conformation states, E and E′. The unprimed enzyme form is catalytically active and cannot react with inhibitor directly. The inhibitor can only react with the inactive primed enzyme form. The active enzyme species must undergo a slow conformational change before reacting with inhibitor. Thus, even in the case where the concentration of inhibitor used is sufficiently large, the active enzyme concentration at reaction initiation and the amount of product finally formed will not be zero. For this model, therefore, a plot of $[P]_\infty$ against 1/[I] will give a straight line with a positive intercept on the $[P]_\infty$ axis. The magnitude of the intercept value depends on the ratio between the rate of enzyme conformational change and that of the modification reaction. Another distinct feature of this model is that in the presence of an irreversible inhibitor, the progress curve of the substrate reaction possesses a double exponential form. When the rate of enzyme conformational change is much faster than the modification reaction, the plot of $[P]_\infty$ against 1/[I] will become a straight line passing through the origin, and the corresponding progress curve will also reduce to a single exponential form. In this situation, the modification reaction of the enzyme by an irreversible inhibitor is identical in form with that of a normal enzyme system. These conclusions can also be obtained from the equations given by Liu and Tsou (1992).

In the study on thiol group modification of aminoacylase by MNP, we found that the plot of $[P]_\infty$ against 1/[MNP] also gives a straight line with intercept on the $[P]_\infty$ axis. In the presence of MNP, however, the enzyme-catalyzed reaction follows a single exponential process. Thus, it can be seen from the discussion given above that neither the competitive complexing inhibition model nor the slow conformation-change model can be used to describe the modification reaction of thiol group in the aminoacylase with MNP. A detailed kinetic study indicates that MNP is a pure noncompetitive complexing inhibitor with respect to aminoacylase, and the noncovalent enzyme−inhibitor complex still has 15% catalytic activity. Thus, when the MNP concentration used is sufficiently large, although enzyme molecules in the solution will be saturated by MNP at the instant of the reaction initiation, the noncovalent enzyme−MNP complexes, EI, can still combine with the substrate and catalyze the substrate reaction to form product before the irreversible modification step occurs. Thus, the results of the present study suggest that (1) MNP is attached at different site from the substrate binding site, and the essential Cys residue may be located between the two sites; (2) the binding of MNP and/or substrate may give rise to a conformational change in the Cys side chain, which causes the reduction in both the turnover number, k_2', and the modification rate, k_{+0}'; (3) the essential thiol group in aminoacylase may participate directly in catalytic reaction step but not in the substrate binding.

ACKNOWLEDGMENT

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APPENDIX

The equations for irreversible inactivation with complex formation before the inactivation step have been given before (Tsou, 1988). On the basis of the Scheme 1, we have

$$[E_T] = [E] + [ES] + [EI] + [ESI]$$

$$[E]_0 = [E_T] + [EI^*] + [ESI^*]$$

As before, it is assumed that the steady state of the substrate reaction is rapidly established and that both [S] and [I] are $\gg$[E]. In addition, it is also assumed that the formation of the EI and ESI complexes are fast reactions relative to the inactivation step. Therefore, the following relations hold at any time:

$$[E] = \frac{K_m[E_T]}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

$$[ES] = \frac{[S][E_T]}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

$$[EI] = \frac{K_m[I][E_T]/K_I}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

$$[ESI] = \frac{[S][I][E_T]/K_I'}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

The rate of enzyme inactivation is given by

$$-\frac{d[E_T]}{dt} = k_{+0}[EI] + k_{+0}'[ESI]$$

$$= \frac{\{(k_{+0}K_m/K_I) + (k_{+0}'[S]/K_I')\}[I][E_T]}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

$$= k_{obs}[E_T] \quad \text{(A1)}$$

Integration of eq A1 with $t = 0$, $[E_T] = [E]_0$ yields

$$[E_T] = [E]_0 \exp(-k_{obst})$$

The rate of product P formation while the enzyme is being modified is

$$\frac{d[P]}{dt} = k_2[ES] + k_2'[ESI]$$

$$= \frac{\{k_2 + (k_2'[I]/K_I')\}[S][E]_0 \exp(-k_{obs}t)}{K_m + [S] + \{(K_m/K_I) + ([S]/K_I')\}[I]}$$

It can be easily shown that the equation for product

concentration at any time t is

$$[P] = [P]_\infty\{1 - \exp(-k_{obs}t)\}$$

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