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Chemical modification of enzymes: Kinetic aspects

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Important information on enzyme ligand interactions can be obtained when analyzing the kinetics of chemical modification reactions. In this article, several kinetic models of inactivation are discussed, along with the determination of enzyme-ligand dissociation constants and of the pK of enzyme reactive groups from chemical modification kinetic data.

Key terms: *inactivation with intermediate complexes; reversible inactivation reactions; pKa.*

INTRODUCTION

This article updates and expands a previous communication (Cardemil, 1987) describing kinetic approaches currently employed for the analysis of enzyme chemical modification reactions. Special reference is made to the use of chemical modification as a tool to obtain information on quantitative aspects of enzyme-ligand interaction.

POSSIBLE KINETIC MECHANISMS FOR THE CHEMICAL MODIFICATON OF ENZYMES

The analysis of the kinetic mechanism of an enzyme chemical modification reaction depends on the conditions of the reaction system. The mathematical treatment is greatly simplified if pseudo-first-order conditions with respect to the modifier are used (that is, if the concentration of the modifier is much higher than the concentration of the enzyme).

Irreversible reactions

If we consider the simplest possible reaction for an enzyme (E) and an inactivator (I) to produce an inactive enzyme-inactivator complex (EI).

$$E + I \xrightarrow{k_1} EI \qquad (1)$$

the reaction rate (v) is given by:

$$v = k_1[E][I] \qquad (2)$$

and if [I] = [E] it simplifies to:

$$v = k_{obs}[E] \qquad (3)$$

where:

$$k_{obs} = k_1[I] \qquad (4)$$

Equation 3 can also be written as:

$$- d[E_a]/dt = k_{obs}[E_a] \qquad (5)$$

where $[E_a]$ is the enzyme concentration at time t. Integration of equation 5 with respect to $[E_a]$ between times 0 and t gives a linear relationship between the natural logarithm of the fractional activity of the enzyme and the time:

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$$Ln[E_a]_t/[E_a]_o = -k_{obs}t \quad (6)$$

so that a plot of the common logarithm of the remaining enzyme activity against time gives a straight line with slope $k_{obs}/2.303$. It is possible, then, to obtain k_1 from a series of determinations of k_{obs} at several different concentrations of I, according to equation 4 (Encinas *et al*, 1990; Coulin *et al*, 1993). Deviations of linearity are expected, however, if the kinetic order of the reaction is not 1, as for example:

$$E + 2I \xrightarrow{k_1} EI_2 \quad (7)$$

where:

$$k_{obs} = k_1 [I]^2 \quad (8)$$

In this particular situation, a straight line can only be obtained by making a plot of k_{obs} against $[I]^2$ (Peters *et al*, 1981; Alvear *et al*, 1989).

The kinetic order of a modification reaction is also commonly obtained from the logarithmic form of equation 8:

$$\log k_{obs} = n \log[I] + \log k_1 \quad (9)$$

where n = kinetic order of the reaction, that is a reflection of the dependence on modifying agent concentration, of the single protein species, modification of which is the rate-limiting step in the overall reaction (Rakitzis, 1985). This relationship has been widely used since first employed by Levy *et al* (1963), although not always properly, as discussed by Jabalquinto *et al* (1983) and by Carlson (1984). It must be pointed out that this relationship holds only for irreversible mechanisms such as that of equation 1, but not for reversible mechanisms or for mechanisms involving the formation of significant amounts of a dissociable complex prior to inactivation as those of equations 12 and 16. Those mechanisms can be identified by appropriate treatment of the data, as will be seen below.

The second-order rate constant k_1 can also be obtained from experiments carried out in conditions where $[E] \approx [I]$, but in this case it must be calculated from the slope of the appropriate second-order plot (Castellan, 1964). For equation 1, the second order plot when equal initial concentrations of enzyme and inactivator are used is the reciprocal of the concentration of remaining enzyme against time, according to

$$1/[E_a] = k_1 t + 1/[E_o] \quad (10)$$

However, if $[I] \neq [E]$ (usually $[I] > [E]$), the appropriate second order plot for mechanism (1) is

$$\frac{1}{[I_o]-[E_o]} Ln \frac{[I_o][E_a]}{[E_o][I_a]} = k_1 t \quad (11)$$

where $[E_a]$ and $[I_a]$ are the enzyme and inactivator concentrations at time t, respectively; in this case, k_1 can be obtained from the slope of the plot of the left-hand side of the equation against t.

These conditions have been employed by Tian *et al* (1985) for the analysis of the inactivation reaction rate of chicken liver fatty acid synthetase by 5,5'-dithiobis-(2-nitrobenzoic acid), and by Lewis *et al* (1989) for similar analyses of the inactivation of cytosolic rat liver phosphoenolpyruvate carboxykinase by N-(7-dimethylamino-4-methylcoumarinyl) maleimide.

Reversible reactions

Let us now consider the mechanism:

$$E + I \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} EI \quad (12)$$

where the reversible formation of an inactive EI complex is described. Introducing the pseudo-first-order condition that [I] - [E], then:

$$E \underset{k_{-1}}{\overset{k_1[I]}{\Leftrightarrow}} EI \quad (13)$$

Now, if one assumes that the concentration of EI at the beginning of the reaction is zero, the rate equation can be easily integrated and simplified by introducing the

equilibrium condition (Frost & Pearson, 1961) to obtain:

$$\text{Ln}\frac{([E]-[E_o])}{([E_o]-[E_e])} = (k_1[I] + k_{-1})t \quad (14)$$

where $[E_o]$ is the initial concentration of E, [E] is the concentration at any time t, and $[E_e]$ is the concentration at equilibrium. As the approach to equilibrium is a first-order process, the observed rate constant from a plot of $\text{Ln}[E]/[E_o]$ versus t will be the sum of the rate constant for the forward and reverse reactions (Strickland & Massey, 1973):

$$k_{obs} = k_1[I] + k_{-1} \quad (15)$$

Therefore, a plot of k_{obs} as a function of [I] should be linear with a slope of k_1, and an extrapolated ordinate intercept of k_{-1} (Cardemil & Eyzaguirre, 1979; Bazaes *et al*, 1993)

Formation of an intermediary complex

This kind of mechanism is the one expected for an affinity label (Plapp, 1982), where a significant amount of dissociable complex is formed between the inactivator and the enzyme prior to the formation of an inactive EI complex:

$$E + I \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} E.I \xrightarrow{k_2} EI \quad (16)$$

For this mechanism, the observed rate of inactivation is given by:

$$v = k_2[E.I] \quad (17)$$

and, provided again that [I] • [E] and assuming that the equilibrium condition $k_2 \bullet k_{-1}$ holds (Brocklehurst, 1979), it can be shown (Kitz & Wilson, 1962) that:

$$k_{obs} = \frac{k_1k_2[I]}{k_1[I] + k_{-1}} \quad (18)$$

and, in reciprocal form:

$$\frac{1}{k_{obs}} + \frac{1}{k_2} + \frac{K_{diss}}{k_2} \times \frac{1}{[I]} \quad (19)$$

where $K_{diss} = k_{-1}/k_1$. Consequently, it can be seen now that for the mechanisms described in equations 1, 12 and 16, the direct plot of kobs as a function of [I] should give, respectively: for equation 1, a straight line passing through the origin, with slope = k_1; for equation 12, a straight line with slope = k_1 and an extrapolated ordinate intercept = k_{-1}; for equation 16, a rectangular hyperbola. This last case is best represented using the reciprocal equation 19, where slope = K_{diss}/k_2 and ordinate intercept = $1/k_2$ (Saavedra *et al*, 1988).

Discrimination among these three mechanisms is possible, then, by plotting k_{obs} versus [I] as show in Figure 1. It should be emphasized, however, that a wide enough range of concentrations of I must be tested in order to clearly differentiate among these mechanisms. For example, if the mechanism of equation 16 applies, but [I] • k_{-1}/k_1, equation 18 reduces to:

$$k_{obs} = \frac{k_2}{K_{diss}}[I] \quad (20)$$

and a type A plot (Fig 1) is obtained, even if an intermediate is involved in the inactivation reaction.

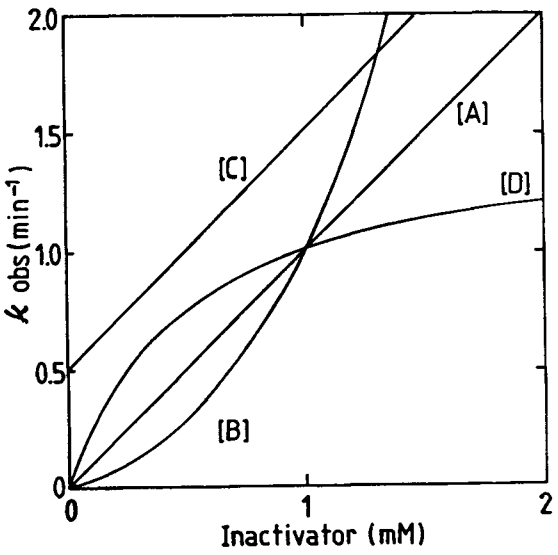

Fig 1. Plot of the variation of kobs as a function of the concentration of inacticator. A, plot of equation 4; B, plot of equation 8; C, plot of equation 15; D, plot of equation 18. The values of the kinetic constants are: k_1 = 1 min^{-1} mM^{-1}; k_{-1} = 1 min^{-1}; k_2 = 0.5 min^{-1}. (From Cardemil, 1987).

DETERMINATION OF ENZYME-LIGAND DISSOCIATION CONSTANTS FROM THE ANALYSIS OF THE KINETICS OF ENZYME INACTIVATION

Once the kinetic mechanism for the chemical modification of an enzyme by an inactivator is known, it is often possible to determine the dissociation constant for the binding of a ligand to the enzyme, provided the ligand modifies the inactivation rate of the enzyme (Kiick *et al*, 1984; Renosco *et al*, 1985). The procedure to be described below provides an alternative way of obtaining valuable information on the binding of substrates or cofactors to enzymes, in addition to other procedures like equilibrium dialysis, titration, spectroscopic methods, etc. (Fersht, 1985).

Irreversible mechanisms

The determination of the dissociation constant for an enzyme-ligand complex from inactivation-protection experiments depends on whether the binding of the ligand to the enzyme completely or partially protects it from its reaction with the inactivator. A general model for this process would be:

$$\begin{array}{ccc} & K_{diss} & \\ E + L & \Leftrightarrow & EL \\ + & & + \\ I & & I \\ \downarrow k_1 & & \downarrow k_2 \\ EI & & ELI \end{array} \qquad (21)$$

where both the enzyme (E) and the enzyme-ligand complex (EL) can react with the inactivator to produce the enzyme-ligand complexes EI and ELI, with rate constants k_1 and k_2, respectively. The binding of L to E is dependent on the magnitude of the dissociation constant K_{diss}. If we assume that the equilibrium between E, L and EL is faster than the reaction of I with E, the inactivation rate of the enzyme in the presence of L can be expressed by:

$$\frac{d[E_a]}{dt} = k_1[E][I] + k_2[EL][I] \qquad (22)$$

where $[E_a]$ = concentration of active enzyme = [E] + [EL].

Dividing equation 22 by $[E_a]$ gives:

$$\frac{d[E_a]}{dt[E_a]} = \frac{k_1[E][I] + k_2[EL][I]}{[E] + [EL]} \qquad (23)$$

and as:

$$K_{diss} = \frac{[E][L]}{[EI]} \qquad (24)$$

if follows that:

$$\frac{d[E_a]}{[E_a]} = \frac{[I](k_1K_{diss} + k_2[L])}{K_{diss} + [L]}\,dt \qquad (25)$$

So, the observed pseudo-first order rate constant for the enzyme inactivation in the presence of a ligand (k_{obs}^{L}) is given by:

$$k_{obs}^{L} = \frac{[I](K_{diss}k_1 + k_2[L])}{K_{diss} + [L]} \qquad (26)$$

and, in reciprocal form:

$$\frac{1}{k_{obs}^{L}} = \frac{K_{diss}}{[I](K_{diss}k_1 + k_2[L]} + \frac{[L]}{[I](K_{diss}k_1 + k_2[L])} \qquad (27)$$

If $k_2 = 0$, that is, if I does not react with EL, the above equation simplifies to:

$$\frac{1}{k_{obs}^{L}} = \frac{1}{[I]k_1} + \frac{[L]}{[I]k_1K_{diss}} \qquad (28)$$

or (see equation 4),

$$\frac{k_{obs}}{k_{obs}^{L}} = 1 + \frac{[L]}{K_{diss}} \qquad (29)$$

Equations 27 and 28 predict a rectangular hyperbola and a straight line, respectively, when $1/k_{obs}^{L}$ is plotted against the concentration of L, thus providing a way to know if I reacts with the EL complex. When equation 28 applies, K_{diss} can be obtained from the extrapolated abscissas intercept of the plot (Mildvan & Leigh, 1964). If equation 29 is employed, K_{diss} is obtained from the reciprocal of the slope. Figure 2 shows ex-

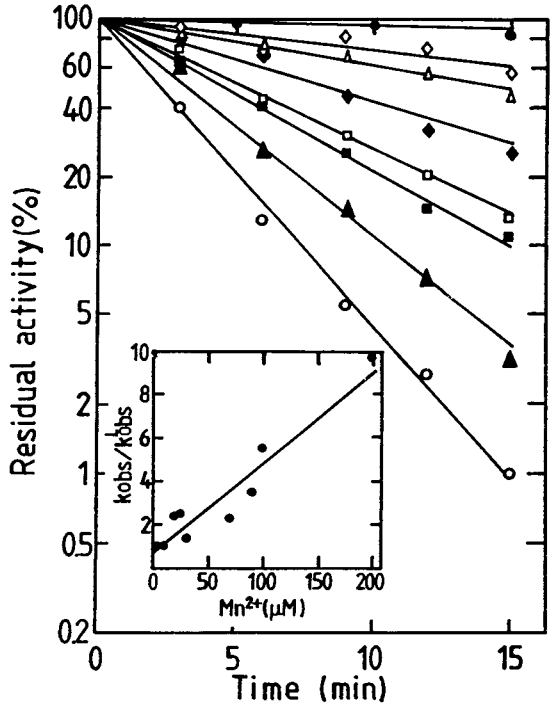

Fig 2. Effect of the concentration of Mn^{2+} on the inactivation rate of Saccharomyces cerevisiae phosphoenolpyruvate carboxykinase by phenylglyoxal. The inset shows the dependence of k_{obs}/k_{obs}^{L} on the concentration of Mn^{2+}. The least-square straight line fit of the data gives a dissociation constant of 23 ± 3 μM, according to equation 29. (From Malebrán and Cardemil, 1987).

perimental data for the determination of the dissociation constant of the *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase-Mn^{2+} complex, according to equation 29. When k_2 is not zero, K_{diss} is best estimated from the following relation introduced by Scrutton and Utter (1965):

$$\frac{k_{obs}^{L}}{k_{obs}} = \frac{k_2}{k_1} + \frac{\left(1 - \frac{k_{obs}^{L}}{k_{obs}}\right)}{[L]} K_{diss} \qquad (30)$$

Here, when k_{obs}^{L}/k_{obs} is plotted as a function of:

$$\frac{\left(1 - \frac{k_{obs}^{L}}{k_{obs}}\right)}{[L]},$$

K_{diss} can be obtained from the slope of the line. Additionally, k_2 may be obtained from the intercept, since k_1 can be independently calculated from equation 4.

Another way of analyzing the data is provided by the relationship:

$$k_{obs}^{L} = k_2[I] + \frac{k_{obs} - k_{obs}^{L}}{[L]} K_{diss} \qquad (31)$$

employed, for instance, by Fujioka and Tanaka (1981) and Jabalquinto *et al* (1983) for the determination of dissociation constants for enzyme-substrate complexes for yeast saccharopine dehydrogenase and chicken liver mevalonate 5-diphosphate decarboxylase, respectively.

Reactions involving the formation of an intermediary complex in the enzyme inactivation process

This situation has been analyzed by Horiike and McCormick (1980) and by Carrillo *et al* (1981). A general scheme for this inactivation mechanism in the presence of a ligand is:

$$\begin{array}{ccc} & K_{diss} & \\ E + L & \rightarrow & EL \\ + & & + \\ I & & I \\ \Downarrow K_{d1} & & \Downarrow K_{d2} \\ E.I & & EL.I \\ \downarrow k_2 & & \downarrow k_2' \\ EI & & ELI \end{array} \qquad (32)$$

where it is assumed again that both E and EL can react with I to produce inactive EI and ELI complexes through the active E.I and EL.I complexes, respectively. K_{d1} and K_{d2} are dissociation constants while k_2 and $k_{2'}$ are rate constants.

A general rate equation for the above mechanism (equation 32) has been derived by Carrillo *et al* (1981) for the situation where both [I] and [L] are large compared to [E], and from that equation it can be deduced that:

$$k_{obs}^{L} = \frac{(k_2 K_{diss} K_{d2} + k_{2'}[L]K_{d1})[I]}{K_{d2}K_{diss}(K_{d1}+[I]) + K_{d1}[L](K_{d2}+[I])} \qquad (33)$$

or, in a reordered form:

$$\frac{k_{obs}^{L}}{k_{obs}} = \frac{k_2'(K_{d1} + [I])}{k_2(K_{d2} + [I])} + K_{diss} \frac{K_{d2}(K_{d1} + [I])}{K_{d1}(K_{d2} + [I])} \times \frac{\left(1 - \frac{k_{obs}^{L}}{k_{obs}}\right)}{[L]} \quad (34)$$

or just:

$$\frac{k_{obs}^{L}}{k_{obs}} = K_{diss} \frac{(K_{d1} + [I])}{K_{d1}} \times \frac{\left(1 - \frac{k_{obs}^{L}}{k_{obs}}\right)}{[L]} \quad (35)$$

for the case where the EL complex does not react with I (that is, for the situation where $k_2' = 0$ and $K_{d2} \rightarrow \infty$).

By comparing equations 34 and 35, it is clear that the extrapolation of a plot of k_{obs}^{L}/k_{obs} versus $(1-k_{obs}^{L}/k_{obs})/[L]$ passes through the origin if the EL complex does not react with I (equation 35); in this case K_{diss} can be calculated from the slope of the plot, since:

$$\text{slope} = K_{diss} (1 + [I]/K_{d1}) \quad (36)$$

provided K_{d1} can be obtained independently as shown in equations 18 and 19. If equation 34 applies, the value of K_{diss} can not be obtained by these graphical procedures.

DETERMINATION OF THE pK_a OF ENZYME REACTIVE GROUPS

The determination of the rate of the chemical modification of an enzyme by an inactivator as a function of pH allows one to identify the pK_a of the reactive group in the enzyme. The determination of pK_a values for specific amino acid residues in proteins by means of chemical modification is a valuable piece of information, since it allows one to verify pK_a values assigned to these residues by other methods. For example, it is often possible to obtain pK_a values for catalytically important groups in enzymes from studies of the pH effect on k_{cat}, K_m, or k_{cat}/K_m (Fersht, 1985). A comparison, therefore, of the pK_a values obtained from both kinetic and chemical modification studies provides a good opportunity for corroborating specific aspects of the chemical mechanism of an enzyme. Using this approach, Willenbrock and Brocklehurst (1984) have postulated a detailed scheme for the protonic dissociation in cathepsin B (a cysteine proteinase) explaining thus the nucleophilic character and catalytic activity of the enzyme.

The mathematical treatment for most cases is identical to that described previously for the determination of K_{diss} for the binding of a ligand. For example, if the reaction of the inactivator with the enzyme is a simple irreversible process, then:

$$\begin{array}{l} \overset{K_A}{E + H^+ \Leftrightarrow EH^+} \\ + \\ I \\ \downarrow k_1 \\ EI \end{array} \quad (37)$$

We can therefore obtain from equation 28 the following relationship:

$$\frac{1}{k} = \frac{1}{k_{max}} + \frac{[H^+]}{k_{max}K_A} \quad (38)$$

where k is the pseudo-first-order inactivation rate constant at a given H^+ concentration, k_{max} is the pH-independent pseudo-first -order inactivation rate constant, and K_A is the ionization constant of the modified residue. A plot of 1/k as a function of $[H^+]$ allows the determination of K_A from the extrapolated abscissas intercept of the graph (Tian *et al*, 1985; Lewis *et al*, 1989).

If the inactivation of the enzyme follows a mechanism that includes the formation of a reversible complex between enzyme and inactivator like:

$$\begin{array}{l} \overset{K_A}{E + H^+ \Leftrightarrow EH^+} \\ + \\ I \\ \Downarrow K_d \\ E.I \\ \downarrow k_1 \\ EI \end{array} \quad (39)$$

The following relation, obtained from equation 35, holds:

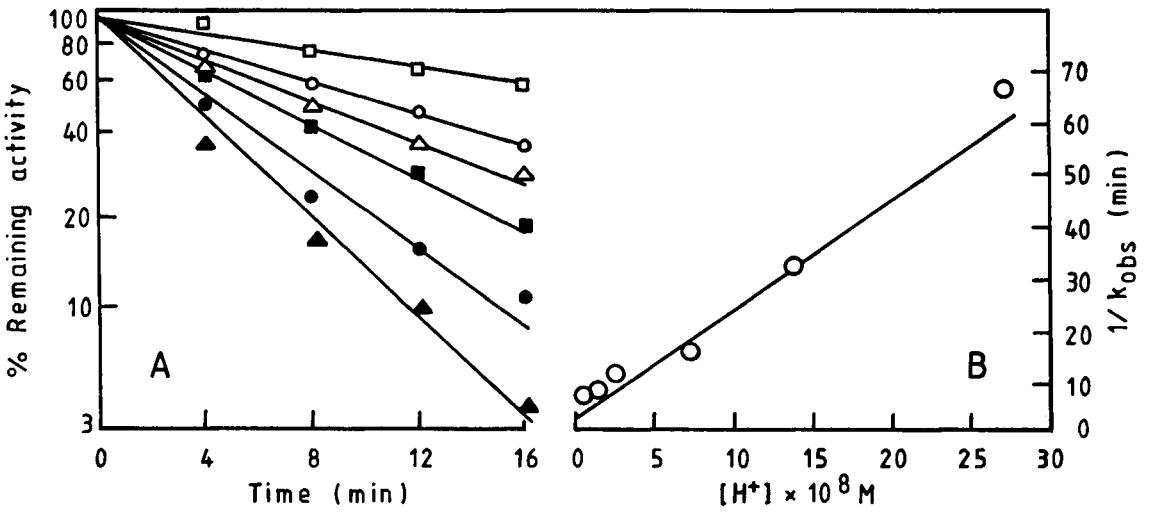

Fig 3. Effect of pH on *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase inactivation by the 2',3'-dialdehyde derivative of ATP. In A, the pseudo-first-order inactivation rate of the enzyme is shown; in B, the reciprocals of the pseudo-first-order inactivation rate constants are plotted against $[H^+]$, according to equation 41. Considering that the inactivator concentration was 66 μM and the previously determined Kd was 60 μM, a pK_a of 8.1 could be calculated. (From Saavedra *et al*, 1988).

$$k = \frac{k_{max}(K_d + [I])}{K_d[H^+]/K_A + K_d + [I]} \qquad (40)$$

or, in reciprocal form:

$$\frac{1}{k} = \frac{1}{k_{max}} + \frac{K_d[H^+]}{k_{max}\, K_A(K_d + [I])} \qquad (41)$$

Thus, a plot of 1/k as a function of $[H^+]$ will yield a straight line with an intercept on the $[H^+]$ axis of $-([I] + K_d)K_A/K_d$. The value of K_A, and hence the pK_a of the ionizable group, can readily be calculated by substituting the known inactivator concentration employed in the experiment, and the previously determined value of Kd. An example of this method is given in Figure 3, where the pKa of a reactive lysine in *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase is calculated from the analysis of the inactivation rate of the enzyme by the 2',3'-dialdehyde derivative of ATP as a function of $[H^+]$.

Equations for more complex cases (i.e., the protonated form of the enzyme also binds the inactivator) can also be derived; the reader is referred to Tipton and Dixon (1979) for details.

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