A simple method for determining kinetic constants of complexing inactivation at identical enzyme and inhibitor concentrations

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Abstract The kinetics of complexing inactivation at identical enzyme and inhibitor concentrations were analyzed and the equations of product generation were derived when the free enzyme concentration is great, larger or smaller than the dissociation constant of inhibitor, K_I. The kinetic constants can be obtained by fitting the derived equations to the progress curve. Numerical examples show that this method is valid and gives satisfactory results.

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Key words: Tight-binding, complexing inactivation; Kinetic constant; Progress curve

1. Introduction

Recent developments showed that irreversible modification of enzyme activity is as important as, if not more important than, reversible inhibition. Affinity labeling has been used extensively for the study of the active sites of enzymes [1–4]. Modifiers that complex specifically to the active sites of enzymes prior to the irreversible modification step appear to have great prospects as therapeutic agents [4,5]. Many highly selective irreversible inhibitors of proteinases developed recently [6] belong to this kind of inhibitors. Most of them possess a very high affinity for enzyme, i.e. so-called tight-binding, complexing irreversible inhibitors with a K_I value being as low as 10^{-9} M [7,8]. The tight-binding, complexing inactivation is often studied by the traditional method taking aliquots for residue activity assay [9]. This method is not only laborious, but also not suitable for fast reaction and cannot avoid the dilution effect on the assay. Although a convenient approach [10,11] has been extensively used to study the enzyme inactivation, it is not applicable to the inactivation at identical enzyme and inhibitor concentrations. Recently, a simple method was adopted to study the modulation of proteinase inhibitors [12–14], but with the disadvantage that the necessary condition of $[E] \ll K_I$ was not considered, so that it was mistakenly applied to the case of $[E] > K_I$ [12,13].

In the present paper, a simple method for the study of the tight-binding, complexing irreversible inhibitors is presented. The relations between the amount of product formation and time at identical enzyme and inhibitor concentrations were derived under the conditions of $[E] \gg K_I$ and $[E] \ll K_I$, respectively. The kinetic constants can be determined by fitting the derived equations to the progress curve.

2. Theory

2.1. Kinetic analysis

An inhibitor (I) inhibits an enzyme (E) in a two-step reaction, first forming a reversible complex, EI, followed by slow transformation to a functionally irreversible complex, EI^*. The inactivation in the presence of substrate (S) is modeled by Scheme 1, where K_I is the dissociation constant of inhibitor from EI, k_i is the inactivation rate constant, ES is the enzyme-substrate complex and K_m, is the Michaelis-Menten constant.

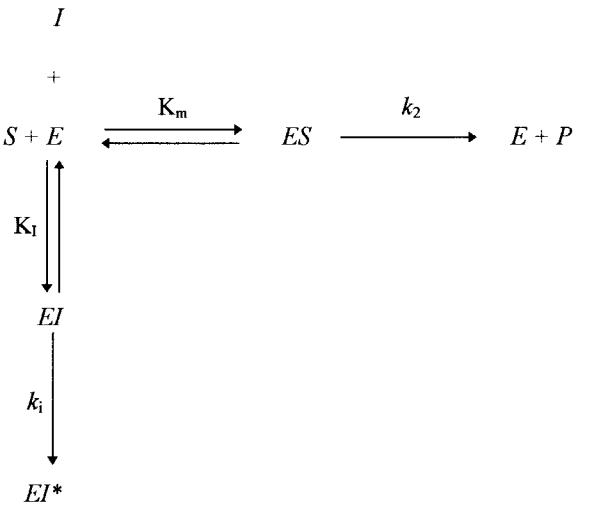
The following assumptions are adopted:

1. Steady states between $E+S$ and ES, $E+I$ and EI are rapidly reached, and the irreversible conversion of EI to EI^* is a slow step, i.e., the rate-limiting step of the inactivation.
2. The substrate concentration is sufficiently high that the depletion of free substrate by binding to the enzyme and that by conversion into product are both negligible.
3. There is no product inhibition.

For inhibitors with $K_I \sim 10^{-9}$ M or less, the experiments must be performed under the condition of comparable enzyme and inhibitor concentrations.

According to the assumptions above and Scheme 1, we have:

$$[I]_0 = [I] + [EI] + [EI^*] \quad (1)$$



Scheme 1.

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$$[E]_0 = [E]_T + [EI^*] \quad (2)$$

$$[E]_T = [E] + [ES] + [EI] \quad (3)$$

$$\frac{[E][S]}{[ES]} = K_m \quad (4)$$

$$\frac{[E][I]}{[EI]} = K_I \quad (5)$$

From Eqs. 1, 2 and 5, we have:

$$[EI] = \frac{[I]_0 - [E]_0 + [E]_T}{1 + \frac{K_I}{[E]}} \quad (6)$$

Substituting Eqs. 4 and 6 into Eq. 3 yields:

$$[E]_T = [E] + [ES] + [EI] =$$

$$\left(1 + \frac{[S]}{K_m}\right)\frac{[E]^2}{K_I} + \left(1 + \frac{[S]}{K_m}\right)[E] + \frac{[I]_0 - [E]_0}{K_I}[E] \quad (7)$$

The velocity of irreversible decrease of $[E]_T$ at any time is given by:

$$\frac{d[E]_T}{dt} = -k_i[EI] \quad (8)$$

By substituting Eqs. 6 and 7 into Eq. 8, we have:

$$\frac{d[E]}{dt} = -k_i \frac{[E]\left\{\left(1 + \frac{[S]}{K_m}\right)[E] + [I]_0 - [E]_0\right\}}{\left(1 + \frac{[S]}{K_m}\right)(2[E] + K_I) + [I]_0 - [E]_0} \quad (9)$$

When $[E]_0$ equals to $[I]_0$, Eq. 9 can be simplified as follows:

$$\frac{d[E]}{dt} = -k_i \frac{[E]^2}{2[E] + K_I} \quad (10)$$

From Eq. 4, we have:

$$[E] = \frac{K_m}{[S]}[ES] = \frac{K_m}{[S]k_2}v \quad (11)$$

Therefore, Eq. 10 can be expressed as:

$$\frac{dv}{dt} = -k_i \frac{v^2}{2v + \frac{[S]k_2}{K_m}K_I} \quad (12)$$

By integrating Eq. 12 with the boundary conditions of $t = 0$, $v = v_0^*$, we obtain:

$$2\ln\frac{v_0^*}{v} + \frac{K_I k_2 [S]}{K_m}\left(\frac{1}{v} - \frac{1}{v_0^*}\right) = k_i t \quad (13)$$

where v and v_0^* are the velocities at any given time and time zero in the presence of inhibitors, respectively.

The relation between v_0^* and $[E]_0$, $[I]_0$ is given by the following equation [15]:

$$v_0^* = \frac{k_2[S]}{2(K_m + [S])}\left([E]_0 - [I]_0 - K_I\frac{[S] + K_m}{K_m} + \sqrt{\left([E]_0 + [I]_0 + K_I\frac{[S] + K_m}{K_m}\right)^2 - 4[E]_0[I]_0}\right)$$

When $[E]_0 = [I]_0$, this can be simplified as follows:

$$\frac{v_0^*}{v_0} = \left(1 + \frac{[S]}{K_m}\right)\frac{K_I}{2[E]_0}\left(-1 + \sqrt{1 + 4\frac{[E]_0}{K_I}\frac{K_m}{K_m + [S]}}\right) = \frac{2}{1 + \sqrt{1 + 4\frac{[E]_0}{K_I}\frac{K_m}{K_m + [S]}}} \quad (14)$$

By solving Eq. 14, we obtain:

$$K_I = \frac{K_m[E]_0 v_0^*}{(K_m + [S])(v_0 - v_0^*)} \quad (15)$$

where

$$v_0 = \frac{k_2[S][E]_0}{K_m + [S]}$$

Integrating Eq. 13 by parts with the boundary conditions of $t = 0$, $[P] = 0$ and $v = v_0^*$ gives:

$$[P] = \int_0^t v\,dt = \frac{2(v_0^* - v)}{k_i} + \frac{K_I k_2 [S]}{K_m k_i}\ln\frac{v_0^*}{v} \quad (16)$$

2.2. Approximation

From Eq. 14, we have:

$$\frac{[E]^*}{K_I} = \frac{1}{2}\left(-1 + \sqrt{1 + 4\frac{[E]_0}{K_I}\frac{K_m}{K_m + [S]}}\right) \quad (17)$$

where $[E]^*$ is the free enzyme concentration at time zero. From Eq. 17, it can be seen that the condition of $[E] \gg K_I$ at time zero can be easily satisfied when:

$$[E]_0 \gg \frac{2(K_m + [S])}{K_m}K_I$$

Thus, Eq. 10 can be simplified as:

$$\frac{d[E]}{dt} = -\frac{k_i}{2}[E] \quad (18)$$

From Eqs. 11 and 18, we have:

$$\frac{dv}{dt} = -\frac{k_i}{2}v$$

and hence

$$v = v_0^* e^{-k_i t/2} \quad (19)$$

By integrating Eq. 19 with the boundary conditions of $t = 0$,

[P] = 0, we can obtain the equation for product formation with time:

$$[P] = \frac{2v_0}{k_i}(1-e^{-k_i t/2}) \quad (20)$$

It should be pointed out that [E] will decrease with time during inactivation, the condition of $[E] \gg K_I$ can only be satisfied during the initial part of the progress curve, and thus only the initial part of the progress curve can be described by Eq. 20.

According to Eq. 17, when:

$$[E]_0 \ll \frac{2(K_m + [S])}{K_m} K_I$$

we have $[E] \ll K_I$, thus Eq. 10 can be simplified as:

$$\frac{d[E]}{dt} = -k_i \frac{[E]^2}{K_I} \quad (21)$$

From Eqs. 11 and 21, we have:

$$\frac{1}{v} = \frac{1}{v_0^*} + \frac{K_m k_i}{k_2 K_I [S]} t \quad (22)$$

and hence

$$[P] = \int_0^t v\, dt = \frac{K_I k_2 [S]}{k_i K_m} \ln\left(1 + \frac{k_i K_m v_0^*}{K_I k_2 [S]} t\right) \quad (23)$$

Clearly, Eqs. 21–23 are valid for the whole progress curve because [E] decreases with time and the condition of $[E] \ll K_I$ is always satisfied.

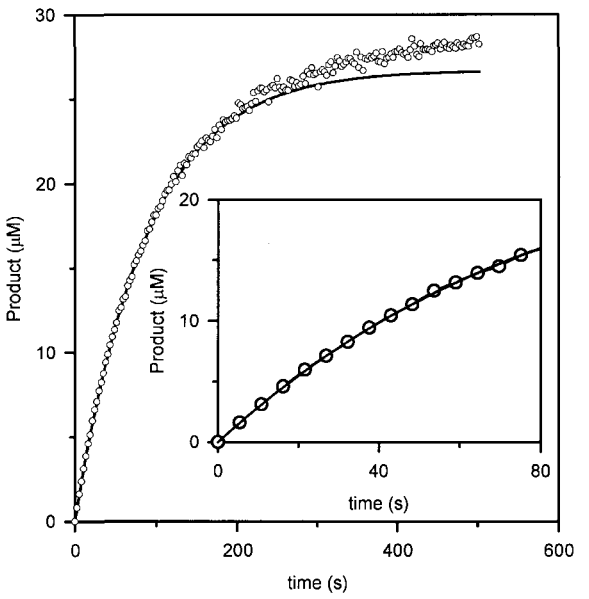

Fig. 1. A simulated progress curve of product formation for the tight-binding, complexing inactivation when $[E] \gg K_I$. The progress curve, (○), represents the values calculated according to Eqs. 13 and 16 with [S] = 2000 μM, $[E]_0 = [I]_0 = 50$ nM, $K_m = 400$ μM, $k_2 = 10$ s^{-1}, $K_I = 0.5$ nM, $k_i = 0.025$ s^{-1}, and a Gaussian-type error of up to 2.5% of the product formed using a random number generator. The smooth curve is the fitted result using a non-linear fitting program. The inset shows the enlarged initial part.

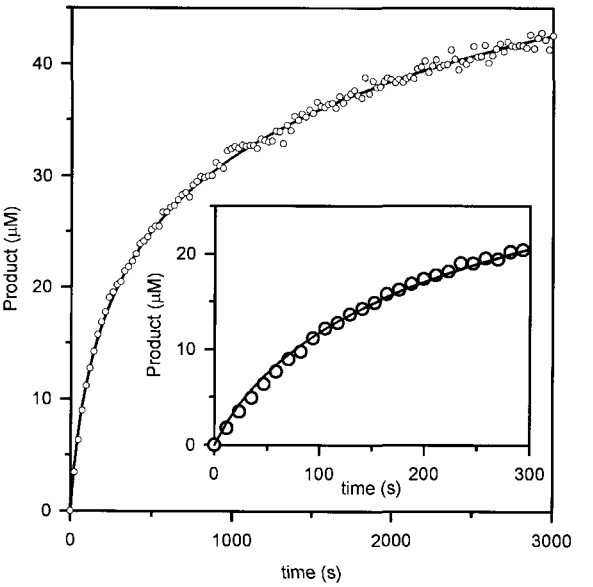

Fig. 2. A simulated progress curve of product formation for the tight-binding, complexing inactivation when $[E] \ll K_I$. The progress curve, (○), represents the values calculated according to Eqs. 13 and 16 with [S] = 4000 μM, $[E]_0 = [I]_0 = 0.05$ nM, $K_m = 400$ μM, $k_2 = 5000$ s^{-1}, $K_I = 0.5$ nM, $k_i = 2.5$ s^{-1}, and a Gaussian-type error of up to 2.5% of the product formed using a random number generator. The smooth curve is the fitted result using a non-linear fitting program. The inset shows the enlarged initial part.

2.3. Determination of the kinetic constants

The kinetic constants can be determined by the following procedures:

1. Determination of v_0, v_0^*, K_m and k_2 from the experiments, where v_0^* can be determined from the tangent line at time zero of the progress curve in the presence of inhibitor.
2. Calculation for K_I according to Eq. 15.
3. Determining the irreversible inactivation rate constant k_i according to Eq. 20 under the condition of:

$$[E]_0 \gg \frac{2(K_m + [S])}{K_m} K_I$$

or to Eq. 23 under the condition of:

$$[E]_0 \ll \frac{2(K_m + [S])}{K_m} K_I$$

Table 1
Parameters determined by the present method under different conditions

	Parameters determined		
	k_i (s^{-1})	K_I (nM)	k_i/K_I (nM^{-1} s^{-1})
(I)	0.025 ± 0.006	0.508 ± 0.005	0.049
(II)	2.518 ± 0.005	0.506 ± 0.003	4.976
	2.785 ± 0.078*		

(I) The conditions are the same as in Fig. 1.
(II) The conditions are the same as in Fig. 2, *determined by plotting method [12–14].

3. Numerical examples

The present method was applied to the simulation of the tight-binding, complexing inactivation by a numerical example under the condition of $[E] \gg K_I$, i.e.,

$$[E]_0 \gg \frac{2(K_m + [S])}{K_m} K_I$$

as shown in Fig. 1. The progress curve was generated with computer according to Eqs. 13 and 16, and then, Eq. 20 was fitted to the initial part using a non-linear fitting program. The inset shows the enlarge part of the initial part of the progress curve with being fitted well. The kinetic constants obtained were listed in Table 1.

A simulated progress curve of the tight-binding complexing inactivation under the condition of $[E] \ll K_I$, i.e.,

$$[E]_0 \ll \frac{2(K_m + [S])}{K_m} K_I$$

was obtained according to Eqs. 13 and 16 as given in Fig. 2. Eq. 23 was well fitted to the whole progress curve. It can be seen that the kinetic constants obtained by the present approach as shown in Table 1 are consistent with the given values (in the legend of Figs. 1 and 2) which are used to generate the progress curves. For comparison, the value of k_i determined from the plot of $1/v$ against t [12–14] is shown in Table 1.

4. Discussion

During the last decade, a systematic study on the kinetics of irreversible modification of enzyme activity has been presented [11]. However, for a tight-binding complexing type inhibitor, the experiment must be performed at comparable or identical enzyme and inhibitor concentrations. Thus the concentration of inhibitor can not be considered as a constant during the modification. In this case, no explicit equation in terms of time can be obtained [15–18]. A numerical method [15] and several graphic methods [16–18] have been proposed to determine the kinetic constants for the slow, tight-binding reversible inhibition. However, the numerical method [15] needs a special computing program; the graphic methods [16–18] rely on the accurate estimation of the initial and steady-state rates and thus large error would probably arise from experiment.

In the present paper, a method was proposed to determine the kinetic constants for tight-binding, complexing inactivation. According to the equations derived under the condition of:

$$[E]_0 \gg \frac{2(K_m + [S])}{K_m} K_I$$

or

$$[E]_0 \ll \frac{2(K_m + [S])}{K_m} K_I$$

the kinetic constants can be obtained from fitting the derived equations to the progress curve using a non-linear fitting program. This is a simple, accurate method because the kinetic constants can be accurately determined from one experiment as shown in the numerical examples.

Recently, a simple method was proposed [12–14], in which the two-step inactivation was treated as one step and then the kinetic constants were determined from the linear plot of $1/v$ against t, but with disadvantage that large error would probably arise from the velocity obtained by the tangent line of the progress curve of product formation. This has been proved by comparing the value of k_i obtained by the plotting method to that by the present method as shown in Table 1. Besides, the necessary condition of $[E] \ll K_I$ for being treated as one step was not considered, so that it was mistakenly applied to the case of $[E] > K_I$ [12,13]. The problem mentioned above will not occur if the method presented in this paper is adopted.

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