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Thermokinetic studies of the irreversible inhibition of single-substrate, enzyme-catalyzed reactions

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Abstract

A thermokinetic ratio method for the irreversible inhibition of single-substrate enzyme-catalyzed reactions is proposed in this paper. By analyzing a measured curve this method can be used to calculate the apparent rate constant of the inhibited reaction without letting the reaction go to completion. Using the LKB-2107 batch microcalorimeter, the arginase-catalyzed hydrolysis of L-arginine in the presence of *p*-chloromercuribenzoate (PCMB) has been studied and PCMB established as an irreversible mixed inhibitor. The second-order rate constants for inhibition of arginase by PCMB in the absence and presence of L-arginine have been determined by this ratio method to be $k_{EI} = 94.4\ M^{-1}\ s^{-1}$ and $k_{ESI} = 35.2\ M^{-1}\ s^{-1}$, respectively, at 298.15 K. Chemical modification with PCMB indicates that arginase contains three reactive cysteinyl residues at most but these residues are not present at the active site of arginase.

Keywords: Arginase; Irreversible inhibition; Microcalorimetry; *p*-Chloromercuribenzoate (PCMB); Thermokinetic ratio method

1. Introduction

At the present time several methods [1–3] are available for kinetic studies of the irreversible inhibition of single-substrate enzyme-catalyzed reactions, but no study of this irreversible inhibition by microcalorimetry has been reported.

Microcalorimetry is a technique that holds much promise for use in the study of enzyme-catalyzed reactions due to its high sensitivity and accuracy. Since the absorp-

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tion or production of heat is an intrinsic property of virtually all enzyme-catalyzed reactions, it should be possible to obtain both kinetic and thermochemical information of this class of reaction with this technique.

Unlike reversible inhibitors, an irreversible inhibitor will remain firmly bound to one of the amino acids of the enzyme and thus act as a marker to enable it to be identified [3]. It seems reasonable to believe that investigation of the irreversible inhibition of enzyme-catalyzed reactions using microcalorimetry may give a clue to the active site structure of enzymes.

This paper suggests ratio equations for thermokinetics and kinetics of a single-substrate, enzyme-catalyzed reaction in the presence of an irreversible inhibitor, which can be used to calculate the apparent rate constant of the reaction by analyzing a measured curve without letting the reaction go to completion, to determine the irreversible inhibitor constants (k_{EI}, k_{ESI}), and to establish the type of irreversible inhibition simultaneously. The hydrolysis of L-arginine catalyzed by arginase in the presence of *p*-chloromercuribenzoate (PCMB) was studied with a batch microcalorimeter to test the validity of this ratio method and PCMB was established as an irreversible mixed inhibitor. The properties of the active site of arginase were investigated by chemical modification combined with microcalorimetry and the experimental results showed that arginase contains three reactive cysteinyl residues at most but that these residues do not belong to the active site of arginase, which solves the problem of whether arginase is a thiol enzyme [4–6].

2. Theory and method

2.1. Thermokinetic ratio equation

Let us investigate the steady-state kinetics of a simple single-substrate, single-intermediate, enzyme-catalyzed reaction in the presence of an irreversible inhibitor, I.

$$\begin{array}{ccc} S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \\ + \qquad + \\ I \qquad I \\ k_{EI} \downarrow \qquad \downarrow k_{ESI} \\ S + EI \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ESI \end{array} \tag{1}$$

where ESI is the inactive ternary complex; k_{EI} and k_{ESI} are the second-order rate constants for inhibition of enzyme (E) activity by the inhibitor in the absence and presence of the substrate (S), respectively, both of which are called the irreversible inhibitor constants.

According to Tsou [1], the reactions above obey first-order reaction kinetics and the concentration of the product (P) increases exponentially with time t:

$$[P] = [P]_\infty (1 - e^{-k_1 t}) \tag{2}$$

$$k_1 = A[\mathrm{I}]_0 \tag{3}$$

$$A = \frac{k_{\mathrm{EI}} K_{\mathrm{m}} + k_{\mathrm{ESI}}[\mathrm{S}]_0}{K_{\mathrm{m}} + [\mathrm{S}]_0} \tag{4}$$

where k_1 is the first-order rate constant, A is the apparent rate constant of the reaction between enzyme and inhibitor in the presence of substrate, K_{m} is the Michaelis constant ($K_{\mathrm{m}} = (k_{-1} + k_2)/k_{+1}$), $[\mathrm{S}]_0$ and $[\mathrm{I}]_0$ are the initial concentrations of substrate and inhibitor respectively and $[\mathrm{P}]_\infty$ is the product concentration at time t_∞

From the thermokinetic equation of a first-order reaction [7], we obtain

$$\frac{\Delta_1}{\Delta_2} = \frac{\mathrm{e}^{-k_1 t_1} - \mathrm{e}^{-kt_1}}{\mathrm{e}^{-k_1 t_2} - \mathrm{e}^{-kt_2}} \tag{5}$$

This is called the thermokinetic ratio equation of a first-order reaction. Here k is the cooling constant of the calorimeter, and Δ_1 and Δ_2 are the peak heights at times t_1 and t_2, respectively. With this equation the value of k_1 can be obtained from the ratio of the calorimetric data (Δ_1/Δ_2) in an experiment without using the total area under the curve; that is, with which the condition that the reaction goes to completion is unnecessary. For the Borchardt–Daniels method [8] and the dimensionless parameter method [7], however, this condition is necessary. This equation is especially suitable for studying the thermokinetics of one-substrate reactions in the presence of irreversible inhibitors, because in some circumstances these reactions do not go to completion [2].

Eq. (5) is also considered as the thermokinetic criterion for the decision that the calorimetric data obey first order kinetics. For a first-order reaction, the value of k_1 calculated by this equation is a constant.

2.2. *Kinetic ratio equation*

For a single-substrate, enzyme-catalyzed reaction in the absence of an inhibitor, we have [3]

$$\frac{1}{V_0} = \frac{K_{\mathrm{m}}}{V_{\max}} \frac{1}{[\mathrm{S}]_0} + \frac{1}{V_{\max}} \tag{6}$$

This is a linear euqation called the Lineweaver–Burk equation in which V_0 is the initial rate of the reaction. The maximum rate, $V_{\max}$, and K_{m} can be calculated from the intercept of $1/V_0$ and the slope $K_{\mathrm{m}}/V_{\max}$, respectively.

Combining Eq. (4) with Eq. (6), we obtain the kinetic ratio (A/V_0) equation of a one-substrate reaction in the presence of an irreversible inhibitor:

$$\frac{A}{V_0} = \frac{k_{\mathrm{EI}} K_{\mathrm{m}}}{V_{\max}} \frac{1}{[\mathrm{S}]_0} + \frac{k_{\mathrm{ESI}}}{V_{\max}} \tag{7}$$

Thus a plot of A/V_0 against $1/[\mathrm{S}]_0$ is linear with a slope of $(k_{\mathrm{EI}} K_{\mathrm{m}})/V_{\max}$ and a y-axis intercept of $k_{\mathrm{ESI}}/V_{\max}$. Then, the values of k_{EI} and k_{ESI} can be calculated from the slope and intercept, respectively, and the type of irreversible inhibition can be established by comparing the value of k_{EI} with that of k_{ESI}.

If ESI cannot be formed, then $k_{ESI} = 0$ and Eq. (7) becomes that for competitive inhibition. If the complex ESI can occur but no EI, then $k_{EI} = 0$ and the equation simplifies to that for uncompetitive inhibition. When $k_{EI} = k_{ESI}$, the equation reduces to that for simple linear noncompetitive inhibition. In the situation where $k_{EI} > k_{ESI}$ or $k_{EI} < k_{ESI}$, the equation becomes that for irreversible mixed inhibition.

Recently, an equation similar to Eq. (7) was given by Wang et al. [9] in their study on the kinetics of the denaturation of the enzyme.

3. Experimental

3.1. Reagents

Doubly distilled water was used throughout. Analytical grade sodium diethylbarbiturate and hydrochloric acid were used for the preparation of the buffer solution. The pH of the solution was adjusted to 9.4 by means of a pH meter, mixing slowly two solutions with concentrations both 0.02 mol dm^{-3}.

Solid arginase with a molecular weight of 110 000, which was extracted from beef liver, was obtained from the Institute of Biophysics of Academia Sinica and purified. An arginase solution of 2.00 mg cm^{-3} was prepared by dissolving the solid arginase in the buffer solution and the stock solution was stored in a refrigerator.

A set of substrate solutions with concentrations 0.100 mol dm^{-3} and 0.0100 mol dm^{-3} and a set of inhibitor solutions with concentrations 2.00×10^{-3} mol dm^{-3} and 2.00×10^{-5} mol dm^{-3} were made by dissolution of analytical grade L-arginine and PCMB, respectively, in buffer solution.

All solutions were freshly prepared before each set of experiments.

3.2. Instrumentation

In order to ensure that inhibitor is fully bound to the enzyme, the PCMB solution was first mixed with the arginase solution in a test tube according to the required concentration and then the tube was immersed in a water bath at a temperature of 310.15 K for 30 min. Subsequently, the reaction heat of hydrolysis of L-arginine catalyzed by arginase in the presence or absence of PCMB was determined at 298.15 K (the accuracy of temperature changes was $\pm$ 0.02 K in 24 h) using a LKB-2107 batch microcalorimeter system. One of the main components of the instrument consists of two separate calorimeter cells (Fig. 1), one of which is the reaction cell and the other a reference cell, each cell being divided into two parts.

4.00 cm^3 of the substrate solution and 2.00 cm^3 of arginase–PCMB mixed solution, already separately diluted to the required concentration by buffer solution, were placed in compartments II and I, respectively. In order to avoid the influence of the heat of mixing on the results of the measurement (this contribution can cause an error of about 10% in the reaction heat), the contents and quantities of both cells were made as identical as possible except that arginase was not added to compartment I of the reference cell. When the microcalorimetry system had been equilibrated and a steady

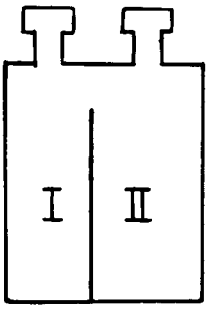


Fig. 1. The calorimeter cell.

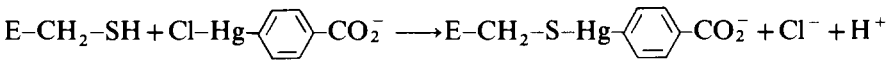
baseline obtained on the recorder, the reaction run was initiated by starting rotation of the calorimeter so as to mix the arginase-inhibitor and substrate solutions. The heat generated in the reaction process was recorded on a chart in the form of the recorder response; the heat capacity of the microcalorimetry system was calibrated by electrical energy and $k = (8.4737 \pm 0.0968) \times 10^{-3}\ s^{-1}$.

4. Results and discussion

4.1. Determination of the irreversible inhibitor constants

In the present report the measurement of the properties of the arginase-catalyzed reaction inhibited by PCMB with a microcalorimeter are described. The reaction investigated is an arginase-catalyzed hydrolysis of L-arginine in the presence of diethylbarbiturate buffer at pH 9.4. This is a pseudo single-substrate enzyme-catalyzed reaction whose products are L-ornithine and urea.

It is well known that mercury has a very strong affinity for sulfur, so it would be expected that mercurial reagents, e.g. PCMB, should bring about highly specific modification of cysteine side-chains in enzymes [10]:



(8)

The results of the determination of the first-order rate constants in two experiments are given in Table 1. It can be seen from Table 1 that the first-order rate constant in each experiment is almost invariable, therefore the arginase-catalyzed reaction in the presence of PCMB was a first-order reaction for L-arginine except in the earlier stage of this reaction. Thus it was concluded that the condition necessary for the utilization of the ratio equations had been satisfied.

The apparent rate constants of the inhibited reactions (calculated by the ratio method) and the initial rates of the uninhibited reactions (represented by $mJ\ s^{-1}$ in the enthalpimetric determination) at different substrate concentrations are listed in

Table 1
First-order rate constants for the hydrolysis of L-arginine catalyzed by arginase in the presence of PCMB at 298.15 K (concentrations in cell: L-arginine, (1). 6.667×10^{-3} mol dm^{-3}, (2). 3.333×10^{-3} mol dm^{-3}; arginase, 0.0167 mg cm^{-3}; PCMB, 1.667×10^{-5} mol dm^{-3})

t_1 in s	t_2 in s	Δ_1 in μV		Δ_2 in μV		$k_1 \times 10^3$ in s^{-1}	
		(1)	(2)	(1)	(2)	(1)	(2)
390	780	20.25	11.55	14.20	7.45	1.05	1.27
390	810	20.25	11.55	13.80	7.25	1.04	1.26
390	840	20.25	11.55	13.45	7.00	1.03	1.25
390	870	20.25	11.55	13.10	6.80	1.02	1.23
390	900	20.25	11.55	12.75	6.60	1.01	1.20
390	930	20.25	11.55	12.45	6.40	1.01	1.20
390	960	20.25	11.55	12.15	6.25	1.00	1.18
390	990	20.25	11.55	11.85	6.10	0.99	1.16
			Mean values			1.02 ± 0.02	1.22 ± 0.04

Table 2. Figs. 2 and 3 show, respectively plots of $1/V_0$ versus $1/[S]_0$ and of A/V_0 versus $1/[S]_0$ at different enzyme concentrations with the relative data (in part) in Table 2. Here the mean standard relative error of these kinetic data (A, V_0) is about 2%. It can be seen from Figs. 2 and 3 that the curves are linear and intersect the abscissa. The slope is proportional to the reciprocal of the enzyme concentration. The results calculated for irreversible inhibitor constants, k_{EI} and k_{ESI}, are given in Table 3. The mean value of k_{EI} (94.4 M^{-1} s^{-1}) is almost three times that of k_{ESI} (35.2 M^{-1} s^{-1}), which shows that PCMB is an irreversible mixed inhibitor of the arginase-catalyzed reaction.

4.2. *Influence of PCMB on arginase activity*

In order to determine the enzyme activity, the substrate concentration must greatly exceed K_m, e.g. $10\,K_m$. Under this condition, V_0, represented by the initial rate of heat evolution, is proportional to the concentration of the enzyme and the enzyme activity

Table 2
Apparent rate constants, A, for the hydrolysis of L-arginine catalyzed by arginase in the presence of PCMB and initial rates, V_0, for these reactions in the absence of PCMB at 298.15 K (concentrations in cell: arginase, 0.0167 mg cm^{-3}; PCMB, 1.667×10^{-5} mol dm^{-3}. $A = k_1/[I]_0$)

$[S]_0 \times 10^3$ in cell in mol dm^{-3}	$k_1 \times 10^3$ in s^{-1}	A in dm^3 mol^{-1} s^{-1}	V_0 in mJ s^{-1}
1.333	1.39	83.4	0.2393
1.667	1.37	82.2	0.2911
2.167	1.32	79.2	0.3415
3.333	1.22	73.2	0.4780
6.667	1.02	61.2	0.6464

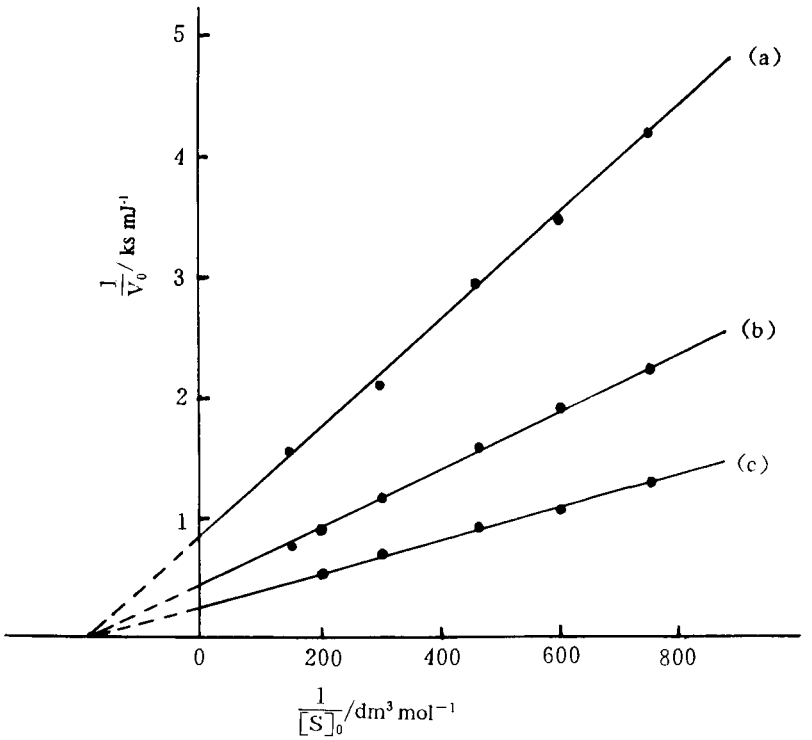


Fig. 2. Lineweaver–Burk plot of $1/V_0$ against $1/[S]_0$ at 298.15 K at enzyme concentrations of (a) 0.0167 mg cm^{-3}, (b) 0.0333 mg cm^{-3}, and (c) 0.0500 mg cm^{-3}.

(EA) can be calculated by [11]

$$EA = \frac{V_0}{\Delta_r H_m} \tag{9}$$

where $\Delta_r H_m$ is the overall molar enthalpy of the reaction.

The results of the measurement of arginase activities at different PCMB concentrations are given in Table 4. It can be seen from Table 4 that when the molar ratio of PCMB to arginase, α, exceeds three the remaining enzyme activity is almost invariant. This means that when 1 mole of arginase is modified by 3 moles of PCMB the degree of inhibition is maximum. According to Eq. (8), it is clear that 1 mole of PCMB reacts with 1 mole of sulfhydryl (SH) groups of cysteine residues in the enzyme. Therefore, we can concluded that arginase contains three reactive cysteinyl residues (or three reactive sulfhydryl groups) at most.

Because the modification of the three cysteinyl residues by PCMB led to only partial (about 50%) loss but not a complete loss of the enzyme activity, according to Palmer [3] we could conclude that these residues are not involved in the catalytic function of arginase. The conclusion that PCMB had a mixed-type inhibitory effect (but not

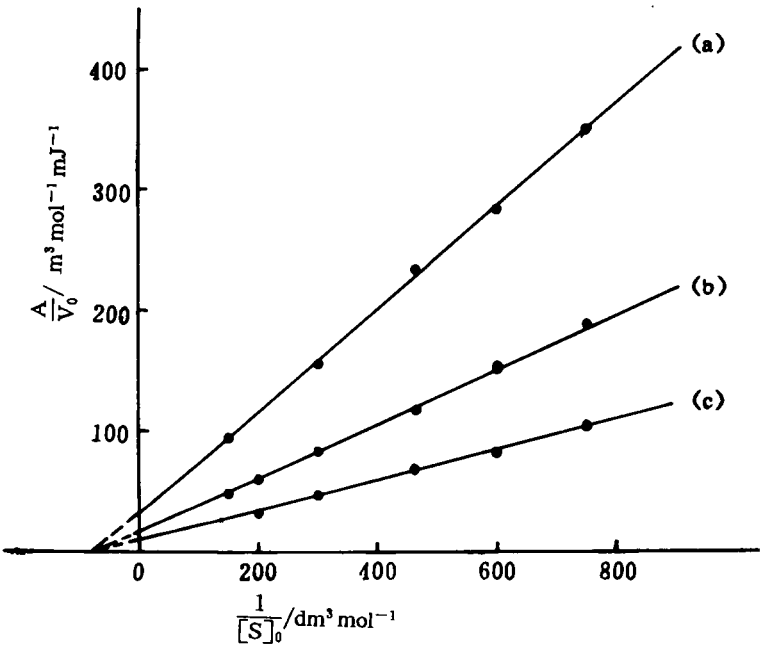


Fig. 3. Ratio plot of A/V_0 against $1/[S]_0$ at 298.15 K at enzyme concentrations of (a) 0.0167 mg cm^{-3}, (b) 0.0333 mg cm^{-3}, and (c) 0.0500 mg cm^{-3}.

Table 3
Second-order rate constants, k_{EI} and k_{ESI}, for inhibition of arginase by PCMB at a concentration in the cell of 1.667×10^{-5} mol dm^{-3} at 298.15 K

Arginase in cell in mg cm^{-3}	$K_m \times 10^3$ in mol dm^{-3}	V_{max} in mJ s^{-1}	k_{EI} in dm^3 mol^{-1} s^{-1}	k_{ESI} in dm^3 mol^{-1} s^{-1}
0.0167[a]	5.29	1.18	95.7	35.4
0.0333	5.24	2.22	95.3	35.5
0.0500	5.38	3.85	92.3	34.7
Mean values	5.30 ± 0.07		94.4 ± 1.9	35.2 ± 0.4

[a] At 310.15 K, $K_m = 2.30 \times 10^{-3}$ mol dm^{-3}.

a competitive inhibition) on the arginase-catalyzed reaction suggested that these cysteinyl residues are not present at the substrate-binding site of arginase, either. Hence, the experimental results showed that these residues do not belong to the active site of arginase; that is, arginase is not a thiol enzyme, which supports the conclusions of Gevorkyan and Davtyan [4]. However, according to Price and Stevens [10] this modification can cause a conformational change in the enzyme affecting substrate-binding (or affecting the catalytic site), which would lead to partial loss of arginase

Table 4
Arginase activities at different PCMB concentrations at 298.15 K (concentrations in cell: arginase, 0.0167 mg cm^{-3}; L-arginine, 6.667×10^{-2} mol dm^{-3}. $\Delta_r H_m = 17.8$ kJ mol^{-1} [12])

PCMB in cell in 10^{-7} mol dm^{-3}	α	EA in 10^{-6} mol min^{-1}	Relative activity in%
0	0	3.924	100
1.667	1	2.685	68.4
3.333	2	2.357	60.1
5.000	3	1.988	50.7
6.667	4	2.001	51.0
8.333	5	1.956	49.8
16.67	10	1.980	50.5
83.33	50	1.930	49.2
166.7	100	2.005	51.1

activity. It would be expected that the three cysteinyl residues might be situated near the active site of arginase.

Acknowledgments

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