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

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Abstract

A thermokinetic reduced extent method for the product inhibition of single-substrate, enzyme-catalyzed reactions is proposed in this paper. By analyzing the calorimetric curves of these reactions, this method can be conveniently used to calculate both kinetic parameters (K_m, K_i and V_m) and molar reaction enthalpy ($\Delta_r H_m$), and to establish the type of product inhibition simultaneously without adding product. The arginase-catalyzed hydrolysis of L-arginine has been studied by microcalorimetry and the product, L-ornithine, has been established as a competitive reversible inhibitor. The kinetic parameters calculated with this method are in agreement with those given in the literature.

Keywords: Arginase; Enzyme-catalyzed reaction; Microcalorimetry; Product inhibition; Thermokinetic reduced extent method

1. Introduction

Calorimetry is a technique that holds much promise for use in the study of enzyme kinetics. Since the absorption 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. Such studies have been undertaken by Kurvits and Siimer [1], Wu et al. [2] and Liu et al. [3], but no

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study of the product inhibition of single-substrate, enzyme-catalyzed reactions by microcalorimetry combined with a thermokinetic reduced extent method has been reported.

Unlike other general forms of inhibition, feedback inhibition by the end-product plays a vital role in metabolic regulation. It seems reasonable to believe that investigation of the product inhibition of enzyme-catalyzed reactions may give clues to the function of the living cell.

In the present report the kinetic reduced extent equations of single-substrate enzyme-catalyzed reactions in the presence of product inhibition and their thermokinetic mathematical models have been suggested, which can be used to calculate both kinetic parameters (K_m, K_i and V_m) and molar enthalpy ($\Delta_r H_m$) and to establish the type of product inhibition simultaneously by analyzing the calorimetric curves of these reactions without adding product. The thermokinetics of hydrolysis of L-arginine catalyzed by arginase were studied with a microcalorimeter and the product, L-ornithine, was established as a competitive inhibitor. The reliability of this method for determination of the thermokinetics of the product inhibition of single-substrate reactions has been verified by the experimental results, and the characteristics of the method have been discussed in detail.

2. Theory and method

2.1. Kinetic reduced extent equations

Let us investigate the steady-state kinetics of a simple single-substrate, single-intermediate, enzyme-catalyzed reaction in the presence of a competitive reversible inhibitor, the product (P).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \tag{1}$$

If the reaction goes to completion and the initial concentration of the product is zero, the extents of reaction at times t and t_∞ may be written respectively as

$$\chi = [P] = [S]_0 - [S] \tag{2}$$

$$\chi_\infty = [P]_\infty = [S]_0 \tag{3}$$

where $[S]_0$ is the initial substrate concentration and [S] is the substrate concentration at time t, [P] and $[P]_\infty$ are the product concentrations at time t and t_∞, respectively.

According to Michaelis–Menten kinetics, we easily prove that

$$\dot{\chi} = \frac{V_m[S]}{K_m(1 + [P]/K_i) + [S]} \tag{4}$$

where $\dot{\chi}$ is the rate of reaction ($\dot{\chi} = d\chi/dt$), K_m is the Michaelis constant ($K_m = k_{-1} + k_2)/k_1$), K_i is the inhibition constant for the product ($K_i = k_{-3}/k_3$) and V_m is the maximum rate ($V_m = k_2[E]_0$).

Inserting Eqs. (2) and (3) into Eq. 4 and performing the integration between limits 0 to χ and 0 to t, we obtain

$$\left(1-\frac{K_m}{K_i}\right)\chi - K_m\left(1+\frac{\chi_\infty}{K_i}\right)\ln\left(1-\frac{\chi}{\chi_\infty}\right) = V_m t \tag{5}$$

The reduced extent of reaction at time t, Φ, can be defined as [3]

$$\Phi = \frac{\chi}{\chi_\infty} = \frac{Q}{Q_\infty} = \frac{\Delta + ka}{kA} \tag{6}$$

where Q is the heat produced before time t, Q_∞ is the total heat effect, Δ is the peak height at time t, a is the peak area before time t, A is the total area under the calorimetric curve and k is the cooling constant of the calorimeter.

Combining Eqs. (5) and (6), we obtain

$$\left(1-\frac{K_m}{K_i}\right)\Phi - K_m\left(\frac{1}{[S]_0}+\frac{1}{K_i}\right)\ln(1-\Phi) = \left(\frac{V_m}{[S]_0}\right)t \tag{7}$$

which is called the kinetic reduced extent equation of a single-substrate, enzyme-catalyzed reaction in the presence of a competitive inhibitor, the product.

Similarly, noncompetitive product inhibition and uncompetitive product inhibition of a single-substrate reaction can be represented by the reduced extent Eqs. (8) and (9), respectively:

$$\left(\frac{[S]_0}{2K_i}\right)\Phi^2 + \left(1-\frac{K_m}{K_i}\right)\Phi - K_m\left(\frac{1}{[S]_0}+\frac{1}{K_i}\right)\ln(1-\Phi) = \left(\frac{V_m}{[S]_0}\right)t \tag{8}$$

$$\left(\frac{[S]_0}{2K_i}\right)\Phi^2 + \Phi - \left(\frac{K_m}{[S]_0}\right)\ln(1-\Phi) = \left(\frac{V_m}{[S]_0}\right)t \tag{9}$$

2.2. *Thermokinetic mathematical models*

In kinetic investigations concerned with an ongoing single-substrate, enzyme-catalyzed reaction system, if a relevant reduced extent Φ_i can be measured at time t_i, three data Φ_1, Φ_2, and Φ_3 at times t that are spread over an interval which is equal to each other, are generally obtained from the calorimetric data with Eq. (6). ($\Delta t = t_3 - t_2 = t_2 - t_1$). From Eq. (7), we obtain

$$\left(1-\frac{K_m}{K_i}\right)(\Phi_2-\Phi_1) - K_m\left(\frac{1}{[S]_0}+\frac{1}{K_i}\right)\ln\left(\frac{1-\Phi_2}{1-\Phi_1}\right) = \left(\frac{V_m}{[S]_0}\right)\Delta t \tag{10}$$

$$\left(1-\frac{K_m}{K_i}\right)(\Phi_3-\Phi_2) - K_m\left(\frac{1}{[S]_0}+\frac{1}{K_i}\right)\ln\left(\frac{1-\Phi_3}{1-\Phi_2}\right) = \left(\frac{V_m}{[S]_0}\right)\Delta t \tag{11}$$

Combining Eqs. (10) and (11), we get

$$\frac{K_m(K_i+[S]_0)}{K_i-K_m}=\frac{2\Phi_2-\Phi_1-\Phi_3}{2\ln(1-\Phi_2)-\ln(1-\Phi_1)-\ln(1-\Phi_3)}[S]_0 \tag{12}$$

$$V_m=\left(\frac{K_m}{\Delta t}\right)\left(1+\frac{[S]_0}{K_i}\right)\frac{(\Phi_3-\Phi_1)\ln(1-\Phi_2)-(\Phi_3-\Phi_2)\ln(1-\Phi_1)-(\Phi_2-\Phi_1)\ln(1-\Phi_3)}{2\Phi_2-\Phi_1-\Phi_3} \tag{13}$$

In order to obtain the kinetic parameters (K_m, K_i and V_m), two experiments are carried out in which the initial substrate concentration in the second experiment is twice that in the first ($[S]'_0 = 2[S]_0$) and other experimental conditions are identical. At times t_1, t_2 and t_3, the reduced extents of reaction in the first and second experiments are Φ_1, Φ_2, Φ_3 and Φ'_1, Φ'_2, Φ'_3, respectively.

Writing

$$y=\frac{2\Phi_2-\Phi_1-\Phi_3}{2\ln(1-\Phi_2)-\ln(1-\Phi_1)-\ln(1-\Phi_3)} \tag{14}$$

$$y'=\frac{2\Phi'_2-\Phi'_1-\Phi'_3}{2\ln(1-\Phi'_2)-\ln(1-\Phi'_1)-\ln(1-\Phi'_3)} \tag{15}$$

and

$$z=\frac{(\Phi_3-\Phi_1)\ln(1-\Phi_2)-(\Phi_3-\Phi_2)\ln(1-\Phi_1)-(\Phi_2-\Phi_1)\ln(1-\Phi_3)}{\Delta t(2\Phi_2-\Phi_1-\Phi_3)} \tag{16}$$

Then, combining Eqs. (14)–(16) with Eqs. (12) and (13), we obtain

$$K_m=\frac{2[S]_0(y-y')}{2y'-y+1} \tag{17}$$

$$K_i=\frac{2[S]_0(y-y')}{2y'-y} \tag{18}$$

$$V_m=\frac{[S]_0yz}{2y'-y+1} \tag{19}$$

When a single-substrate, enzyme-catalyzed reaction is taking place in a conduction calorimeter, the molar reaction enthalpy is [3]:

$$\Delta_r H_m=\frac{KA}{V[S]_0} \tag{20}$$

where K is the apparatus constant of the calorimeter and V is the total volume of the reacting system (in this paper, $V = 6.00\,cm^3$).

Eqs. (17)–(20) are called the thermokinetic mathematical model of a single-substrate, enzyme-catalyzed reaction inhibited competitively by the product.

Other types of product inhibition can be treated by similar methods.

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 of 0.02 mol dm^{-3}.

The solid arginase 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 0.0100 mol dm^{-3} substrate solution was made by dissolution of analytical grade L-arginine in buffer solution.

All solutions were freshly prepared before each set of experiments.

3.2. Instrumentation

The reaction heat of hydrolysis of L-arginine catalyzed by arginase was determined at 298.15 K 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 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, the contents and quantities in both cells were

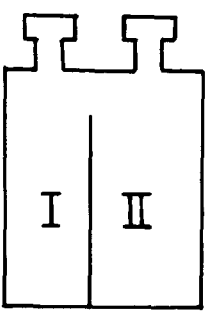

Fig. 1. The calorimeter cell.

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 baseline obtained on the recorder, the reaction run was initiated by starting rotation of the calorimeter so as to mix the arginase and substrate solutions. The heat generated in the reaction process was recorded on a chart in the form of the recorder response.

4. Results

The arginase-catalyzed hydrolysis of L-arginine (the substrate) in the presence of diethylbarbiturate buffer at pH 9.4 was studied to test the validity of this reduced extent method. The reaction almost went to completion and obeyed typical Michaelis–Menten kinetics even in the presence of the reaction product, L-ornithine [4], which competitively inhibited arginase [5,6]. Therefore, this reduced extent method can be used to analyze the calorimetric curves of the reaction. Calibrated by electrical energy, $K = 15.374\,\mathrm{mJ\,mV^{-1}\,s^{-1}}$ and $k = 8.4982 \times 10^{-3}\,\mathrm{s^{-1}}$. The molar reaction enthalpies and the kinetic parameters calculated by this method from the calorimetric data are listed in Tables 1, 2 and 3.

From these tables, it can be seen that the values of measurements of K_m, K_i, V_m, and $\Delta_r H_m$ have better repeatability and self-consistency and V_m is proportional to the enzyme concentration. Furthermore, the kinetic parameters (K_m, K_i) calculated by this method are in agreement with literature values and the accuracy of these constants is demonstrably better than those in the literature [7,8]. Therefore, the correctness and validity of this reduced extent method applied to the studies of thermokinetics of product inhibition of single-substrate, enzyme-catalyzed reactions should be consider-

Table 1
Hydrolysis of L-arginine catalyzed by arginase with concentration 0.0167 mg cm^{-3} in cell

No.	$10^3[S]_0$ in mol dm^{-3}	$-y$	$10^4 z$ in s^{-1}	A in mV s	$10^3 K_m$ in mol dm^{-3}	$10^3 K_i$ in mol dm^{-3}	$10^5 V_m$ in mol dm^{-3} s^{-1}	$\Delta_r H_m$ in kJ mol^{-1}
1	0.8333	3.342	13.05	5.889	4.91	1.24	1.07	18.1
2	1.667	2.341	9.144	11.538	5.25	1.28	1.10	17.7
3	3.333	1.832	5.854	23.094	5.12	1.26	1.09	17.8
4	6.667	1.580	3.406	46.128	–	–	–	17.7
5	0.8333	3.227	12.96	5.841	4.98	1.21	1.09	18.0
6	1.667	2.273	9.088	11.610	4.90	1.20	1.07	17.8
7	3.333	1.798	5.745	23.196	5.25	1.24	1.11	17.8
8	6.667	1.554	3.342	45.885	–	–	–	17.6
9	0.8333	3.106	12.78	5.802	5.22	1.17	1.14	17.8
10	1.667	2.198	9.025	11.439	5.08	1.16	1.12	17.6
11	3.333	1.747	5.684	23.250	5.16	1.17	1.13	17.9
12	6.667	1.520	3.307	49.926	–	–	–	18.0
			Average values		5.10 ± 0.14	1.21 ± 0.04	1.10 ± 0.03	17.8 ± 0.2

Table 2
An example of analysis of the calorimetric curve of an arginase-catalyzed reaction (no. 2 in Table 1)

i	a_i in mV s	$10^3\Delta_i$ in mVΦ_i		Data group	Δt in s	$-y$	$10^4 z$ in s^{-1}
1	1.994	15.85	0.3345	(Φ_2, Φ_3, Φ_4)	120	2.425	9.275
2	2.916	15.35	0.4093	(Φ_3, Φ_4, Φ_6)	120	2.310	9.162
3	4.101	13.05	0.4885	(Φ_6, Φ_7, Φ_8)	120	2.284	9.125
4	5.112	10.95	0.5547	$(\Phi_7, \Phi_8, \Phi_{10})$	120	2.407	9.200
5	5.220	10.70	0.5615	(Φ_2, Φ_4, Φ_7)	240	2.323	9.165
6	5.993	8.950	0.6106	(Φ_3, Φ_6, Φ_8)	240	2.253	9.106
7	6.765	7.050	0.6582	$(\Phi_4, \Phi_7, \Phi_{10})$	240	2.284	9.133
8	7.449	5.250	0.6991	(Φ_1, Φ_5, Φ_9)	360	2.745	9.291
9	7.509	5.000	0.7018	$(\Phi_2, \Phi_6, \Phi_{10})$	360	2.296	9.140
10	8.057	3.550	0.7345	$(\Phi_5, \Phi_9, \Phi_{11})$	360	2.081	8.839
11	9.024	1.000	0.7923	Average values		2.341 ± 0.170	9.144 ± 0.124

Table 3
Kinetic parameters for the hydrolysis of L-arginine catalyzed by arginase

Arginase in mg cm^{-3} in cell	$10^3 K_m$ in mol dm^{-3}	$10^3 K_i$ in mol dm^{-3}	$10^5 V_m$ in mol dm^{-3} s^{-1}
0.0167	5.10	1.21	1.10
0.0333	5.04	1.23	2.15
0.0500	5.19	1.28	3.41
Average values	5.11	1.24	–
Literature values [7,8]	5; 5.6	1.3	–

ed as proved. The experimental results also mean that the product, L-ornithine, is indeed a competitive inhibitor of the arginase-catalyzed reaction.

5. Discussion

5.1. Reduction of the thermokinetic reduced extent method

As in the case of uninhibited reactions ($K_i \to \infty$), Eqs. (7)–(9), and Eqs. (12) and (13) reduce, respectively, to the kinetic reduced extent equation and the thermokinetic mathematical model of single-substrate reactions uninhibited by the product [3]:

$$\Phi - \left(\frac{K_m}{[S]_0}\right)\ln(1-\Phi) = \left(\frac{V_m}{[S]_0}\right)t \tag{21}$$

$$K_m = y[S]_0 \tag{22}$$

$$V_m = K_m z = yz[S]_0 \tag{23}$$

In the event that $K_i = K_m$, Eq. (7) reduces to the form of a simple first-order equation [9], the term Φ being lost.

5.2. Establishment of the type of product inhibition

The thermokinetic reduced extent method proposed in this paper can be used to calculate kinetic parameters and to ascertain whether the enzyme is inhibited by the product with the calorimetric data from two experiments (condition: $[S]'_0 = 2[S]_0$) simultaneously without adding product. This method can also be used to establish the type of product inhibition with the calorimetric data from three experiments (conditions: $[S]''_0 = 2[S]'_0 = 4[S]_0$). If the enzyme is not inhibited by the product, we have the relation: $y = 2y'$. The enzyme-catalyzed reaction in the presence of product competitive inhibition disobeys this relation but obeys the relation: $y + 2y'' = 3y'$. Other types of product inhibition disobey the two relations.

5.3. Advantages of the thermokinetic reduced extent method

Compared with the traditional initial rate method [2], this reduced extent method has two advantages. The first advantage is that it utilizes the entire calorimetric curve, not only the initial part of the curve, which leads to a notable decrease in the number of experiments. The second advantage is that it utilizes the product yielded during the reaction (this approaches the case in the living cell); that is, with it the addition of product into the reaction system is unnecessary. For the traditional initial rate method, however, this addition and eight experiments at least must be satisfied. This reduced extent method is especially suitable for studying the product inhibition of one-substrate reactions in the case of the product being difficult to obtain (e.g. the product is unstable, or expensive, or difficult to purify).

In addition, the accuracy of this method is also better than other experimental approaches because the kinetic data (K_m, K_i, and V_m) can be calculated with it to three places of significant digits and the standard relative error of these data is small (about 3%).

In conclusion, the reduced extent method suggested in this paper is simple and its validity and feasibility have been proved. It is evident that this method is only suitable for the single-substrate, enzyme-catalyzed reactions that can go to completion.

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