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MICROCALORIMETRIC STUDIES ON THE PRODUCT INHIBITION OF HYDROLYSIS OF *L*-ARGININE CATALYZED BY BOVINE LIVER ARGINASE

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Abstract

A new thermokinetic reduced extent method for the product inhibition of single substrate enzyme-catalyzed reactions is proposed and compared with the traditional initial rate method in this paper. The arginase-catalyzed hydrolysis of *L*-arginine to *L*-ornithine and urea was studied at 37°C in 40 mM sodium barbiturate–HCl buffer solution (pH=9.4). Michaelis constant (K_m) for arginine and maximum velocity (V_m) of the reaction were determined by initial method and thermokinetic method. The activation of exogenous manganese to this reaction was also studied. The product inhibition constant (K_P), which cannot be obtained directly from the initial rate method, was determined by thermokinetic without adding *L*-ornithine to the reaction system. When the concentration of Mn^{2+} in cell is 0.1 mM, the enzyme gets its full activity. Incubation arginase with appropriate concentration of Mn^{2+} resulted in increased V_{max} and a higher sensitivity of the enzyme to product with no change in the K_m for arginine. We suggest that the exogenous manganese ions in solution have just recovered the activity of arginase, which was lost in dissolving and dilution, but no effect on the mechanism of the reaction.

Keywords: bovine liver arginase, inhibition, *L*-arginine, Mn^{2+}, thermokinetics

Introduction

Arginases (EC 3.5.3.1) are ubiquitous in nature, having been found in bacteria, fungi, plants, reptiles and mammals. In mammals, arginase has been detected in many different tissues having a complete and an incomplete urea cycle, such as liver, kidney, red blood cells, brain, mammary gland, gastrointestinal tract and vitreous humor [1–3]. In the urea cycle of mammals, arginase is a very important enzyme which specifically catalyzes the hydrolysis of *L*-arginine to *L*-ornithine and urea. Urea is the principal metabolite for disposal of nitrogen as a neutral and non-toxic waste product formed during

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amino acid metabolism in mammals. *L*-ornithine serves as a biosynthetic precursor to *L*-proline and the polyamines such as putrescine, spermine (in eucaryotes) and spermidine (in prokaryotes). These polyamines are found in high concentration in actively growing cells where they act as growth factors. It is believed that they play a role in controlling rates of nucleic acid biosynthesis. Thus, *L*-ornithine formation may be the main function of arginase in cells that have incomplete urea cycles [4]. Meanwhile, the product *L*-ornithine is the reversible competition inhibitor [5–7]. Unlike other general forms of inhibition, this feedback inhibition by the end-product plays a vital role in metabolic regulation. Traditional methods of studying this product inhibition must introduce the product into the reaction system and distinctly the original reaction system was destroyed. The present work utilized the product yielded during the reaction; it much more approaches the case in the living cell.

Bovine liver arginase is an enzyme with trimer; each subunit contains a double Mn (II, II) cluster core. Mn^{2+} ions are thought to activate a metal coordinated water molecule, generating the hydroxide ion that nucleophilically attacks the guanidine carbon of arginine [8–10]. Arginine hydrolysis is achieved by a metal–activate solvent molecule that symmetrically bridges the two Mn^{2+} ions. Binding of arginine is coupled to breaking of the μ–aquo bridge, leaving the water molecule bound to only one of the ions, and proton transfer from this aquo ligand to the N^{δ}-guanidino atom of the substrate forms the metal-bound nucleophilic hydroxide. In the Mn (II, II) cluster, one of the Mn^{2+} ions binding with the enzyme is reversible. After the loss of one Mn^{2+} ion in dissolving and dilution of arginase, the activity of the enzyme will decrease much more [11, 12]. In present work, Michaelis constant (K_m) for arginine and maximum velocity (V_m) of the reaction were determined by traditional initial rate method and thermokinetics, and the inhibition constant of product (K_P) was also determined by thermokinetic method. The influence of these biochemical constants of the reaction by exogenous Mn^{2+} ions has also been discussed.

Microcalorimetric methods provide in-situ, on-line, quasi-continuous, non-invasive and accurate measurements of thermodynamic and kinetic data of the reaction without constraint on solvent, spectral electrochemical and other properties of the reaction systems involved. Owing to their advantages, these methods have received increasing attention from researchers [13–15].

Theory

Velocity equation of arginase reaction

Because one of the substrates – water is the solvent, we can respect the reaction of *L*-arginine hydrolysis catalyzed by arginase as a single substrate enzyme reaction. As to the inhibition of the product, the mechanism of this reaction can be described as below:

$$E+S \overset{k_1}{\leftrightarrow} ES \quad (1)$$

$$ES \overset{k_2}{\rightarrow} E+P \quad (2)$$

$$E+P \xleftrightarrow{K_P} EP \tag{3}$$

in which E, S and P is arginase, *L*-arginine and *L*-ornithine, respectively, ES is the complex between enzyme and substrate, EP is the complex of enzyme and product. In the steady state concentration of ES, the velocity equation of this reaction can be easily obtained:

$$V=-\frac{d[S]}{dt}=\frac{V_m[S]}{K_m(1+[P]/K_P)+[S](1-K_m/K_P)} \tag{4}$$

where K_m and V_m is the Michaelis constant and maximal velocity of arginase, K_P is the inhibitory constant of product, they are respectively expressed as:

$$K_m=\frac{k_{-1}+k_2}{k_1};\quad V_m=k_2[E_t] \tag{5}$$

$$K_P=\frac{[E][P]}{[EP]} \tag{6}$$

in which $[E_t]=[E]+[ES]+[EP]$ is the total concentration of enzyme.

The thermokinetic equation of arginase reaction

Substituting $[P]=[S_0]-[S]$ into Eq. (4), and integral the equation from $t=0$ to $t=t$, the kinetic equation can be obtained:

$$K_m\left(1+\frac{[S_0]}{K_P}\right)\ln\frac{[S]}{[S_0]}+\left(1-\frac{K_m}{K_P}\right)([S]-[S_0])=-V_m t \tag{7}$$

where $[S_0]$ and $[S]$ are concentration of substrate when time is 0 and t. If the reaction reduced extent Φ_t is defined, it is

$$\Phi_t=\frac{[S_0]-[S_t]}{[S_0]} \tag{8}$$

And substituting it into Eq. (7), we can get the kinetic equation, which is expressed as reduced extent:

$$-\frac{\ln(1-\Phi_t)}{\Phi_t}=\frac{V_m}{K_m(1+[S_0]/K_P)}\frac{t}{\Phi_t}-\frac{[S_0](1-K_m/K_P)}{K_m(1+[S_0]/K_P)} \tag{9}$$

In the conduction calorimeter, the heat produced by a process is given by Tian equation, that is

$$Q_t=Ka_t+\Lambda\Delta_t;\quad Q_\infty=KA_\infty \tag{10}$$

in which Q_t and Q_∞ is the heat of exothermic (or endothermic) reaction at time t and $t\to\infty$, a_t and A is area before t and the total area enclosed by a thermal curve, Δ_t is the peak height of the curve at t, K and Λ are apparatus constants that are obtained by

electrical energy calibration. So the reduced extent of the reaction occurring in the calorimetric system can be expressed as:

$$\Phi_t = \frac{[S_0]-[S_t]}{[S_0]} = \frac{Q}{Q_\infty} = \frac{Ka_t + \Lambda\Delta_t}{KA_\infty} = \frac{a_t + \tau\Delta_t}{A_\infty} \tag{11}$$

where $\tau=\Lambda/K$, which has a dimension of time, is called time constant, and it can be gained by the linear-fit of $-\ln\Delta_t$ *vs*. *t* for electrical energy calibration curve as described in [16]. So (9) is the thermokinetic equation of enzymatic reaction.

Method of determination of K_m, V_m and K_p

Initial rate method of determination of K_m and V_m

For a Michaelis–Menton enzyme, in a definite enzyme total concentration, the relation of initial reaction velocity and initial substrate concentration is fit for Lineweaver–Burk double reciprocal equation:

$$\frac{1}{V_0} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S_0]} \tag{12}$$

The initial velocity of the reaction V_0 can be calculated from the initial exothermic velocity as

$$V_0 = -\frac{\Omega_0}{\Delta_r H_m V} \tag{13}$$

and

$$\Omega_t = \frac{\partial Q_t}{dt} \tag{14}$$

In Eq. (13), $\Delta_r H_m$ is the mole reaction enthalpy, which is determined to be –17.2 kJ mol^{-1} in this experiment according to the method in [17]. *V* is the volume of the reaction system; it is 6 mL in this experiment. In Eq. (14), Ω_t is the exothermic velocity at time *t*, the initial exothermic velocity Ω_0 is the slop of linear-fit of Q_t *vs. t* in the initial period of the reaction, in this period before the Φ_t less than 0.1, neglecting the product inhibition is thought as no much more influence on the results.

By analyzing the calorimetric curve of the reaction according to Eqs (10), (13) and (14) in a definite total enzyme and initial substrate concentration, we could gain a series data of V_0 for different $[S_0]$. The values of K_m and V_m could be gained through the Lineweaver–Buek double reciprocal plot according to Eq. (12).

Thermokinetic method of determination of K_m, V_m and K_p

According to Eq. (10), in a definite total enzyme and initial substrate concentration, the plot of $-\ln(1-\Phi_t)/\Phi_t$ *vs*. t/Φ_t must be linear. Defining the *y*-axis intercept and slop as *A* and *B*, respectively, that is

$$A = \frac{[S_0](1-K_m/K_P)}{K_m(1+[S_0]/K_P)};\ B = \frac{V_m}{K_m(1+[S_0]/K_P)} \tag{15}$$

The reciprocal of the slop B^{-1} is:

$$\frac{1}{B}=\frac{K_m}{V_m K_P}[S_0]+\frac{K_m}{V_m} \tag{16}$$

The y-axis intercept a and slop b of the second plot of B^{-1} *vs.* $[S_0]$ are

$$a=\frac{K_m}{V_m};\ b=\frac{K_m}{V_m K_P} \tag{17}$$

Combining Eqs (15) and (17) we obtain

$$K_P=\frac{a}{b} \tag{18}$$

$$V_m=\frac{1}{b-A/B[S_0]} \tag{19}$$

$$K_m=aV_m \tag{20}$$

Materials and methods

Doubly distilled water was used throughout the experiment. Analytical grade sodium barbiturate and muriatic acid were used for the preparation of the buffer solution. The value of pH of the buffer was adjusted to 9.4 at 37°C by means of pH meter, dripping slowly of 0.1 M HCl to sodium barbiturate solutions with concentrations of 40 mM.

The solid bovine liver arginase was purchased from Washington Biochemical Cooperation and has not been further purified before it is used. Arginase stock solution with concentration 9.6 mg mL^{-1} was prepared by dissolving the solid enzyme in buffer and stored in a refrigerator for use.

Biochemical grade *L*-arginine was used as substrate without further purification. Required concentration of substrate solutions were made by dissolving *L*-arginine in the buffer. Analytical grade $MnSO_4$ was used as the activation reagent in this experiment. The solution was prepared by boiled doubly distilled water before each calorimetric experiment and was titrated by H_2O_2 solution with determined concentration.

The heat of reaction was determined by an LKB-2107 batch microcalorimeter system, in which there are two pairs of gemel batch micro-reactor: one is used as react cell and the other is reference cell. The details of the performance and the structure of instrument were introduced in the previous work [18] and we will not describe them here.

In the enzymatic reaction experiments, different volume of substrate stock was diluted to 10 mL, 4 mL of which was taken to react cell (II) and reference cell (II), respectively, and 2 mL of enzyme solution was taken to react cell (I); 2 mL of buffer solution was added into reference cell (I). In the activation experiment, the same concentration of $MnSO_4$ solution was added into each cell. With the exception of enzyme the concentration and volume of other samples were the same in react cell (I) as in reference cell (I). After the system had gotten balance at 37°C, and the Mixing Start

button of the apparatus was pressed, the calorimeter turned 360°C and reversed. The voltage signal was recorded by means of LKB-2210 dual-pen integrating recorder.

Results

The arginase-catalyzed hydrolysis of *L*-arginine (the substrate) in the presence of sodium barbiturate–HCl buffer (pH=9.4) at 37°C almost went to completion and obeyed typical Michaelis–Menton kinetics even in the presence of the reaction product, *L*-ornithine, which competitively inhibited arginase. Therefore, the biochemical constants of this reaction can be determined by the methods described above. An example of analyzing the calorimetric curve is given in Table 1 and the linear plot of $-\ln(1-\Phi_t)$ *vs.* t/Φ_t is given in Fig. 1. The second plot of B^{-1} *vs.* $[S_0]$ is given in Fig. 2 when the total concentration of enzyme was 0.0032 mg mL^{-1} in the absence of exogenous of Mn^{2+} and the concentration of Mn^{2+} was 0.1 mM. Figure 3 is the Lineweaver–Burk double reciprocal plot of the reaction. The calculation values of K_m, V_m and K_P are listed in Tables 2 and 3.

Table 1 An example of analysis of the calorimetric curve an arginase-catalyzed reaction[a, b]

t/s	Δ_t/mV	a_t/mV s^{-1}	Φ_t
105	48	2979.907	0.210
222	58	9459.340	0.396
324	52	15113.315	0.516
414	44	19445.314	0.598
516	36	23513.274	0.674
636	28	27341.278	0.743
708	24	29213.303	0.777
800	20	31232.902	0.815
906	16	33144.483	0.850
978	14	34224.497	0.870
1056	12	35238.497	0.889
1146	10	36228.497	0.907

[a]Electrical calibration constants: K=4.1306°C·10^{-3} mJ mV^{-1} s^{-1}; Λ=0.491 mJ mV^{-1}; τ=118.87 s, total area A_∞=41223.49 mV s^{-1}.
[b]Total concentration of arginase in cell is 0.0032 mg mL^{-1}, initial concentration of arginine is 1.65 mM, [Mn^{2+}]=0.1 mM

From the figures, it can be seen that the better linear relations were obtained either by initial rate method or by thermokinetic method for this reaction. These results indicate that the reaction of arginase-catalytic hydrolysis of arginine obeys the kinetic mechanism described in Eqs (1) to (3), and the inhibition of the product, *L*-ornithine, to the reaction is indeed the reversible competition type. From the tables, it

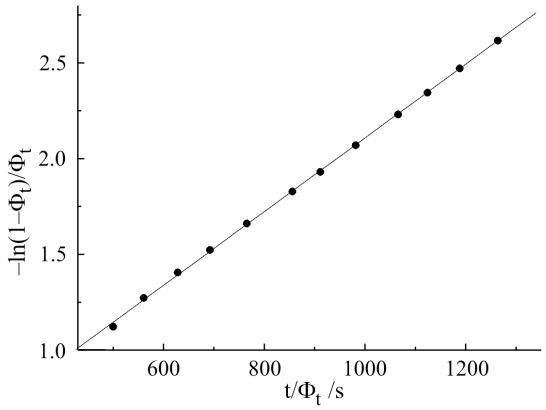

Fig. 1 Linear relationship of $-\ln(1-\Phi_t)/\Phi_t$ and t/Φ_t; r=0.9998, B=19.3·10^{-4}

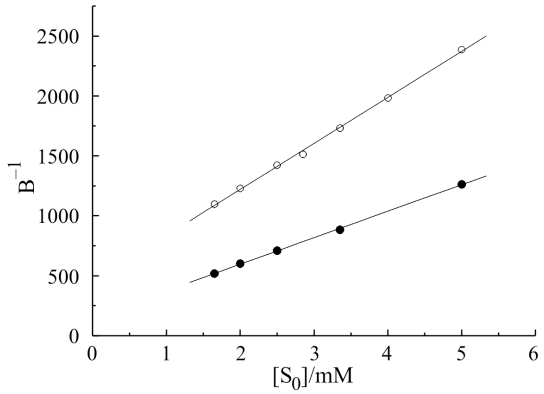

Fig. 2 Plot of B^{-1} *vs*. $[S_0]$; $[E_t]$=0.0032 mg mL^{-1}, ○ – C_{Mn}^{2+}=0, ● – C_{Mn}^{2+}=0.1 mM

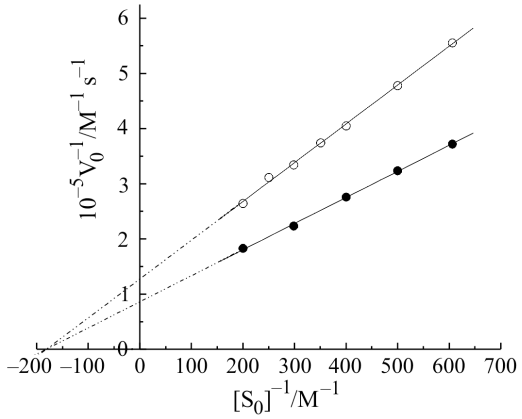

Fig. 3 Lineweaver–Burk double reciprocal plot of the reaction $[E_t]$=0.0032 mg mL^{-1}, ○ – C_{Mn}^{2+}=0, ● – C_{Mn}^{2+}=0.1 mM

Table 2 Linear fit constant A, B for Eq. (9) and a, b for Eq. (16) and the calculated values of K_m, V_m, K_P [a]

$[S_0]$/mM	A	10^4B	K_m/mM	10^2V_m/mM s^{-1}	A	10^4B	K_m/mM	10^2V_m/mM s^{-1}
		$[Mn^{2+}]$=0				$[Mn^{2+}]$=0.1 mM		
1.65	0.4442	9.1252	5.13	1.13	0.6054	19.3000	5.03	3.24
2	0.4828	8.1302	5.23	1.15	0.6282	16.6000	4.89	3.15
2.5	0.5169	7.0239	5.08	1.12	0.6704	14.0877	5.07	3.27
2.85	0.5505	6.6023	4.98	1.10	–	–	–	–
3.35	0.5756	5.7708	5.28	1.16	0.7182	11.3179	4.92	3.18
4	0.5977	5.0443	5.19	1.14	–	–	–	–
5	0.6177	4.1896	5.11	1.13	0.7497	7.9107	4.97	3.21
	Average values		5.14	1.13			4.98	3.21

[a]The linear fit constants a and b are 453.6447 and 383645.3049 according to Eq. (16) in the absence of Mn^{2+}; and 154.9483 and 220918.1661 when the concentration of Mn^{2+} in cell is 0.1 mM

can be seen that the values of measurements of K_m, V_m and K_P have better repeatability and self-consistency and are in agreement with literature values.

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

Method	K_m/ mM	10^2V_m/ mM s^{-1}	K_P/ mM	K_m/ mM	10^2V_m/ mM s^{-1}	K_P/ mM
		$[Mn^{2+}]=0$			$[Mn^{2+}]=0.1$ mM	
Thermokinetic	5.14	1.13	1.18	4.98	3.21	0.70
Initial rate	5.53	0.79	–	5.50	1.16	–
Literature	5[a];5.6[b];5.11[c]	–	1.3[a];1.24[c]	–	–	–

[a][19]; [b][20]; [c][18]

In the thermokinetic method, almost the entire calorimetric curve, in which the reduced extent Φ_t of the reaction is 0.2 to 0.9, was used to determine the parameters K_m, V_m and K_P. But the initial rate method has just used the previous period of the calorimetric curve, in which the reaction reduced extent is less than 0.1 and the inhibition of the product to the reaction was always ignored, so it can only give the parameters K_m and V_m. This new method using more direct thermal information from the process would give more reliable kinetic information.

Conclusions

Since the thermokinetic methods are based on the analysis of the full calorimetric curve obtained from the high sensitive and accurate microcalorimeter by reduced extent method. Compared with the initial rate method, the advantage is that it utilizes more heat messages in the entire calorimetric curve for studying the reactions. Therefore, this method could express the mechanism more exactly than initial rate method and then it could give more results. It is especially suitable for studying the product inhibition in the case of the product being difficult to obtain (e.g. the product is unstable, or expensive, or difficult to purify).

Arginase is one of the enzymes that contained a double metal core, and an Mn(II)–Mn(II) cluster is necessary for arginase activity. Much of the enzyme activity is lost when one of the Mn^{2+} is apart from the enzyme. The exogenous Mn^{2+} will recover the enzyme activity. Meanwhile, with the increase of active enzyme concentration, the concentration of EP increases at definite reaction extent. So arginase becomes more sensitive to the inhibition of product. These results indicate that the inhibition of *L*-ornithine to arginase occurs by competing the active center with arginine and it must combine with the double metal core of the enzyme.

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References

1 T. Gotoh, T. Sonoki, A. Nagasaki, K. Terada, M. Takiguchi and M. Mori, FEBS Lett., 395 (1996) 119.
2 S. M. Morris Jr., D. Bhamidipati and D. Kepka-Lenhart, Gene, 193 (1997) 157.
3 M. F. Gursu, Arch. Med. Res., 32 (2001) 432.
4 G. C. Dismukes, Chem. Rev., 96 (1996) 2909.
5 N. Carvajal, M. Acoria, J. P. Rodriguez, M. Fernandez and J. Martinez, Biochim. Biophys. Acta, 70 (1982) 146.
6 L. C. Wright, C. J. Brady and R. W. Hinde, Phytochemistry, 20 (1981) 2641.
7 T. S. R. Subrahmanyan and R. Raghupathi, Indian J. Biochem. Biophys., 23 (1986) 359.
8 Z. F. Kanyo, L. R. Scolnick, D. E. Ash and D. W. Christianson, Nature, 383 (1996) 554.
9 M. C. Bewley, P. D. Jeffrey, M. L. Patchett, Z. F. Kanyo and E. N. Baker, Structure, 7 (1999) 435.
10 R. S. Reczkowski and D. E. Ash, Arch. Biochem. Biophys., 312 (1994) 31.
11 C. N. Pace, A. Buonanno and J. Simmons-Hansen, Anal. Biochem., 109 (1980) 261.
12 N. Carvajal, M. Salas and V. Lopez, J. Inorg. Biochem., 77 (1999) 163.
13 X. Y. Xie, Z. Y. Wang, D. Q. Wu, S. S. Qu and C. X. Wang, J. Therm. Anal. Cal., 70 (2002) 589.
14 X. Li, Y. Liu, J. Wu, H.-G. Liang and S.-S. Qu, J. Therm. Anal. Cal., 67 (2002) 589.
15 E. Utzig, J. Therm. Anal. Cal., 54 (1998) 391.
16 X. Y. Xie, Z. Y. Wang and C. X. Wang, Acta Chemica Sinca (in Chinese), 61 (2003) 331.
17 Y. Liang, C. X. Wang and D. Q. Wu, Chem. J. Chinese Universities (in Chinese), 16 (1995) 744.
18 Y. Liang, C. X. Wang, D. Q. Wu and S. S. Qu, Thermochim. Acta, 268 (1995) 27.
19 R. Gopalakrishna and B. Nagarajan, Indian J. Biochem. Biophys., 15 (1978) 488.
20 S. V. Chubaryan, L. R. Tumanyan and R. O. Torchyan, Biol. Zh. Arm., 36 (1983) 233.