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Thermokinetic Study on the Reversible Competitive Inhibition of Bovine Liver Arginase

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A new thermokinetic reduced extent method for studying of the reversible competitive inhibition of single substrate enzyme-catalyzed reactions was proposed in this paper. The reaction that arginase-catalyzed hydrolysis of *L*-arginine to *L*-ornithine and urea and the inhibition of this reaction by the product, *L*-ornithine, and exogenous *L*-lysine were studied at 37 ℃ in 40 mmol•L^{-1} sodium barbiturate-HCl buffer solution (pH＝9.4). Michealis constant K_m for arginine and maximum velocity V_m of the reaction were determined to be 5.14 mmol•L^{-1} and 1.13×10^{-2} mmol•L^{-1}•s^{-1}, respectively. The product inhibition constant K_P and inhibitory constant K_I of *L*-lysine were determined to be 1.18 and 5.6 mmol•L^{-1}, respectively. All the results have better repeatability and self-consistency and are in agreement with literature values. This new method using more direct thermal information from the process would give more reliable kinetic information than the traditional initial rate method.

Keywords bovine liver arginase, reversible competitive inhibition, *L*-arginine, *L*-lysine, thermokinetics

Introduction

Arginase (EC 3.5.3.1) is a widespread and very important enzyme in mammals, which specifically catalyzes the hydrolysis of *L*-arginine to urea and the non-protein amino acid *L*-ornithine, a key step in the urea cycle.[1] Urea is the principal metabolite for disposal of nitrogen as a neutral and nontoxic waste product formed during 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 spermindine (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.[2] Recent studies have established the presence in mammals of at least two distinct arginase genes coding for immunologically distinct iso-forms.[2-4] One of them (AI) is located in the cytoplasm and strongly expressed in the liver whereas the extrahepatic arginase, AII, was found in mitochondria with a wider tissue distribution.[3,4] Despite the diversity of living organisms in which they were found, arginases exhibit a high sequence identity and highly similar physicochemical properties.[5,6] All arginases require divalent metal ions for their catalytic activity, and their highest activity is reached in the presence of Mn(II) ions.[7,8]

Amino acids like *L*-valine, *L*-lysine and *N*-hydroxy-*L*-arginine inhibit arginase activity and switch off the EPR signal of the binuclear center probably by removing a bridging ligand or by increasing the inter-manganese separation.[9,10] The second class of inhibitors, such as borate ions and hydroxylamine, only produces modest changes in the EPR spectra, resulting in a simplification of the spectrum by conversion to species with a relatively narrow distribution of Mn—Mn separations.[9,11]

In the previous researches, microcalorimetric method has been acknowledged as a highly effective method for studying the kinetics of enzymatic reaction and inhibition,[12-14] but few data are presently available about the inhibition of arginase. In the present work, we studied the inhibition of bovine liver arginase (BLA) by *L*-ornithine, one of the products of the reaction, and *L*-lysine by means of a new thermokinetic reduced extent method. These amino acids were thought to be the reversible competitive inhibitors of arginase.

Experimental

Velocity equation of arginase reaction in the presence of reversible competitive inhibition

Because one of the substrates, water, is the solvent, the reaction of *L*-arginine hydrolysis catalyzed by arginase can be respected as a single substrate enzyme reaction. In the presence of the reversible competitive inhibition, the mechanism of the reaction can be described

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as:

$$E+S \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} ES \xrightarrow{k_2} E+P \quad (1)$$

$$E+P \xleftrightarrow{K_P} EP \quad (2)$$

$$E+I \xleftrightarrow{K_I} EI \quad (3)$$

where E, S and P are arginase, substrate and product, respectively, I is the reversible competitive inhibitor, ES the complex between enzyme and substrate, EP the complex of enzyme and product, and EI the complex of enzyme and inhibitor. In the steady state concentration of ES, the velocity equation of the reaction in the presence of reversible competitive inhibition can be easily obtained:

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

where K_m and V_m are the Michealis constant and maximal velocity of arginase, K_P is the inhibitory constant of product, K_I the inhibitory constant of reversible inhibitor, and they are respectively expressed as

$$K_m=\frac{k_{-1}+k_2}{k_1}, \quad V_m=k_2[E_t] \quad (5)$$

$$K_P=\frac{[E][P]}{[EP]}, \quad K_I=\frac{[E][I]}{[EI]} \quad (6)$$

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

Thermokinetic equation of arginase reaction in the presence of reversible competitive inhibition

Substituting $[P]=[S_0]-[S]$ to Eq. (4) and integrating the equations from $t=0$ to $t=t$, the kinetic equation in the presence of reversible competitive inhibitor can be obtained:

$$K_m(1+\frac{[S_0]}{K_P}+\frac{[I]}{K_I})\ln\frac{[S]}{[S_0]}+(1-\frac{K_m}{K_P})\cdot([S]-[S_0])=-V_mt \quad (7)$$

in which [I] is the concentration of inhibitor, $[S_0]$ and [S] are concentration of substrate when time is 0 and t. Define the reaction reduced extent Φ_t,

$$\Phi_t=\frac{[S_0]-[S_t]}{[S_0]} \quad (8)$$

and substitute it into Eq. (7), the kinetic equation can be expressed as reduced extent:

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

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

$$Q_t=Ka_t+\Lambda\Delta_t, \quad Q_\infty=KA_\infty \quad (10)$$

in which Q_t and Q_∞ are the heat of exothermic (or endothermic) reaction at time t and $t\to\infty$, a_t and A_∞ are area before t and the total area enclosed by a thermograph, Δ_t is the peak height of the thermograph at t, and K and Λ are apparatus constants. So the reduced extent of the reaction occurring in the calorimetric system can be expressed as

$$\Phi_t=\frac{[S_0]-[S_t]}{[S_0]}=\frac{Q}{Q_\infty}=\frac{Ka_t+\Lambda\Delta_t}{KA}=\frac{a_t+\tau\Delta_t}{A} \quad (11)$$

$\tau=\Lambda/K$, which has a dimension of time, is called time constant. It can be obtained by the linear-fit of $-\ln\Delta_t$ against t for electrical energy calibration curve as described in reference.[15] So Eq. (9) is the thermokinetic equation of enzymatic reaction in the presence of reversible competitive inhibitor.

Method of determination of K_P and K_I

According to Eq. (9), at a definite total enzyme and initial substrate concentration, the plot of $-\ln(1-\Phi_t)/\Phi_t$ against t/Φ_t should be linear. Define that the y-axis intercept is A and the slope is B, that is:

$$A=-\frac{[S_0](1-K_m/K_P)}{K_m(1+[S_0]/K_P+[I]/K_I)}$$

$$B=\frac{V_m}{K_m(1+[S_0]/K_P+[I]/K_I)} \quad (12)$$

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

$$\frac{1}{B}=\frac{K_m}{V_m}+\frac{K_m}{V_mK_P}[S_0]+\frac{K_m}{V_mK_I}[I] \quad (13)$$

At a definite total enzyme concentration, the y-axis intercept a and slope b of the second plot of B^{-1} vs. $[S_0]$ in the absence of inhibitor are:

$$a=\frac{K_m}{V_m}, \quad b=\frac{K_m}{V_mK_P} \quad (14)$$

At a definite total enzyme and initial substrate concentration, the y-axis intercept c and slope d of the second plot of B^{-1} vs. [I] are

$$c=\frac{K_m}{V_m}\left(1+\frac{[S_0]}{K_P}\right), \quad d=\frac{K_m}{V_mK_I} \quad (15)$$

So, the product inhibitory constant and the reversible

competitive inhibitory constant K_I can be calculated from the equations below:

$$K_P=\frac{a}{b} \tag{16}$$

$$K_I=\frac{c/d}{1+[S_0]/K_P} \tag{17}$$

The Michealis constant K_m and maximal velocity V_m can also be calculated from the equations:

$$V_m=\frac{1}{b-A/B[S_0]} \tag{18}$$

$$K_m=aV_m \tag{19}$$

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 ℃ by means of pH meter, dripping slowly 0.1 mol•L^{-1} HCl to sodium barbiturate solutions with concentrations of 0.04 mol•L^{-1}.

The solid bovine liver arginase was purchased from Washington Biochemical Cooperation and was not further purified before use. 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 and *L*-lysine were used as substrate and reversible competitive inhibitor, respectively, without further purification. Required concentration of substrate and inhibitor solutions were made by dissolving *L*-arginine and *L*-lysine in the buffer.

The heat of reaction was determined by an LKB-2107 batch microcalorimeter system, in which there are two pairs of twins batch micro-reactors: one was used as reaction cell and the other was reference cell. The details of the performance and the structure of instrument were introduced in the previous work[16] which will not be described them here.

In the enzymatic reaction experiments, different volume of substrate stock was diluted to 10 mL, 4 mL of which was taken to reaction cell II and reference cell II respectively, and 2 mL of enzyme solution was taken to reaction cell I. 2 mL of buffer solution was added into reference cell I. In the inhibitory experiment, the same concentration of lysine solution was added into reaction cell I and reference cell I. With the exception of enzyme the concentration and volume of other samples were the same in reaction cell I as in reference cell I. After the system reached equilibrium at 37 ℃, and the Mixing Start button of the apparatus was pressed, the calorimeter turned 360° and reversed. The voltage signal was recorded by means of an LKB-2210 dual-pen integrating recorder.

Results and discussion

Results

The arginase-catalyzed hydrolysis of *L*-arginine (the substrate) in the presence of sodium barbiturate-HCl buffer (pH=9.4) at 37 ℃ almost went to completion and obeyed typical Michaelis-Menton kinetics even in the presence of the reaction product, *L*-ornithine, and exogenous competitive inhibitor, *L*-lysine. 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 one of the linear plots of $-\ln(1-\Phi_t)/\Phi_t$ against t/Φ_t in the absence and presence of lysine is given in Figure 1. The second plot of B^{-1} vs. $[S_0]$ in the absence of lysine is given in Figure 2 when the total concentration of enzyme was 0.032 mg•mL^{-1}. The second plot

Table 1 An example of analyzing the calorimetric curve obtained from one of arginase-catalyzed reactions[a,b]

t/s	Δ_t/mV	a_t/(mV•s)	Φ_t
240	57	8861.112	0.219
282	57	11255.111	0.253
318	56	13289.054	0.280
398	52	17630.653	0.334
459	48	20660.702	0.370
528	44	23834.702	0.408
606	40	27110.660	0.447
693	36	30416.698	0.487
792	32	33782.664	0.528
912	28	37382.663	0.572
1059	24	41204.663	0.619
1254	20	45494.663	0.672
1500	16	49922.680	0.728
1860	12	54962.680	0.792

[a] Electrical calibration constants: $K=4.1306\times10^{-3}$ mJ/(mV•s), $\Lambda=0.491$ mJ/mV, $\tau=118.87$ s, total area $A_\infty=71132.679$ mV•s. [b] Total concentration of arginase in cell was 0.032 mg•mL^{-1}, and initial concentration of arginine was 2.85 mmol•L^{-1}.

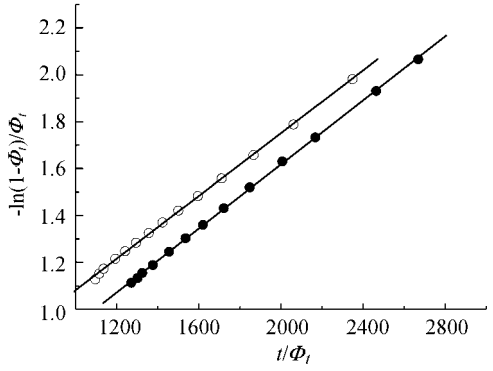

Figure 1 Linear relationship of $-\ln(1-\Phi_t)/\Phi_t$ and t/Φ_t. ○: $[E_t]=0.032$ mg•mL^{-1}, $[S_0]=2.85$ mg•mL^{-1}, $c_{lysine}=0$; ●: $[E_t]=0.027$ mg•mL^{-1}, $[S_0]=2.5$ mg•mL^{-1}, $c_{lysine}=4.00$ mg•mL^{-1}.

of B^{-1} vs. [I] was given in Figure 3 when the total concentration of enzyme was 0.027 mg•mL^{-1} and the initial substrate concentration was 2.5 mmol•L^{-1}. The calculation values of K_m, V_m, K_P and K_I are listed in Tables 2 and 3.

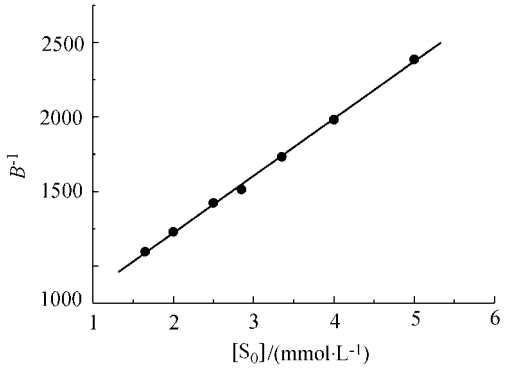

Figure 2 Plot of B^{-1} vs. $[S_0]$ in the absence of lysine at $[E_t]$= 0.032 mg•mL^{-1}.

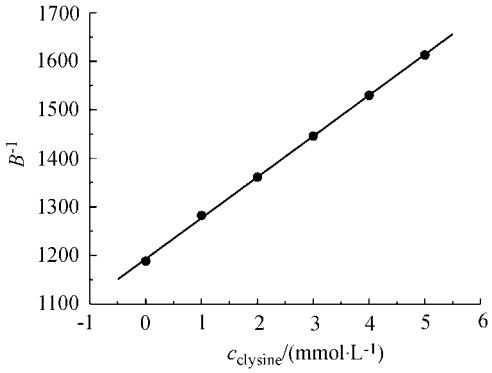

Figure 3 Plot of B^{-1} vs. c_{lysine} at $[E_t]$=0.027 mg•mL^{-1} and $[S_0]$=2.5 mmol•L^{-1}.

Discussion

It can be seen from Figure 1 to Figure 3 that better linear relationships were obtained by thermokinetic method for this reaction. These results indicate that the reaction of arginse-catalyzed hydrolysis of arginine obeys the kinetic mechanism described in Eqs. (1)—(3), and the inhibition of the product, *L*-ornithine, and the exogenous inhibitor, *L*-lysine to the reaction is indeed the reversible competition type. It can be seen from Tables 2 and 3 that the values of measurements of K_m, V_m, K_P and K_I have good repeatability and self-consistency and are in agreement with literature values.

In the thermokinetic method, almost the entire calorimetric curve, in which the reduced extent Φ_t of the reaction is 0.2—0.9, was used to determine the parameters K_m, V_m, K_P and K_I. But the initial rate method has just used the initial period of the calorimetric curve, in which the reaction reduced extent was 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 used more direct thermal information from the process and therefore would give more reliable kinetic information.

Table 2 Linear fit constants *A* and *B* for Eq. (9) and the calculated values of K_m and V_m in the absence of lysine [a]

$[S_0]$/(mg•mL^{-1})	*A*	10^4B	K_m/(mg•mL^{-1})	10^2V_m/(mg•mL^{-1}•s^{-1})
1.65	0.4442	9.1252	5.13	1.13
2.00	0.4828	8.1302	5.23	1.15
2.50	0.5169	7.0239	5.08	1.12
2.85	0.5505	6.6023	4.98	1.10
3.35	0.5756	5.7708	5.28	1.16
4.00	0.5977	5.0443	5.19	1.14
5.00	0.6177	4.1896	5.11	1.13
Average values			5.14	1.13

[a] The linear fit constants *a* and *b* are 453.6447 and 383645.3049 according to Eqs. (13) and (14) in the absence of lysine.

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

Parameter	K_m/(mmol•L^{-1})	10^2V_m/(mmol•L^{-1}•s^{-1})	K_P/(mmol•L^{-1})	K_I/(mmol•L^{-1})
Thermokinetic	5.14	1.13	1.18	5.6
Literature	5[a], 5.6[b], 5.11[c]	—	1.3[a], 1.24[c]	4.3[d]

[a] Reference 17. [b] Reference 18. [c] Reference 19. [d] Reference 20.

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