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MICROCALORIMETRIC STUDIES ON THE NON-COMPETITIVE INHIBITION OF BOVINE LIVER ARGINASE

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Abstract

Two methods, e.g. initial rate method and thermokinetic reduced extent method were presented for studies on non-competitive inhibition. Arginase-catalyzed the hydrolysis of *L*-arginine to *L*-ornithine and urea and the inhibition of this reaction by sodium fluoride were studied in the absence and presence of exogenous of Mn^{2+} at 37°C in 40 mM sodium barbiturate-hydrochloric acid buffer solution (pH 7.4). Both methods were successively used to determine the values of K_I. The advances and disadvantages of each method were compared in this paper. Exogenous Mn^{2+} could result in more sensitivity of arginase to F^{-1}. Since the inhibition of arginase by F^{-1} depends on the pH values of the reaction system and behave as a non-competitive inhibition, it probably due to its small volume and high electronic density allow it access to the activity site of the enzyme and replaces of μ-OH_2 (or μ-OH) as the bridge ligand with Mn(II, II) cluster. However, further studies are necessary to determine the modes of interaction of F^{-1} with bovine liver arginase.

Keywords: bovine liver arginase, *L*-arginine, non-competitive inhibition, sodium fluoride, 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 *L*-ornithine and urea, a key step in the urea cycle [1]. 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 is strongly expressed in the liver whereas the extrahepatic arginase, AII, is found in mitochondria with a wider tissue distribution [3, 4]. Despite the diversity of living organisms in which they are found, arginases exhibit a high sequence identity and

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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 (NOHA) 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]. A 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 narrower distribution of Mn–Mn separations [9, 11].

In the previous work, 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 accessibility of the Mn(II, II) cluster of arginase to molecules different from *L*-arginine. In present works, we studied the inhibition of bovine liver arginase (BLA) by fluoride, which is the only inorganic anion can inhibit arginase and be sorted to the third class of inhibitor of this enzyme. The influence on the inhibition by exogenous Mn^{2+} was also studied.

Theory

Velocity equation and thermokinetic equation of arginase reaction in presence of reversible non-competitive inhibition

Since the product, *L*-ornithine, is a competitive reversible inhibitor of the enzyme, the velocity equation of the reaction in the presence of non-competitive inhibition is:

$$V = -\frac{\mathrm{d}[S]}{\mathrm{d}t} = \frac{V_\mathrm{m}[S]}{K_\mathrm{m}(1+[I]/K_\mathrm{I}+[P]/K_\mathrm{P})+[S](1+[I]/K_\mathrm{I}-K_\mathrm{m}/K_\mathrm{P})} \tag{1}$$

where K_m and V_m is the Michaelis constant and maximal velocity of arginase, K_P is the inhibitory constant of product. K_I is the inhibitory constant of non-competitive reversible inhibitor. It is expressed as

$$K_\mathrm{I} = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]} \tag{2}$$

Substituting $[P]=[S_0]-[S]$ to Eq. (1) and the reaction reduced extent, $\Phi_t = ([S_0]-[S_t])/[S_0]$, to the integral form of Eq. (1), we can get the thermokinetic equation of enzymatic reaction in the presence of reversible non-competitive inhibitor.

$$-\frac{\ln(1-\Phi_\mathrm{t})}{\Phi_\mathrm{t}} = \frac{V_\mathrm{m}}{K_\mathrm{m}(1+[S_0]/K_\mathrm{P}+[I]/K_\mathrm{I})}\frac{t}{\Phi_\mathrm{t}} - \frac{[S_0](1+[I]/K_\mathrm{I}-K_\mathrm{m}/K_\mathrm{P})}{K_\mathrm{m}(1+[S_0]/K_\mathrm{P}+[I]/K_\mathrm{I})} \tag{3}$$

Thermokinetic method of determination of K_I

According to Eq. (3), with a definite total enzyme and initial substrate concentration, the plot of $-\ln(1-\Phi_t)/\Phi_t$ *vs.* t/Φ_t must be linear. Definite the slope is *B*, that is:

$$B=\frac{V_m}{K_m(1+[S_0]/K_P+[I]/K_I)} \quad (4)$$

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

$$\frac{1}{B}=\frac{K_m}{V_m}\left(1+\frac{[S_0]}{K_P}\right)+\frac{K_m}{V_m K_I}[I] \quad (5)$$

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

$$c=\frac{K_m}{V_m}\left(1+\frac{[S_0]}{K_P}\right); \qquad d=\frac{K_m}{V_m K_I} \quad (6)$$

So, the inhibitory constant K_I can be calculated from the equation below:

$$K_I=\frac{c/d}{1+[S_0]/K_P} \quad (7)$$

The product inhibitory constant K_p could be obtained by the linear plot B^{-1} *vs*. $[S_0]$ in the absence of exogenous inhibitor as described in our previous work [13].

Materials and methods

Preparation of substrate and enzyme stock solutions were same to [13], the pH of the buffer was adjusted to 7.4 at 37°C by addition of 0.1 M HCl to the sodium barbiturate solution (40 mM). Analytical grade NaF was used as the inhibitory. The stock solutions were prepared by dissolving it in the buffer. $MnSO_4$ (analytical grade) solutions were prepared by boiled doubly distilled water before each calorimetric experiment. In the activation and inhibition experiments, the same concentrations of $MnSO_4$ or NaF solution were added to each cell. With the exception of enzyme, the concentration and volume of other samples were the same in the reference cell (*I*) as in the reaction cell (*I*). LKB-2107 batch microcalorimeter system was used to determine the heat of reaction, the method of other calorimetric experiments were also same to [13].

Results and discussions

It is well known that the reaction of hydrolysis of *L*-arginine catalysed by bovine liver arginase depends on the pH values of the system and of which the optimal pH is 9.4 [2]. In this experiment, we discover that the inhibition of NaF to bovine liver arginase also depends on the pH of the system: the inhibitory rate decreases with the pH increases, for example, the inhibitory rate are 0.37 and 0.029 when the pH of the reaction system are 7.4 and 9.4, respectively. Considered the demands of reaction and inhibition, we selected the pH was 7.4 in this experiment.

Evidence of the inhibition type

In the initial period of the reaction, if the product inhibition could be ignored, the similar Lineweaver–Burk double reciprocal equation of Eq. (5) can be described as:

$$\frac{1}{V_0}=\frac{1}{V_m}\left(1+\frac{[I]}{K_I}\right)+\frac{K_m}{V_m}\left(1+\frac{[I]}{K_I}\right)\frac{1}{[S_0]} \tag{8}$$

In the conduction calorimeter, the initial velocity of the reaction V_0 can be calculated as described in [15]. For make sure the inhibition type of NaF to bovine liver arginase, a series data of V_0 in different initial substrate concentration were determined when the concentration of NaF in the reaction system are 0, 1.00 and 1.67 mM, respectively. The double reciprocal plot of V_0^{-1} *vs*. $[S_0]^{-1}$ was shown in Fig. 1. We can see from the figure that each data set has a better linear relation and they have an intersection point in the reverse abscissa. This result indicates that the relation of V_0^{-1} and $[S_0]^{-1}$ accord with Eq. (8) and the inhibition of NaF to arginase is one of the reversible non-competitive inhibitions.

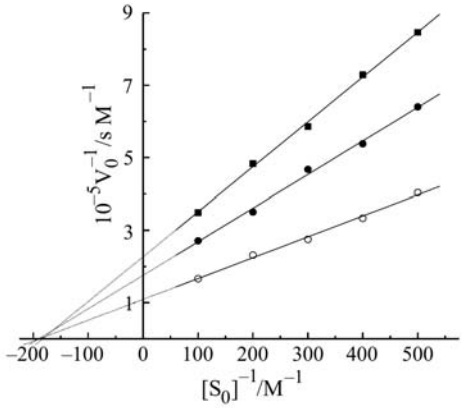

Fig. 1 The Lineweaver–Burk double reciprocal plot of the reaction in different concentration of inhibitor. ○ – C_{NaF}=0; ● – C_{NaF}=1.00 mM; ■ – C_{NaF}=1.67 mM

Determination of K_I by initial rate method

Equation (8) can be changed to the following form:

$$\frac{1}{V_0}=\frac{1}{V_m K_I}\left(1+\frac{K_m}{[S_0]}\right)[I]+\frac{1}{V_m}\left(1+\frac{K_m}{[S_0]}\right) \tag{9}$$

With no change of total enzyme and initial substrate concentration, different values of V_0 with different concentrations of NaF solutions were determined in the absence of exogenous Mn^{2+} ions and the concentration of Mn^{2+} was 0.167 mM. The plots of V_0^{-1} *vs*. [*I*] according to Eq. (9) in the two conditions were shown in Fig. 2. The values of K_I, which calculated from the slope and the *y*-axis intercept, were listed in Table 1.

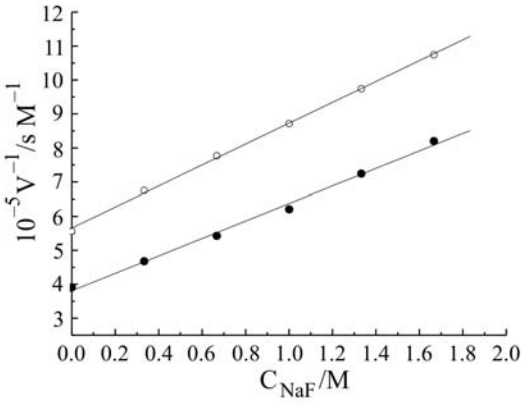

Fig. 2 The plot of V_0^{-1} *vs.* [*I*] in the absence and presence of Mn^{2+}.
○ – $C_{Mn^{2+}}$=0; ● – $C_{Mn^{2+}}$=0.167 mM

Table 1 The inhibitory constant K_I of arginase by fluoride

Method	K_I/mM	
	$[Mn^{2+}]$=0	$[Mn^{2+}]$=0.167 mM
Initial rate	1.84	1.48
Thermokinetic	0.51[a]	0.38[b]
Literature	1.3±0.5[c]	

[a]Calculated according to Eq. (19): [S_0]=2 mM, K_P=1.62 mM, *c*=1245.59, *d*=1097079.48;
[b]Calculated according to Eq. (19): [S_0]=2 mM, K_P=1.33 mM, *c*=1011.03, *d*=1052381.69;
[c][19]

Determination of K_I by thermokinetic method

An example of analyzing the calorimetric curve was given in Table 2 and the linear plot of $-\ln(1-\Phi_t)/\Phi_t$ *vs.* t/Φ_t was shown in Fig. 3 in the presence of the inhibitor NaF. The plot of B^{-1} *vs.* [*I*] was shown in Fig. 4 when the total concentration of enzyme was 0.0533 mg mL^{-1} and the initial concentration of substrate was 2.00 mM in the absence of exogenous of Mn^{2+} and the concentration of Mn^{2+} was 0.167 mM. The calculation values of K_I according to Eq. (7) were also listed in Table 1.

Discussion

Both methods have been successively used to determine the K_I of a non-competitive inhibition of arginase. The advantage of the initial rate method is, it can determine the K_I and provide the evidence of distinction of the inhibition type simultaneously, but it always ignored the inhibition of the product. The advantage of the thermokinetic method is, it could give the values of K_I and K_P, but it cannot distinguish the competitive type of the inhibition. The differential of the K_I values obtained from different methods in Table 1, must be resulted from consideration of the product inhibition or not.

Table 2 An example of the calorimetric curve analysis obtained from one of arginase-catalyzed reactions in the presence of fluoride[a,b]

t/s	Δ_t/mV	a_t/mV s^{-1}	Φ_t
250.2	44.2	8275.889	0.200
306.0	44.2	10742.248	0.237
354.0	43.5	12847.091	0.266
477.0	39.5	17951.591	0.335
591.0	35.5	22226.553	0.391
714.0	31.5	26347.086	0.445
852.0	27.5	30418.056	0.499
1029.0	23.5	34931.555	0.558
1245.0	19.5	39575.576	0.620
1530.0	15.5	44563.076	0.687
1734.0	13.5	47521.061	0.727
1926.0	11.5	49921.073	0.759
2214.0	9.5	52945.062	0.801
2634.0	7.5	56515.061	0.850

[a]Electrical calibration constants: K=4.1306·10^{-3} mJ mV s^{-1}; Λ=0.491 mJ mV^{-1}; τ=118.87 s, total area A_∞=67492.065 mV s^{-1}
[b]Total concentration of arginase in cell is 0.0533 mg mL^{-1}, initial concentration of arginine is 2.00 mM, [Mn^{2+}]=0.167 mM.

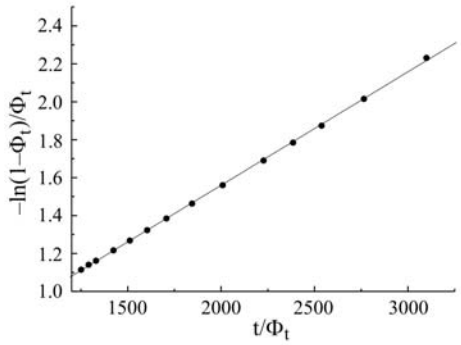

Fig. 3 Linear relationship of $-\ln(1-\Phi_t)/\Phi_t$ and t/Φ_t; C_{NaF}=0.67 mM, $C_{Mn^{2+}}$=0.167 mM, r=0.9999, B=5.98·10^{-4}

Quite recently, the crystal structures of fully activated rat liver (RLA) and *Bacillus caldovelox* arginases have been published [16, 17]. The active site of these enzymes involves a Mn(II, II) cluster with two histidine residues, a μ-OH_2 (or μ-OH) bridge and both μ-1,3-O,O'-carboxylate and μ-1,1-O,O-carboxylate bridges as Mn^{II} ligands. Metal ions are thought to activate a metal-coordinated water molecule generating a hydroxide ion that nucleophilically attacks the guanidino carbon of *L*-arginine [2, 16–19]. Fluoride

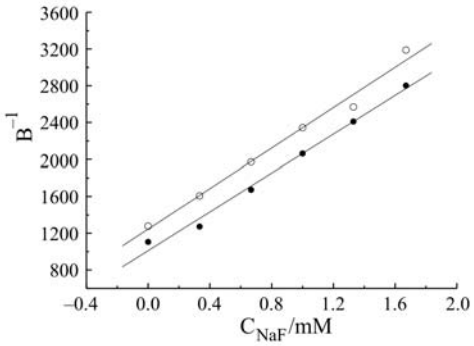

Fig. 4 Plot of B^{-1} *vs*. [*I*]; [E_t]=0.0533 mg mL^{-1}, [S_0]=2.00 mM. ○ – $C_{Mn^{2+}}$=0, ● – $C_{Mn^{2+}}$=0.167 mM

ions could inhibit the activity of arginase probably because of its small volume and high electronic density allow it to access the activity site of the enzyme and replaces of μ-OH_2 (or μ-OH) as the bridge ligand with Mn(II, II) cluster. This interaction would occur between F^{-1} and free arginase, *E* or *L*-arginine-arginase complex, ES. Therefore, the inhibition of F^{-1} to arginase is one of the non-competitive inhibitions. Exogenous Mn^{2+} could result in more sensitivity of arginase to this inhibitor.

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