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Thermochimica Acta 414 (2004) 19–23
thermochimica acta
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Thermokinetic studies on the activation of bovine liver arginase by manganese ions

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Received 5 July 2003; received in revised form 4 November 2003; accepted 10 November 2003

Abstract

The activation of bovine liver arginase, which catalyzes the hydrolysis of L-arginine to L-ornithine and urea, by manganese ions was studied by thermokinetic methods at 37 °C in 40 mM sodium barbiturate–HCl buffer solution (pH 9.4). Full activation of arginase, by incubation with 0.1 mM Mn^{2+}, resulted in increased of V_{max}, and a higher sensitivity of the enzyme to product and L-lysine inhibition, with no change in the K_m for arginine. Upon addition of 0.1 mM Mn^{2+} to the reaction, the inhibitory constants of product (K_P) and L-lysine (K_I) decreased from 1.18 to 0.70 mM and from 5.60 to 3.10 mM, respectively. We suggest that the exogenous manganese ions in reaction recovered the activity of arginase, which was lost in dissolving and dilution, without effecting on the mechanism of the reaction.

Keywords: Bovine liver arginase; Activation; L-Lysine; Mn^{2+}; Thermokinetics

1. 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 specifically catalyzes the hydrolysis of L-arginine to L-ornithine and urea. L-Ornithine formation may be the main function of arginase in cells that have incomplete urea cycles [4].

Bovine liver arginase is a trimeric enzyme, each subunit containing a Mn(II, II) cluster core. In the Mn(II, II) cluster, one of the Mn^{2+} ions is bound reversibly. After losing of one Mn^{2+} ions in dissolving and dilution of arginase, the activity decreases [5,6]. In the present work, the influence of exogenous Mn^{2+} ions on bovine liver arginase activity, Michealis constant (K_m), maximum velocity (V_m), inhibition constant of product (and K_P), L-lysine (K_I) were studied by thermokinetics.

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doi:10.1016/j.tca.2003.11.004

2. Theory

2.1. Velocity equation of arginase reaction in the presence of a reversible competitive inhibitor

Because one of the substrates, water, is the solvent, the reaction of L-arginine hydrolysis catalyzed by arginase is described as a single substrate enzyme reaction. The mechanism of this reaction can be described as.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad (1)$$

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

$$E + P \overset{K_P}{\rightleftharpoons} EP \quad (3)$$

$$E + I \overset{K_I}{\rightleftharpoons} EI \quad (4)$$

in which E, S, P, and I are arginase, L-arginine, L-ornithine, and inhibitor, respectively. ES is the complex between enzyme and substrate, EP is the complex of enzyme and product, and EI is the complex of enzyme and inhibitor. In the steady state concentration of ES, the velocity equation of

this reaction can be easy obtained:

$$V = -\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_m(1+[I]/K_I+[P]/K_P)+[S](1-K_m/K_P)} \quad (5)$$

where K_m and V_{max} are the Michealis constant and maximal velocity, and K_P and K_I are the binding constants of product and exogenous inhibitor, respectively.

$$K_m = \frac{k_{-1}+k_2}{k_1}; \quad V_{max} = k_2[E_t] \quad (6)$$

$$K_P = \frac{[E][P]}{[EP]}; \quad K_I = \frac{[E][I]}{[EI]} \quad (7)$$

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

2.2. The thermokinetic equation of the arginase reaction in the presence of reversible competitive inhibitor

Substituting $[P] = [S_0] - [S]$ into Eq. (5), and integrating the equation from $t = 0$ to t, the kinetic equation is:

$$K_m\left(1+\frac{[S_0]}{K_P}+\frac{[I]}{K_I}\right)\ln\frac{[S]}{[S_0]}+\left(1-\frac{K_m}{K_P}\right)([S]-[S_0]) = -V_{max}t \quad (8)$$

where [I] is the concentration of inhibitor, $[S_0]$ and [S] the concentrations of substrate when time is 0 and t. The reaction reduced extent Φ_t is defined as:

$$\Phi_t = \frac{[S_0]-[S_t]}{[S_0]} \quad (9)$$

When substituted into Eq. (8), we can get the kinetic equation expressed as reduced extent:

$$-\frac{\ln(1-\Phi_t)}{\Phi_t} = \frac{V_{max}}{K_m(1+[S_0]/K_P+[I]/K_I)}\frac{t}{\Phi_t} - \frac{[S_0](1-K_m/K_P)}{K_m(1+[S_0]/K_P+[I]/K_I)} \quad (10)$$

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

$$Q_t = Ka_t + \Lambda\Delta_t; \quad Q_\infty = KA \quad (11)$$

where Q_t and Q_∞ are the heats of reaction at time t and $t \to 8$, a_t and A are areas before t and the total area enclosed by the curve, Δ_t is the peak height of the curve 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

$$\Phi_t = \frac{[S_0]-[S_t]}{[S_0]} = \frac{Q}{Q_\infty} = \frac{Ka_t+\Lambda\Delta_t}{KA} = \frac{a_t+\tau\Delta_t}{A} \quad (12)$$

$\tau = \Lambda/K$, which has a dimension of time, can be obtained from a linear-fit of $-\ln\Delta_t$ against t for the electrical energy calibration curve as described in reference [7]. Therefore, Eq. (10) is the thermokinetic equation of the enzymatic reaction in the presence of reversible competitive inhibitor.

3. Materials and methods

Doubly distilled water was used throughout the experiment. Analytical grade sodium barbiturate and muriatic acid were used for preparation of the buffer solution. The pH of the buffer (40 mM) was adjusted to 9.4 at 37 °C by means of a pH meter.

Crystalline bovine liver arginase was purchased from Worthington Biochemical Corporation and was not further purified before use. Arginase stock solution (9.6 mg ml^{-1}) was prepared by dissolving the solid enzyme in the buffer and stored in a refrigerator.

Biochemical grade L-arginine and L-lysine were used as substrate and inhibitor without further purification. The required concentrations of substrate and inhibitor solutions were made by dissolving in the buffer. $MnSO_4$ (analytical grade) solutions were prepared in boiled, doubly distilled water before each calorimetric experiment and was titrated with H_2O_2 solution to determine the concentration.

The heat of reaction was determined with an LKB-2107 batch microcalorimeter system. In the enzymatic reaction experiments, different volumes of substrate stock solutions were diluted to 10 ml, and 4 ml placed in both the reaction reference cells, 2 ml of enzyme solution was placed in the other side of the reaction cell, and 2 ml of buffer solution was added to the other side of the reference cell. In the activation and inhibition experiments, the same concentrations of $MnSO_4$ or lysine solution were added to each cell. With the exception of enzyme, the concentrations and volumes of other samples were the same in the reference and reaction cells. After the system was equilibrated at 37 °C, and the mixing start button was pressed, the calorimeter was turned 360° and reversed to mix the solutions in the reaction and reference cells. The voltage signal was recorded by means of LKB-2210 dual-pen integrating recorder.

4. Results

4.1. Influence of Mn^{2+} on arginase activity

With the definite concentration of enzyme, the activity of arginase, ACT, expressed as μmole of arginine hydrolyzed in one minute, can be calculated by

$$\mathrm{ACT} = -\frac{1}{\Delta_r H_m}\frac{\partial Q_t}{dt} = -\frac{\Omega_t}{\Delta_r H_m} \quad (13)$$

$$\Omega_t = \frac{\partial Q_t}{dt} \quad (14)$$

In Eq. (13), $\Delta_r H_m$ is the molar reaction enthalpy of this reaction, which is determined to be 17.2 ± 0.2 kJ mol^{-1} in this

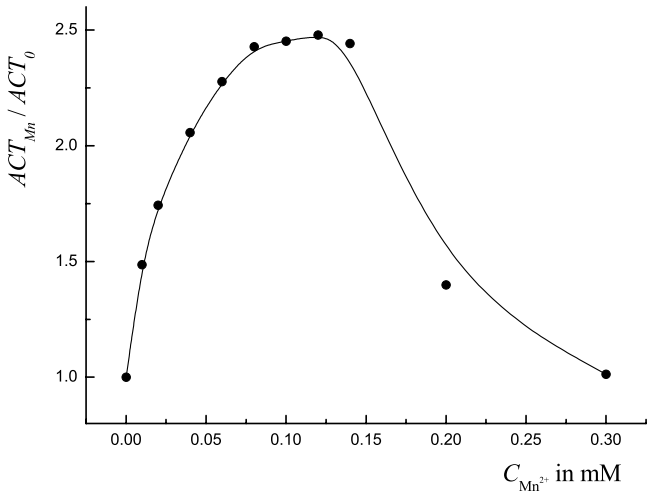

Fig. 1. Influence of Mn^{2+} ions on the relative enzyme activity. $[E_t] = 0.016\,mg\,ml^{-1}$, $[S_0] = 5\,mM$.

experiment according to the method in reference [8]. Ω_t is the velocity at time t, the initial velocity Ω_0 is the slope of a linear-fit of Q_t against t in the initial period of the reaction, in the period, Ω_t is less than 0.1, neglecting product inhibition has no influence on the results. In the present experiments, the activity of arginase in different exogenous Mn^{2+} concentrations with an initial substrate concentration of 5 mM and total enzyme concentration of $0.016\,mg\,ml^{-1}$. The relative enzyme activity ACT_{Mn}/ACT_0 is given by Fig. 1. ACT_{Mn} and ACT_0 are the arginase activity in the presence and absence of Mn^{2+}, respectively.

4.2. Influence of Mn^{2+} on K_m and V_{max} of arginase for arginine

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

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S_0]} \tag{15}$$

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

$$V_0 = \frac{\Phi_0}{\Delta_r H_m V} \tag{16}$$

where V is the volume of the reaction system (6 ml in this experiment). Fig. 2 gives the Lineweaver–Burk double reciprocal plots in the presence and absence of exogenous Mn^{2+} ions with the same total arginase concentration.

From the slope and y-axis intercept, V_{max} and K_m for each condition can be calculated. The results are listed in Table 1. The results of Fig. 2 and Table 1 show that the appropriate concentrations of exogenous Mn^{2+} result in an increase of V_{max} and no influence on K_m. it indicates the association of Mn^{2+} recovers the activity of the enzyme, but does not change the mechanism of the reaction.

Table 1
The biochemical parameters of arginase in the absence and in the presence of saturate concentrations of Mn^{2+} ions

$[Mn^{2+}]$ (mM)	A_{Mn}/A_0	$10^3 V_m$ (mM s^{-1})	K_m (mM)	K_P (mM)	K_I (mM)
0	1	7.86 ± 0.33	5.53 ± 0.34	1.18 ± 0.07	5.60 ± 0.22
0.1	2.45	11.64 ± 0.49	5.50 ± 0.58	0.70 ± 0.05	3.10 ± 0.04

4.3. Influence of Mn^{2+} on the product inhibition constant of arginase

According to Eq. (10), for a definite total enzyme and initial substrate concentration, the plot of $-\ln(1-\Phi_t)/\Phi$

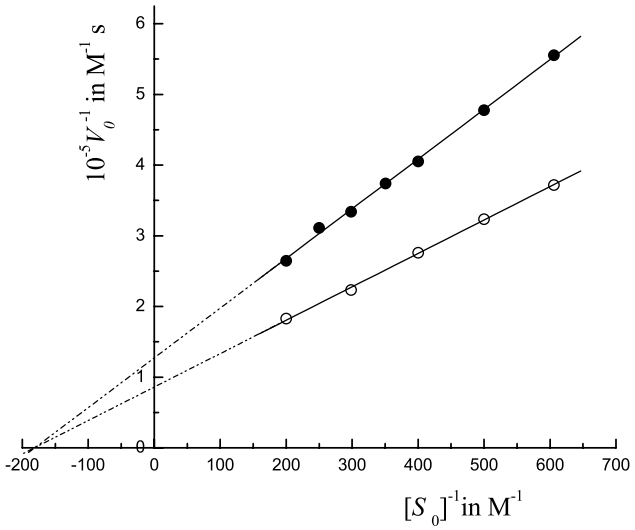

Fig. 2. Lineweaver–Burk double reciprocal plot of arginase for arginine. $[E_t] = 0.0032\,mg\,ml^{-1}$, (●) activation absence, and (○) $C_{Mn^{2+}} = 0.1\,mM$.

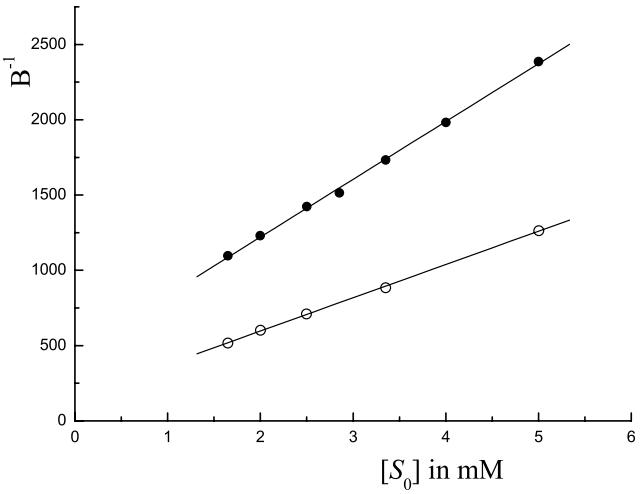

Fig. 3. Linear plot of the slope in Eq. (10) vs. initial concentration of arginine. $[E_t] = 0.0032\,mg\,ml^{-1}$, and (●) activation absence, and (○) $C_{Mn^{2+}} = 0.1\,mM$.

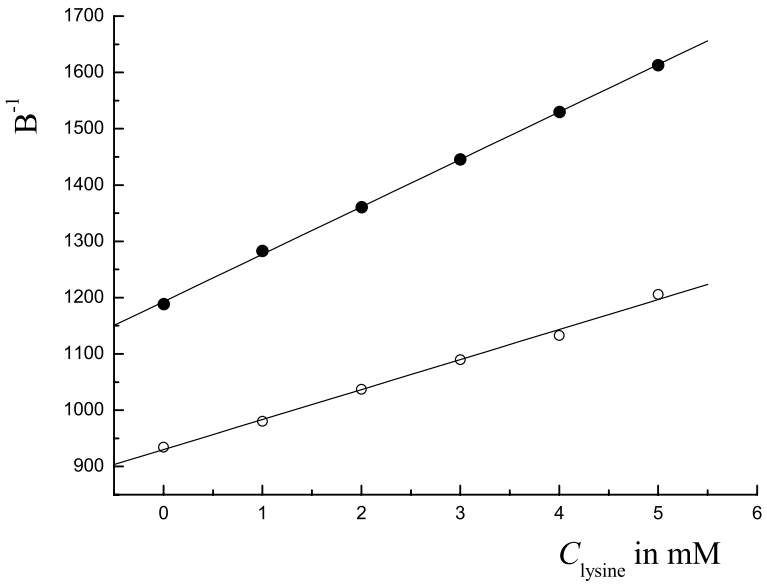

Fig. 4. Linear plot of the slope in Eq. (10) vs. concentration of lysine. $[E_t] = 0.0032\,mg\,ml^{-1}$, $[S_0] = 2.5\,mM$ (●) $C_{Mn^{2+}} = 0.1\,mM$, and (○) activation absence.

against t/Φ_t must be linear. The reciprocal of the slope B^{-1} in the absence of exogenous inhibitor is:

$$\frac{1}{B} = \frac{K_m}{V_{max} K_P}[S_0] + \frac{K_m}{V_{max}} \quad (17)$$

According to Eq. (17), the linear relationship between B^{-1} and initial substrate concentration was obtained in the presence and absence of Mn^{2+} ions. The product inhibitory constant K_P can be calculated by the quotient of slope and y-axis intercept of the plots. Fig. 3 shows the results in the absence and presence of saturating concentrations of Mn^{2+} ions. The calculated values of K_P are listed in Table 1.

4.4. Influence of Mn^{2+} on the inhibitory constant of exogenous reversible competitive inhibitor

With fixed total enzyme and initial substrate concentrations, treating the thermograms with different L-lysine concentrations according to the method described in Section 4.3, the relationship between the slope B and inhibitor concentration is:

$$\frac{1}{B} = \frac{K_m}{V_{max} K_I}[I] + \frac{K_m}{V_{max}}\left(1 + \frac{[S_0]}{K_P}\right) \quad (18)$$

The quotient of the y-axis intercept and the slope of the plot of B^{-1} versus inhibitor concentration [I] is $K_I(1 + [S_0]/K_P)$. The inhibitory constant K_I can be calculated by combining this formula and the values of $[S_0]$ and K_P. Fig. 4 shows the plot of B^{-1} against [I], and the calculated values of K_I are listed in Table 1 in the absence and in the presence of saturating concentrations of Mn^{2+} ions.

5. Conclusions

Arginase is one of the enzymes that contains a double metal core; a Mn(II)–Mn(II) cluster is necessary for arginase activity. Much of the enzyme activity is lost when one of the Mn^{2+} ions is removed from the enzyme. The addition of exogenous Mn^{2+} recovers the enzyme activity. It can be seen in Fig. 1 that at low Mn^{2+} concentrations, the relative enzyme activity increases with the increase of exogenous Mn^{2+} ion and reaches a plateau at 0.08–0.14 mM Mn^{2+} because the arginase is saturated by Mn^{2+} ions. At high Mn^{2+} concentration, increasing exogenous Mn^{2+} concentration decreases the relative enzyme activity. The reason may be inhibition by Mn(III) from the oxidation of Mn^{2+} in a basic solution.. This result is similar to the studies of human vitreous humor arginase [3] but the saturation concentration of Mn^{2+} found here is much lower than the literature value (5 mM). Two reasons for the difference may be the commercial arginase used in the experiments may have been mixed with some Mn(II) salt and the time in this study for preincubating arginase with Mn^{2+} (2 h) is much longer than the optimal time (10 min in [3]).

The activation of arginase by Mn^{2+} increases the active enzyme concentration, and enhances the maximal velocity of the reaction. The Michealis constant and reaction mechanism are not changed. Meanwhile, with the increase of active enzyme concentration, the concentrations of EP and EI increase. So, arginase becomes more sensitive to the inhibition of product and an exogenous reversible inhibitor. These results indicate that the inhibition of arginase by L-ornithine and L-lysine occurs by competing for the active center with arginine.

Acknowledgements

These projects are supported by National Nature Sciences Foundation of China (No. 30070200) and Hubei Provincial Department of Education (No. 2003A0009).

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