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Exploring antibiotic resistant mechanism by microcalorimetry II

Determination of thermokinetic parameters of imipenem hydrolysis with metallo-β-lactamase ImiS

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Abstract In an effort to understand the reaction of antibiotic hydrolysis with B2 metallo-β-lactamases (MβLs), the thermodynamic parameters of imipenem hydrolysis catalyzed by metallo-β-lactamase ImiS from *Aeromonas veronii bv. sobria* were determined by microcalorimetric method. The values of activation free energy $\Delta G^{\theta}_{\neq}$ are 86.400 ± 0.043, 87.543 ± 0.034, 88.772 ± 0.024, and 89.845 ± 0.035 kJ mol^{-1} at 293.15, 298.15, 303.15, and 308.15 K, respectively, activation enthalpy $\Delta H^{\theta}_{\neq}$ is 18.586 ± 0.009 kJ mol^{-1}, activation entropy $\Delta S^{\theta}_{\neq}$ is −231.34 ± 0.12 J mol^{-1} K^{-1}, apparent activation energy E is 21.084 kJ mol^{-1}, and the reaction order is 1.5. The thermodynamic parameters reveal that the imipenem hydrolysis catalyzed by metallo-β-lactammase ImiS is an exothermic and spontaneous reaction.

Keywords Microcalorimetry · Metallo-β-lactamase ImiS · Antibiotic hydrolysis · Thermokinetic parameters

Introduction

β-lactam antibiotics have been used for several decades, and the bacterial resistance to antibiotics is growing clinical concern [1, 2]. Bacterial resistance to β-lactam-containing antibiotics is accomplished by the production of β-lactamases, which hydrolyze the β-lactam ring of β-lactam-containing antibiotics [3], such as imipenem as shown in Scheme 1 [4]. The β-lactamases have been categorized into four distinct groups. Group A, C, and D β-lactamases utilize an active site serine as a nucleophile in the hydrolysis reaction. The remaining group B enzymes, often called metallo-β-lactamases (MβLs), require one or two Zn (II) ions per monomer for full catalytic activity [5, 6]. The MβLs have been further divided into three subclasses, B1, B2, and B3 [7]. Both the B1 and B3 subclasses have a broad-spectrum profile (including penicillins, cephalosphorins, and carbapenems) and, in vitro, require one or two zinc (II) ions for maximal enzymatic activity. In contrast, subclass B2 enzymes have a narrow substrate profile, hydrolyzing carbapenems almost exclusively, and exhibit maximal activity when bound to only one Zn (II). These B2 enzymes are produced by various species of *Aeromonas* and are active only in the monozinc form [8]. As of now, no clinically approved MβL inhibitor is available.

The microcalorimetry is a powerful method to probe the pathway and mechanism of chemical reaction [9]. With the microcalorimetry, Zhao et al. investigated the effects of two ginsenosides Rg1 and Rb1 on splenic lymphocytes growth [10], Garcia-Cuello et al. design and built a determination of the differential heat of adsorption [11], and Yang et al. explored inhibition of two cephalosporins on *E. coli* [12]. Recently, we reported the determination of thermokinetic parameters of penicillin G hydrolysis catalyzed by MβL L1 from *Stenotrophomonas maltophilia* with microcalorimetric method [13]. In order to further probe the carbapenem hydrolysis with the B2 subclass MβLs, based on over-expression and purification of MβL ImiS,

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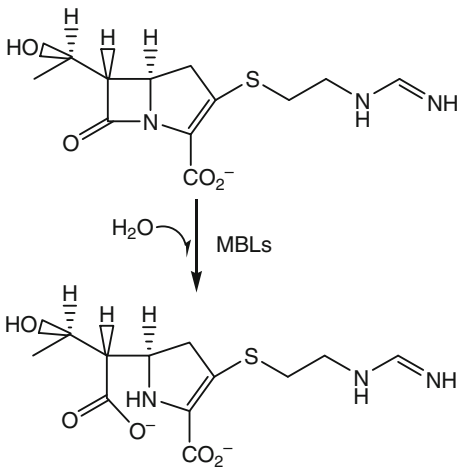

Scheme 1 Imipenem hydrolysis catalyzed with MβLs

this article first reports the determination of thermokinetic parameters of imipenem (a representative of carbapenems) hydrolysis catalyzed by ImiS from *Aeromonas veronii bv. sobria* by microcalorimetric method.

Materials and methods

Overexpression and purification of ImiS The Over-expression and purification of ImiS were carried out as previous literature described [14]. The plasmid containing the gene for ImiS, pET-26b-ImiS, was used to transform into BL21 (DE3) *E. coli* cells. A overnight pre-culture of *E. coli* BL21 (DE3) containing the pET-26b-ImiS plasmid in LB (Luria–Bertani) medium was used to inoculate 4 × 1 L of LB medium containing 25 μg/mL kanamycin and 100 μM $ZnSO_4{\cdot}7H_2O$. The cells were allowed to grow at 37 °C with shaking until the cells reached an optical density at 600 nm of 0.6–0.8. Protein production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were shaken at 37 °C for 3 h. The cells were collected by centrifugation (15 min at 7000 rpm) and resuspended in 30–40 mL of 50 mM Tris, pH 7.0, containing 500 mM NaCl. The cells were ruptured and the cell debris was removed by centrifugation (30 min at 15000 rpm). The cleared supernatant was dialyzed versus 2 L of 50 mM Tris, pH 7.0, overnight at 4 °C, centrifuged (30 min at 15000 rpm) to remove insoluble matter, and loaded onto an equilibrated SP-Sepharose column, equilibrated with 300 mL of 50 mM Tris, pH 7.0. Bound proteins were eluted from the column with a linear gradient of 0–500 mM NaCl in 50 mM Tris–HCl, pH 7.0, at a flow rate of 2 mL/min. The gradient increased at a rate of approximately 1% per minute, and 6 mL fractions were collected. Fractions containing ImiS were identified by SDS-PAGE and concentrated with an Amicon ultrafiltration cell equipped with an YM-10 cellulose membrane. ImiS concentrations were determined using Beer's law and an extinction coefficient of 37250 M^{-1} cm^{-1} at 280 nm [15].

Calorimetric experiments The calorimetric experiments at 293.15, 298.15, 303.15, and 308.15 K were performed on Micro-DSCIII (Setaram, France) microcalorimeter. Before collection of the thermokinetic data, the enthalpies of KCl aqueous solution (spectral purity) was measured with 17.266 ± 0.074 kJ/mol at 298.15 K, which matches the literature value of 17.241 ± 0.018 kJ/mol [16], the relative deviation is 0.14%. Standard α-Al_2O_3 was used for calibration of the heat capacity, the sample mass was 320.60 mg, and the standard molar heat capacity *Cp* (α-Al_2O_3) was 79.44 kJ mol^{-1} K^{-1} at 298.15 K, which matches the literature value of 79.02 kJ mol^{-1} K^{-1} [17], the relative deviation is 0.53%. These data indicate that the calorimetric system is accurate and reliable.

Results and discussion

Metallo-β-lactamase ImiS was purified with a SP-Sepharose column eluted with a linear gradient of 0–500 mM NaCl in 50 mM Tris–HCl, pH 7.0, the SDS-PAGE gels showed (Fig. 1) that the protein has an molecular mass of 25 kDa. The yield of ImiS was 5 mg per liter culture. The typical thermodynamics curves are obtained during imipenem hydrolysis catalyzed by MβL ImiS at 293.15, 298.15, 303.15, and 308.15 K, and the collected experimental data are listed in Table 1. Based on the

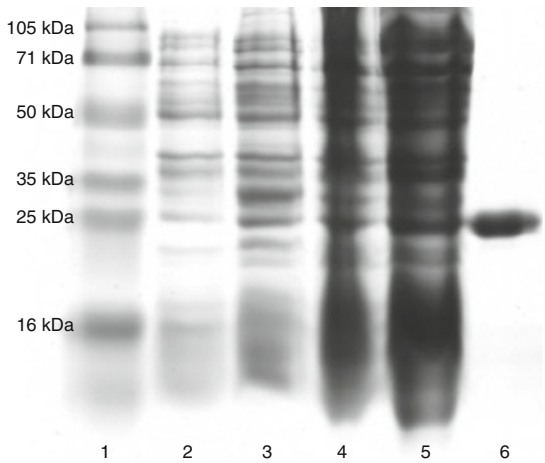

Fig. 1 SDS-PAGE gel of recombinant ImiS *Lane 1*, molecular weight makers; *lane 2*, boiled cell fraction of BL21 (DE3) *E. coli* cells containing the pET26b-ImiS plasmid before induction; *lane 3*, boiled cell fraction of BL21 (DE3) *E. coli* cells containing the pET26b-ImiS plasmid after a 4 h induction period with 1 mM IPTG; *lane 4*, crude protein after ultrasonication and centrifugation; *lane 5*, crude protein after overnight dialysis; and *lane 6*, purified ImiS

Table 1 Collected thermokinetic data of imipenem hydrolysis catalyzed by metallo-β-lactamase ImiS

293.15 K			298.15 K		
t/s	H_i/H	dH_i/dt	t/s	H_i/H	dH_i/dt
275	0.0175	0.0056	276	0.0127	0.0051
285	0.0893	0.0053	286	0.0835	0.0048
295	0.1702	0.0045	296	0.1646	0.0042
305	0.2510	0.0039	306	0.2460	0.0035
315	0.3278	0.0033	316	0.3223	0.0030
325	0.3990	0.0027	326	0.3923	0.0025
335	0.4641	0.0023	336	0.4560	0.0021
345	0.5229	0.0019	346	0.5132	0.0018
355	0.5756	0.0016	356	0.5645	0.0015
365	0.6227	0.0014	366	0.6101	0.0013
303.15 K			308.15 K		
t/s	H_i/H	dH_i/dt	t/s	H_i/H	dH_i/dt
272	0.0474	0.0044	272	0.0222	0.0053
282	0.1194	0.0040	282	0.0982	0.0049
292	0.1981	0.0035	292	0.1814	0.0042
302	0.2763	0.0030	302	0.2634	0.0035
312	0.3503	0.0025	312	0.3406	0.0029
322	0.4186	0.0021	322	0.4116	0.0025
332	0.4806	0.0017	332	0.4756	0.0020
342	0.5363	0.0015	342	0.5329	0.0017
352	0.5860	0.0012	352	0.5838	0.0014
362	0.6300	0.0010	362	0.6287	0.0012

H_0 -2.4267 J g^{-1}/293.15 K; -1.8773 J g^{-1}/298.15 K; -1.5206 J g^{-1}/303.15 K; -1.5032 J g^{-1}/308.15 K

thermokinetic Eqs. 1–4 [18], the calculated thermokinetic parameters of imipenem hydrolysis with ImiS are listed in Table 2.

$$\ln\left(\frac{1}{H}\frac{dH_i}{dt}\right) = \ln k + n\ln\left(1-\frac{H_i}{H_0}\right) \quad (1)$$

$$\ln k = \ln A - \frac{E}{RT} \quad (2)$$

$$\Delta G^{\theta}_{\neq} = RT\ln\frac{RT}{Nhk} \quad (3)$$

$$\ln\frac{k}{T} = -\frac{\Delta H^{\theta}_{\neq}}{RT} + \frac{\Delta S^{\theta}_{\neq}}{R} + \ln\frac{k_B}{h} \quad (4)$$

In the equations, H_0 is the total heat of reaction (corresponding to the area under the T/K curve); H_i, the reaction heat at some time t (corresponding to the area under the curve at time t); dH_i/dt, the rate of heat production at time t; k, rate constant; n, reaction order; A, pre-exponent; E, apparent activation energy; R, gas constant; T, absolute temperature; N, Avogadro constant; h, Planck constant; $\Delta G^{\theta}_{\neq}$, activation free energy; $\Delta H^{\theta}_{\neq}$, activation enthalpy; $\Delta S^{\theta}_{\neq}$, activation entropy; k_B, Boltzmann constant.

For the imipenem hydrolysis reaction catalyzed by metallo-β-lactamase ImiS, the following thermodynamic parameter were obtained, the values of activation free energy $\Delta G^{\theta}_{\neq}$ are 86.400 ± 0.043, 87.543 ± 0.034, 88.772 ± 0.024, and 89.845 ± 0.035 kJ mol^{-1} at 293.15, 298.15, 303.15, and 308.15 K, respectively, $\Delta H^{\theta}_{\neq}$ is 18.586 ± 0.009 kJ mol^{-1} and $\Delta S^{\theta}_{\neq}$ is -231.34 ± 0.12 J mol^{-1} K^{-1}. The reaction order is 1.5, and with the increasing of temperature, the reaction rate constant k increases from 2.4558 to 3.7789×10^{-3} s^{-1}. The apparent activation energy of the reaction E is 21.084 kJ mol^{-1}. In summary, the activation energy and the activation enthalpy of this reaction are smaller, while the activation entropy of this reaction is high. These data indicate that the hydrolysis reaction of imipenem catalyzed by metallo-β-lactamase ImiS is an exothermic reaction and it is spontaneous in the temperature range 293.15–308.15 K.

Based on the steady-state kinetic studies, Walsh proposed a mechanism of metallo-β-lactamase L1 catalyzing nitrocefin hydrolysis: E+S $\rightleftharpoons$ ES $\rightleftharpoons$ EI$\rightarrow$E+P primarily, enzyme E, and substrate S form complex ES, subsequently generate a tetrahedral intermediate EI, and finally EI releases the product P, and the enzyme. In which the cleavage of the β-lactam amide bond is the rate-determining step for breakdown of the majority of β-lactam

Table 2 Thermokinetic parameters of imipenem hydrolysis catalyzed by metallo-β-lactamase ImiS

T/K	Equation 1			Equation 2			Equation 3	Equation 4		
	$k/\times 10^{-3}$ s^{-1}	n	R^2	E/kJ/mol	LnA	R^2	$\Delta G^{\theta}_{\neq}$/kJ/mol	$\Delta H^{\theta}_{\neq}$/kJ/mol	$\Delta S^{\theta}_{\neq}$/J/mol/K	R^2
293.15	2.4558	1.5143	0.9991	21.084	2.6396	0.9934	86.400 ± 0.043	18.586 ± 0.009	-231.34 ± 0.12	0.9916
298.15	2.8549	1.5317	0.9990				87.543 ± 0.034			
303.15	3.1917	1.5529	0.9993				88.772 ± 0.024			
308.15	3.7789	1.5753	0.9994				89.845 ± 0.035			

R^2 linear correlation coefficient

substrates [19]. Thermodynamically, clearly, the cleavage of β-lactam ring also is the master exothermic step of hydrolysis of the β-lactam substrates. According to the mechanism, the thermodynamic parameter $\Delta H^{\theta}_{\neq}$ is larger, the kinetic parameter K_{cat} of enzyme is larger, and the hydrolysis reaction is faster. Recently, we studied imipenem hydrolyses with ImiS [20] and penicillin G hydrolyses with L1 [21], the kinetic results showed that the K_{cat} values were 2.15 and 15.80 s^{-1}, respectively, which are agreement with the thermodynamic results reported in this paper (18.586 $\pm$ 0.009 kJ mol^{-1}) and in previous our paper (24.02 kJ mol^{-1}) corresponding to the enzymes and substrates [13]. These data imply that the thermodynamic parameters help understand the hydrolysis mechanism of β-lactam substrates with MβLs better. In this article, the thermokinetic data of imipenem hydrolysis with ImiS, a B2 subclass MβLs, have been determined, and also, we will study the thermokinetics of antibiotic hydrolysis with CcrA belonging to B1 subclass MβLs. Based on all these thermokinetic data gained and to be gained with a representative MβL from each of the distinct subclasses, we will try to identify a common mechanistic aspect of the enzymes, which will be used to guide inhibitor design efforts.

Conclusions

In this article, we first report the thermokinetic parameters of imipenem hydrolysis catalyzed with metallo-β-lactamase ImiS from *Aeromonas veronii bv. sobria* by microcalorimetrey, which include the activation free energy $\Delta G^{\theta}_{\neq}$, activation enthalpy $\Delta H^{\theta}_{\neq}$, activation entropy $\Delta S^{\theta}_{\neq}$, and apparent activation energy E. These thermokinetic parameters are helpful to better understand the reaction and mechanism of antibiotic hydrolysis catalyzed by MβLs. Also, the information gleaned in these studies will be helpful to understand other hydrolyses that belong to the β-lactamase superfamily.

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