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# Exploring antibiotic resistant mechanism by microcalorimetry

## Determination of thermokinetic parameters of metallo-β-lactamase L1 catalyzing penicillin G hydrolysis

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**Abstract** In an effort to probe the reaction of antibiotic hydrolysis catalyzed by B3 metallo-β-lactamase (MβL), the thermodynamic parameters of penicillin G hydrolysis catalyzed by MβL L1 from *Stenotrophomonas maltophilia* were determined by microcalorimetric method. The values of activation free energy $\Delta G^{\theta}_{\neq}$ are 88.26, 89.44, 90.49, and 91.57 kJ mol$^{-1}$ at 293.15, 298.15, 303.15, and 308.15 K, respectively, activation enthalpy $\Delta H^{\theta}_{\neq}$ is 24.02 kJ mol$^{-1}$, activation entropy $\Delta S^{\theta}_{\neq}$ is −219.2511 J mol$^{-1}$ K$^{-1}$, apparent activation energy $E$ is 26.5183 kJ mol$^{-1}$, and the reaction order is 1.0. The thermodynamic parameters reveal that the penicillin G hydrolysis catalyzed by MβL L1 is an exothermic and spontaneous reaction.

**Keywords** Microcalorimetry · Metallo-β-lactamases · L1 · Antibiotic hydrolysis · Thermokinetic parameters

## Introduction

The overuse of antibiotics in the clinical setting has resulted in a large number of bacteria with metallo-β-lactamases (MβLs) including recently reported New Delhi metallo-β-lactamase 1 (NDM-1), which is resistant to most commonly used antibiotics such as penicillin, cephalosporin, and carbapenem families [1–5]. These antibiotic resistant bacteria exhibit their activity by using MβLs to hydrolyze the β-lactam ring of β-lactam containing antibiotics, such as penicillin G [6]. The β-lactamases are divided into A, B, C, and D group [7], and the MβLs are group B enzymes which are Zn(II)-dependent [7], and the MβL L1 is a B3 group enzyme, it has been shown to bind 2 Zn(II) ions per monomer [8]. In an effort to probe the hydrolysis reaction of β-lactam containing antibiotics catalyzed by MβLs, much kinetic data have been reported [9–12], and these data were obtained by steady-state kinetic methods, but there have not been related thermokinetic data reported.

The microcalorimetry is a powerful method to probe the pathway and mechanism of chemical reaction [13], and it is used in many fields [14–24]. With the microcalorimetry, recently, Kong et al. investigated the activity of berberine [14], Zhao evaluated the activity of berberine on *Shigella dysenteriae* [17], and Yang et al. explored inhibition of two cephalosporins on *E. coli* [19]. However, there was no report on MβL catalyzing penicillin hydrolysis. In order to better understand the procedure of β-lactam containing antibiotics hydrolysis catalyzed by MβLs, this paper first reports the determination of thermokinetic parameters of penicillin G hydrolysis catalyzed by MβL L1 from *Stenotrophomonas maltophilia* by microcalorimetric method.

## Experimental

Penicillin G sodium salt was purchased from Sigma. Cacodylic acid sodium salt trihydrate was obtained from Amresco. MβL L1 was overexpressed and purified by using the procedure as described previously [12]. The L1 purity was identified by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and confirmed by MALDI-TOF (matrix-assisted laser desorption ionization, time-of-flight) mass spectrum. The calorimetric experiments

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at 293.15, 298.15, 303.15, and 308.15 K were performed on RD496-III microcalorimeter as described previously [25]. Before collection of the thermokinetic data, the calorimetric constant of calorimeter was measured by the Joule effect at four different temperatures and the constants are 63.72 ± 0.031, 63.48 ± 0.037, 62.90 ± 0.041, and 62.57 ± 0.030 μV/mW at 293.15, 298.15, 303.15, and 308.15 K, respectively. The enthalpies of KCl aqueous solution (spectral purity) was measured with 17.238 ± 0.048 kJ $mol^{-1}$ at 298.15 K, which matches the literature value of 17.241 ± 0.018 kJ $mol^{-1}$ [26], the accuracy is 0.02% and the precision is 0.3%, this indicates that the calorimetric system is accurate and reliable [25].

The buffer for microcalorimetric experiments was prepared as previously reported one for steady-state kinetics [12, 27]. A 0.5 mL of 30 μM L1 sample in 50 mM Tris buffer (pH 8.5) and 4.0 mL of penicillin G sodium salt (5 mM) in 50 mM cacodylate buffer, pH 7.0, containing 100 μM $ZnCl_2$ were put into the stainless steel sample cell separately [28]. After equilibrium, the containers of L1 and substrate were pushed down simultaneously. As a result, the L1 was mixed with the substrate, and the thermogram was recorded.

## Results and discussion

The typical thermograms are obtained during penicillin G hydrolysis catalyzed by Mβ L1 at 293.15, 298.15, 303.15, and 308.15 K, and the collected experimental data are listed in Table 1. Based on the thermokinetic Eqs. 1–4 [28] and the collected data listed in Table 1, the calculated thermokinetic parameters of penicillin G hydrolysis catalyzed by Mβ L1 are listed in Table 2.

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

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

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

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

$H_0$, 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 penicillin G hydrolysis reaction catalyzed by Mβ L1, the following thermodynamic parameter were obtained, the values of activation free energy $\Delta G^{\theta}_{\neq}$ are 88.26, 89.44, 90.49, and 91.57 kJ $mol^{-1}$ at 293.15, 298.15, 303.15, and 308.15 K, respectively, $\Delta H^{\theta}_{\neq}$ is 24.02 kJ $mol^{-1}$ and $\Delta S^{\theta}_{\neq}$ is −219.2511 J $mol^{-1}$ $K^{-1}$. The reaction order is around 1.0, and the data shown that the reaction rate constant $k$ increases from 1.1431 to 1.9295 × $10^{-3}$ $s^{-1}$ with increasing of reaction temperature from 293.15 to 308.15 K. The apparent activation energy of the reaction $E$ is 26.5183 kJ $mol^{-1}$. These data indicate that the hydrolysis reaction of penicillin G catalyzed by Mβ L1 is an exothermic reaction and it is spontaneous in the temperature range 293.15–308.15 K [28]. With the steady-state kinetic method, Crowder et al. reported the $K_m$ value with 38–278 μM [9–12]. Based on the gained thermokinetic data, we tried to employ the reduced-extent method [29] to calculate $K_m$ and result is 1419 μM, this maybe due to limitation of the reduced-extent method.

**Table 1** The collected thermokinetic data of penicillin G hydrolysis catalyzed by Mβ L1

| 293.15 K | | | 298.15 K | | |
|---|---|---|---|---|---|
| $t$/s | $H_i/H_0$ | $dH/dt/\times10^{-4}$ J $s^{-1}$ | $t$/s | $H_i/H_0$ | $dH/dt/\times10^{-4}$ J $s^{-1}$ |
| 700 | 0.398 | 9.071 | 440 | 0.419 | 9.011 |
| 740 | 0.427 | 8.526 | 470 | 0.455 | 8.344 |
| 780 | 0.456 | 8.078 | 500 | 0.488 | 7.764 |
| 820 | 0.484 | 7.638 | 530 | 0.520 | 7.297 |
| 860 | 0.510 | 7.236 | 560 | 0.551 | 6.824 |
| 900 | 0.536 | 6.870 | 590 | 0.579 | 6.408 |
| 940 | 0.560 | 6.522 | 620 | 0.606 | 5.998 |
| 980 | 0.584 | 6.179 | 650 | 0.631 | 5.615 |
| 1020 | 0.606 | 5.872 | 680 | 0.654 | 5.347 |
| 1060 | 0.627 | 5.557 | 710 | 0.676 | 5.005 |
| 303.15 K | | | 298.15 K | | |
| $t$/s | $H_i/H_0$ | $dH/dt/\times10^{-4}$ J $s^{-1}$ | $t$/s | $H_i/H_0$ | $dH/dt/\times10^{-4}$ J $s^{-1}$ |
| 400 | 0.305 | 8.145 | 300 | 0.212 | 7.117 |
| 440 | 0.340 | 7.640 | 340 | 0.244 | 6.817 |
| 480 | 0.373 | 7.202 | 380 | 0.275 | 6.504 |
| 520 | 0.406 | 6.810 | 420 | 0.305 | 6.280 |
| 560 | 0.437 | 6.497 | 460 | 0.333 | 5.962 |
| 600 | 0.468 | 6.168 | 500 | 0.361 | 5.746 |
| 640 | 0.497 | 5.815 | 540 | 0.388 | 5.515 |
| 680 | 0.524 | 5.487 | 580 | 0.413 | 5.293 |
| 720 | 0.551 | 5.194 | 620 | 0.438 | 5.060 |
| 760 | 0.576 | 4.935 | 660 | 0.461 | 4.867 |

$H_0$ = 1.311 J (293.15 K), 1.147 J (298.15 K), 0.7161 J (303.15 K), 0.4663 J (308.15 K)

**Table 2** Thermokinetic parameters of penicillin G hydrolysis catalyzed by M$\beta$L L1

| $T$/K | Equation 1 | | | Equation 2 | | | Equation 3 | Equation 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $k/\times 10^{-3}\mathrm{s}^{-1}$ | $n$ | $R^2$ | $E$/kJ mol$^{-1}$ | Ln$A$ | $R^2$ | $\Delta G^{\theta}_{\neq}$/kJ mol$^{-1}$ | $\Delta H^{\theta}_{\neq}$/kJ mol$^{-1}$ | $\Delta S^{\theta}_{\neq}$/J mol$^{-1}$ K$^{-1}$ | $R^2$ |
| 293.15 | 1.1431 | 1.0113 | 0.9989 | 26.5183 | 4.0939 | 0.9964 | 88.26 | 24.02 | −219.2511 | 0.9957 |
| 298.15 | 1.3290 | 0.9968 | 0.9983 | | | | 89.44 | | | |
| 303.15 | 1.6170 | 1.0017 | 0.9985 | | | | 90.49 | | | |
| 308.15 | 1.9295 | 0.9984 | 0.9993 | | | | 91.57 | | | |

$R^2$ linear correlation coefficient

## Conclusions

In this paper, we first report the thermokinetic parameters of penicillin G hydrolysis catalyzed by metallo-$\beta$-lactamase L1 from *Stenotrophomonas maltophilia* 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, combining with the relative kinetic data, are helpful to understand the reaction procedure and mechanism of antibiotic hydrolysis catalyzed by M$\beta$Ls, and the method maybe used for other metalloenzymes.

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## References

1. Wang Z, Fast W, Valentine AM, Benkovic SJ. Metallo-beta-lactamase: structure and mechanism. Curr Opin Chem Biol. 1999;3(5):614–22.
2. Krishna B. New Delhi metallo-beta-lactamases: a wake-up call for microbiologists. Indian J Med Microbiol. 2010;28(3):265–6.
3. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010;10(9):597–602.
4. Anzellotti A, Farrell N. Zinc metalloproteins as medicinal targets. Chem Soc Rev. 2008;37(8):1629–51.
5. Fischbach MA, Walsh CT. Antibiotics for emerging pathogens. Science. 2009;325(5944):1089–93.
6. Fisher JF, Meroueh SO, Mobashery S. Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. Chem Rev. 2005;105(2):395–424.
7. Bush K, Jacoby GA. Updated functional classification of {beta}-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.
8. Crowder MW, Spencer J, Vila AJ. Metallo-beta-lactamases: novel weaponry for antibiotic resistance in bacteria. Acc Chem Res. 2006;39(10):721–8.
9. Garrity JD, Carenbauer AL, Herron LR, Crowder MW. Metal binding Asp-120 in metallo-beta-lactamase L1 from *Stenotrophomonas maltophilia* plays a crucial role in catalysis. J Biol Chem. 2004;279(2):920–7.
10. Hu Z, Spadafora LJ, Hajdin CE, Bennett B, Crowder MW. Structure and mechanism of copper- and nickel-substituted analogues of metallo-beta-lactamase L1. Biochemistry. 2009;48(13):2981–9.
11. Carenbauer AL, Garrity JD, Periyannan G, Yates RB, Crowder MW. Probing substrate binding to metallo-beta-lactamase L1 from *Stenotrophomonas maltophilia* by using site-directed mutagenesis. BMC Biochem. 2002;3:4–16.
12. Crowder MW, Walsh TR, Banovic L, Pettit M, Spencer J. Overexpression, purification, and characterization of the cloned metallo-beta-lactamase L1 from *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother. 1998;42(4):921–6.
13. LeBlond C, Wang J, Larsen RD, Orella CJ, Forman AL, Landau RN, et al. Reaction calorimetry as an in situ kinetic tool for characterizing complex reactions. Thermochim Acta. 1996;289(2):189–207.
14. Kong W, Li Z, Xiao X, Zhao Y, Zhang P. Activity of berberine on *Shigella dysenteriae* investigated by microcalorimetry and multivariate analysis. J Therm Anal Calorim. 2010;102(1):331–6.
15. Zhao Y, Wang J, Shan L, Li R, Yan D, Xiao X. Activity of ginsenoside Rh on the growth of mice splenic lymphocytes investigated by microcalorimetry and factor analysis. J Therm Anal Calorim. 2010. doi:10.1007/s10973-010-1146-5.
16. Dragoi B, Rakic V, Dumitriu E, Auroux A. Adsorption of organic pollutants over microporous solids investigated by microcalorimetry techniques. J Therm Anal Calorim. 2010;99(3):733–40.
17. Zhao Y, Yan D, Wang J, Zhang P, Xiao X. Anti-fungal effect of berberine on Candida albicans by microcalorimetry with correspondence analysis. J Therm Anal Calorim. 2010;102(1):49–55.
18. Wang J, Cheng D, Zeng N, Xia H, Fu Y, Yan D, et al. Application of microcalorimetry and principal component analysis. J Therm Anal Calorim. 2010;102(1):137–42.
19. Yang L, Sun L, Xu F, Zhang J, Zhao J, Zhao Z. Inhibitory study of two cephalosporins on *E. coli* by microcalorimetry. J Therm Anal Calorim. 2010;100(2):589–92.
20. Lago N, Legido J, Paz Andrade M, Arias I, Casás L. Microcalorimetric study on the growth and metabolism of Pseudomonas aeruginosa. J Therm Anal Calorim. 2010. doi:10.1007/s10973-010-1175-0.
21. López-Fonseca R, Landa I, Elizundia U, Gutiérrez-Ortiz MA, González-Velasco JR. Thermokinetic modeling of the combustion of carbonaceous particulate matter. Combust Flame. 2006;144(1–2):398–406.
22. Völker S, Rieckmann T. Thermokinetic investigation of cellulose pyrolysis-impact of initial and final mass on kinetic results. J Anal Appl Pyrolysis. 2002;62(2):165–77.
23. Illeková E, Svec P, Miglierini M. Thermokinetic analysis of the multistep crystallization of a NANOPERM-type ribbon. J Non-Cryst Solids. 2007;353(32–40):3342–7.
24. Bouchoux G, Buisson DA. Gas phase basicity of $X(CH_2)_3Y$(X, Y = OH, $NH_2$) by the thermokinetic method. Int J Mass Spectrom. 2006;249–250:412–9.

25. Ji M, Liu M, Gao S, Shi Q. A new microcalorimeter for measuring thermal effects. Instrum Sci Technol. 2001;29(1):53–7.
26. Marthada V. The enthalpy of solution of SRM 1655 (KCl) in $H_2O$. J Res NBS Standards. 1980;85(6):467–74.
27. Hu Z, Periyannan G, Bennett B, Crowder MW. Role of the Zn1 and Zn2 sites in metallo-beta-lactamase L1. J Am Chem Soc. 2008;130(43):14207–16.
28. Gao S, Chen S, Hu R, Li H, Shi Q. Derivation and application of thermodynamic equations. Chin J Inorg Chem. 2002;18(4):362–6.
29. Liu J-S, Zeng X-C, Tian A-M, Deng Y. Application of a reduced-extent method to thermokinetic studies of enzyme-catalyzed reactions. Thermochim Acta. 1995;253:275–83.