Chinese Journal of Chemistry, **2004**, *22*, 252—255 **Article**

Kinetic Studies on Na^+/K^+-ATP_{ase} by Using Thermokinetic Method

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Na^+/K^+-ATP_{ase} (EC 3.6.1.3) is an important membrane-bound enzyme. By using microcalorimetry, the thermokinetic method was developed to kinetic studies on Na^+/K^+-ATP_{ase} for the first time. Compared with other ones, the method provided accurate measurements of not only thermodynamic data but also the kinetic data. At 310.15 K and pH＝7.4, the molar reaction enthalpy Δ_rH_m was measured as (−40.408±1.9) kJ • mol^{-1}. The Michaelis constant K_m was determined to be $(0.479\pm0.020)\times10^{-3}$ mol • L^{-1} and consistent with literature figure which is about 0.5×10^{-3} mol • L^{-1}. The maximum velocity V_{max} obtained was (0.681±0.026) μmol P_i • min^{-1} • mg $protein^{-1}$. All of the data have good repeatability and self-consistency. The reliability of thermokinetic method was verified by the experimental results and further confirmed by colorimetric studies. Moreover, the effect of enzyme pre-dilution on its activities was also investigated.

Keywords Na^+/K^+-ATP_{ase}, microcalorimetry, thermokinetic method, kinetic, pre-dilution

Introduction

Na^+/K^+-ATP_{ase} (ATP phosphohydrolase, EC 3.6.1.3) is an important membrane-bound enzyme. It catalyzes the hydrolysis of ATP, and uses the energy from the reaction to maintain the low internal sodium and high internal potassium concentrations characteristic of most animal cells. So it has been convincingly identified with the sodium pump. The kinetics of Na^+/K^+-ATP_{ase} has been extensively researched by classical methods such as spectrophotometry, $^{32}\gamma$-ATP isotopic determination and colorimetry.[1-7] Kinetic studies on Na^+/K^+- ATP_{ase} using ATP in millimolar concentration range of those required for optimal hydrolytic activity *in vivo* reveal K_m values ranging from 0.1 mmol • L^{-1} to 1.0 mmol • L^{-1}.[5,7]

Compared with classical ones, the thermokinetic method can provide *in-situ*, online, quasi-continuous, non-invasive and accurate measurements of not only thermodynamic data but also the kinetic data of the reaction under investigation. Moreover, there is also no constraint on both the solvent and the spectral, electrochemical, or other properties of the reaction systems involved.

Owing to these advantages, the thermokinetic method has received increasing attention from researchers in many fields. Since the absorption or production of heat is an intrinsic property of all enzyme-catalyzed reactions, microcalorimetry as the most powerful tool of the thermokinetic method has been extensively used in the study of enzyme-catalyzed reactions.[8-13] But microcalorimetry employed to the kinetic studies on Na^+/K^+-ATP_{ase} has not been reported yet. In this paper, microcalorimetric studies on Na^+/K^+-ATP_{ase} were carried out under mimetic physiological conditions (T＝310.15 K, pH＝7.4, ATP in millimolar concentration range). The thermokinetic method was developed to kinetic studies on Na^+/K^+-ATP_{ase} for the first time. By analyzing the calorimetric curves of ATP hydrolysis reactions, not only the thermodynamic data Δ_rH_m but also the kinetic data K_m and V_m were produced. All of the data obtained had good repeatability and self-consistency. The reliability of thermokinetic method was verified by the experimental results and further confirmed by colorimetric studies. Moreover, the effect of Na^+/K^+-ATP_{ase} pre-dilution on its activities was also investigated.

Experimental

Reagent

Na^+/K^+-ATP_{ase} (ATP phosphohydrolase, EC 3.6.1.3) from porcine cerebral cortex was purchased from Sigma Corporation. Protein concentration was determined by the method of Lowry *et al.*[14] using bovine serum albumin as the standard.

Other reagents were of analytical grade. All solutions were prepared with doubly distilled water or buffer.

Instrument

LKB-2107 batch microcalorimeter system combines

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a micro-batch reactor (BR) with a conduction calorimeter. UV-1601 spectrophotometer manufactured by Shimadzu (Japan) was used.

Methods

Microcalorimetric method: The calorimetric curves were obtained at 310.15 K (37.00 ℃) using an LKB-2107 batch microcalorimeter system. Before each calorimetric experiment, all reagent solutions were diluted to the required concentrations with buffer solution (composition in millimolar: Tris-HCl, 100; NaCl, 100; KCl, 10; $MgCl_2$, equal to the concentration of ATP; pH =7.4). Detailed experimental procedures were the same as previously described.[8,9]

Colorimetric method: In order to verify the results obtained by microcalorimetry, colorimetric method as a representative classical method was also used in this work. Furthermore, the effect of Na^+/K^+-ATP_{ase} predilution on its activities was studied by this method. Experiments were performed on a UV-1601 spectrophotometer.

Na^+/K^+-ATP_{ase} activities were measured by determining the amount of released inorganic phosphate (P_i) based on the malachite green assay of Kallner[15] with modifications. Each enzyme preparation was incubated in buffer solution at 37 ℃ for about 20 min before the assay. Standard curve of inorganic phosphate (P_i) was obtained under the same experimental conditions by using KH_2PO_4 as standard. The enzyme activity was expressed as micromoles of P_i released per mg of protein per min.

In kinetic studies, initial velocities were estimated by selecting brief reaction periods (5 min), over which P_i production was linear with time. By using initial rate method, the kinetic parameters K_m and V_m can be determined.

Results

Measurements of kinetic parameters K_m and V_{max} by calorimetric method

When a thermal change of a chemical reaction takes place in a batch conduction calorimeter, the relation between input function Q and output function Δ should obey Tian's equation, so we can obtain the following equations:[8]

$$Q=K\cdot(A_t+\tau\cdot\Delta_t) \quad (1)$$

$$Q_\infty=K\cdot A \quad (2)$$

where τ is the time constant of reaction system and K the proportionality constant, Q the heat liberated before time t, Q_∞ the total heat effect. As Figure 1 shows that Δ_t is the peak height at time t, A_t is the peak area up to time t, and A is the total area under the thermogram.

Defining the substrate conversion ratio $\Phi_t=([S]_0-[S]_t)/[S]_0$, where $[S]_0$ and $[S]_t$ stand for the substrate concentrations at the beginning and time t, then Φ_t can be described as:

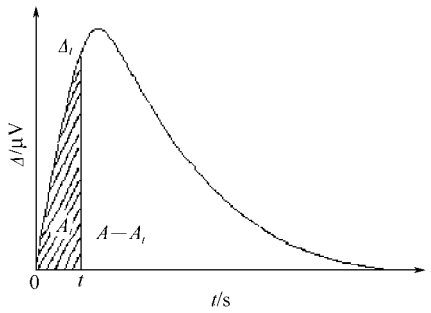

Figure 1 A typical calorimetric curve of LKB2107.

$$\Phi_t=\frac{[S]_0-[S]_t}{[S]_0}=\frac{Q}{Q_\infty}=\frac{\Lambda\cdot\Delta_t+KA_t}{KA}=\frac{\tau\cdot\Delta_t+a_t}{A} \quad (3)$$

where τ ($=\Lambda/K$) is a constant with time dimension. An electric calibration can be run after each experiment to determine this parameter. From the calorimetric curve of the calibration, a series of points behind the peak are chosen. For these points, the system is under a natural cooling procedure, so they should obey the equation: $\ln\Delta=\ln\Delta_0-t/\tau$. By plotting $\ln\Delta$ against t, the parameter τ can be obtained from the slope of the linear curve. At the same time, the parameter K mentioned above can be determined through dividing the heat of calibration by area under the calibration curve.

It was pointed out that when Mg^{2+} and ATP were varied together at a 1∶1 molar ratio in a millimolar concentration range about cellular levels *in vivo*, the kinetics of Na^+/K^+-ATP_{ase} should obey a simple Michaelis-Menton equation:[3]

$$v=-\frac{d[S]}{dt}=\frac{V_m[S]}{K_m+[S]} \quad (4)$$

For the above equation, it is easy to prove that

$$\frac{\ln(1-\Phi_t)}{t}=\frac{[S]_0}{K_m}\cdot\frac{\Phi_t}{t}-\frac{V_m}{K_m} \quad (5)$$

where $[S]_0$ stands for the substrate concentration at the beginning, K_m the Michaelis constant and V_{max} the maximum velocity of an enzyme-catalyzed reaction.

Thus, by choosing a series of points from calorimetric curves and then plotting $\ln(1-\Phi_t)/t$ against Φ_t/t, from the slope and the intercept of the linear curve, the kinetic parameters can be obtained. Table 1 and Figure 2 showed an example of analyzing a calorimetric curve. Furthermore, the results obtained under different substrate concentrations are listed in Table 2.

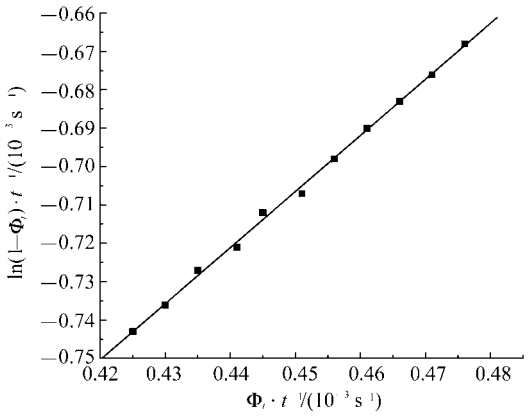

Figure 2 An example of thermogram analysis.

Table 1 An example of analyzing a microcalorimetric curve [a]

t/s	Δ_t/ mV	A_t/ (V • s)	Φ_t	$\Phi_t \cdot t^{-1}$/ (10^{-3} s^{-1})	$\ln(1-\Phi_t) \cdot t^{-1}$/($10^{-3}$ s^{-1})
1080	5.9	6.41	0.5138	0.476	−0.668
1140	5.75	6.74	0.5370	0.471	−0.676
1200	5.50	7.07	0.5595	0.466	−0.683
1260	5.40	7.37	0.5809	0.461	−0.690
1320	5.25	7.67	0.6019	0.456	−0.698
1380	5.10	7.97	0.6229	0.451	−0.707
1440	4.90	8.24	0.6413	0.445	−0.712
1500	4.75	8.52	0.6609	0.441	−0.721
1560	4.60	8.77	0.6782	0.435	−0.727
1620	4.50	9.03	0.6966	0.430	−0.736
1680	4.35	9.27	0.7132	0.425	−0.743

[a] Results: $K_m = 0.499 \times 10^{-3}$ mol • L^{-1}, $V_{max} = 6.82 \times 10^{-7}$ mol • L^{-1} • s^{-1} = 0.655 μmol P_i • min^{-1} • mg $protein^{-1}$, correlation coefficient R = 0.9991, molar reaction enthalpy $\Delta_r H_m = -39.883$ kJ • mol^{-1}. Other parameters: τ = 97.09 s, A = 13.59 V • s, $[S]_0 = 7.30 \times 10^{-4}$ mol • L^{-1}, $[E]_t = 6.25 \times 10^{-2}$ mg protein • mL^{-1}.

All experiments were carried out for several times. It was shown that the data of K_m and V_{max} have good repeatability and self-consistency. At 310.15 K and pH = 7.4, the Michaelis constant K_m was measured as $(0.479 \pm 0.020) \times 10^{-3}$ mol • L^{-1} and consistent with literature figure of about 0.5×10^{-3} mol • L^{-1}.[3,4] The maximum velocity V_{max} was determined to be (0.681 ± 0.026) μmol P_i • min^{-1} • mg $protein^{-1}$.

Colorimetric determination of kinetic parameters

For a Michaelis-Menton equation

$$v_0 = \frac{V_{max}[S]_0}{K_m + [S]_0} \quad (6)$$

where v_0 stands for the initial rate, $[S]_0$ the initial substrate concentration, K_m the Michealis constant, and V_{max} the maximum velocity. By inversion, the following equation can be obtained:

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]_0} \quad (7)$$

By plotting $1/v_0$ against $1/[S]_0$, the kinetic parameters K_m and V_{max} can be gotten from the slope and the intercept of the linear curve (Table 3).

Table 2 Results obtained under different substrate concentrations in kinetic parameter measurements by thermokinetic method[a]

$10^3[S]_0$/ (mol • L^{-1})	$10^3 K_m$/ (mol • L^{-1})	V_{max}/ (μmol P_i • min^{-1} • mg $protein^{-1}$)	R
0.181	0.476	0.664	0.9955
0.363	0.489	0.699	0.9945
0.450	0.465	0.683	0.9940
0.688	0.475	0.687	0.9980
0.730	0.499	0.655	0.9991
0.908	0.470	0.698	0.9965
Average value	0.479 ± 0.020	0.681 ± 0.026	0.9963 ± 0.0028

[a] Other parameters: t = 37.0 ℃, pH = 7.4, $[E]_t = 6.25 \times 10^{-2}$ mg protein • mL^{-1}.

Table 3 The data of double-reciprocal plot in kinetic parameter determinations by colorimetric method[a]

$10^4[S]_0$/ (mol • L^{-1})	$10^{-4}[S]_0^{-1}$/ (L • mol^{-1})	v_0/(μmol P_i • min^{-1} • mg $protein^{-1}$)	v_0^{-1}/(min • mg protein • μmol^{-1} P_i)
0.91	1.099	0.109	9.174
1.36	0.735	0.159	6.289
1.81	0.552	0.203	4.926
2.27	0.441	0.218	4.587
3.63	0.275	0.281	3.559
9.10	0.110	0.451	2.219

[a] Results: $K_m = 0.466 \times 10^{-3}$ mol • L^{-1}, $V_{max} = 0.682$ μmol P_i • min^{-1} • mg $protein^{-1}$, correlation coefficient R = 0.9960. Other parameters: t = 37.0 ℃, pH = 7.4, $[E]_t = 6.25 \times 10^{-2}$ mg protein • mL^{-1}.

Results showed that K_m (0.466×10^{-3} mol • L^{-1}) and V_{max} (0.682 μmol P_i • min^{-1} • mg $protein^{-1}$) are consistent with the values obtained by the thermokinetic method. The reliability of thermokinetic method being used to kinetic studies on Na^+/K^+-ATP_{ase} was further confirmed.

Measurement of the molar reaction enthalpy $\Delta_r H_m$

For a chemical reaction:

$$Q_\infty = n\Delta_r H_m \quad (8)$$

Where $\Delta_r H_m$ is the molar reaction enthalpy, Q_∞ the total heat effect of the reaction, n the number of moles of initial substrate. Combining Eq. (8) with Eq. (2), we obtain

$$\Delta_r H_m = KA/n \quad (9)$$

where K stands for the proportionality constant and can be obtained from calibration experiment.

The molar reaction enthalpy $\Delta_r H_m$ of ATP hydrolysis reaction is (-40.408 ± 1.9) kJ • mol^{-1} in the condition of 310.15 K and pH=7.4 (Table 4). All experiments were at least in duplicate. Results showed good repeatability and self-consistency.

Table 4 Molar reaction enthalpy, $\Delta_r H_m$, of ATP hydrolysis reaction at 310.15 K and pH=7.4

$10^3[S]_0$/(mol • L^{-1})	$-Q_\infty$/J	$-\Delta_r H_m$/(kJ • mol^{-1})
0.091	0.0152	41.889
0.136	0.0211	38.714
0.182	0.0291	39.960
0.363	0.0609	41.968
0.908	0.1504	41.415
1.815	0.2795	38.504
Average value		40.408 ± 1.9

[a] Other parameters: $V = 4.00$ mL, $[E]_t = 6.25 \times 10^{-2}$ mg protein • mL^{-1}.

Discussion

In microcalorimetric experiments, there would produce undetectable heat effect if certain volume (microliter) of Na^+/K^+-ATP_{ase} solution (5 U/mL) were pre-diluted to 2 mL as usual. In this occasion, the enzymatic reactions cannot be carried out successfully. In order to clarify the phenomenon, the effect of Na^+/K^+-ATP_{ase} pre-dilution on its activities was investigated by colorimetric method (Table 5). Results showed that excessive pre-dilution greatly decreases the enzyme activities. That is to say, the enzyme activity is reduced as the enzyme concentration is reduced. There is dilution inactivation phenomenon occurred in Na^+/K^+-ATP_{ase}.

The similar phenomenon of enzyme dilution inactivation has been described previously.[16,17] It was shown to be associated with the dissociation of the active tetrameric form of the enzyme into inactive dimmers and monomers.

It was pointed out that the minimal functional unit of Na^+/K^+-ATP_{ase} is a heterodimer, composed of two noncovalently linked subunits termed α and β.[18] Thus, it can be concluded that excessive pre-dilution seemingly destroyed the minimal functional unit and probably resulted in the dissociation of the active heterodimer form into α and β inactive monomers. So, appropriate initial concentration of Na^+/K^+-ATP_{ase} should be an indispensable factor in microcalorimetric experiments. In this work, just small volume (microlitre) of enzyme solutions with high concentration (4—5 U/mL) was used and undiluted in microcalorimetric studies.

Table 5 Colorimetric studies on the effect of enzyme pre-dilution on its activities[a]

$10^4[S]_0$/(mol • L^{-1})	V/μL	m	x/%
3.63	25	1	100
3.63	25	10	<75
3.63	25	50	<10
3.63	25	100	<5
7.30	25	1	100
7.30	25	10	<50
7.30	25	50	<5

[a] Annotation: V stands for the volume of 10 mg protein • mL^{-1} Na^+/K^+-ATP_{ase}, m the number of enzyme pre-dilution, x the percentage of original enzyme activities that pre-diluted enzymes possessed under the same conditions.

References

1 Plesner, L.; Plesner, I. W. *Biochim. Biophys. Acta* **1981**, *643*, 449.
2 Hexcum, T.; Samson, F. E.; Himes, R. H. *Biochim. Biophys. Acta* **1970**, *212*, 322.
3 Robinson, J. D. *Biochim. Biophys. Acta* **1974**, *341*, 232.
4 Robinson, J. D. *Biochim. Biophys. Acta* **1976**, *429*, 1006.
5 Robinson, J. D. *Biochemistry* **1967**, *6*, 3250.
6 Rossi, R. C.; Garrahan, P. J. *Biochim. Biophys. Acta* **1989**, *981*, 85.
7 Robinson, J. D.; Flashner, M. S. *Biochim. Biophys. Acta* **1979**, *549*, 145.
8 Wang, Z.-Y.; Wang, C.-X.; Qu, S.-S. *Thermochim. Acta* **2000**, *360*, 141.
9 Wang, Z.-Y.; Liu, Y.-W.; Wang, C.-X.; Zhou, S.-H.; Qu, S.-S. *Acta Chim. Sinica* **2001**, *59*, 492 (in Chinese).
10 Liang, Y.; Wu, Y.-X.; Li, D.-H.; Wang, C.-X.; Qu, S.-S.; Zou, G.-L. *Thermochim. Acta* **1997,** *307*, 149.
11 Hüttl, R.; Bohmhammel, K.; Pritzkat, K.; Wolf, G. *Thermochim. Acta* **1993**, *229*, 205.
12 Hüttl, R.; Bohmhammel, K.; Pritzkat, K.; Wolf, G. *Thermochim. Acta* **1995**, *250*, 1.
13 Oehlschläger, K.; Hüttl, R.; Wolf, G. *Thermochim. Acta* **1996**, *271*, 41.
14 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
15 Kaller, A. *Clin. Chim. Acta* **1975**, *59*, 35.
16 Khew-Goodall, Y. S.; Johannssen, W.; Attwood, P. V.; Wallace, J. C.; Keech, D. B. *Arch. Biochem. Biophys.* **1991**, *284*, 98.
17 Attwood, P. V.; Johannssen, W.; Chapman-Smith, A.; Wallace, J. C. *Biochem. J.* **1993**, *290*, 583.
18 Blanco, G.; Koster, J. C.; Sanchez, G.; Mercer, R. W. *Biochemistry* **1995,** *34*, 319.

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