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BINDING AND REACTION CALORIMETRIES IN Na,K ATPase - ATP SYSTEM

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SUMMARY

A highly sensitive liquid flow micro reaction calorimetry system based on the heat conduction principle with multi-thermomodules, applicable to suspended solutions has been developed. This calorimetry system has been well suited for measurements on the enzyme kinetics of suspended membrane proteins.

Binding and reaction studies were carried out by using it on the suspended Jørgensen's Na,K-ATPase - membrane sample with cations (Na^+, K^+, Mg^{2+}), ADP, ATP-γ-S and ATP. Evaluated values of enthalpy for cation bindings, and evaluated thermodynamic quantities of the bindings of substrate analogues ADP and ATP-γ-S were obtained definitely. Binding stability of this protein with these analogues was strong and enthalpic nearly to that in almost globular protein-ligand binding systems.

The biphasic Lineweaver-Burk plot on the reaction heat, which is proportional to the velocity of generated heat, of this ATPase with ATP was found calorimetically as a function of ATP concentration. Apparent values of the Michaelis constant Km were obtained to be about 7 μmol and 1 mmol for low- and high- ATP affinity sites, respectively. Evaluated values of reaction heat with ATP concentration coincide with the theoretical values when the free energy change of ATP decomposition is assumed to be 21 kJ/mol.

INTRODUCTION

Na-pump, Na,K-ATPase which is an electrogenic pump protein plays a role in transporting across the cell membrane. A large number of enzymatic and pump mechanical studies has been done since this ATPase was discovered (ref.1). Recently, primary structures of subunits originated from several animal organs were being elucidated. On the higher structure and the pump mechanism, many approaches to make clear will be necessitated at present.

In order to understand better the kinetic and energy transforming mechanisms of energy conversion proteins, an extremely high sensitive flow conduction microcalorimetry system (ref.2) which is applicable to membrane-protein suspensions has been developed by us and the study by using it was proved useful for quantitative evaluating the binding and reaction behaviors of cations, ATP and it's analogues. Calorimetric study in combination with measurements of other physicochemical properties in especial is possible to have some progressive knowledges for the kinetic and energy transformation mechanisms, and it is un-

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der investigation in this ATPase - ATP system.

METHODS

Apparatus

Flow type calorimetry has several advantages over batch calorimetry. The advantages consist of following subjects: (1) simple for operation, (2) unnecessity of equilibration time prior to experiment, (3) no vapor space, etc.(ref. 3). Subjects (2) and (3) are needful for measurements of biological materials and of extreemly small generated heat. Moreover, it is indispensable to give a uniform solvent atmosphere circumference of solute particles in the case of suspended medium as membrane-protein or cell suspension. Progress of heat detector sensitivity and smooth passage of sample solution or suspension remain problems. A highly sensitive flow microcalorimetry system, which is applicable to suspended solutions and based on the heat conduction principle with multi-thermomodules, was made by way of experiment (ref.2). Distinctive features of this system are uses of zigzaged thermoplastic pipe (Fig. 1, internal diameter; 0.5-0.8mm) which was fixed directly on the surface of modules, of an autosampler for suspension (Fig. 2) and of nonactive liquid Fluorinert in calorimeter cell. Accuracy of this system was made sure by measuring of the heat of dilution of sodium chloride, potassium chloride, urea and sucrose aqueous solutions.

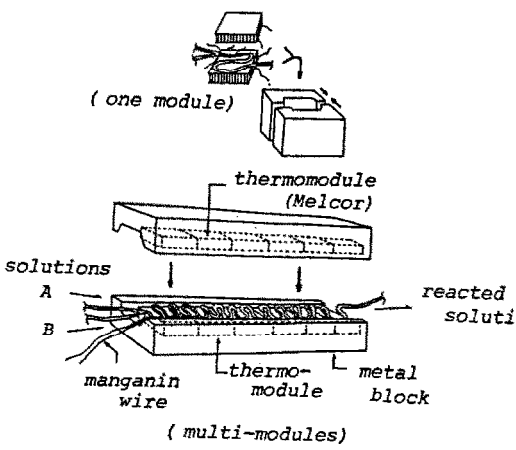


Fig. 1. Sensor portion.

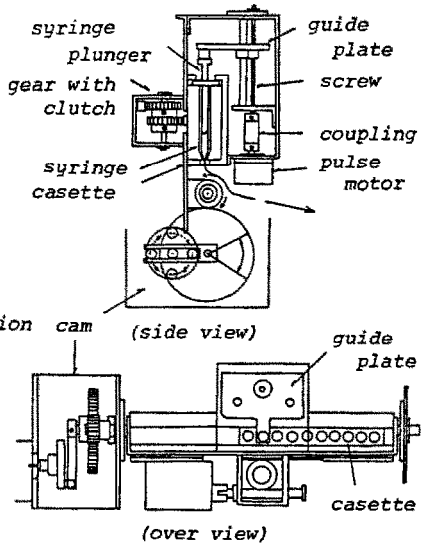


Fig. 2. An autosampler for suspension.

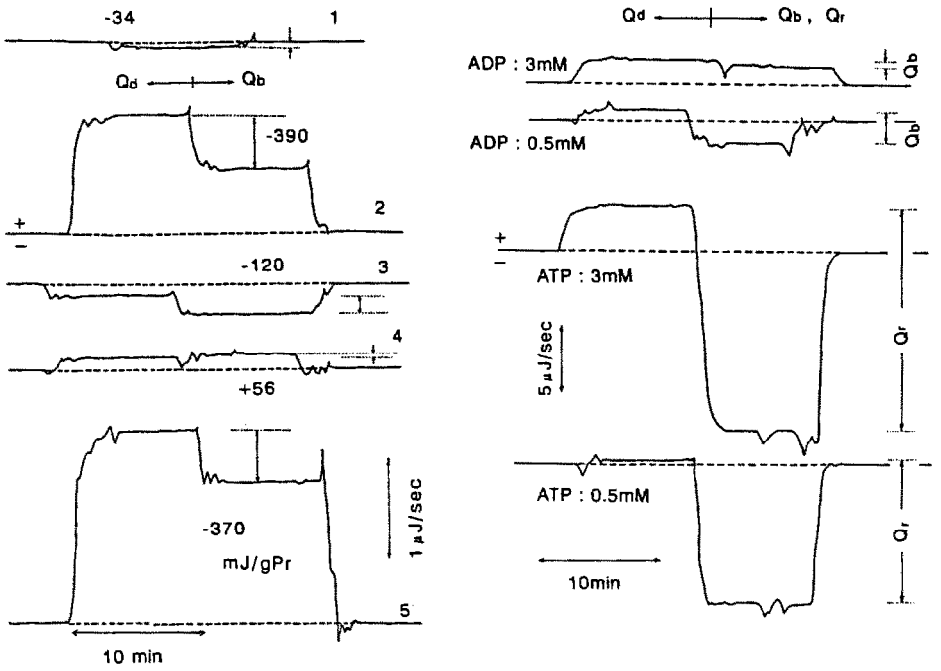


Fig. 3. *(left)* Examples of thermal response I; dilution of Na,K-ATPase(1), and interactions of that with 150mM NaCl(2), 15mM KCl(3), 3mM $MgCl_2$(4) and the mixed salts(5), respectively. Qd; heat of dilution, Qb; heat of binding.
Fig. 4. *(right)* Examples of thermal response II; ADP binding and ATP reaction with Na,K-ATPase. Qr; heat of reaction.

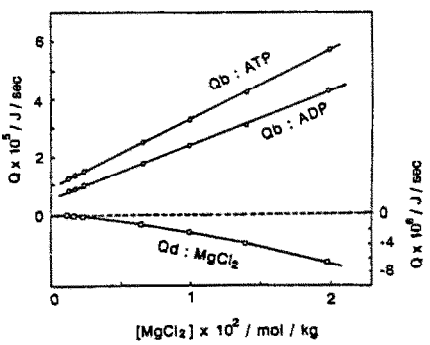
Materials and Procedures

The Na,K-ATPase sample was prepared from sheep kidney according to the method of Jørgensen (ref. 4) and calorimetry has been almost performed in a solution system [150mM NaCl, 15mM KCl, 3mM $MgCl_2$, 0.5mM EDTA and 0.05-0.08% protein (specific activity; S.A. 150-1,000 mol Pi/mg h), in 20mM Imidazole-HCl; pH 7.40] which should be satisfied the ionic requirement for the functional activity of this pump protein, at 298.15K.

Values of generated electric power between 10^{-8}- 10^{-5} J/sec have been measured. Content of protein (Pr conc.) was analyzed by the method of Lowry (ref. 5) and the value of S.A. was determined according to ordinary method (ref. 6), at 310K. All chemicals were of reagent grade, and the flow rate into two mixing tubes were carried out at about each 6 ml/h.

RESULTS AND DISCUSSION

From Fig. 3 to Fig. 9, evaluated values of heats of binding and reaction in in this Na,K-ATPase system (ref. 7) are shown. They were all evaluated subtract-



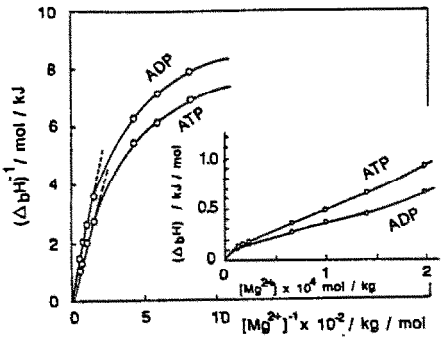


Fig. 5. *(left)* Observed values of the heat of binding (complex formation) and that of dilution in ADP- and ATP- $MgCl_2$ systems, in imidazole buffer, at pH 7.40.
ADP; m=3.8203x10^{-2}, Qd=-6.77x10^{-7} J/sec (ΔdH=-10.2J/mol),
ATP; m=3.3950 " , Qd=+3.94x10^{-6} " (ΔdH=+670 ").
Fig. 6. *(right)* Molar heats of binding in ADP- and ATP- Mg^{2+} systems as a function of Mg^{2+}concentration, and double reciprocal plot of the data.

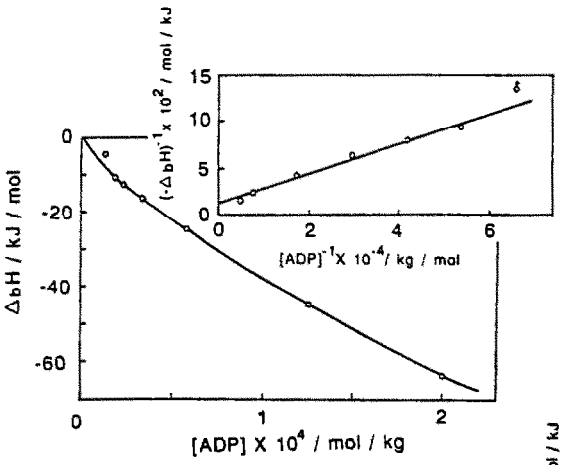


Fig. 7. Molar heat of binding of Na,K-ATPase with ADP as a function of ADP concentration, and double reciprocal plot of the data.
Pr conc. 0.939mg/ml, S.A. 925.2μmol Pi/mg h.

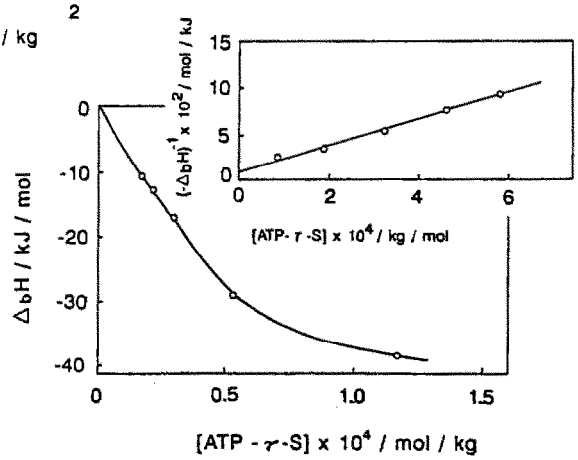


Fig. 8. Molar heats of binding of Na,K-ATPase with ATP -γ-S as a function of ATP-γ-S concentration, and double reciprocal plot of the data.
Pr conc. and S.A.; the same as those in Fig. 7.

TABLE 1

Evaluated thermodynamic quantities of ligand bindings, at 298.15K.

System	$-\Delta G^{o}$ (kJ mol^{-1})	$-\Delta H^{o}$ (kJ mol^{-1})	$-\Delta S^{o}$ (J $mol^{-1}K^{-1}$)
ADP - Mg^{2+}	8.6	13.3	74
ATP - Mg^{2+}	8.5	18.7	91
Na,K-ATPase-ADP	23.4 ±1	78.1 ±2	187 ± 5
Na,K-ATPase-ATP-γ-S	18.2 ±2	109 ±3	305 ±17

ing dilution effects. As shown in Fig. 3, even in the case of very small heat change it was possible to measure obviously because of high sensitivity and base line stability of this calorimetric measurement. This result has an expectation of being able to investigate the binding behavior in turn over of this ATPase.

Interactions of metal ions with nucleoside phosphates have been studied and it makes clear that metal complexing reactions occur *via* multi-step process. Double reciprocal plot in Fig. 6 shows this complex formations are understood to be multi-steps for reason of non-linear plot without in low Mg^{2+} concentration region. Data of our experiment coincide with reported values determined from another method (ref. 8). In the case of binding of Na,K-ATPase with substrate analogues ADP and ATP-γ-S, the reciprocal plots accepted apparently linear relations for monotonic step process, though it is known that ATP-γ-S participates partly in phosphorization of this ATPase. Evaluated thermodynamic quantities of binding in both systems are shown in Table 1. It is assumed in this study that binding stability of this protein with these analogues is strong and enthalpic nearly to that in almost globular protein-ligand binding systems (refs. 9,10).

The most notable result is that the heats of reaction in Na,K-ATPase - ATP system showed two reaction types with ATP concentration. The numerical data are presented in Table 2, and relation between evaluated heat and ATP concentration is shown in Fig. 9. Data are presented in the Lineweaver-Burk form in Fig. 10. In this calorimetry, heat of reaction is proportional to velocity of generated heat. Many authors have presented data on this activity that suggest a biphasic this plot, and the same biphasic behavior (Michaelis consts. Km= 7μM and 1mM) was revealed calorimetrically in this work. This reason has been considered as suggestive evidence for high- and low-affinity sites for ATP in this ATPase - ATP reaction mechanism (refs. 11,12,13). Theoretical free energy changes in this reaction system can be calculated from the values of Km and S.A., and it is shown in Fig. 11. Values of S.A. decrease to a third at 25°C from those at 37°C (ref. 14). Directly measured values of ATP decomposition are not found up to the

TABLE 2

Evaluated values of heat of reaction in Na,K-ATPase - ATP system, at 298.15K.

Medium: 150mM NaCl, 15mM KCl, 3mM $MgCl_2$, 0.5mM EDTA in 20mM Imidazole-HCl Buffer, at pH 7.40.

[I] ATPase: Pr.conc. 0.821mg/ml, S.A. 259.0µmol Pi/mg h.
Flow rate: 1.6673×10^{-3} ml/sec, Q_d(ATPase): -5.3×10^{-8} J/sec.

No.	ATP conc.* (mol/kg)	$-Q_d \times 10^7$ (J/sec)	$-Q_r \times 10^6$ (J/sec)	$-\Delta rH$ (J/g Pr)	$-\Delta rH$ (kJ/mol ac.Pr)**
1	1.195×10^{-6}	0.00	0.411	0.301	5.81×10^2
2	1.327×10^{-5}	0.23	0.530	0.388	7.49 "
3	1.722x "	0.27	2.24	1.64	3.16×10^3
4	2.512x "	0.80	3.15	2.30	4.44 "
5	4.739x "	1.10	3.67	2.68	5.18 "
6	2.001×10^{-4}	1.34	4.94	3.61	6.97 "
7	4.001 "	1.49	5.70	4.17	8.05 "

[II] ATPase: Pr.conc. 0.510mg/ml, S.A. 149.0µmol Pi/mg h.
Flow rate: 1.6554×10^{-3} ml/sec, Q_d(ATPase): -5.3×10^{-8} J/sec.

8	5.001×10^{-4}	1.92	6.89	8.12	2.72×10^4
9	1.002×10^{-3}	4.17	14.2	17.2	5.76 "
10	2.007 "	21.9	19.7	25.8	8.66 "
11	3.001 "	42.1	21.1	29.9	1.00×10^5

* Final concentration.

** Net values of heat of reaction for enzymatic active Na,K-ATPase based on S.A. and M.W. 131,000.

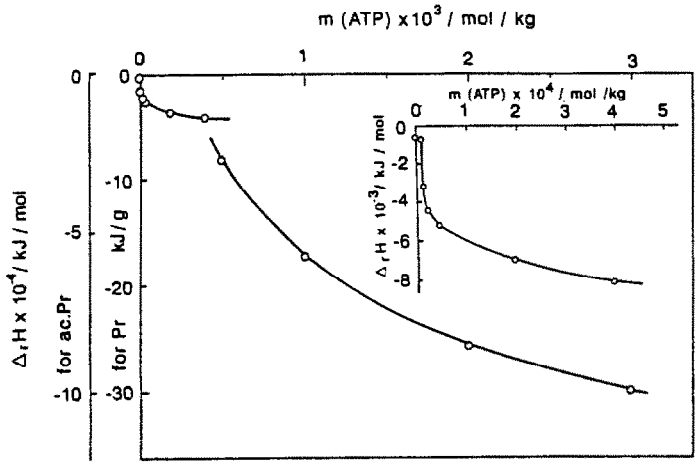


Fig. 9. Evaluated values of the heat of reaction in Na,K-ATPase - ATP system, at 298.15K. Detailes of measured media and numerical values are shown in Table 2.

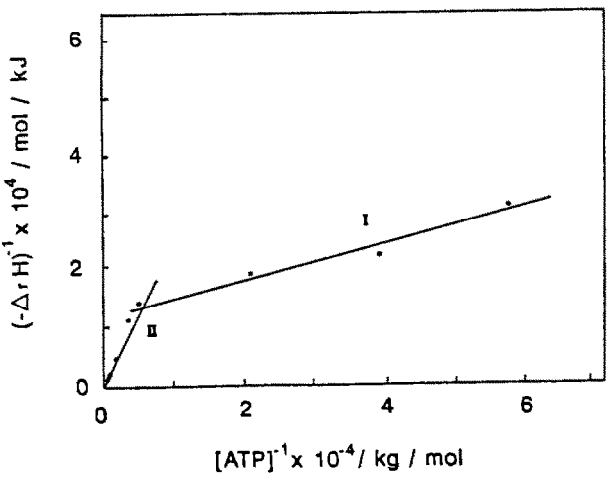


Fig.10. Substrate kinetics in Na,K-ATPase - ATP system: data are presented in double reciprocal Lineweaver-Burk form.

present. In this figure, dotted lines 1 and 2 are shown those to be calculated theoretically on the basis of ΔG^{o}= 31 and 21 kJ/mol, respectively. It may be supposed that the value of enthalpy change ΔH^{o} should be used instead of ΔG^{o} in this case. Evaluated values of reaction heat coincided in appearance with the values which the free energy change ΔG^{o} of ATP decomposition is assumed to be 21 kJ/mol(5 kcal/mol).

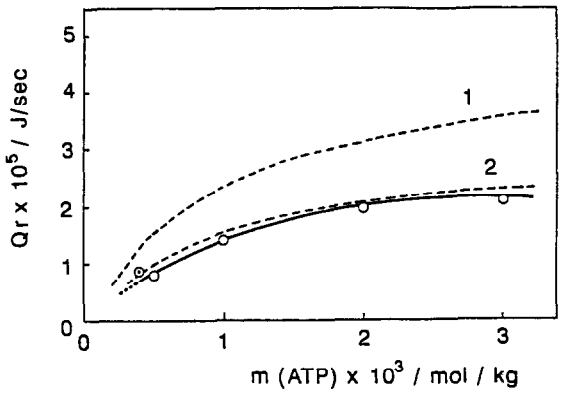


Fig.11. Reaction heats (velocity of generated heat) in Na,K-ATPase - ATP system as a function of ATP concentration, at 298.15K; theoretical values are shown as dotted lines; 1. ΔG^{o}=31 kJ/mol, 2. 21 kJ/mol.

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