Binding Properties of Adenosine Deaminase Interacted with Theophylline

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Thermodynamic studies were carried out to evaluate the binding of theophylline on adenosine deaminase (ADA) in 50 mM sodium phosphate buffer pH 7.5, at 300 K, using isothermal titration calorimetry (ITC). A simple method for determination of binding isotherm in the drug–ADA interaction was applied using ITC data. ADA has two binding sites for theophylline, which show positive cooperativity in its sites. The intrinsic association equilibrium constants are 6 and 52 mM^{-1} in the first and second binding sites, respectively. Hence, occupation of the first site has produced an appreciable enhancement by 8.7 of the binding affinity of the second site. The molar enthalpies of binding are −12.2 and −14.9 kJ/mol in the first and second binding sites, respectively.

Key words adenosine deaminase; theophylline; cooperativity; isothermal titration calorimetry; calorimetric method

Adenosine deaminase (ADA) is a glycoprotein (34.5 kDa) consisting of a single polypeptide chain of 311 amino acids,[1,2] which catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine nucleosides to their respective inosine derivative nucleosides and ammonia with a rate enhancement of 2×10^{12} relative to the nonenzymatic reaction.[3] Catalysis requires a Zn^{2+} cofactor.[4] The enzyme is widely distributed in vertebrates, invertebrates and mammals including humans. Aberrations in the expression and function of ADA have been implicated in several disease states such as severe combined immuno deficiency (SCID), which is characterized by impaired B- and T-cell-based immunity resulting from an inherited deficiency in ADA.[5,6] Higher level of ADA in the alimentarytract and decidual cells of the developing fetal-maternal interface put ADA among those enzymes performing unique roles related to grows rate of cells, embryo implantation, and other undetermined functions.[7,8] The inhibition of adenosine deaminase in brain would allow an accumulation of adenosine, which produces vasodilation, and increase of cerebral blood flow. Therefore the decrease of enzyme activity would potentiate the sedative actions of adenosine in interneuronal communication of the central nervous system.[9]

ADA has a (α/β) barrel structure motif. The active site of ADA resides at the C-terminal end of the β barrel, in a deep oblong-shaped pocket. A pentacoordinated Zn^{2+} cofactor is embedded in the deepest part of the pocket. The zinc ion is located deep within the substrate binding cleft and coordinated in a tetrahedral geometry to His 15, His 17, His 214, and Asp 295. A water molecule, which shares the ligand coordination site with Asp 295, is polarized by the metal giving rise to a hydroxylate ion that replaces the amine at the C6 position of adenosine through a stereo specific addition–elimination mechanism.[10] Mutation studies of amino acids in the proposed active site near the zinc binding site in the adenosine deaminase confirmed the essential role of these residues in catalysis.[11—13]

ADA can hydrolyze the substituent in 6 position of a variety of substituted purine nucleosides. The enzyme' hydrolytic capabilities have been exploited to convert lipophilic 6-substituted purine nucleosides to products which show anti-HIV (human immunodeficiency virus) activity.[14,15]

Understanding the interaction of ADA with its effectors at molecular level will be important in the development of the next generation of pharmaceutical agents that act as inhibitors, activator, or substrates. Isothermal titration calorimetry (ITC) gives valuable information about biomacromolecule-ligand interaction and enzyme inhibition.[16—24] Following our previous research on modified histidine residues[25] and inhibition study of ADA by inosine,[26] caffeine[27] and acetaminophen,[28] in this work we describe the binding properties of ADA interacted with theophylline studied by isothermal titration calorimetry using a new data analysis approach. Theophylline at low doses under long-term conditions increases the ADA activity.[29] Also, there is reference reporting inhibition of ADA by theophylline at high doses.[30]

Experimental

Materials Adenosine deaminase (ADA; type IV) from calf intestinal mucosa and theophylline were obtained from Sigma. All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. Phosphate solution at 50 mM concentration, pH=7.5, was used as a buffer.

Methods The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made of stainless steel. Theophylline solution (2 mM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 ml ADA, 2.0 or 5.0 μM, in phosphate buffer (50 mM), pH=7.5. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of drug solution into the perfusion vessel was repeated 20 times, and each injection included 10 μl reagent. It has been spent a time of 15 min for recording the heat of each injection, although the equilibrium reached much sooner than that time. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the theophylline solution was measured as described above except that ADA was excluded. Also, the heat of dilution of

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the protein solution was measured as described above except that the buffer solution was injected to the protein solution in the sample cell. The enthalpies of drug and protein solutions dilution were subtracted from the enthalpy of ADA–theophylline interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

The molecular weight of ADA was taken to be 34500.[31)]

Theoretical

In general, there will be "g" sites for binding of ligand molecules per protein macromolecule and ν is defined as the average moles of bound ligand per mole of total protein. At any constant heat value due to the binding of ligand molecules per mole of protein (ΔH), the free concentration of ligand (L^{free}) and ν are also constant at equilibrium on both two curves of ΔH against total concentration of either ligand or protein obtained in two titration experiments at two different concentrations of a protein (such as Fig. 2). Ligand molecules exist in two forms of free and bound. Hence, $L^{free}=L-L^{bound}$, where L and L^{bound} are the total and bound concentration of ligand, respectively. The titration calorimetric experiment has been carried out in two concentrations of the protein, shown by **1** and **2**. Equality of L^{free} at any constant value of ΔH on both titration curves (such as Fig. 2) results the equation:

$$L_1-L_1^{bound}=L_2-L_2^{bound} \tag{1}$$

By applying $\nu=L^{bound}/M$, where M is the total concentration of the protein, and equality of ν at any constant value of ΔH on both titration curves, it can be deduced Eq. 2 from Eq. 1.

$$L_1-\nu M_1=L_2-\nu M_2 \tag{2}$$

This equation can be rearranged to give Eq. 3.

$$\nu=\frac{L_2-L_1}{M_2-M_1} \tag{3}$$

Then L^{free} can be calculated by Eq. 4, which obtains from substitution of ν from Eq. 3 into the equation $L^{free}=L_1-\nu M_1$.

$$L^{free}=\frac{L_1M_2-L_2M_1}{M_2-M_1} \tag{4}$$

One can then calculate ν and L^{free} from Eqs. 3 and 4, respectively. This procedure is repeated over the range of ΔH values that span the titration curves, thus yielding a full range of values of ν and L^{free}. In this way, one can obtain a binding isotherm for protein ligand interaction by two calorimetric titration curves at two different concentration of a protein. This analytical approach has some similarities in the concept with the Halfman and Nishida spectroscopy method.[32)]

Results and Discussion

The raw data obtained from isothermal titration calorimetry of ADA interaction with theophylline (as a ligand) in two different concentrations of the protein are shown in Fig. 1. Figure 1a shows the heat of each injection and Fig. 1b shows the heat of related to each total concentration of theophylline, $[Theo]_t$. These raw calorimetric data can be used to show the heat of binding of theophylline molecules per mole of ADA (ΔH) *versus* total concentration of theophylline, Fig. 2a, or *versus* total concentration of the protein, Fig. 2b. Using Eqs. 3 and 4, one can obtain a binding isotherm as shown in Fig.

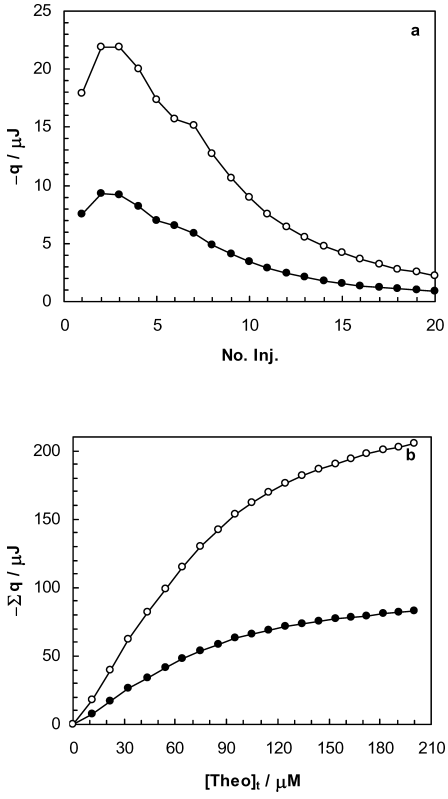

Fig. 1. (a) The Heat of Theophylline Binding on ADA for 20 Automatic Cumulative Injections, Each of 10 μl, of Theophyline, 2 mM, into the Sample Cell Containing 1.8 ml ADA Solution at Two Initial Concentrations of 2 μM (●) and 5 μM (○) and (b) the Heat of Binding *versus* Total Concentration of Theophylline, Calculated from Fig. 1a

3a or the Scatchard plot,[33)] $\nu[Theo]_f$ *versus* ν, as shown in Fig. 3b, where $[Theo]_f$ is the free concentration of theophylline.

The shapes of the Scatchard plots are clearly characteristic of different types of cooperativity.[34,35)] A concave downward curve, as shown in Fig. 3b, describes a system with positive cooperativity. For obtaining approximated values of binding parameters, it might be possible to fit the binding data to the Hill equation,[36)]

$$\nu=\frac{g(K[Theo]_f)^n}{1+(K[Theo]_f)^n} \tag{5}$$

where K and n are the binding constant and Hill coefficient, respectively. The binding data for the binding of theophylline to ADA have been fitted to the Hill equation using a computer program for nonlinear least-square fitting.[37)] The results are: $g=2$, $K=17.6\,mM^{-1}$ and $n=1.47$. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 3b. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites (K_1 and K_2) has been introduced from the Scatchard plot.[38)] It has been shown that, in the limit as ν approaches 0, $\nu/[Theo]_f=2K_1$ and when $\nu=1$, or at half-saturation, $\nu/[Theo]_f=(K_1K_2)^{1/2}$. Thus, K_1 can be obtained from the ordinate intercept of a Scatchard plot and K_2 is derived from the value of $\nu/[Theo]_f$ at half-saturation. The results obtained from Fig. 3b are $K_1=6\,mM^{-1}$ and $K_2=52\,mM^{-1}$. A check for the validity of the binding con-

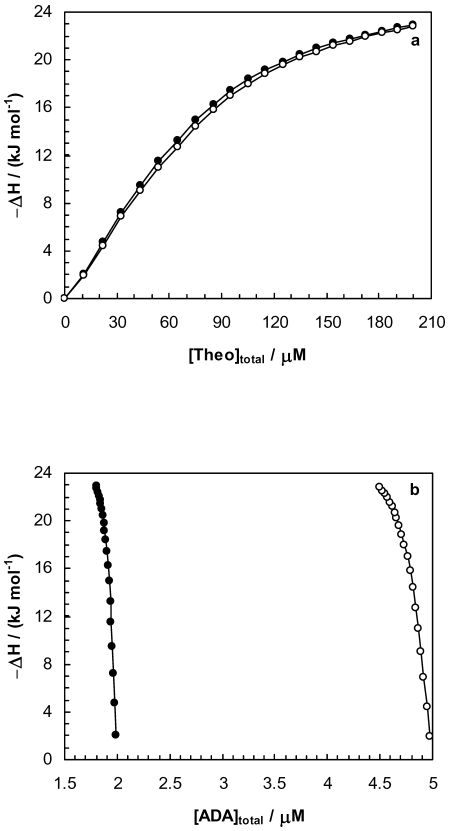

Fig. 2. (a) The Heat of Binding Theophylline per Mole of ADA (ΔH) *versus* Total Concentration of Theophylline, Calculated from Fig. 1b and (b) the Heat of Binding Theophylline per Mole of ADA (ΔH) *versus* Total Concentration of ADA

The initial concentration of ADA was 2 μM (●) and 5 μM (○).

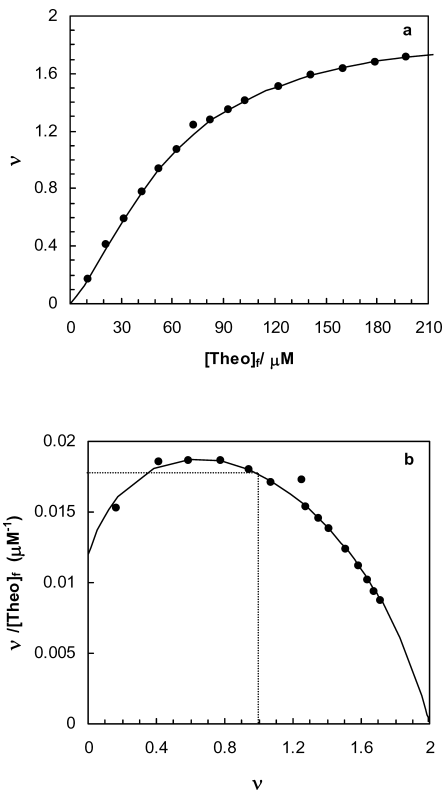

Fig. 3. The Binding Isotherm (a) and the Scatchard Plot (b) of Binding Theophylline by ADA at 27 °C

The best-fit curve of the experimental binding data was transformed to both binding isotherm and the Scatchard plot using Eq. 5 with $g=2$, $K=17.6\,\mathrm{mM}^{-1}$ and $n=1.47$.

stants can also be done by a nonlinear least squares of data fitting to the Adair equation for system with two binding sites (including ν, K_1, K_2 and $[\mathrm{Theo}]_f$),[39] which the results for K_1 and K_2 values are as the same as above. So, occupation of the first site has produced an appreciable enhancement 8.7 of the binding affinity of the second site. Moreover, values of ΔH in different values of ν (obtained from Fig. 2) give the molar enthalpies of binding -12.2 and -14.9 kJ/mol in the first and second binding sites, respectively.

The new calorimetric method described in this paper allows obtaining the binding isotherm for measurement of the complete set of thermodynamic parameters in protein drug binding studies. The binding isotherm for drug–protein interaction can be easily obtained by carrying out titration calorimetric experiment in two different concentrations of protein. An enhancement by 8.7 of the binding affinity of the second site due to the binding of theophylline on the first binding site may be followed by a fine conformational change in ADA leading to the activation of the protein at low doses of the drug, as reported before.[29] Hence, the second site may be related to the inhibition of ADA that is filled at higher doses of the drug, according to the observation reported before.[30]

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References

1) Doddona P. E., Schewach D. S., Kelly W. N., Argos P., Markham A. F., Orkin S. H., *J. Biol. Chem.*, **259**, 12101—12106 (1984).
2) Chang Z., Nygaard P., Chinualt A. C., Kellems R. E., *Biochemistry*, **30**, 2275—2280 (1991).
3) Frick L., Neela J. P., Wolfenden R., *Bioorg. Chem.*, **15**, 100—108 (1987).
4) Wilson D. K., Rudolph F. B., Quiocho F. A., *Science*, **252**, 1278—1284 (1991).
5) Herschfeld M. S., Mitchell B. S., "The Metabolic and Molecular Basis of Inherited Disease." ed. by Scriver C. R., Beaudet A. L., Sly W. S., Valle D., McGraw-Hill, New York, 1995, pp. 1725—1768.
6) Herschfield M. S., *Semin. Hematol.*, **35**, 291—298 (1998).
7) Gan T. E., Dadonna P. E., Mitchell B. S., *Blood*, **69**, 13176—13180 (1987).
8) Hong L., Mulholland J., Chinsky J. M., Knudsen T. B., *Biol. Reprod.*, **44**, 83—93 (1991).
9) Phillis J. W., Wu P. H., *Prog. Neurobiol.*, **16**, 187—239 (1981).
10) Wilson D. K., Quiocho F. A., *Biochemistry*, **32**, 1689—1693 (1993).
11) Bhaumik D., Medin J., Gathy K., Coleman M. S., *J. Biol. Chem.*, **268**, 5464—5470 (1993).
12) Mohamedali K. A., Kurz L. C., Rudolph F. B., *Biochemistry*, **35**, 1672—1680 (1996).
13) Sideraki V., Mohamedali K. A., Wilson D. K., Chang Z., Kellems R. E., Quiocho F., Rudolph F. B., *Biochemistry*, **35**, 7862—7872 (1996).
14) Barchi J. J., Jr., Marquez V. E., Driscoll J. S., Ford H., Jr., Mitsuya H., Shirasaka T., Aoki S., Kelly J. A., *J. Med. Chem.*, **34**, 1647—1655 (1991).
15) Ford H., Jr., Siddiqui M. A., Driscoll J. S., Marquez V. E., Kelly J. A., Mitsuya H., Shirasaka T., *J. Med. Chem.*, **38**, 1189—1195 (1995).
16) Saboury A. A., Bordbar A. K., Moosavi-Movahedi A. A., *J. Chem. Thermodyn.*, **28**, 1077—1082 (1996).
17) Saboury A. A., Moosavi-Movahedi A. A., *J. Enzyme Inhib.*, **12**, 273—279 (1997).
18) Saboury A. A., *Thermochim. Acta*, **320**, 97—100 (1998).
19) Ghadermarzi M., Saboury A. A., Moosavi-Movahedi A. A., *Polish J. Chem.*, **72** 2024—2029 (1998).
20) Saboury A. A., Sarri-Sarraf N., Saeidian S., *Thermochim Acta*, **381**,

147—151 (2002).
21) Sarri-Sarraf N., Saboury A. A., Moosavi-Movahedi A. A., *J. Enz. Inhib. Med. Chem.*, **17**, 203—206 (2002).
22) Saboury A. A., *Biologia*, **57**, 221—228 (2002).
23) Saboury A. A., *J. Chem. Thermodyn.*, **17**, 1975—1981 (2003).
24) Saboury A. A., *J. Therm. Ana. Cal.*, **72** 93—103 (2003).
25) Ataei G., Moosavi-Movahedi A. A., Saboury A. A., Hakimelahi G. H., Hwu J. Ru.,Tsay S. C., *Int. J. Biol. Macromol.*, **27**, 29—30 (2000).
26) Saboury A. A., Divsalar A., Ataei G., Moosavi-Movahedi A. A., Housaindokht M. R., Hakimelahi G. H., *J. Biochem. Mol. Biol.*, **35**, 302—305 (2002).
27) Saboury A. A., Divsalar A., Ataie G., Amanlou M., Moosavi-Movahedi A. A., Hakimelahi G. H., *Acta Biochim. Pol.*, **50**, 849—855 (2003).
28) Ataie G., Safarian S., Divsalar A., Saboury A. A., Moosavi-Movahedi A. A., Ranjbar B., Cristalli G., Mardanian S., *J. Enz. Inhib. Med. Chem.*, **19**, 71—78 (2004).
29) Bandyopadhyay B. C., Poddar M. K., *Meth. Find. Exp. Clin. Pharmacol.*, **19**, 181—184 (1997).
30) Singh L. S., Sharma R., *Mol. Cell. Biochem.*, **204**, 127—134 (2000).
31) Brady T. G., O'Sullivan W., *Biochim. Biophys. Acta*, **132**, 127—137 (1967).
32) Halfman C. J., Nishida T., *Biochemistry*, **11**, 3493—3498 (1972).
33) Scatchard G., *Ann. N.Y. Acad. Sci.*, **50**, 660—662 (1949).
34) Saboury A. A., Moosavi-Movahedi A. A., *Biochem. Educ.*, **22**, 48—49 (1994).
35) Bordbar A. K., Saboury A. A., Moosavi-Movahedi A. A., *Biochem. Educ.*, **24**, 172—176 (1996).
36) Hill A. V., *J. Physiol.*, **40**, iV—Vii (1910).
37) James M. L., Smith G. M., Wolford J. C., "Applied Numerical Methods for Digital Computer," 3rd Edn., Harper and Row Publisher, New York, 1985.
38) Galley W. C., Bouvier M., Clas S.-D., Brown G. R., ST-Pierre L. E., *Biopolymers*, **27**, 79—83 (1988).
39) Edsall J. T., Gutfreund H., "Biothermodynamics," Chapter 5, John Wiley & Sons Ltd., New York, 1983.