Kinetic and Thermodynamic Study on the Inhibition of Adenosine Deaminase by Theobromine

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Kinetic and thermodynamic studies were made on the binding of theobromine on the activity of adenosine deaminase (ADA) in 50 mM sodium phosphate buffer pH 7.5 at 300 K, using UV spectrophotometry and isothermal titration calorimetry (ITC). Theobromine acts as a competitive inhibitor. A graphical fitting method was used for determination of binding constant and enthalpy of inhibitor binding by using ITC data. The dissociation-binding constant is equal to 318 μM by the calorimetry method, which agrees well with the value of 311 μM for the inhibition constant that was obtained from the spectroscopy method. The molar enthalpy and entropy of binding for theobromine are -15.80 kJ/mol^{-1} and 14.30 J K^{-1}mol^{-1}, respectively. So, the binding process for theobromine on ADA is spontaneously by both enthalpy and entropy driven.

Key Words: Adenosine deaminase, Theobromine, Inhibition, Isothermal titration calorimetry.

INTRODUCTION

Adenosine deaminase (ADA) is a monomeric protein (34.5 kDa), which catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine nuclcosides to their respective inosine derivatives nucleosides and ammonia[1]. Catalysis requires a Zn^{2+} cofactor[2]. This enzyme is present in virtually all human tissues, but the highest levels are found in the lymphoid system such as lymph nodes, spleen and thymus. 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[4, 5]. Higher level of ADA in the aiimentary tract and decidual cells of the developing

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fetal-maternal interface put ADA among those enzymes performing unique roles related to growth rate of cells, embryo implantation and other undetermined function[6, 7]. ADA is widely distributed in the brain and one important function of this enzyme is probably associated with regulation of the extracellular level of adenosine and 2′-deoxyadenosine in contact with cerebral blood vessels. The inhibition of adenosine deaminase in brain would allow an accumulation of adenosine, which would produce a 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[8].

ADA is a glycoprotein that consists of a single polypeptide chain of 311 amino acids. It was sequenced in 1984[9]. The primary amino acids sequence of ADA is highly conserved across species[10]. ADA has an (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[11]. 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[12–14]. 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[15, 16].

Understanding the interaction of ADA with its inhibitors and its substrates at molecular level will be important in the development of the next generation of pharmaceutical agents that act as inhibitors or substrates. Following our previous research[17–20], in this work the inhibitory effect of theobromine on the enzymatic reaction of ADA by spectrophotometry and calorimetric methods have been described. Theobromine ($C_7H_8N_4O_2$, MW = 180.17) is the principal alkaloid of the cacao bean, which contains 1.5–3% of the base. Also present in cola nuts and in tea.

EXPERIMENTAL

Adenosine deaminase (type IV, from calf intestinal mucosa) and theobromine were obtained from Sigma. The other related highest grade chemicals were ascertained from different chemical sources. The solutions were prepared in doubly distilled water.

Enzyme Assay: Enzyme activity was assayed by UV-Vis spectrophotometry, using a Shimadzu-3100 instrument, following the decrease in absorbance at 265 nm due to the conversion of adenosine to inosinc based on the Kaplan method[21], using the change extinction coefficient of –8400 M^{-1} cm^{-1} for adeno-

sine. The standard assay mixture had a final volume of 1 mL. The concentration of the enzyme in the assay mixture was 0.94 nM. Assays were carried out in 50 mM sodium phosphate buffer, pH = 7.5. Enzyme activities were measured over at least seven different concentrations of adenosine and the assays were repeated at least three times. Adenosine concentration range was between 0.4 and 2.8 K_m. Care was taken to use appropriate experimental conditions to keep enzyme reaction linearity during the first minute of the reaction.

Isothermal Titration Calorimetry: Isothermal titration microcalorimetric experiments were performed with a 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 from stainless steel. Theobromine solution (1 mM) was injected by use of a Hamilton syringe into a stirred calorimetric titration vessel, which contained 1.8 mL of enzyme solution, 20 μM, including 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 theobromine solution into the perfusion vessel was repeated 20 times and each injection included 50 μL reagent. 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 theobromine solution was measured as described above except ADA was excluded. Also, the heat of dilution of the protein solution was measured as described above except 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-theobromine interaction as previous reports[22-24]. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular weight of ADA was taken to be 34,500[25].

RESULTS AND DISCUSSION

Fig. 1 shows double reciprocal Lineweaver-Burk plots for ADA at different fixed concentrations of theobromine, at pH = 7.5 and 300 K. The value of V_{max} is unchanged by theobromine, but that the apparent Michaelis constant (K'_m) value is increased, this confirms the competitive inhibition of drug on ADA. The values of K'_m at any fixed concentration of theobromine were obtained from Fig. 1 and plotted vs. concentrations of inhibitor in the inset of the figure, named secondary plot, to obtain inhibition constant (K_I). Results are:

$$K_m = 39\ \mu M \qquad K_I = 311\ \mu M$$

The Michaelis-Menten constant (K_m) obtained from these experiments is identical with that of a previous report[18-20].

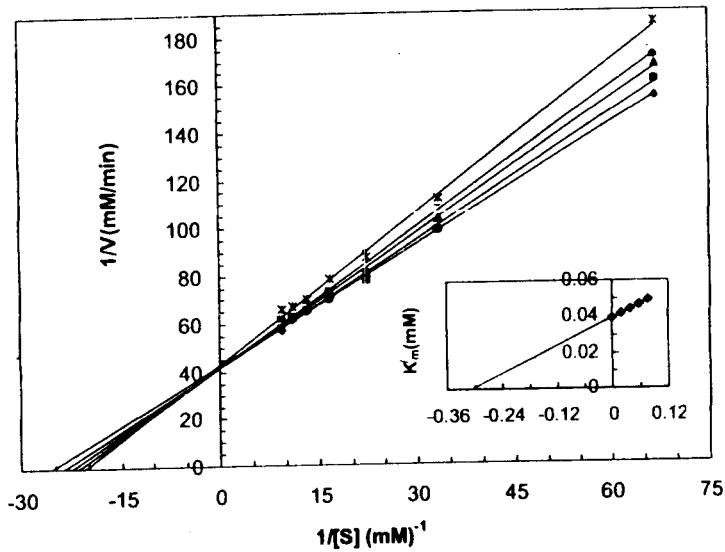

Fig. 1. Double reciprocal Lineweaver-Burk plots for kinetics of ADA at pH = 7.5 and T = 300 K in the presence of different fixed concentrations of theobromine: 0 μM (♦), 18.75 μM (■), 37.50 μM (▲), 56.25 μM (●) and 75.00 μM (*). In the inset a secondary plot of 1/[S]-axis intercepts versus [I] is shown; S and I are substrate and inhibitor, respectively.

By titration of a solution containing an enzyme (E) with a solution of inhibitor (I), the equilibrium reaction moves toward increasing concentration of EI complex The heat value of the reaction depends on the concentration of the EI complex. Thus, the reaction under consideration can be written:

$$EI \rightleftharpoons E + I \quad K_I = [E][I]/[EI] \tag{1}$$

and also

$$[I]_{total} = [I] + [EI] \tag{2}$$

$$[E]_{total} = [E] + [EI] = (K_I[EI]/[I]) + [EI] \tag{3}$$

Equation (2) can be solved for [I] and this then substituted into equation (3), which can then be rearranged to give a quadratic equation of which the only real root is:

$$[EI] = \{(B + K_I) - [(B + K_I)^2 - C]^{1/2}\}/2 \tag{4}$$

where

$$B = [E]_{total} + [I]_{total}, \quad C = 4[E]_{total}[I]_{total} \tag{5}$$

The sum of heat evolutions following the i-th titration step, q_i, can be expressed as

$$q_i = \Delta H^\circ V_i[EI]_i \tag{6}$$

where V_i is the volume of the reaction solution and ΔH° is the enthalpy of binding. Combination of equations (4) and (6) will lead to

$$\Delta H^\circ = 1/A_i\{(B_i + K_I) - [(b_i + K_I)^2 - C_i]^{1/2}\} \quad (7)$$

where

$$A_i = V_i/2q_i \quad (8)$$

A_i, B_i and C_i can be calculated in each injection; so equation (7) contains two unknowns, K_I and ΔH°. A series of reasonable values for K_I is inserted into equation (7) and corresponding values for ΔH° are calculated and a graph ΔH° vs. K_I is constructed. Curves of all titration steps will intersect in one point, which represents the true value for ΔH° and K_I[23, 24].

The data obtained from isothermal titration microcalorimetry of ADA interaction with theobromine is shown in Fig. 2. Figure 2a shows the heat of each injection and Fig. 2b shows the heat related to each total concentration of

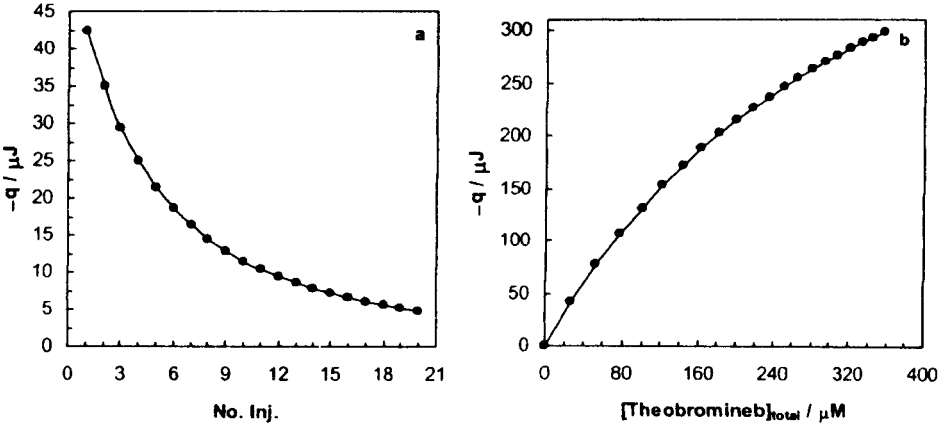

Fig. 2. (a) The heat of theobromine binding on ADA for 20 automatic cumulative injections, each of 50 μL, of theobromine solution, 1 mM, into the sample cell containing 1.8 mL ADA solution at a concentration of 20 μM. (b) The heat of binding vs. total concentration of theobromine, calculated from Fig. 2a.

theobromine. The plots of ΔH° vs. K_I, according to equation (7), for first 10 injections are shown in Fig. 3. The intersection of curves gives:

$$K_I = 318\ \mu M, \qquad \Delta H^\circ = -15.80\ kJ\ mol^{-1}$$

Good conformity of the dissociation binding constant (K_I) obtained from thermodynamic and kinetic studies is observed. Also, calorimetric measurements showed that the interaction between ADA and theobromine is an exothermic process.

The standard Gibbs free energy change of binding theobromine on ADA can be calculated using inverse K_I value as the association binding constant, $K = 1/K_I = 3144.7\ M^{-1}$, in equation (9):

$$\Delta G^\circ = -RT \ln K = -20.09\ kJ\ mol^{-1} \quad (9)$$

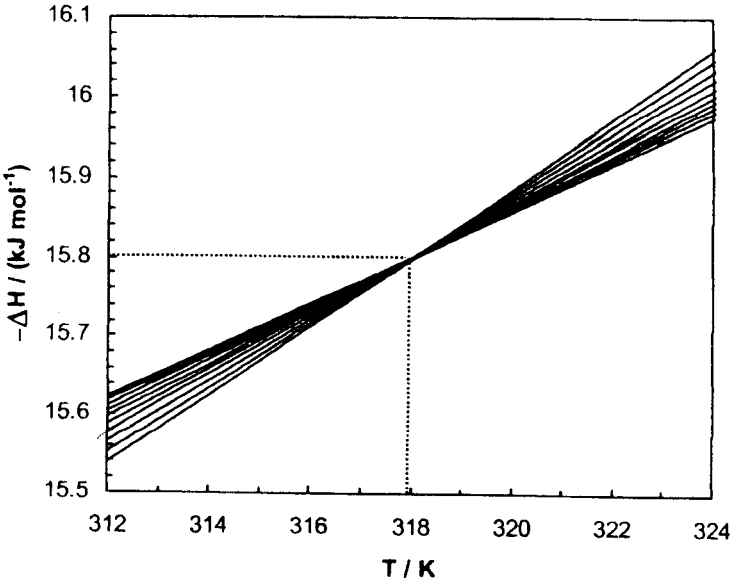

Fig. 3. $\Delta H°$ versus K_I for first 10 injections in the reasonable values of K_I, according to equation (7). The coordinates of intersection point of curves give true valuees for $\Delta H°$ and K_I.

Also the standard entropy change of binding theobromine on ADA can be calculated according to the equation (10):

$$\Delta S° = (\Delta H° - \Delta G°)/T = +14.30 \text{ J K}^{-1}\text{mol}^{-1} \quad (10)$$

Therefore, the drug binding process is both enthalpy and entropy driven. The negative $\Delta H°$ and positive $\Delta S°$ values cause binding process spontaneously.

Conclusion

It is concluded that ADA is competitively inhibited by theobromine at experimental condition. The exothermic process of theobromine binding to ADA leads to increase of inhibition constant when the temperature increases. Moreover, the interaction between ADA and theobromine is spontaneously by both enthalpy and entropy driven.

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