Acta Biochimica Polonica

Vol. 50 No. 3/2003

849–855

QUARTERLY

Inhibition study of adenosine deaminase by caffeine using spectroscopy and isothermal titration calorimetry✪

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Received: 22 April, 2003; revised: 14 July, 2003; accepted: 02 September, 2003

Key words: adenosine deaminase, caffeine, inhibition, calorimetry

Kinetic and thermodynamic studies were made on the effect of caffeine on the activity of adenosine deaminase in 50 mM sodium phosphate buffer, pH 7.5, using UV spectrophotometry and isothermal titration calorimetry (ITC). An uncompetitive inhibition was observed for caffeine. A graphical fitting method was used for determination of binding constant and enthalpy of inhibitor binding by using isothermal titration microcalorimetry data. The dissociation-binding constant is equal to 350 μM by the microcalorimetry method, which agrees well with the value of 342 μM for the inhibition constant that was obtained from the spectroscopy method. Positive dependence of caffeine binding on temperature indicates a hydrophobic interaction.

Adenosine deaminase (ADA, EC 3.5.4.4), a key enzyme in purine metabolism, catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively (Shanjukumar & Sharma, 2000). It is present in virtually all human tissues, but the highest levels are found in the lymphoid system such as lymph nodes, spleen and thymus (Cristalli *et al.,* 2001). Different studies have shown that ADA is not only a cytosolic enzyme, but can be found also as an ecto-enzyme (Franco *et al.,* 1997). In addition, ecto-ADA binds directly to at least three different cell surface molecules,

✪The financial support by the University of Tehran and Shahid Beheshti University of Medical Science is gratefully acknowledged.

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Abbreviations: ADA, adenosine diaminase; AIDS, acquired immunodeficiency syndrome; ITC, isothermal titration calorimetry; SCID, severe combined immunodeficiency disease.

human CD26 (a lymphocyte activation marker) and A1 and A2B adenosine receptors (Herrera *et al.,* 2001; Martin *et al.,* 1995). In humans and other mammals, ADA exists as two distinct isoenzymes, which have been termed ADA1 and ADA2 (Niedzwicki & Abernethy, 1991). A deficiency of ADA activity is associated with a form of severe combined immunodeficiency disease (SCID) (Giblett *et al.,* 1972; Valentine *et al.,* 1977), while a 40- to 70-fold increase of its activity is associated with a form of chronic nonspherocytic hemolytic disease (Daddona & Kelley, 1978). Enzyme abnormalities have been reported also in some leukaemia diseases (Murray *et al.,* 1985), in acquired immunodeficiency syndrome (AIDS) (Murray *et al.,* 1985; Wilson *et al.,* 1991), in tuberculosis (Banales *et al.,* 1999), in Parkinson's disease (Chiba *et al.,* 1995) and in stress (Miyahara *et al.,* 1998). It has been suggested that modulating ADA activity may be a target for chemotherapy. Therefore, ADA inhibitors may be used both as drugs and as codrugs in combination with certain anticancer or antivirial agents which are adenosine analogues (Glazer, 1980; Lee *et al.,* 1984).

ADA is a glycoprotein consisting of a single polypeptide chain of 311 amino acids. It was sequenced in the year 1984 (Daddona *et al.,* 1984). The primary amino-acids sequence of ADA is highly conserved across species (Chang *et al.,* 1991). Studies on the crystal structure of mouse ADA show that the protein is composed of an eight-stranded α/β motif with five additional α-helices, and the active site is located at the β-barrel COOH terminal end (Wilson *et al.,* 1991). The crystal structure has also revealed that ADA is a metalloenzyme that complexes one mole of Zn^{2+} per mole of protein. The zinc ion is located deep within the substrate binding 3 cleft and coordinated in a tetrahedral geometry to three Nε 2 atoms of His-15, His-17 and His-214, and the Oδ 2 of 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 group at the C-6 position of adenosine through a stereospecific addition-elimination mechanism. Experiments confirm that in the active enzyme, zinc plays a critical role in catalysis (Bhaumik *et al.,* 1993). It has been reported that secondary metal binding site(s) are available that allow some metal ions to inhibit the holoenzyme (Cooper *et al.,* 1997).

Caffeine, or 1,3,7-trimethylxanthine, is a purine alkaloid structurally related to uric acid (Arnaud, 1987); it is a key component of many popular drinks, most notably tea and coffee (Ashihara & Crozer, 2001). Caffeine has been applied to the removal of tannin from the reaction mixture, both in the presence or in absence of protein (Mejbaum-Katzenellenbogen *et al.,* 1959; Mejbaum-Katzenellenbogen & Dobryszycka, 1962). The procedure of precipitating proteins by tannin followed by subsequent regeneration by caffeine from protein–tannin compounds was found not to change the properties of the antigen–antibody reaction or the production of antibodies *in vivo*. The effects of exogenous adenosine are very often opposite to those of methylxanthines (Berne *et al.,* 1983). Methylxanthines such as caffeine, are both adenosine receptor antagonists and phosphodiesterase inhibitors. Caffeine has been shown to have effects on the leukocyte function (Gail *et al.,* 1995). The function of ADA is critical in controlling the effect of adenosine in many systems. Adenosine is an endogenous antihypoxic and anticonvulsant, as well as a modulator of platelet aggregation, lipolysis, glycogenolysis, blood flow, and neurotransmission (McIlwain, 1983; Stone, 1989). Therefore, modulation of ADA with the use of highly specific inhibitors might modify the action of endogenous adenosine under various physiological and pathological conditions (Agarwal, 1982; Centelles *et al.,* 1988). In this study, we investigated the inhibitory effect of caffeine on the enzymatic reaction of ADA at two temperatures by spectroscopy and calorimetric methods.

MATERIALS AND METHODS

Adenosine deaminase (type IV) from calf intestinal mucosa, adenosine and caffeine were obtained from Sigma. Other chemicals were of highest grade available from chemical sources. Solutions were prepared in doubly distilled water.

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 inosine based on the Kaplan method (Kaplan, 1955), using the change extinction coefficient of 8400 M^{-1} cm^{-1} for adenosine. 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 0.25 and 2.5 K_m. Care was taken to use appropriate experimental conditions to keep enzyme reaction linearity during the first minute of the reaction.

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 (multijunction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Caffeine solution (2 mM) was injected by use of a Hamilton syringe into a stirred calorimetric titration vessel, which contained 1.8 ml of enzyme solution, 0.75 mg/ml, 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 inosine solution into the perfusion vessel was repeated 25 times, and each injection included 15 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 inosine solution was measured as described above except enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of enzyme–caffeine interaction. The enthalpy of dilution of the enzyme is negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

In all calculations the relative molecular mass of ADA was taken to be 34 500 (Brady & O'Sullivan, 1967).

RESULTS AND DISCUSSION

Figure 1 shows double reciprocal Lineweaver-Burk plots for ADA at different fixed concentrations of caffeine, at pH = 7.5 and 27°C. Both values of maximum velocity

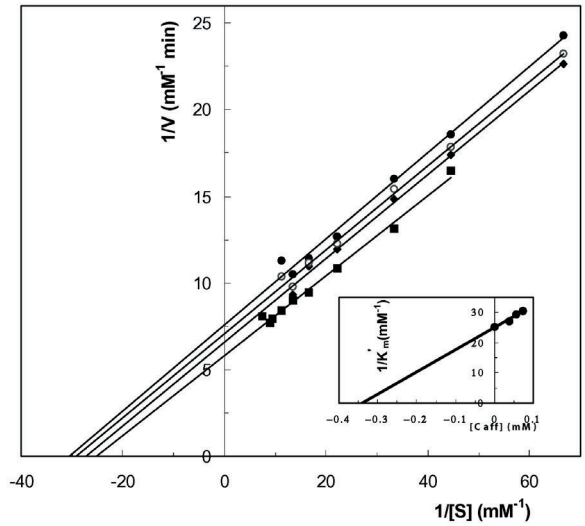

Figure 1. Double reciprocal Lineweaver-Burk plots for the kinetics of ADA at pH = 7.5 and 27°C in the presence of different fixed concentrations of caffeine: 0 μM (■), 37.50 μM (◆), 56.25 μM (○), and 75.00 μM (●).

In the inset a secondary plot of 1/[S]-axis intercepts *versus* [I] is shown; S and I are substrate and inhibitor, respectively.

(V_{max}) and apparent Michaelis constant (K'_m) are decreased by increasing the concentration of caffeine. This confirms the uncompetitive nature of the inhibition of ADA by caffeine. The values of K'_m at any fixed concentration of caffeine were obtained from Fig. 1 and plotted *versus* concentrations of caffeine in Fig. 1, named secondary plot, to obtain inhibition constant (K_I). At 27°C, the results are as follows:

$$K_m = 38\ \mu M \qquad K_I = 342\ \mu M$$

The Michaelis-Menten constant (K_m) obtained from these experiments is identical with that of a previous report (Moosavi-Movahedi *et al.,* 1993; Saboury *et al.,* 2002). Figure 2 shows double reciprocal Lineweaver-Burk plots for ADA at different fixed concentrations of caffeine, at pH = 7.5 and 37°C. At this temperature, the values of V_{max} and K'_m are also decreased by increasing the concentration of caffeine, which confirms the uncompetitive nature of the inhibition of ADA by caffeine. The results obtained from the secondary plot (shown in the inset of Fig. 2b) at 37°C are as follows:

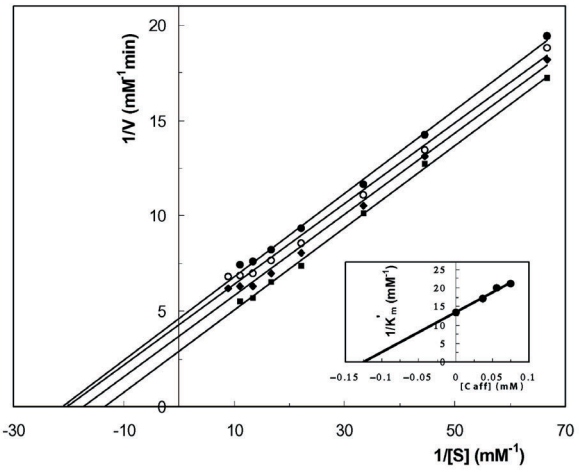

Figure 2. Double reciprocal Lineweaver-Burk plots for the kinetics of ADA at pH = 7.5 and 37°C in the presence of different fixed concentrations of caffeine: 0 μM (■), 37.50 μM (◆), 56.25 μM (○), and 75.00 μM (●).

In the inset a secondary plot of 1/[S]-axis intercepts *versus* [I] is shown; S and I are substrate and inhibitor, respectively.

$$K_m = 74.1\ \mu M \qquad K_I = 126\ \mu M$$

Increasing the temperature by 10°C leads to a decrease of K_I value, which means that caffeine binding to ADA is an endothermic process.

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:

$$EI \Leftrightarrow E+I \qquad K=[E][I]/[EI] \qquad (1)$$

and also

$$[I]_{total} = [I] + [EI] \qquad (2)$$

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

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:

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

where

$$B = [E]_{total} + [I]_{total} \qquad C = 4[E]_{total}[I]_{total} \qquad (5)$$

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

$$Q_i = \Delta H\ V_i\ [EI]_i \qquad (6)$$

where V_i is the volume of the reaction solution and ΔH is the enthalpy of binding.

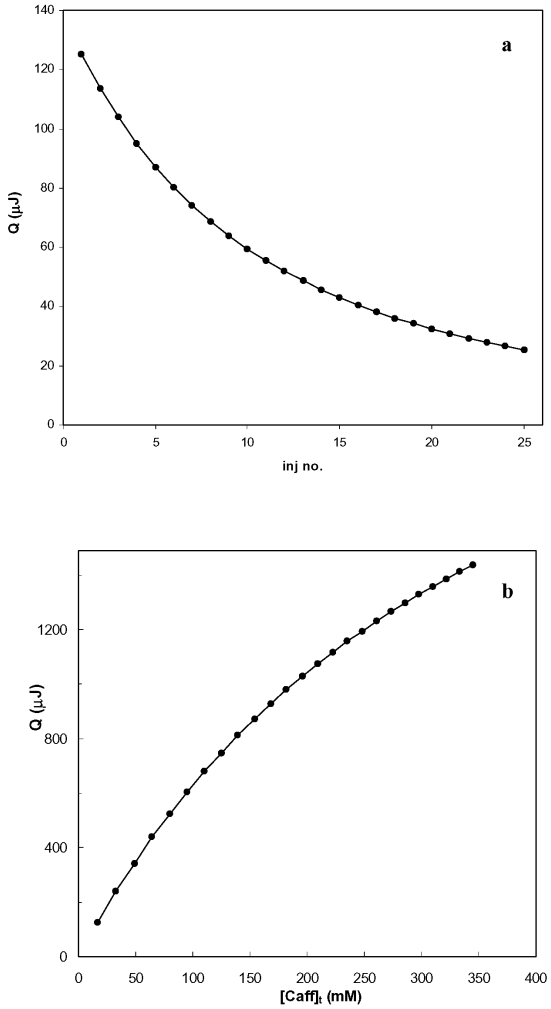

Figure 3. (a) The heat of caffeine binding to ADA for 25 automatic cumulative injections, each of 15 μl of caffeine solution, 2 mM, into sample cell containing 1.8 ml ADA solution at a concentration of 0.75 mg/ml (21.7 μM). (b) The heat of the binding *versus* total concentration of caffeine, calculated from Fig. 3a.

All calorimetric measurements were done at pH = 7.5 and 27°C.

Combination of equations (4) and (6) will lead to

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

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 unknown, K and ΔH. A series of reasonable value for K is inserted into equation (7) and corresponding values for ΔH are calculated and a graph H *versus* K is constructed. Curves of all titration steps will intersect in one point, which represents the true value for ΔH and K.

The data obtained from isothermal titration microcalorimetry of ADA interaction with caffeine is shown in Fig. 3. Figure 3a shows the heat of each injection and Fig. 3b shows the heat related to each total concentration of caffeine. The plots of ΔH *versus* K, according to equation (7), for first 8 injections are shown in Fig. 4. The intersection of curves gives:

$$K = 350\ \mu\text{M} \quad \Delta H = +75.0\ \text{kJ/mol}$$

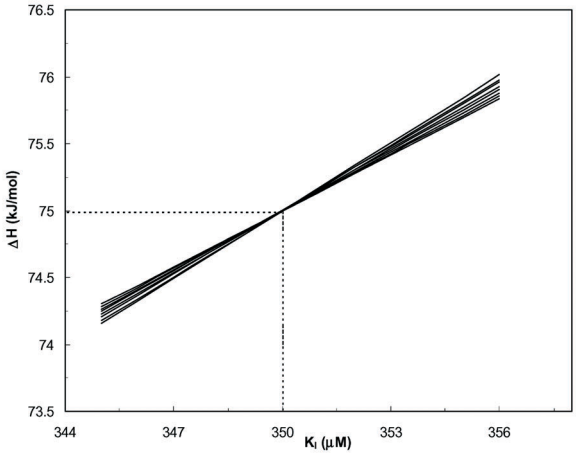

Figure 4. ΔH *versus* K for the first 8 injections at reasonable values of K, according to equation (7).

The coordinates of intersection point of curves give the true value for ΔH and K.

A good conformity of the dissociation binding constant (K) obtained from thermodynamic and kinetic studies is observed. Also, calorimetric measurements showed that the

interaction between ADA and caffeine is an endothermic process.

CONCLUSION

It is concluded that ADA is uncompetitively inhibited by caffeine at two temperatures of 27°C and 37°C. The endothermic process of caffeine binding to ADA leads to a decrease of inhibition constant when the temperature increases. Moreover, the interaction between ADA and caffeine might be a hydrophobic interaction, as increasing the temperature improves the binding.

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