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Kinetic and Structural Analysis of the Inhibition of Adenosine Deaminase by Acetaminophen

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Kinetic and thermodynamic studies have been made on the effect of acetaminophen on the activity and structure of adenosine deaminase in 50 mM sodium phosphate buffer pH 7.5, at two temperatures of 27 and 37°C using UV spectrophotometry, circular dichroism (CD) and fluorescence spectroscopy. Acetaminophen acts as a competitive inhibitor at 27°C (K_i = 126 μM) and an uncompetitive inhibitor at 37°C (K_i = 214 μM). Circular dichroism studies do not show any considerable effect on the secondary structure of adenosine deaminase by increasing the temperature from 27 to 37°C. However, the secondary structure of the protein becomes more compact at 37°C in the presence of acetaminophen. Fluorescence spectroscopy studies show considerable change in the tertiary structure of the protein by increasing the temperature from 27 to 37°C. Also, the fluorescence spectrum of the protein incubated with different concentrations of acetaminophen show different inhibition behaviors by the effector at the two temperatures.

Keywords: Adenosine deaminase; Acetaminophen; Competitive inhibition; Uncompetitive inhibition; Circular dichroism; Fluorescence

INTRODUCTION

Adenosine deaminase (ADA) is an enzyme indispensable to the purine metabolic pathway and the maintenance of a competent immune system. ADA is a monomeric protein (34.5 kDa), which catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine nucleosides to their respective inosine derivatives nucleosides and ammonia with a rate enhancement of 2×10^{12} relative to the nonenzymatic reaction.[1] Catalysis requires a Zn^{2+} cofactor.[2] The enzyme is widely distributed in vertebrates, invertebrates and mammals including humans. The 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.[3] 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 levels of ADA in the alimentary tract and decidual cells of the developing fetal-maternal interface put ADA among those enzymes performing unique roles related to the growth rate of cells, embryo implantation, and other undetermined functions.[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 vasodilation and increase in cerebral blood flow. Therefore the decrease in enzyme activity would potentiate the sedative actions of adenosine in interneuronal communication of the central nervous system.[8]

ADA is a glycoprotein, sequenced in 1984,[9] that consists of a single polypeptide chain of 311 amino acids. The primary amino acid sequence of ADA is

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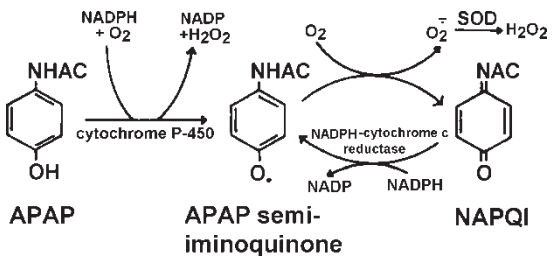
highly conserved across species.[10] ADA has a (α/β) barrel structure motif and the active site of ADA resides at the C- terminal end of the β barrel, in a deep oblong-shaped pocket; a penta-coordinated Zn^{2+} cofactor is embedded in the deepest part of the pocket. The zinc ion is located deep within 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 adenosine deaminase confirmed the essential role of these residues in catalysis.[12–14]

ADA can hydrolyze the substituent in the 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]

The widely used analgesic drug acetaminophen causes severe hepatic toxicity when ingested in large amounts.[17] The hepatotoxicity of acetaminophen might be due to the cytochrome P-450 mediated one-electron oxidation to the semi-iminoquinone radical.[18] This radical species might then redox cycle through *N*-acetyl-*p*-benzoquinone imine (NAPQI) leading to the formation of reactive oxygen radicals (Scheme 1). In model experiments, oxidation of acetaminophen by horseradish peroxidase led to the formation of a reactive intermediate, which could become covalently bound to proteins.[19,20] In this case the reactive species were concluded to be NAPQI and hydroquinone. Kinetic studies of the decomposition of NAPQI in aqueous solutions of acetaminophen indicated a comproportionation reaction leading to semi-iminoquinone radical formation.[21] In the absence of other reactive solutes the radical decays rapidly by second order kinetic with a rate constant of $(2.2 \pm 0.4) \times 10^9\ M^{-1}\ sec^{-1}$.[22] NAPQI can react with sulfhydryl groups such as GSH[23] and protein-thiols.[24] The covalent binding of NAPQI to cell proteins is considered the initial step in a chain of events eventually leading to cell necrosis.[25]



SCHEME 1

Understanding the interaction of ADA with its inhibitors and its substrates at molecular level is important in the development of the next generation of pharmaceutical agents that act as inhibitors or substrates. Following our previous research,[26,27] in this work we describe the kinetic and structural evaluation of the interaction of ADA with acetaminophen at two different temperatures.

MATERIAL AND METHODS

Materials

Adenosine deaminase (type IV, from calf intestinal mucosa), adenosine, and acetaminophen were obtained from Sigma. The other related chemicals, of the highest grade, were obtained from different industrial sources. The solutions were prepared in doubly distilled water.

Methods

Enzyme Assay

Enzymatic activities were assayed by UV-Vis spectrophotometry with a Shimadzu-3100 instrument, based on The Kaplan Method to follow the decrease in absorbance at 265 nm resulting from the conversion of adenosine to inosine.[28] This method uses the change in extinction coefficient of adenosine ($8400\ M^{-1}\ cm^{-1}$), on conversion to inosine by the catalytic activity of the enzyme. The concentration of enzyme in the assay mixture 50 mM sodium phosphate buffer, pH 7.5, was 0.94 nM with a final volume of 1 ml. Activities were measured using at least seven different concentrations of adenosine and the assays were at least in triplicate. The adenosine concentration range used is between 0.25–2.5 K_m. Care was taken to use experimental conditions where the enzyme reaction was linear during the first minute of the reaction.

Fluorescence Spectroscopy

Fluorescence spectroscopy was performed using a Hitachi fluorescence spectrophotometer model MPF-4 and a 1-cm path length fluorescence cuvette. The excitation wavelength was adjusted to 290 nm and emission spectra were recorded for all of the samples in the range of 300 to 400 nm. Samples of 5.78 μM ADA were in 50 mM standard phosphate buffer, pH 7.5. All spectra were normalized for protein concentration.

Circular Dichroism (CD) Measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan). The solutions for far-UV

investigations contained a constant concentration of acetaminophen (5 mM) as well as protein (0.25 mg/ml). The results were expressed in molar ellipticity [θ] (deg cm^2 dmol^{-1}) based on a mean amino acid residue weight of 111 (MRW). The molar ellipticity was determined as $[\theta]_\lambda = (100 \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 dmol^{-1},[29] and a JASCO standard non-hydroscopic ammonium, (+)-10-camphorsulfonate assuming $[\theta]_{290.5} = 7910$ deg cm^2 dmol^{-1}.[29] The noise in the data was smoothed by using the JASCO J-715 software. This software uses the fast Fourier-transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes. The JASCO J-715 software was used to predict the secondary structure of the protein according to the statistical method.[30,31]

RESULTS

Kinetic Studies

Figure 1 shows a double reciprocal plot for the ADA-adenosine system in which three different concentrations of acetaminophen (37.50–75.00 μM) are incubated with the enzyme-substrate complex at 27°C. The single crossover point indicates competitive inhibition of adenosine deaminase by acetaminophen due to the penetration of the inhibitor molecules (acetaminophen) into the active site of the enzyme. Inset of the figure depicts a plot of the apparent Michaelis constant, K_m', values against the concentrations of acetaminophen used leading to $K_i =$ 126 μM at 27°C.

Figure 2 shows a Lineweaver–Burk plot for the ADA–adenosine system where three different concentrations of acetaminophen (56.25–112.50 μM) are incubated with it at 37°C. The parallel lines of the figure illustrates the typical uncompetitive inhibition of ADA by acetaminophen at this temperature. This type of inhibition indicates that at 37°C the inhibitor molecules (acetaminophen) should be bound to ADA binding sites, remote from the enzyme's catalytic site. Inset of the Figure indicates a reciprocal plot of the apparent Michaelis constant, K_m', against the acetaminophen concentrations from which results $K_i = 214$ μM.

Structural Analysis

CD Investigations

Figure 3 shows the UV–CD spectra registered for native ADA at two different temperatures of 27 and 37°C. The two recorded spectra represent two minima at wavelengths of 222 and 210 nm. These two wavelengths can be utilized for monitoring the secondary structural alterations for the protein, which might occur as the conditions alter. It can be

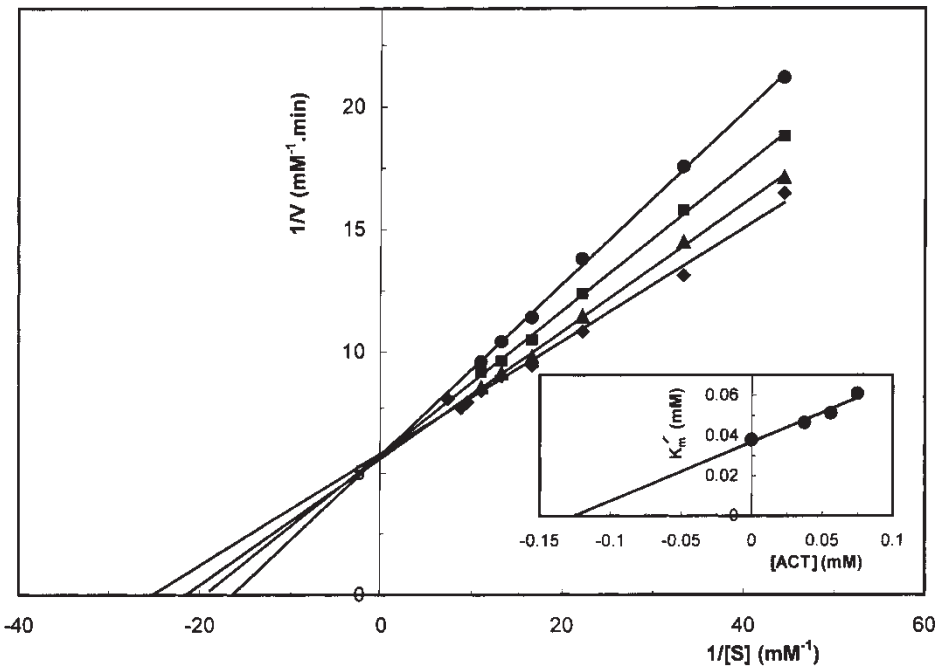


FIGURE 1 Double reciprocal Lineweaver–Burk plots for the kinetics of ADA at pH = 7.5 and T = 27°C in the presence of different fixed concentrations of acetaminophen: 0 μM (◆), 37.50 μM (▲), 56.25 μM (■), and 75.00 μM (●). In the inset a secondary plot of 1/[S] − axis intercepts versus [I] is shown; S and I are substrate and inhibitor, respectively.

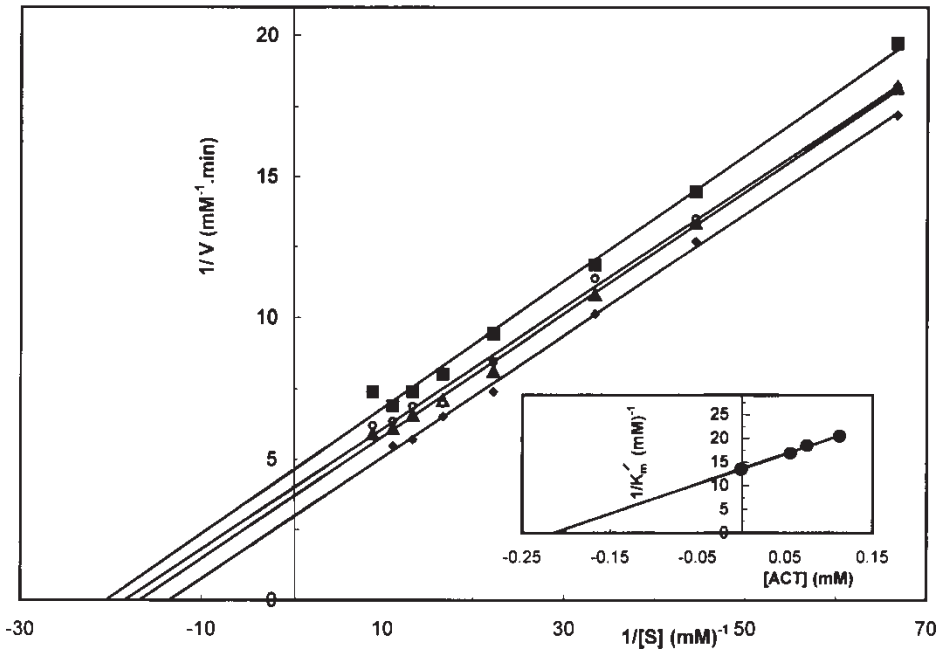


FIGURE 2 Double reciprocal Lineweaver–Burk plots for kinetics of ADA at pH = 7.5 and T = 37°C in the presence of different fixed concentration of acetaminophen: 0 μM (◆), 56.25 μM (▲), 75 μM (○), and 112.5 μM (■). In the inset a secondary plot, $1/K'_m$ versus [I]; K'_m and I are the apparent Michaelis constant and inhibitor, respectively.

seen that at 27 and 37°C, little difference is registered between the two spectra confirming the non-occurrence of any rigorous secondary structural alterations for the native enzyme at the two cited temperatures (Table 1). Alternatively, from Figures 4a and 4b it can be seen that incubation of the enzyme at a constant concentration of acetaminophen (5 mM) has induced more secondary structural alterations, especially at 27°C (see Table 1).

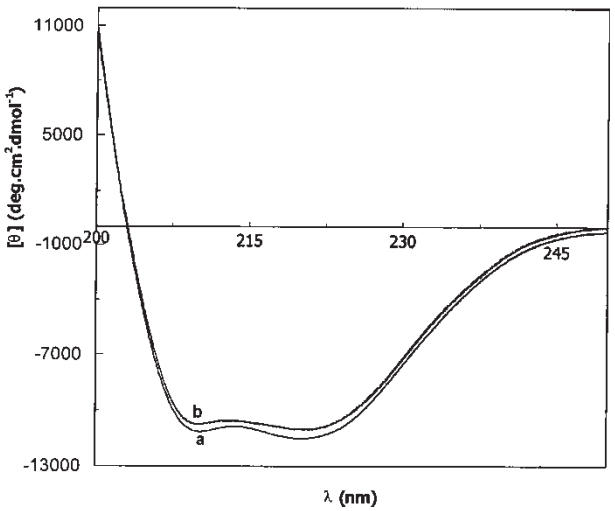


FIGURE 3 The UV–CD spectra for the native structure of ADA (0.25 mg/ml) at pH = 7.5 and two different temperatures of 27 (a) and 37°C (b).

Fluorescence Studies

Here, fluorescence technique is used for determination of temperature-induced conformational changes occurring on the ADA structure at the tertiary level. Figures 5a and 5b show the fluorescence spectra of the native as well as those three enzyme structures, formed upon incubation of the protein with three different concentrations of acetaminophen at 27 and 37°C. The fluorescence technique has revealed that in contrast with the structural constancy of the secondary structure of the native enzyme (Figure 3), the native structure of adenosine deaminase exhibits rigorous conformational alterations with a change in temperature from 27 to 37°C (Figures 5a and 5b). In fact, at 27°C the intensity of the intrinsic fluorescence recorded for native ADA is about 50 a.u. (Figure 5a) which has been changed to about 30 a.u. at 37°C (Figure 5b). Interestingly, it can be seen that similar to the findings of the CD investigations, incubation of acetaminophen with adenosine deaminase can induce tertiary structural alterations that are manifested as the fluorescence fluctuations recorded at the maximum point of the spectra near 350 nm. The pattern of the fluorescence fluctuations are ompletely different for the temperatures 27 and 37°C showing the different effects of acetaminophen on the enzyme's structure at the two cited temperatures. For example, it can be seen that at 27°C the most fluorescence quenching is recorded for an acetaminophen concentration of 37.5 μM whereas at 37°C the greatest quenching occurrs at an acetaminophen

TABLE 1 Alteration of secondary structure of ADA, incubated in 5 mM concentration of acetaminophen (ACT), at two temperatures of 27 and 37°C

Secondary Structure	ADA (27°C)	ADA + ACT (27°C)	ADA (37°C)	ADA + ACT (37°C)
Helix	44.0	37.6	42.9	40.2
β Structures	13.7	2.5	13.5	18.4
Random coil	42.4	59.9	43.6	41.4

concentration of 56.25 μM. Moreover, at 37°C little shifts are registered at the maximum point of each fluorescence spectra (Figure 5b). These shifts are absent in the recorded fluorescence spectra at 27°C in agreement with our hypothesis concerning the different site of action for acetaminophen on the ADA structure at the two temperatures of 27 and 37°C.

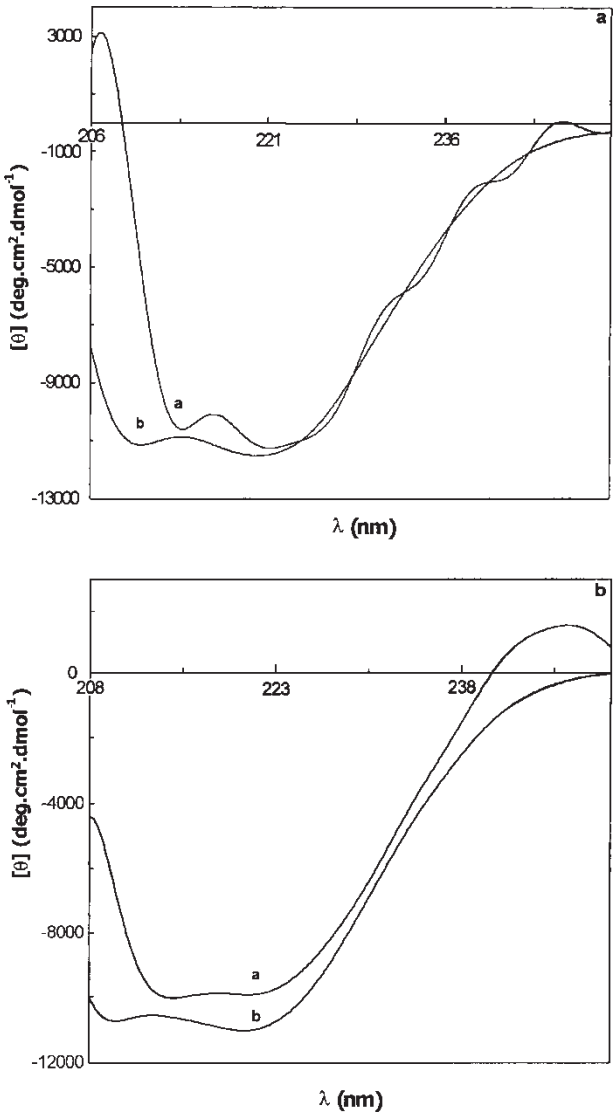


FIGURE 4 (a) The UV–CD spectra for the native structure of ADA (0.25 mg/ml) at pH = 7.5 and 27°C (a) in the presence of a fixed concentration of acetaminophen 5 mM and (b) in the absence of acetaminophen. (b) The UV–CD spectra for the native structure of ADA (0.25 mg/ml) at pH = 7.5 and T = 37°C (a) in the presence of a fixed concentration of acetaminophen (5 mM) and (b) in the absence of acetaminophen.

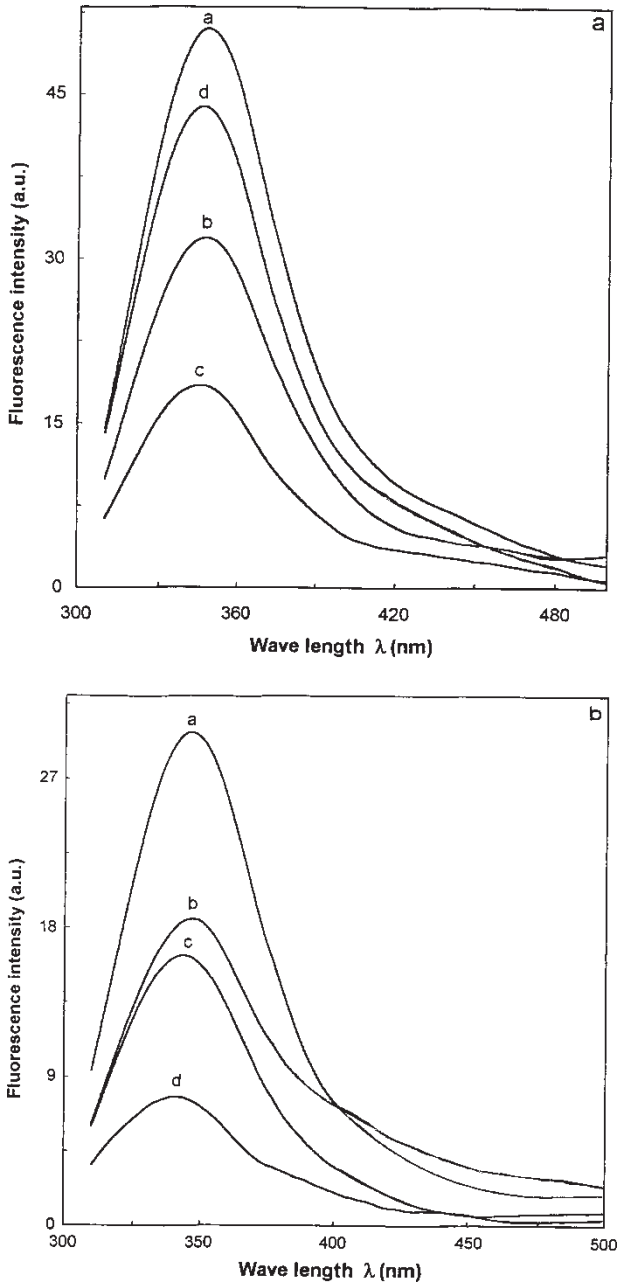


FIGURE 5 (a) The fluorescence spectra of ADA (0.94 nm) at pH = 7.5 and T = 27°C in the presence of different fixed concentrations of acetaminophen: 0 μM (a), 37.50 μM (b), 56.25 μM (c), and 75.00 μM (d), after dialysis versus phosphate buffer. (b) The fluorescence spectra of ADA (0.94 nm) at pH = 7.5 and T = 37°C in the presence of different fixed concentrations of acetaminophen: 0 μM (a), 37.50 μM (b), 56.25 μM (c), and 75.00 μM (d), after dialysis versus phosphate buffer.

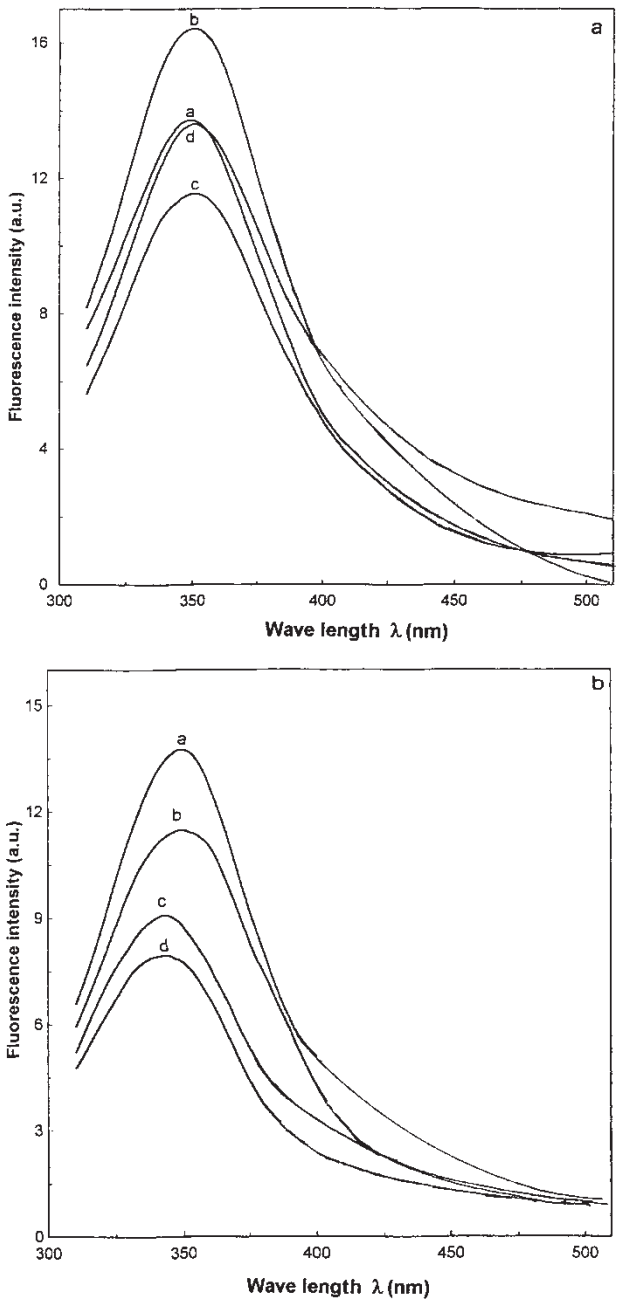


FIGURE 6 (a) The fluorescence spectra of ADA (0.94 nm) at pH = 7.5 and T = 27°C in the presence of different fixed concentrations of acetaminophen: 0 μM (a), 37.50 μM (b), 56.25 μM (c), and 75.00 μM (d), after dialysis versus phosphate buffer containing β-mercaptoethanol (12.5 mM). (b) The fluorescence spectra of ADA (0.94 nm) at pH = 7.5 and T = 37°C in the presence of different fixed concentrations of acetaminophen: 0 μM (a), 37.50 μM (b), 56.25 μM (c), and 75.00 μM (d), after dialysis versus phosphate buffer containing β-mercaptoethanol (12.5 mM).

Interestingly, fluorescence studies for those ADA structures which are made in the presence of β-mercaptoethanol as well as with different concentrations of acetaminophen manifested different fluctuation patterns confirming a different site of action for acetaminophen molecules at the two temperatures of 27 and 37°C (Figures 6a and 6b).

Defining the Role of the Cysteinyl Residues

Here, the possible effect of acetaminophen on the enzyme's cysteinyl residues has been checked using the chemical reagent β-mercaptoethanol. This reagent is commonly used as a potent reducing agent to reduce the oxidized form of the protein sulfydryl groups. Adenosine deaminase contains five cysteinyl residues, two of which are placed near the active site of the enzyme (Cys 262 and Cys 153).[32] Table 2 shows that dialysis of the native enzyme (in the absence of acetaminophen) against a buffer containing β-mercaptoethanol is followed by a significant decrease in the enzyme's activity. This decrement of activity can be seen clearly by comparing the V_{max} values of 0.108 and 0.117 mM min^{-1} (after dialysis in the presence of β-mercaptoethanol) with those equivalent values of 0.138 and 0.168 mM min^{-1} (before dialysis in the absence of β-mercaptoethanol), which are obtained at 27 and 37°C, respectively. The cited finding indicates the important structural or functional role of the cysteinyl residues especially in their oxidized forms. Performing of the same dialysis of ADA in the presence of β-mercaptoethanol as well as different concentrations of acetaminophen (37.5–75 μM) has revealed that at the two temperatures of 27 or 37°C there is no additional decrement for the enzyme activity in comparison with the individual existence of β-mercaptoethanol confirming the absence of any effect exerted by acetaminophen on the cysteinyl resides (Table 2). The concentration of the enzyme was checked in all samples before and after dialysis and there was not any rigorous difference between them. Moreover, the "Wilcoxon signed ranks test" statistical examination performed by an SPSS program has been applied to check the meaningful difference between all column values in Table 2. The P value for each column has been calculated and it is less than 0.05 showing the significant difference between the columns values.

DISCUSSION

Regarding the kinetic data, it can be concluded that at two different temperatures of 27 and 37°C two tertiary conformations exist for ADA. In fact, the inhibition of ADA by acetaminophen is competitive and uncompetitive at 27 and 37°C, respectively confirming the existence of two possible conformers for ADA (Figures 1 and 2). Alternatively, two enzyme conformers are also in keeping with two different fluorescence fluctuation patterns at 27 and 37°C (Figures 5a and 5b). With respect to these figures it can be seen that when the acetaminophen concentration is changed between the range of 0–75 μM, at 27°C the lowest and the highest

TABLE 2 Comparison of ADA activity at two different temperatures of 27 and 37°C in buffer and buffer containing β-mercaptoethanol

[ACT] (μM)	V_{max} (mM/min) Buffer 27°C (Before dialysis)	V_{max} (mM/min) Buffer 27°C (After dialysis)	V_{max} (mM/min) Buffer 37°C (Before dialysis)	V_{max} (mM/min) Buffer 37°C (After dialysis)	V_{max} (mM/min) Buffer + Mercaptoethanol 27°C (After dialysis)	V_{max} (mM/min) Buffer + Mercaptoethanol 37°C (After dialysis)
0.00	0.138	0.120	0.168	0.125	0.108	0.117
37.50	0.125	0.114	0.156	0.131	0.109	0.115
56.25	0.117	0.118	0.133	0.130	0.105	0.117
75.00	0.110	0.115	0.120	0.137	0.105	0.120

Note: Each value in the Table is an average from two experimental results. The maximum standard error for these values was ± 0.003. Statistical calculations for significance performed by an SPSS program to check the meaningful difference between all column values shows less than 0.05 for the P value.

fluorescence spectra are obtained for the acetaminophen concentrations of 37.5 and 0 μM, respectively. But, on the other hand, at 37°C the lowest and the highest fluorescence spectra are obtained at the acetaminophen concentrations of 56.25 and 37.5 μM, respectively. It should be noted here that at the zero concentration of acetaminophen the recorded fluorescence intensity for native ADA at the two temperatures of 27 and 37°C are completely different indicating the presence of two ADA conformers even in the absence of any acetaminophen molecules (Figures 5a and 5b). Regarding Figure 5b it can be seen that beside the difference in the fluorescence fluctuation patterns there are also little shifts in the maximum point of the fluorescence spectra at 37°C when compared to those at 27°C in which no equivalent shifts are registered. This finding shows the essential difference in the acetaminophen-binding site on ADA at 37°C, which was not exposed to acetaminophen molecules at 27°C. In terms of the β-mercaptoethanol experiment it can be deduced that the cysteinyl residues in the structure of ADA did not show any rigorous influence upon the interaction with acetaminophen because the decrement in enzyme velocity (with respect to the velocity decrement in the presence of β-mercaptoethanol) has not been changed significantly in the absence or presence of acetaminophen (Table 2).

Acknowledgements

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