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Full Length Research Paper

Study of sensitivity and ability of adenosine deaminase in response to pre-unfolding and especially pathological temperatures via changing the enzyme structure and activity

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Adenosine deaminase (ADA) is an important enzyme of the purine metabolic pathway, which catalyzes the conversion of adenosine and deoxyadenosine to their respective inosine derivatives plus ammonia, in a rapid and irreversible reaction. In this work, we studied the structural and kinetic properties of bovine ADA as a function of temperature in the range, 20 - 80°C by circular dichroism (CD) and UV-spectrophotometric techniques, as well as by measuring its activity in this temperature range. The results suggest that thermal unfolding of ADA occurs at temperatures above 60°C, while the enzyme undergoes detectable conformational changes during pre-unfolding heating. These changes affect the kinetics of reaction catalyzed by ADA. The relation between enzyme activity and structural changes is discussed.

Key words: Adenosine deaminase (ADA), UV-Vis spectrophotometry, Conformational changes, Circular dichroism (CD), Kinetic study.

INTRODUCTION

Adenosine deaminase (ADA, EC 3.5.4.4) is a $(/)_8$ enzyme, which deaminates (deoxy)adenosine to (deoxy)inosine irreversibly (Cristalli et al., 2001). ADA is widely distributed in mammalian tissues and its role is critical in proliferation, maturation and function of lymphoid cells (Cristalli et al., 2001; Harutyunyan et al., 2005). Adenosine modulates the immune system and inhibits as inflammation via reduction of cytokine biosynthesis and neutrophil functions; therefore, ADA is involved in inflammation process. Its role in inflammation and malignancy has been studied experimentally and the results have been showed that ADA enzyme may play a role in inflammatory diseases of the pancreas (Ibis et al., 2007). Inhibition of adenosine deaminase results in a significant attenuation of intestinal inflammation (Antonioli et al., 2007). Aberrations in the expression and function of ADA have been implicated in several disease states such as severe combined immune deficiency (SCID), which is characterized by impaired B- and T-cell-based immunity resulting from an inherited deficiency in ADA (Cristalli et

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al., 2001). Mammalian ADA, like murine ADA and especially bovine ADA, are very similar to human ADA. The similarity of bovine ADA and its human counterpart is ~98.6%; therefore, it is reasonable to assume that these two enzymes behave very similarly. Since the three-dimensional structure of murine (Wilson et al., 1991) and bovine ADA (Terasaka et al., 2004) are highly similar, it is generally assumed that the human counterpart is also of the same structure.

Binding and kinetic properties of ADA have been intensely studied with the interaction of its substrates and inhibitors (Cristalli et al., 2001). Some groups (Lucacchini et al., 1982; Castro et al., 1998; Ford et al., 2000) have also investigated the mutual effects of these interactions and ADA conformers (Lucacchini et al., 1982; Castro et al., 1998; Ford et al., 2000). However, relatively few studies have ever considered the thermodynamic and structural properties of mammalian ADA.

Anderson and Britt (2002) have reported detailed equilibrium unfolding thermodynamic analyses for ADA conformers. They suggested that ADA undergoes an abrupt conformational transition at ~29ºC. Moreover, Bodnar and Britt (2006) studied the conformational change of ADA by differential scanning calorimetry; they suggested that this conformational change has an unfolding quality that appears to be on the direct path to the physiological-temperature conformer. Cooper et al. (1997) demonstrated that divalent cations are important for ADA activity, but their removal does not influence its structural and binding characteristics. Finally, Shu and Frieden (2004, 2005) addressed the subject of ADA structure/stability-activity relationship with the application of ^{19}F NMR spectroscopy for a variety of conditions and mutants.

Here, the structural and kinetic propertie of ADA in the temperature range of 20^{o} –80°C was studied. The results suggest that detectable conformational changes occur in the structure of ADA during pre-unfolding heating that affect the kinetics of reaction catalyzed by ADA.

The main aim of this study is assessment of ADA sensitivity in response to pathological temperatures of body via switching of the enzyme to a more active structure.

MATERIALS AND METHODS

Bovine adenosine deaminase was purchased from Sigma (USA). Other substances were of reagent grade and were purchased from Merck (Germany) . If not indicated, phosphate buffer (10 mM, pH=7.0) was used in the study.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter (Japan). Each experiment was repeated three times to ensure the reproducibility of the results. Results are expressed as ellipticity, θ (degree cm^{2}dmol^{-1}), based on a mean amino acid residue weight (MRW). This value was assumed to be 113 Da for ADA. The molar ellipticity was determined as:

$$\theta_\lambda = (\theta \quad 100\ MRW/cl) \qquad (1)$$

Where c is the protein concentration in mM, l is the length of light path in cm and θ is the measured ellipticity in degree at a given wavelength. The data was smoothed using the Jasco J-715 software (version 1.10.02), which includes a fast Fourier-transform noise reduction routine. The concentration of the protein solution was 0.3 mg/ml for CD experiments. Tris buffer, 50 mM, pH 7.5, was used for these experiments. Percentage of secondary structures was calculated using CDSSTR within the CDPro software package (this software is available at http://lamar.colostate.edu/sreeram/CDpro) (Sreerama et al., 2000).

UV- spectrophotometric study

The UV-Visible spectra of ADA were recorded on Unico spectrophotometer (USA). The spectra from 250 nm to 300 nm were read at different temperatures ranging from 20 to 50°C. ADA was incubated for 5 min at each temperature in Tris buffer, 50 mM and pH 7.5.

Enzyme activity assay

Enzyme activity analyses were performed by a UV 3100 recording spectrophotometer (Shimadzu, Japan) . The assay was based on the decrease in the absorbance of the reaction mixture at 265 nm due to the conversion of adenosine to inosine (Kaplan, 1955). The standard assay mixture had a final volume of 1 ml. The reaction mixtures contained 1.5×10^{-2} units of ADA and 0.112 mM adenosine in phosphate buffer. One enzyme unit was considered as the amount of enzyme which is able to catalyze 1 mol adenosine per minute. The buffer and enzyme solutions were pre-incubated at the assay temperature for 5 min. Each assay was repeated three times.

RESULTS

CD spectroscopy results suggest structural transitions for ADA before denaturation

In order to investigate the relation between structure of ADA and temperature, the far-UV CD spectra of this enzyme in the temperature range of 20 – 80°C were obtained. The CD spectra in the temperature ranges of 20 – 60°C and 60 – 80°C are illustrated in the Figures 1 and 2, respectively. Clearly, the CD spectra in the Figure 1 undergo complex changes and show “fluctuations” with increasing temperature. This behavior is not easy to interpret. CD spectra in the Figure 2, however, show more regular variations by the temperature change. The ambiguous thermal behavior of ADA is studied by analysis of $_{222nm}$ as a function of temperature because the alpha helix changes are well detectable in the 222nm (Figure 3). By comparison Figures 1, 2 and 3, it appears that denaturation process occurs above 60°C, while ADA structure undergoes some conformational changes below this temperature. The amounts of secondary structure of ADA in the studied temperatures are tabulated in the Table 1.

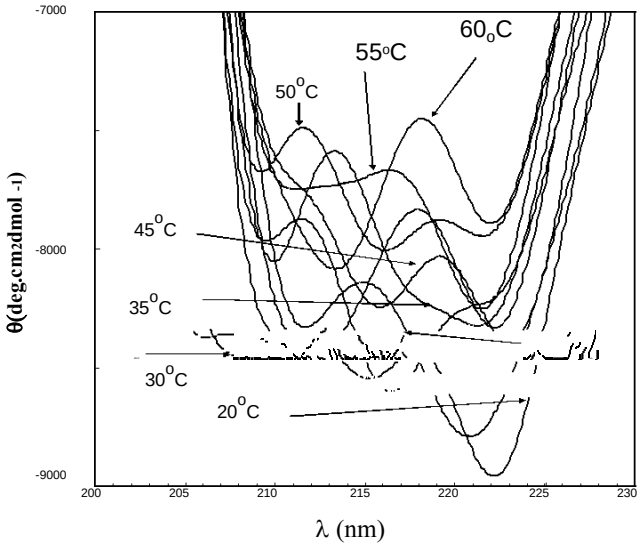

Figure 1. The Far- UV CD spectra of ADA in the range of 20-60°C in 50 mM Tris buffer, pH 7.5.

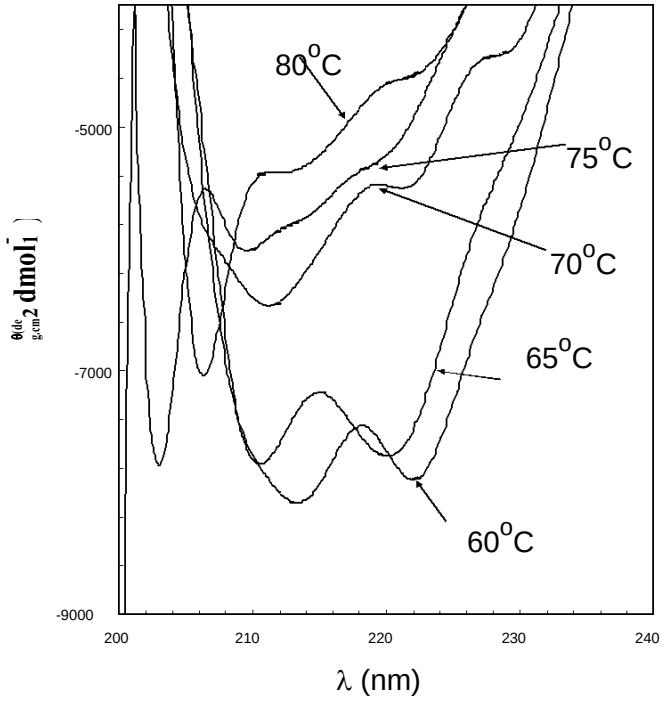

Figure 2. The Far-UV CD spectra of ADA in the range of 60 - 80°C in 50 mM Tris buffer, pH 7.5.

UV-Visible spectroscopy results suggest structural transitions

For better analysis of structural changes of ADA in temperatures below 60°C, UV-Vis spectrophotometric spectra were obtained in this range (Figure 4). Absorbance of the protein at 280 nm (the absorbance of

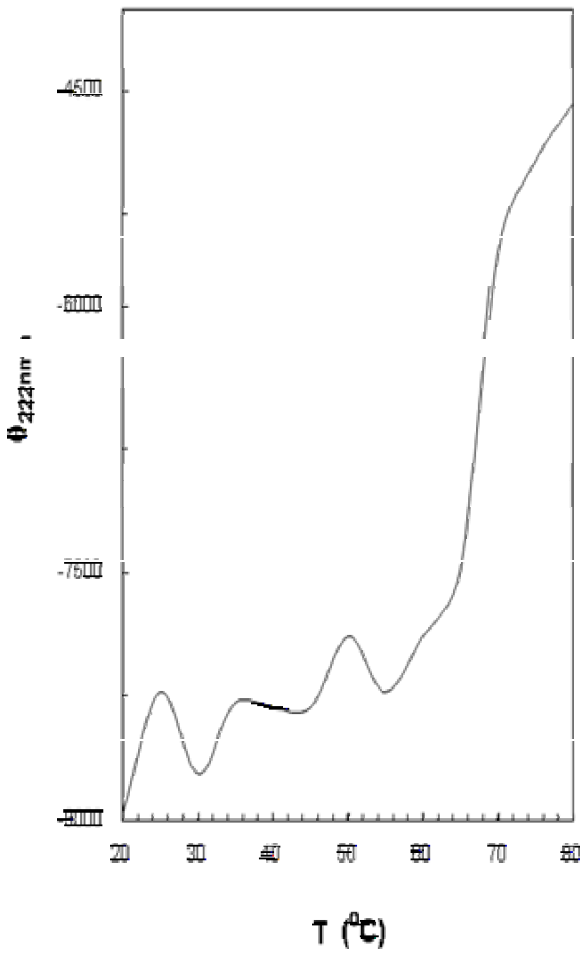

Figure 3. CD parameter of ADA at 222nm as a function of temperature.

aromatic amino acids in proteins) was recorded in the applied temperatures (Figure 5A). Like the CD spectroscopy experiment, small but reproducible structural transitions of ADA are observed here.

Arrhenius plot shows jumps that coincide with structural transitions

We assumed that conformational changes detected by CD and also UV-Vis spectroscopy may affect ADA activity. In order to investigate this probable effect, ADA activity was evaluated as a function of temperature (Figure 6) . According to Figure 6, ADA specific activity increases with arising temperature until 60°C and decreases above 60°C. Figure 7 illustrates the Arrhenius plot for this experiment, in which natural logarithm of activity is plotted versus the inverse of absolute temperature.

In an Arrhenius plot when the data points lie on a line, the slope is equal to:

Table 1. Percentage of ADA secondary structures at 20 - 80°C range of temperature in Tris buffer 50 mM, pH 7.5.

Temperature, °C -	Helix percentage -	structure percentage
25	38±0.03	21±0.02
30	33±0.03	21±0.02
35	36±0.02	22±0.03
40	35±0.04	22±0.02
45	31±0.03	22±0.03
50	37±0.02	19±0.03
55	32±0.04	22±0.03
60	33±0.04	24±0.03
65	35±0.05	22±0.04
70	20±0.06	25±0.06
75	17±0.08	
80	3±0.50	

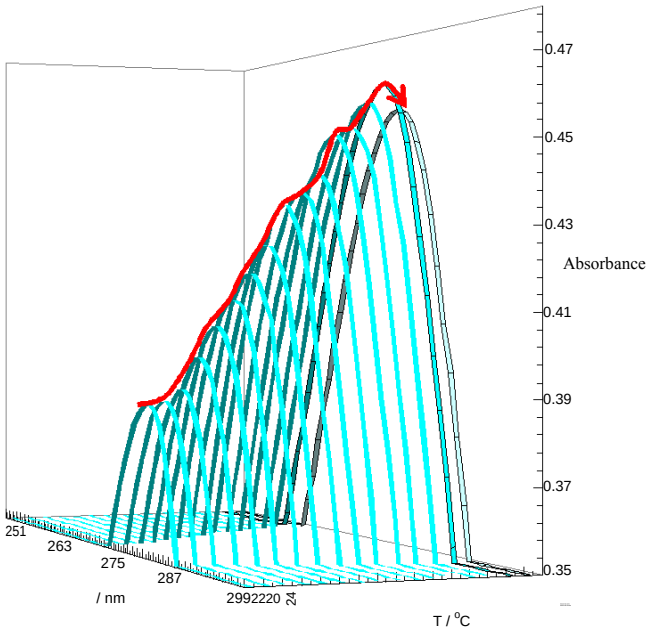

Figure 4. ADA UV spectra in range of 20-50 °C in Tris buffer, 50 mM, pH 7.5.

$$m = -\Delta E_a / R \qquad (2)$$

Where ΔE_a is the activation energy of reaction and R is the universal gas constant. Kinetic constant, k , can be calculated as:

$$k = \exp(-\Delta E_a / RT) \qquad (3)$$

In Figure 7, two major jumps are observed during 40 - 45°C and 53 - 56°C transitions. Kinetic parameters for the three linear segments in Figure 7 are shown in Table 2.

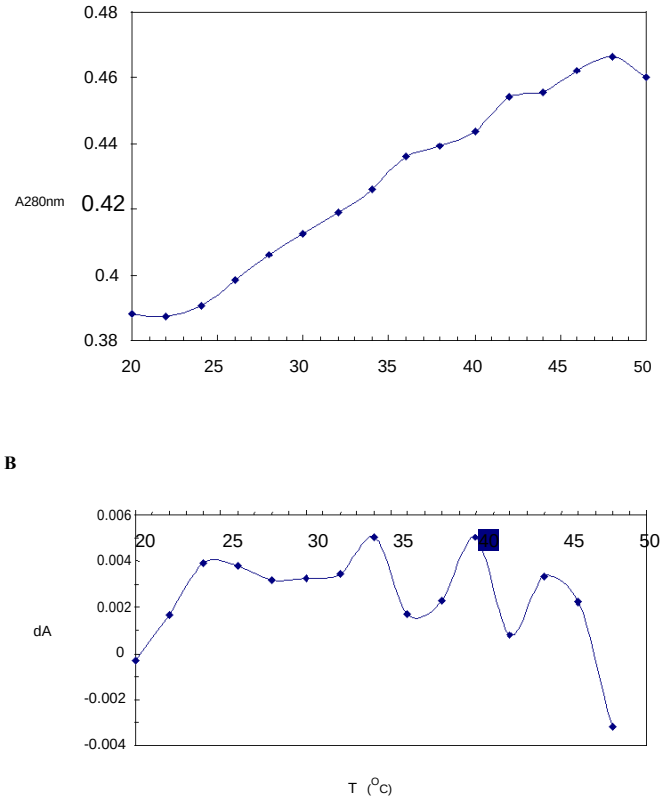

Figure 5. (A) ADA absorbance in 280 nm in the range of 20 - 50°C in 50 mM Tris buffer, pH 7.5; (B) ADA absorbance changes in 280 nm (the amount of absorbance at certain degree of temperature minus absorbance of the preceding temperature) in the range of 20 - 50°C in 50 mM Tris buffer, pH 7.5.

DISCUSSION

As it is depicted in the Figures 2 and 3, the regular changes of CD parameter and spectra at above 60°C can

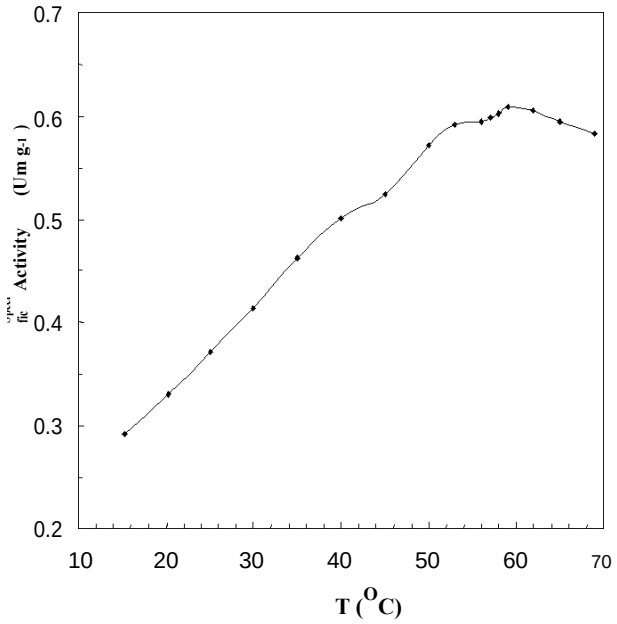

Figure 6. Activity profile of ADA as a function of temperature.

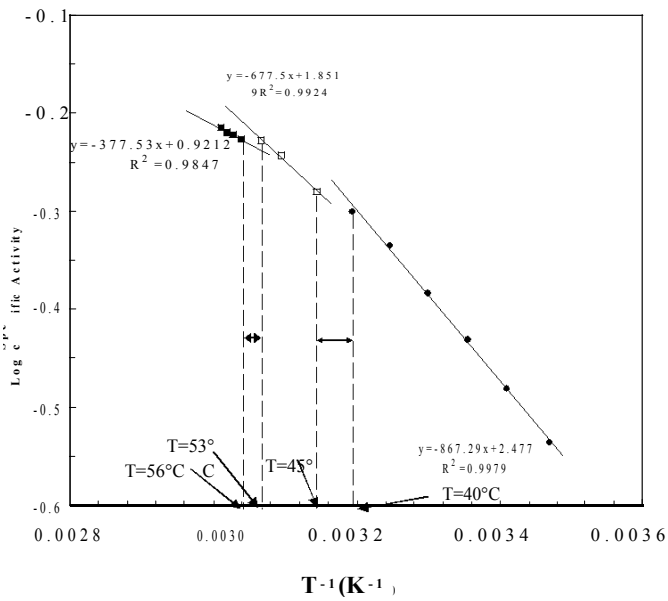

Figure 7. Arrhenius plot as the logarithm of variation of ADA activity as a function of inverse of absolute temperature.

be interpreted as occurrence of protein denaturation. Here we assumed that the irregular fluctuations, in the CD spectra and also $_{222nm}$ for ADA in the range of 20 - 60°C of temperature as pre-unfolding temperatures, refer to the conformational changes (Figures 1 and 3). Pre-unfolding temperatures are some temperatures that ADA molecule does not denature. These temperatures may not be temperatures of human body. According to this study, ADA does not denature up to 60°C, therefore by 60°C are defined as pre-unfolding temperatures.

It is previously reported that a transition occurs at ~29°C in the structure of bovine ADA (Bodnar and Britt, 2006). In addition, at ~30°C coformycin binding to bovine ADA is slightly higher than its expected value based on a smoothed trend (Castro and Britt, 1998). Moreover, in a study by intrinsic fluorescence spectroscopy, Anderson and Britt (2002) reported conformational change in bovine ADA during the 27-31°C transition. All these observations are consistent with the first conformational change observed in this study. It is shown that amount of helicity changes about 5% for 25-30°C transition (Table 1) and 30°C is a critical point in the Figure 3. If we adopt 30°C as the critical point for structural transition, by considering Figure 3 and Table 1, the 40 - 45°C and also 50 - 55°C transitions might be critical points for conformational changes of ADA. Previously it was reported (Rezaei-Tavirani et al., 2006) that such fluctuations in the CD experiments refer to the conformational change for human serum albumin at the pre-denaturation temperatures.

The UV-Visible spectra are also in line with the above hypothesis. Heating up to 48°C results in a gradual increase of the protein light absorbance (Figure 4). A closer look at the absorbance values at 280nm, however, reveals small fluctuations in the trend of absorbance increase as a function of temperature (Figure 5A). Plotting the absorbance change at each temperature point relative to its preceding temperature point (Figure 5B) can show the fluctuation with better resolution. It should be noted that a drop in dA value in curve in Figure 5B, shows a sudden decrease in the rate of protein conformational change. This is presumably equivalent to entering a new (semi)stable state. In other words, the system has just passed a conformational transition.

There are three obvious local minima in Figure 5B. The first one occurs at ~28°C, which is consistent with the detected transitions in other studies (Castro and Britt, 1998; Anderson and Britt, 2002; Bodnar and Britt, 2006) and in our CD experiments. The second minimum occurs about the physiological temperature, that is ~36°C. This means that ADA is in a relatively stable state at this temperature. The last observed minimum of this curve occurs at 42°C, which is the temperature of pathogenic fever. This point coincides with the 40 45°C transition observed in CD studies. This observation suggests a special role for ADA when facing the pathogenic fever conditions. Interestingly, this critical transition is also reflected in the activity change of ADA (see below).

Figure 6 shows that with the increase in temperature up to 60°C, ADA activity increases, while above 60°C, ADA activity decreases. Basically, for most enzymes, increasing activity is observed below a certain temperature threshold, and it is followed by a decrease in the enzyme activity due to the beginning of thermal protein denaturation (van Holde et al., 1998). Note that the increase in ADA activity does not show a smooth trend. To study

the changes that occur in the activity increase of ADA, its corresponding Arrhenius plot is given in Figure 7.

Nonlinearity in Arrhenius plot can appear as a curvature (Allen et al., 1990), a break (Massey et al., 1966), or a jump (Biosca et al., 1983). In general, temperature-induced conformational changes in soluble enzymes and/or phase changes in proteins result in a break or a jump in Arrhenius plot. In Figure 7, the Arrhenius plot is a nonlinear curve. Clearly, it has no considerable curvature, but it seems that it can be considered as a combination of at least three linear parts which are characterized by their specific equations and slopes. Two major jumps are observed during 40 45°C and 53 56°C transitions. These two transitions are in agreement with the second and third transitions in the CD spectra. The 40 45°C transition is in agreement with the observed jump in UV-Visible spectrum. It should be noted that the observed activity decreases above 60°C (data not shown). This effect can be considered as a consequence of ADA unfolding in this region.

Kinetic parameters for the three linear segments in Figure 7 are shown in Table 2. Based on k values extrapolated at 38.5°C (bovine body temperature), one may conclude that the low-temperature conformer is about 1.8 and 4.8 times less active compared to the middle- and the high-temperature conformers, respectively. The three conformers are of dissimilar activation energies; therefore, structural properties of their binding sites and/or active sites are different.

This study improves the knowledge of physicochemical properties of ADA and prepares enzyme activating probability through interactions with ligands (for example drugs).

Considering the importance of the disease in which ADA activity is reduced, SCID (severe combined immune deficiency), accessibility to more active conformers of the enzyme would be useful to overcome this problem by using some ligands such as drugs.

Conclusion

According to this study it could be concluded that ADA before thermal denaturation experiences three distinct transitional states. These critical transitions are occurred in the range of temperatures that corresponds to 27 31°C and 40 45°C and 53 56°C. The second transitional state corresponds to the pathological condition (fever) in body and it is accompanied with formation of a more active conformer.

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