A study on the activity and thermal stability of adenosine deaminase in the presence of spermine

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ABSTRACT

Adenosine Deaminase is an aminohydrolase (EC 3.5.4.4) which participates in the purine metabolism where it degrades either adenosine or 2'-deoxyadenosine producing inosine or 2'-deoxyinosine, respectively. The enzyme contains a parallel alpha/beta -barrel motif with eight central beta strands and eight peripheral alpha helices. ADA is located both in the cytosol and on the cell membrane. Since spermine, a natural metabolite, exists in all cells and tissues and its effect on the cell proliferation and enzyme regulation have been reported, thermal inactivation of the ADA and spermine regulatory effect on the ADA activity have been investigated in this study. Percentage of ADA activity in the presence and absence of spermine (1000 µM) in Tris buffer 50 mM, pH 7.5 at physiologic and pathologic temperatures have been reported in the present study. Thermal inactivation curves for ADA in the absence and presence of spermine (1000 µM) in different temperatures ranging from 55 ^{o}C to 70 ^{o}C have been drawn. Our data showed that spermine activates the enzyme in the low concentrations of adenosine at 37 ^{o}C. However, it inhibits ADA activity at 42 ^{o}C in the same concentrations of substrate. It is concluded that spermine regulatory effect depends on combined influence of temperature and adenosine concentration. Furthermore, thermal stability of the enzyme also depends on temperature in presence of spermine. Binding site of spermine on the enzyme has been identified by docking analysis.

Keywords: Adenosin deaminase; Activity; stability; Spermine; Temperature

INTRODUCTION

Adenosine Deaminase (ADA) is a cytosolic enzyme, which has been the object of considerable interest, mainly because in human a congenital defect in the enzyme causes severe combined immunodeficiency disease (SCID). ADA is an aminohydrolase (EC 3.5.4.4) which participates in the purine metabolism where it degrades either adenosine or 2'-deoxyadenosine producing inosine or 2'- deoxyinosine, respectively. The enzyme is widely distributed and many of its biochemical properties have been studied in different species [1]. The product of human ADA gene consists of 363 amino acids (41 kDa) and there is a high degree of amino acids sequence conservation amongst species. The enzyme contains a parallel α/ß-barrel motif with eight central β strands and eight peripheral α helices, which is a common structure found in 1/10 of known enzymes [2]; it also contains five additional helices.

Chang et al. (1991) proposed that catalytic functions are carried out by Cys 262, Asp 295, Asp 296, and His 214 of the mammalian adenosine deaminases. The zinc ion is coordinated by His 15, His 17, His 214 and Asp 295. In humans, the highest ADA activity is found in thymus and other lymphoid tissues (~800 IU/mg), the lowest in erythrocytes (~1 IU/mg) [3]. ADA is located both in the cytosol and on the cell membrane. Recent evidence suggests that ecto-ADA had extra-enzymatic and co-stimulatory functional roles. ADA modulates ligand binding and signaling through A1R on DDT1MF-2 cells, a smooth muscle cell line [4-5]. ADA seems to be necessary for the high affinity binding of agonists to A1R [6-7]. The human enzyme is genetically polymorphic and is found in 2 electrophoretically distinct forms termed ADA-1 and ADA-2 [8]. In addition three variants that exhibit apparent molecular weight values in different ranges have

been described among vertebrates [9-10]. These forms have been designated A (M.W. > 200,000), B (M.W. ~ 100,000), and C (M.W ~ 35,000). Tissues of higher mammals generally lack the B form and are characterized by varying ratios of A and C. Human erythrocytes [8] contain almost exclusively the type C form, whereas other tissues contain varying amounts of the high-molecular-weight species [11].

Serum ADA levels increase in pancreatic disorders, especially in pancreatic cancer. It may be a serum marker for the diagnosis of pancreatic cancer [12]. ADA is involved in some diseases such as: Behçet's disease (BD) [13], tuberclosis [14], brain tumor [15], lung cancer and mesothelioma [16].

Spermine is one of the natural metabolites of the human body. It was introduced for the first time as one of the compounds in the sperm and soon after scientists realized that spermine exists in all cell types as a member of polyamins family [17]. The role of polyamines in the cell proliferation has been determined as well as their role in other cell processes [18, 19].These kinds of components have positive charge, therefore, their interaction with negative charge of the macromolecules such as DNA [20, 21], proteins [22] and enzymes [23] for regulating the function and stabilizing the structure have been reported. These findings and also this fact that polyamines functions are firmly controlled by several regulating mechanisms represent their important role in the cell function [24, 25].

Polyamines also play an important role in stress conditions especially in thermal stress [26-27]. Therefore, their role as a natural metabolites could be important in physiologic response to environmental stresses.

Previous findings indicate that concentration of polyamines increases in colon cancer, lung, prostate and breast cancer [28-33]. Furthermore, the relation between adenosine deaminase, (the enzyme that exists in all cell types) and different type of cancers [34-36] suggests that there might be a relation between ADA and poly amine functions. These molecules participate in the regulation of glutamate receptor function and also some of the ion channels, especially calcium channel [37-41]. According to these statements and the fact that polyamines are able to regulate the protein and enzyme functions [23, 42], in the present study the regulatory effect of spermine (as a final product of poly amine biosynthesis pathway and as the most important poly amine in the body) on the ADA activity has been investigated *in vitro*. Moreover, based on polyamine roles in thermal stability of enzymes [43], and also the importance of thermal stability changes of adenosine deaminase by biologic ligands [44-45], in the present study, the authors have investigated the effect of spermine on thermal stability of adenosine deaminase.

MATERIALS AND METHODS

Calf intestine adenosine deaminase was purchased from Roche. Adenosine (as substrate), spermine and Tris base were purchased from Sigma.

Enzyme assay

Adenosine deaminase was assayed after an overnight dialysis against 50 mM, pH 7.5 Tris buffer to removed excess glycerol molecules as preservative in commercial product. The enzyme assays were done by measuring the decreased absorption of substrate in 265 nm and using Spectronic 601 UV-Vis spectrophotometer instrument. The enzyme solution (94 Unit/ml) was prepared in 1 ml final volume of standard mixture. Enzyme activity was measured in 12 different concentrations of adenosine from 2×10^{-2} μM to 38×10^{-2} μM in 50 mM pH 7.5 Tris buffer and every point was repeated three times. The averages were illustrated as the relation of enzyme activity in the presence of spermine 1000 μM to enzyme activity in the absence of spermine in term of percentage at 37 ^{o}C and 42 ^{o}C. Polyamines have a wide concentration range in body tissues (17-18) therefore here the best responding concentration of spermine for this enzyme (1000 μM) is selected by some simple experiments. The enzyme activity in the absence of spermine (a_0) was assayed as a function of substrate concentrations. This experiment was carried out for the enzyme in the presence of spermine 1000 μM (a_1). The percentage of activity changes (ΔAct %) in the presence of spermine was calculated by equation 1:

$$\Delta Act\ \% = 100(a_1 - a_0)/a_0 \qquad (1)$$

Thermal inactivation of ADA*:*

Thermal inactivation of Adenosine deaminase was studied in the range of 55 ^{o}C to 70 ^{o}C (minimum and maximum temperatures of irreversible inactivation for ADA during 40 minutes) in the absence and presence of

spermine. The final mixture of sample was composed of 94 Unit/ml enzyme, spermine 1000 µM and Tris buffer 50 mM, pH 7.5. The sample mixture was incubated about 40 minutes in different temperatures from 55 to 70 °C and enzyme activity was assayed every 5 minutes in one fixed concentration of adenosine which corresponded to V_{max}. Thermal inactivation constant (k_i) was calculated from slope of the linear part of the absolute logarithm of residual activity against incubation time.

Docking analysis

Hex is interactive graphics for calculating and displaying feasible docking modes or pairs of protein and DNA molecules. Hex reads protein and DNA molecular structures from PDB-format files. Up to three PDB files can be loaded into hex at any one time. These are treated as a receptor, a ligand and a reference complex. Using this graphic program (Hex), force energy (the binding energy of ligand) and shape energy were calculated and the spermine binding site on the enzyme and the involved amino acid residues was predicted.

RESULTS

Adenosine deaminase, an enzyme distributed in the human tissues [11], was considered as good marker of cell mediated immunity [46]. It plays a crucial role in lymphocyte proliferation and differentiation [47], and shows its highest activity in T- lymphocytes [48], therefore, study of ADA activity in physiological and pathological temperatures (infectious conditions) would be helpful for understanding the mechanism of immune system indirectly in such conditions and in the presence of spermine as an important metabolite which is involved in some cell processes.

Figure 1 shows the percentage of changes in enzyme activity induced by of 1000 µM spermine at 37 °C as well as 42 °C.

The docking analysis by HEX software revealed that spermine has a binding site on the enzyme molecule which is composed of α-helices and coils related to amino acids 105-107, 214 and 265-287, in addition the binding parameters of E_{force} (binding energy of ligand), E_{shape} (energy content of the protein) and E_{total} ($E_{force}+E_{shape}$) are -31.5, -120.6 and -152.1 J, respectively (Figure 2).

Because of polyamines role in thermal stability of enzymes [43] and the importance of adenosine deaminase stability as an important enzyme in immune processes and critical enzyme in purine catabolism, the effect of spermine on the thermal stability of ADA has been investigated here.

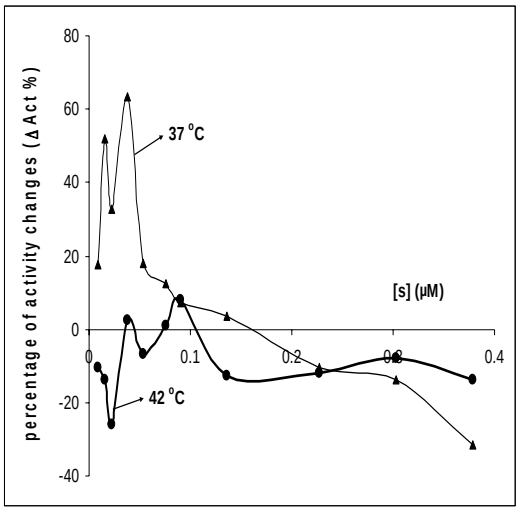

Figure 1. The relation of ADA activity in the presence of spermine (1000 µM) to the enzyme activity in the absence of spermine in term of percentage of activity changes at 37 °C and 42 °C

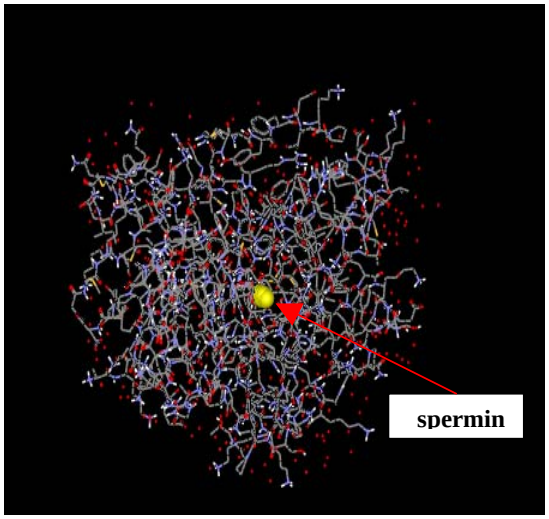

Figure 2. The docking analysis for binding site of a spermine molecule on adenosine deaminase molecule

Natural logarithm of residual activity against incubation time in different temperatures in the absence of spermine and presence of 1000 µM spermine is represented in the figures 3 and 4 respectively.

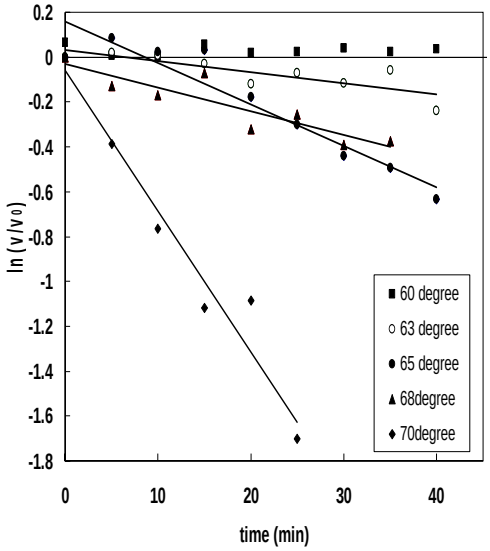

Figure 3. Natural logarithm of residual activity against incubation time in the absence of spermine

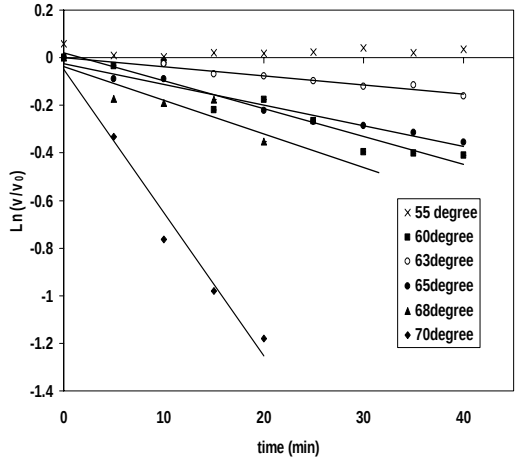

Figure 4. Natural logarithm of residual activity against incubation time for different temperatures in the presence of 1000 μM spermine

The thermal inactivation of the enzyme was studied in different temperatures from 55 ^{o}C to 70 ^{o}C in the absence and presence of 1000 μM spermine. The temperatures below 55 ^{o}C were overlooked, because the slopes of the curves were zero or near zero. The slope of ADA inactivation curves is considered as thermal inactivation constant (k_i). Figure 5 shows changes of k_i against temperature in the presence and absence of spermine.

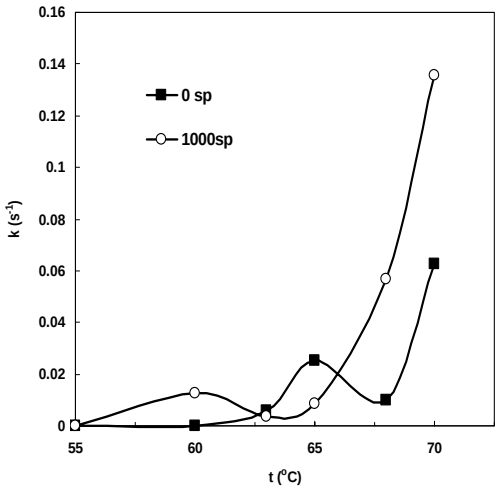

Figure 5. The changes of thermal inactivation constant against temperature in the absence and presence of spermine

DISCUSSION

As it is depicted in the figure 1, spermine induces 63% activation and 30% inhibition in the ADA activity at 37 ^{o}C. At 42 ^{o}C, the values are 8% and 25%, respectively. The standard deviation of each point was not more than 10% of related average.

At 37 ^{o}C, spermine can induced ADA activity (up to 60 percent of the activity in the absence of spermine) in the substrate concentrations below 0.25 μM. But in higher concentrations of substrate, spermine decreases ADA activity (maximum inhibition is about 30 percent). It can be deduced that spermine effect on ADA activity is depend on substrate concentration at this temperature. The observation may be related to probable conformational changes in the enzyme molecules due to substrate binding to the binding site(s) other than active site [49].

Despite of 37 ^{o}C, spermine mostly inhibits ADA activity (maximum 25% inhibition) in almost all substrate concentrations (except of some substrate concentrations that ADA has been activated less than 8%). It seems that, at 42 ^{o}C, changes in ADA activity by spermine are less dependent to substrate concentration than 37 ^{o}C. Moreover, spermine effect at physiologic temperature is reversed in comparison with pathologic temperature in the most applied concentrations of adenosine. The maximum difference between two temperatures is observed in concentrations bellow 0.1 μM

adenosine which corresponds with adenosine concentration in plasma and adipose tissue. Interestingly, the maximum activation effect of spermin (63%) at physiological temperature is belonging to mentioned concentration ranges of substrate. On the other hand, spermine inhibits the enzyme activity about 20% at pathologic temperature in the same range of substrate. This may have a physiological or pathological importance. These interpretations are true apart from the fact that the activity changes are reversible or irreversible.

The results show that spermine can activate or inhibit ADA activity depend on two factors: temperature and substrate concentration. We have already showed that temperature changes can induce conformational changes in ADA molecules [50]. On the other hand, a mentioned before, substrate molecules can bind to the binding site(s) other than active site and change its function probably due to inducing a conformational change in the enzyme molecule. The enzyme conformational change, in turn, may affect on the binding of spermine on its binding site and/or its effectiveness on ADA enzymatic activity change.

Thermal inactivation curve is usually a logarithmic curve in most of the enzymes and k_i raises in logarithmic manner with temperature increase [51]. Therefore, thermal inactivation curve of ADA deviates from normal manner. k_i of ADA increases at 65 ^oC in the absence of spermine, It means that ADA is stabilized abruptly at low and high temperatures of 65 ^oC. The presence of 1000 μM spermine does not eliminate this abrupt increment, but moderates its intensity and shifts it to low temperatures (this kind of behavior has not be reported for other protein). Spermine destabilizes ADA by increasing k_i at high temperatures as well as at 60 ^oC but stabilizes the enzyme at 65 ^oC. Spermin has not any obvious effect on ADA stability at 55 and 63 ^oC. It seems that spermine affects on ADA stability by a temperature dependent manner. This observation is compatible with its effect on ADA activity (Figure 1). Therefore, it is concluded that the quality of spermine effect on thermal inactivation depends on temperature. The results of the study on the spermine effect on the ADA activity and thermal inactivation propose that ADA may have a flexible structure and its conformation is sensitive to environment which caused various activity and stability under the physical (e.g. temperature) and chemical (e.g. binding of some ligands and substrate) conditions. Since temperature, substrate and spermine concentrations are changed under several stresses and diseases [26-32], alteration in activity and stability of ADA [12-16] (as a key enzyme in purine metabolism especially in immune system) may have important roles in clinical issues.

According to docking analysis, it is concluded that spermine as a natural metabolite could bind to ADA and spermine binding might affect the ADA activity and ADA stability as well**.**

Spermine as a natural ligand activates ADA enzyme at physiological temperature in the certain concentrations of subestrate. The regulatory effect of spermine on ADA activity diminishes at pathological temperatures. Spermin also effects on ADA stability on the temperature depending manner.

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