Studies on 5′-AMP Deaminase and its Inhibition by some Methylated 5′-AMP Analogues

[1,2]K. F. AZHAR* [2]K. A. KHAN AND [2]SIRAJUDDIN
[1]*PCSIR Scientific Information Centre Karachi-75280, Pakistan*
[2]*PCSIR Laboratories Complex, Karachi-75280, Pakistan*

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Summary: 5′-AMP deaminase has been isolated from rabbit muscles and structure is already established. The enzyme has been used for preliminary inhibition studies using N^1-methyl-5′-AMP methylester and N^6- methyl-5′-AMP-metylester as inhibitors. Both the derivatives were found to be of semitightbinding type and behaved as weak competitive inhibitors.

Introduction

Adenosine deaminase is an important enzyme involved in nucleic acids metabolism (purine metabolism) in living systems. Increase or decrease of this enzyme leads to certain abnormal conditions. The deficiency of this enzyme is linked to impaired immune function with variable degree of influence on the combined immunodeficiency disease. SCID is a disorder characterized by low or absent circulatory T and B lymphocytes [1]. An increase in the activity of adenosine deaminase and adenosine monophosphate deaminase has also been reported in various types of leukemia and tumor growth [2, 3]. Inorganic pyrophosphate and polyphosphates have acted as potent inhibitors of purified AMP deaminase from yeast [4]. AMP-deaminase from stomach smooth muscles was isolated and physico-chemical properties of the purified enzyme were investigated. The enzyme had an activity optimum at pH 6.5, and poorly deaminated the substrate analogues tested [5]. It is reported that therapeutic action of a number of adenosine analogues is limited due to their facile conversion into less active inosine due to the action of adenosine deaminase, hence deaminase inhibitors are of interest [6]. Much work on the syenthesis of adenosine analogues for andenosine deaminase inhibitors has been reported [7], but little information is available about the synthesis and evaluation of 5′-AMP deaminase inhibitors. A number of methylated 5′-AMP derivatives possessing methyl substituents on adenine, ribose and phosphate moieties of 5′-AMP have been prepared and characterized in our laboratories [8, 9]. Inhibitory properties of two of them i.e. N^1-methyl-5′-AMP methyl-ester and N^6-methyl-5′-AMP methyl-ester are described in this publication.

Results and Discussion

The method followed for the synthesis of N^1methyl-5′-AMP-methylester and N^6-methyl-5′-AMP-methylester is reported in literature [10]. However, the compounds were further characterized by ^{1}H-NMR spectroscopy, the relevant data are described in the experimental part. The method used for extraction of enzyme was reported in [11]. Rabbit muscles from hind limbs and back were extracted with $NaHCO_3$ solution, absorbed over alumina Cγ, eluted with sodium phosphate and finally fractionated with ammonical ammonium sulphate solution. The partially purified enzyme preparation was used for kinetic studies for the enzyme as well as for the inhibition studies. The enzyme activity was determined by following the change of O.D at 265 nm due to the conversion of 5′-AMP to 5′-IMP in succinate buffer of pH 5.9 [12]. The unit of enzyme activity is defined as that amount which causes an initial rate of change in optical density at 265nm of 0.001 per minute under the above condition. From 500 gms of rabbit muscle 52000 units of partially purified AMP deaminase was obtained. In order to calculate the specific activity of enzyme the protein content was determined by Lowry's method [13]. The specific activity of enzyme isolated was 260 units/mg of protein. The content was further characterized by determination of Michealis Menton constant (Km), effect of pH, and effect of inhibitors. The Km value was found to be 6.2×10^{-5} M, this and other values are in close agreement with those reported in the literature [11].

In order to determine the class of inhibitor, i.e. readily reversible, semitight binding or tight binding, enzyme assays were carried out to determine the time

*To whom all correspondence should be addressed.

course of the reaction with and without preincunbation of the inhibitor with the enzyme [14, 1 5].

The effect of incubation for both the inhibitors i.e. N^1-mehtyl-5′-AMP-methylester and N^6-methyl-5′-AMP methylester is shown in Fig. 1 and 2, respectively. Both the inhibitors show very slight inhibition for about 1 minute in the absence of incubation but after this period steady state conditions were reached. After 15 min. incubation pronounced inhibition was exhibited right from the start of reaction this indicated that both inhibitors are slow to equilibrate and are classed as semitight-binding type of inhibitors [14].

The type of inhibition i.e. competitive or non-competitive was determined for both the compounds by using classical Lineweaver-Burk plot [16] in which reaction velocities at a fixed concentration of inhibitor were determined at varying substrate concentrations [s]. A plot of 1/v against [1/s] is shown in Fig 3 and 4 for both compounds, respectively. As is evident from the results, although 1/v is same for inhibited and uninhibited reactions, the Km varies with inhibitor concentration. This type of kinetics where the inhibitor raises the apparent Km of substrate is characteristic of a competitive inhibitor. The inhibition constants Ki were found to be 3.1×10^{-5} M and 2.35×10^{-5} M for N^1-methyl and N^6-methly-5′AMP-methylester, respectively. Therefore, results indicate that both the inhibitors are of semitightbinding type, the inhibition is competitive in nature and Ki values suggest that both the compounds are weak type of inhibitors.

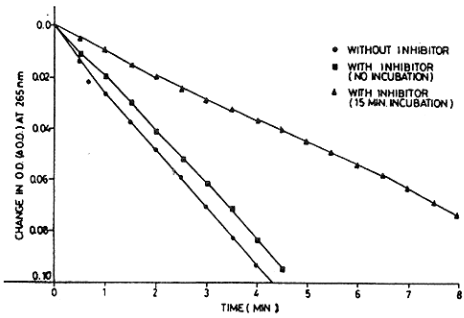

Fig 1. Effect of Incubation on Inhibition of AMP deaminase by N^1-methyl-5′-AMP-methyl-ester

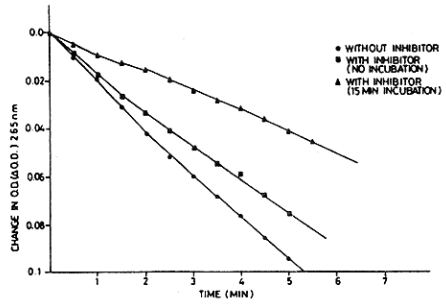

Fig 2. Effect of Incubation on Inhibition of AMP deaminase by N^6-methyl-5′-AMP methylester

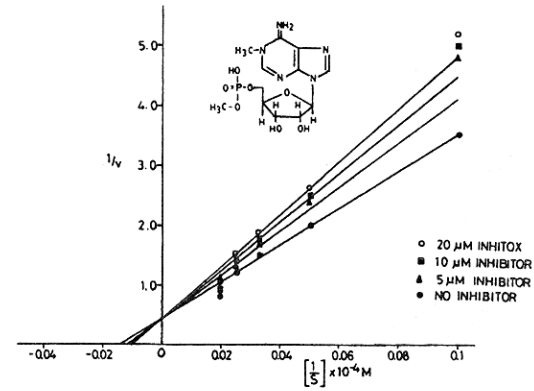

Fig. 3: Lineweaver-Burk Plot for the Inhibition of 5′-AMP deaminase by N^1-methyl-5-AMP methylester

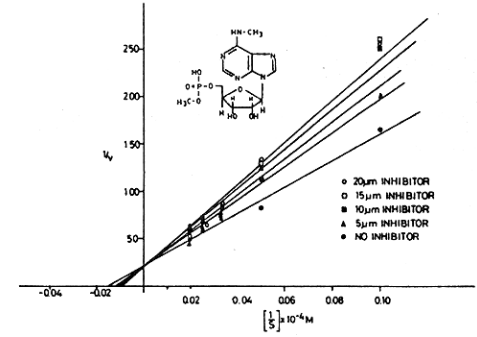

Fig. 4: Lineweaver-Burk Plot for the Inhibition of 5′-AMP deaminase by N^6-methyl-5′-AMP methylester

Experimental

5-AMP was purchased from Fluka, A.G. Switzerland. UV spectra were determined on Perkin Elmer Lambda 4C- Spectrophotometer. ^{1}H-NMR were recorded on a BRUKER AM 300 Spectrometer. Paper electrophoresis was carried out on Whatman No.1 strips (44 x 10 cm) in 0.5 M phosphate buffer pH 8.0 at 400 volts and 12 amp for 2 h, (electrophoretic mobility at pH 8.6 is half that of 5′-AMP indicating it to be a 5′-AMP- alkylester).

Synthesis of N^1-Methyl-5′-AMP-methylester and N^6-Methyl-5′-AMP-methylester.

N^1-Methyl-5′-AMP-methylester was prepared according to the method reported in [10] 5′AMP (500 mg) in distilled water (15 ml) was treated with dimethylsulphate (0.75 ml) and the mixture was stirred at room temperature for three h at pH 6.8-7.4. The product of R_f 0.54 in solvent system isopropanol: ammonium hydroxide: water (7:1:2 v/v) was obtained as an amorphous solid (100 mg). U.V. spectrum pH1, λ_{max} 256nm, λ_{min} 230 nm; pH12, λ_{max} 258 nm, λ_{min} 231 nm. Paper electrophoresis in phosphate buffer pH8.0, 2.5 cm (5′-AMP, 5.0 cm). ^{1}H-HMR (300 MHz, D_2O): δ 8.55 (s, 1H, H8), 8.34 (s,1H, H-2), 6.16 (1H, d, *J*=3Hz, H-1′), 3.55 (3H, s, O-CH_3), 3.39 (3H, s, N-CH_3).

N^6-Methyl-5′-AMP-methylester:

To N^1-methylester (50 mg) in water ammonium hydroxide was added and the reaction was left at room temperature for 24 h at pH 12, the solution was concentrated and separated by preparative paper chromatography. The product of R. 0.57 in the solvent system described for N^6-derivative was obtained as an amorphous powder (30 mg). U.V. spectrum pH1, λ_{max} 260 nm, λ_{min} 234, pH12 λ_{max} 264 nm, λ_{min} 234nm. Paper electrophoresis, phosphate buffer pH8.0, 2.5 cm (5′-AMP, 5.0 cm). ^{1}H-NMR (300 MHz, D_2O): δ 8.22 (s, 1H, H-8), 8.14 (s, 1H, H-2), 5.76 (d, 1H, *J*=2.4Hz, H-1′), 3.37 (s, 3H, N-CH_3), 3.19 (s, 3H, O-CH_3).

Isolation of AMP deaminase:

The method followed for the isolation of enzyme from rabbit muscles was reported [7]. Rabbit muscles (500 g) from hind limbs and back, stored over ice was ground and washed with 0.85% NaCl solution (4x500 ml) for 20 min, squeezing through cheese cloth every time, deaminase was extracted from the tissues with 2% cold $NaHCO_3$ solution (500 ml) and filtered in a cold chamber using Whatman No. 12 filter paper. Alumina C_γ was added to $NaHCO_3$ extracted with continues stirring and suspension was kept at 8°C for 30 min. and then centrifuged at 4°C. The supernatant was discarded and deaminase was eluted at 25°C with 1M Na_2HPO_4 buffer (50 ml).

The eluate was kept in cold and bulk of crystallized Na_2HPO_4 is discarded. To the eluate (45 ml) ammonium sluphate solution (167 ml, pH7.6) was added at 0°C. to obtain 0.27 saturation. The precipitate was collected, the supernatant was brought to 0.45 saturation by addition of NH_4SO_4 solution (110 ml), the precipitate was collected and combined with precipitate of 0.27 saturation. Finally, the precipitate was dissolved in 0.1 N Na_2HPO_4 solution (20 ml). This enzyme was used for inhibition studies.

Assay of Enzyme Activity:

The spectrophotometric method was used, which is based upon the deamination of 5′-AMP to 5′-IMP

$$5'\text{-AMP} + H_2O \xrightarrow{\text{AMP- deaminase}} 5'\text{-IMP} + NH_3$$

At 265 nm the molar extinction of 5′-IMP is only 40% of that of 5′- AMP. The change in O.D at 265 nm was observed and recorded. A 0.04 M solution of 5′-AMP was prepared and diluted with succinate buffer pH5.9 (1:1000 ml) to obtain 40μm solution of AMP. To 3 ml of this solution 0.1 ml of diluted (1:120 ml), enzyme solution was added. After mixing the O.D at 260 nm was recorded at 1 min. intervals.

Unit of Enzyme Activity:

A unit of enzyme activity is defined as "amount which causes an initial rate of change in O.D at 265 nm of 0.001 per min. under the above conditions".

Determination of Km Value

Solutions of 5'AMP were prepared in succinate buffer 0.05 M, pH 5.9 in concentrations of 10, 20, 30, 40 and 50 μM in tube #. 1 to 5, respectively. To each tube enzyme solution 0.1 ml

(2.6 unit) was added in a total volume of 3 ml of each sample. After mixing the decrease in absorbance was measured at 265 nm at 1 min. intervals. The initial velocity, v, corresponding to each concentration [s] was determined and the reciprocals of velocity 1/v was plotted against reciprocal concentration [1/s] according to Lineweaver-Bulk plot to estimate the Km and maximum velocity of the enzyme.

Effect of Pre-incubation of Inhibitor and Enzyme on the Reaction Velocity:

The velocity of the reaction was measured with and without pre incubation of the enzyme and inhibitor according to following method.

Incubation Time (mins.)	Vol. of inhibitor (ml)	Vol. of Enzyme (ml)	Buffer (ml)	Substrate (ml)	Total Vol. (ml)
0.00	0.00	0.10	2.25	0.75	3.00
0.00	0.30	0.10	1.85	0.75	3.00
15.00	0.30	0.10	1.85	0.75	3.00

In the absence of incubation the reaction was started by the addition of enzyme, while when the enzyme was incubated with inhibitor, the reaction was started by addition of substrate. The velocity of reaction was measured as rate of change of OD / 0.5 min. at 265 nm.

Inhibition of AMP deaminase by N^1-methyl-5′-AMP-methylester and N^6-methyl-5′-AMP-methylester:

The velocities of the reaction of enzyme with varying substrate concentration were recorded in the presence and absence of varying inhibitor concentrations. The reciprocal of initial velocity 1/v was plotted against reciprocal of substrate concentration [1/s], (Figs. 3 & 4). The Ki was determined by formula and also by plotting slopes against corresponding inhibitor concentrations.

$$Km\ (app) = Km\ (1 + [I] / Ki)$$

Where

Km (app) = Km of the substrate in the presence of inhibitor Km = Km in the absence of inhibitor

[I] = Concentration of inhibitor

Ki = Inhibition constant

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