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Study of the competitive inhibition of adenosine deaminase by *erythro*-9-(2-hydroxy-3-nonyl)adenine using capillary zone electrophoresis

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Abstract

This article describes how capillary zone electrophoresis was used to evaluate the inhibitory behavior of EHNA or *erythro*-9-(2-hydroxy-3-nonyl)adenine on the enzymatic deamination of adenosine by adenosine deaminase. The technique used for carrying out the assay was electrophoretically mediated micro analysis (EMMA). A fused-silica capillary was filled with phosphate buffer containing the inhibitor at a concentration that was adequate to inhibit the enzyme. After subsequent injection of the enzyme adenosine deaminase and the substrate adenosine, enzymatic reaction, electrophoretic separation and quantification of substrate and reaction product all took place inside the capillary. Reaction velocities were estimated from the peak area of the reaction product inosine. Michaelis–Menten and Lineweaver–Burk plots were constructed and the inhibitory constant K_i of EHNA was determined by comparison of the K_m of adenosine deaminase in the presence of inhibitor with the K_m in a solution without inhibitor.

Keywords: Adenosine deaminase; Enzymes; Hydroxynonyladenine; Capillary zone electrophoresis; Electrophoretically mediated micro analysis; Competitive inhibition

1. Introduction

Adenosine deaminase (ADA) catalyzes the irreversible hydrolytic deamination of adenosine to inosine and of 2′-deoxyadenosine to give 2′-deoxyinosine. This reaction plays an important role in purine metabolism and in the differentiation and maturation of lymphoid system cells. Hereditary reduced or absent adenosine deaminase activity causes lymphopenia and severe combined immunodeficiency disease (SCID) [1]. The discovery that ADA activity is related to the development of several diseases such as lymphocytic leukaemia and acquired immunodeficiency syndrome (AIDS), has stimulated the interest in studying the enzyme. The inhibition of this enzyme is the object of many investigations with the aim of finding ADA inhibitors that are lymphocytotoxic and may play a role in chemotherapy of human lymphoid malignancies [2–4]. ADA inhibitors may be used both as drugs and as codrugs in combination with adenine nucleoside analogues and might also have application in immunosuppressive therapy and in the study of the genetic deficiency of the enzyme. In fact, ADA inhibitors have shown marked potentiation of the cytotoxic effects of some adenosine analogues. Molecules that have been under study as ADA

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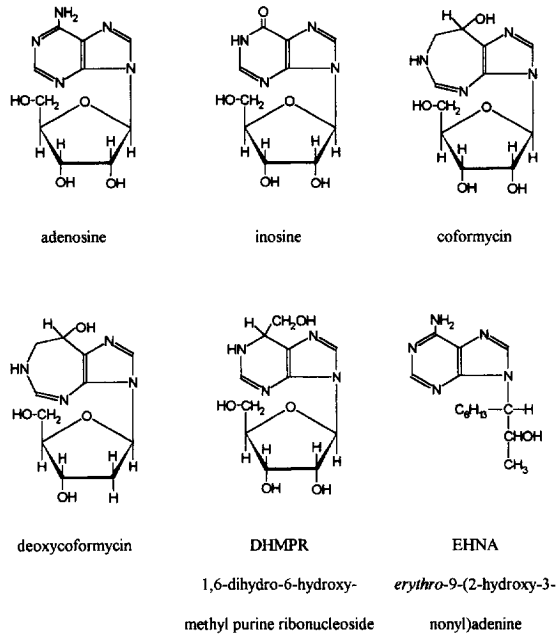

Fig. 1. Structures of substrate, reaction product and some inhibitors of adenosine deaminase.

inhibitors all show a structural resemblance with adenosine, as can be seen from Fig. 1.

Recently, a new application for the evaluation of enzymatic reactions in capillary electrophoresis was proposed, electrophoretically mediated micro analysis (EMMA) [5]. In this technique, substrate and enzyme are introduced into the capillary as distinct plugs, the first analyte injected being the one with the lower electrophoretic mobility. Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities. Enzymatic reaction takes place, in absence or presence of an electric field, and finally the resultant reaction product and the unreacted substrate are electrophoretically transported towards the detector, where they are individually detected. EMMA has so far been described for the enzymatic analysis of both enzymes [6–9] and substrates [10,11]. In previous work [12], we applied the EMMA technique to determine the Michaelis constant of the irreversible deamination of adenosine to inosine. This paper deals with the evaluation of the inhibitory behaviour of a known ADA inhibitor using the EMMA technique.

2. Experimental

2.1. Instrumentation

The capillary electrophoresis instrument was built in-house from commercially available components. Electric fields were applied with a Spellman CZE 1000 R high voltage power supply (Plainview, NY, USA), using two hollow platinum electrodes (Boschmans, Antwerp, Belgium). Detection was achieved with a Spectra 100 variable UV–Vis detector, equipped with an on-capillary cell, both purchased from Thermo Separation Products (Fremont, CA, USA). Data were collected on a HP 3396 Series II integrator (Hewlett-Packard, Avondale, PA, USA) and stored on a 386 personal computer, running the HP Peak 96 software. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D. and 375 μm O.D. were utilized. The capillaries were of 44.7 cm total length with an effective length (distance from injection to detection) of 26.0 cm. At both cathodic and anodic sides, the capillary was led through the electrodes which were dipped in the vials containing the buffer solution. A Plexiglass box with the following dimensions was constructed: length 720 mm, width 105 mm and height 95 mm. This plexi housing was built on top of the UV detector and contained cathodic buffer vial, anodic sample or buffer vial, electrodes, capillary and UV detection cell. A removable cover protects the analyst from exposure to high voltage.

2.2. Chemicals

Adenosine was obtained from Acros Chimica (Geel, Belgium). Adenosine deaminase (EC 3.5.4.4.) from calf spleen and EHNA [*erythro*-9-(2-hydroxy-3-nonyl)adenine] were purchased from Sigma (St. Louis, MO, USA). Analytical grade monobasic and dibasic sodium phosphate were from Merck (Darmstadt, Germany). All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through a 0.2 μm nylon membrane filter

(Euro-Scientific, Lint, Belgium or Alltech, Deerfield, IL, USA).

2.3. Electrophoresis procedure

As the running buffer, a 100 m*M* solution of sodium phosphate at pH 7.5, containing 12.5 n*M* EHNA was used throughout the experiments. The capillaries were rinsed with 0.1 *M* NaOH, water and buffer solution prior to use. Injections were performed electrokinetically by placing the sample vial in the anodic position and applying a voltage for a certain time. All assays were performed without temperature control at ambient temperature.

3. Results and discussion

3.1. Basic equations of enzyme kinetics

A simple enzymatic reaction can be schematically displayed as

$$E+S \overset{K_m}{\rightleftharpoons} ES \overset{k_{cat}}{\rightarrow} E+P \quad (1)$$

where E stands for enzyme, S for substrate, ES for enzyme–substrate complex and P for reaction product. We assume that the concentration of enzyme is negligible compared with that of the substrate and that no reverse reaction from E+P to ES takes place, i.e., the second reaction step is irreversible, characterized by the catalytic turnover number k_{cat}.

The kinetics of an enzymatic reaction under these conditions can be described by the Michaelis–Menten equation:

$$v = \frac{V_{max}[S]}{[S] + K_m} \quad (2)$$

where v and V_{max} are the initial and maximum velocity of the reaction, [S] is the substrate concentration and K_m is the Michaelis constant, equal to the substrate concentration at half the maximum velocity. Plotting against [S] gives a hyperbolic curve, referred to as the Michaelis–Menten plot. A more common way of representing the Michaelis–Menten equation is the double reciprocal or Lineweaver–Burk equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

Plotting $1/v$ against $1/[S]$ yields a linear relationship from which the value of K_m can be directly derived from the intercept on the abscissa. Because of this advantage, the Lineweaver–Burk plot is generally preferred for analyzing data graphically.

As well as being irreversibly inactivated by heat or denaturing agents, enzymes may be reversibly inhibited by the binding of inhibitors. There are four types of inhibition, depending on the nature of binding between inhibitor and enzyme. If the inhibitor and the substrate compete for the same active site on the enzyme, the inhibitor is said to be a competitive inhibitor. In the case of simple Michaelis–Menten mechanism, an additional equilibrium must be considered:

$$\begin{array}{ccccc} E+S & \overset{K_m}{\rightleftharpoons} & ES & \overset{k_{cat}}{\longrightarrow} & E+P \\ \Big\Vert\, K_i & & & & \\ EI & & & & \end{array} \quad (4)$$

where I is the inhibitor, EI is the enzyme–inhibitor complex and K_i is the inhibitory constant. In the presence of a competitive inhibitor at a concentration [I], Eq. 2 can be rewritten as

$$v = \frac{V_{max}[S]}{[S] + K_m(1 + [I]/K_i)} \quad (5)$$

K_m is apparently increased by a factor of $(1+[I]/K_i)$ and this allows for calculation of K_i.

Besides competitive inhibitors, three other types of reversible inhibition can occur, namely noncompetitive, uncompetitive and mixed inhibition [13]. These types of inhibition can be discriminated by the way they affect K_m and k_{cat} and may just as well be assessed by the EMMA technique.

3.2. Electrophoretically mediated micro analysis (EMMA)

When two analytes with different electrophoretic mobility are injected subsequently as distinct plugs into a capillary, the faster migrating zone will

interpenetrate the slower one, on condition that the first analyte injected is the one with the lower electrophoretic velocity. If substrate and enzyme are injected under these conditions, enzymatic reaction takes place inside the capillary. A schematic representation of the process in the capillary is depicted in Fig. 2. Turning off the voltage at moment (c) or (d) allows the enzymatic reaction to proceed and reaction product to accumulate due to the amplifying nature of an enzymatic reaction. Reapplying the voltage drives substrate and reaction product electrophoretically towards the detector where they are individually detected.

3.3. EMMA of adenosine deaminase to determine K_i of EHNA

In previous experiments with EMMA of adenosine deaminase, the K_m for the deamination of adenosine to inosine was estimated to be $5.3 \cdot 10^{-5}$ *M* [12], which was within the range 2.5 to $7.4 \cdot 10^{-5}$ *M*,

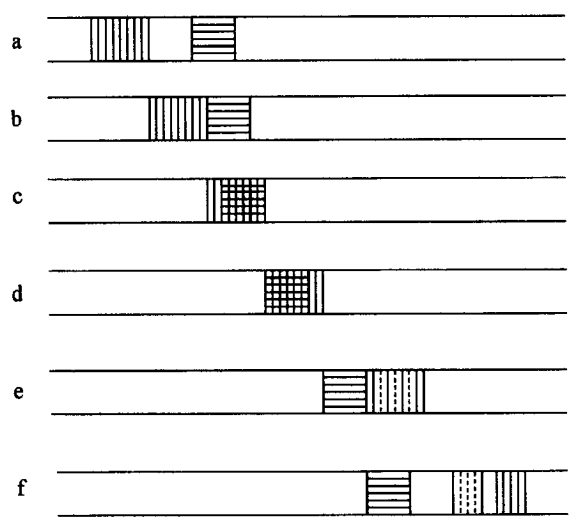

Fig. 2. Schematic illustration of electrophoretic mixing and enzymatic reaction of substrate and enzyme. Horizontal bars represent zone with lower electrophoretic mobility, in our case the enzyme; vertical bars symbolize zone with higher electrophoretic mobility, i.e., the substrate; vertical dotted lines stand for the reaction product. (a); two distinct zones with an intermediate buffer plug, (b); first contact between the two zones, (c); full interpenetration, (d); beginning disengagement, (e); full disengagement, (f); separation of substrate and reaction product.

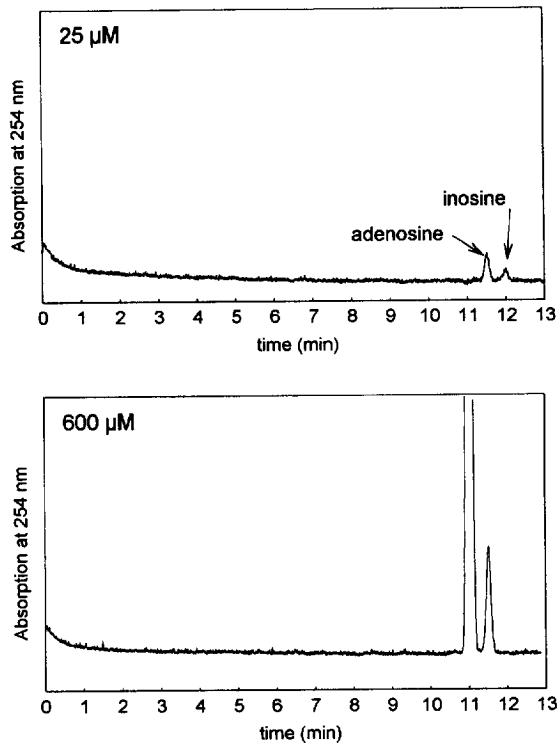

Fig. 3. Electropherograms of enzymatic conversion of 2 adenosine concentrations. Capillary: fused-silica, $L = 44.7$ cm, $l = 26.0$ cm, I.D. $= 75$ μm; background electrolyte: 100 m*M* sodium phosphate pH 7.5, containing 12.5 n*M* EHNA; injection: adenosine deaminase 14.4 n*M* for 10 s at 2.0 kV, phosphate buffer 100 m*M* pH 7.5 with 12.5 n*M* EHNA for 10 s at 2.0 kV, and adenosine 25 or 600 μ*M* for 20 s at 2.0 kV; separation voltage: 5.0 kV (current = 73 μA); 35 s into the electrophoretic run, voltage is turned off for 5 min; electropherogram is started when voltage is applied again; detection: UV at 254 nm.

reported in the literature. The assays made use of the same buffer system that is used in the present work, except that no inhibitor was added. The inhibitory behavior of EHNA, a competitive inhibitor with high affinity, was quantitatively assessed. A Michaelis–Menten analysis was conducted, in order to determine the Michaelis constant of adenosine deaminase in the presence of EHNA. Due to the high affinity of EHNA for the enzyme, an inhibitor concentration in the nanomolar range sufficiently inhibited the enzyme. Therefore EHNA was dissolved in the running buffer to give a concentration of 12.5 n*M*, which is in the same order of magnitude than the expected K_i. As experiments by Agarwal et

al. revealed [14], 10 min of preincubation of the enzyme with the inhibitor were required to establish an equilibrium between both. If the enzymatic reaction is started by adding ADA that has not been preincubated with the inhibitor, a significant amount of substrate may be converted to product before the equilibrium is reached between the enzyme and the inhibitor. Therefore we preincubated enzyme as well as substrate in buffer containing 12.5 n*M* EHNA at least 10 min prior to injection into the capillary. Moreover, when substrate reacted with the enzyme-inhibitor complex, the reaction was completely inhibited initially. This inhibition was slowly released and after 2 to 3 min steady state conditions were achieved [14]. These findings of Agarwal et al. [14] brought us to the following procedure: Consecutive injection of enzyme and substrate was done and 35 s into the electrophoretic run, a 5 min zero potential amplification was conducted to allow the reaction to proceed till steady state reaction conditions were attained. The time after which voltage is turned off (35 s) corresponds with the point where enzyme and substrate zones are fully engaged (Fig. 2 (c)) and can be calculated from the electrophoretic mobilities and the width of the injection zone of the analytes [12]. Electrophoretic mobilities of enzyme and substrate are expected not to have changed in the presence of EHNA at pH 7.5, even after preincubation. Therefore the calculations done in ref. [12] were adopted here. Turning off the voltage and allowing the enzyme to convert more substrate also allowed to obtain reasonably high inosine peaks. Table 1 summarizes the injection parameters that were chosen. A series of 6 adenosine concentrations (25, 50, 100, 200, 400 and 600 μM) was analyzed in triplicate and the reaction

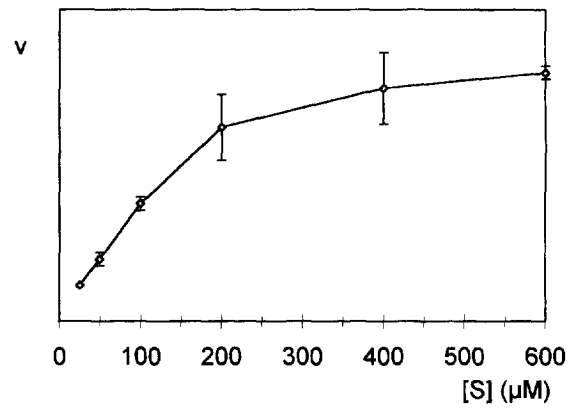

Fig. 4. Michaelis–Menten plot for the enzymatic deamination of adenosine to inosine, inhibited by 12.5 n*M* EHNA. Error bars represent the estimated standard deviation on the mean of three analyses.

velocity for each concentration was determined from the corrected peak area of inosine. In Fig. 3, typical electropherograms of the highest and the lowest concentration adenosine are shown from the in-capillary conversion. Fig. 4 shows the Michaelis–Menten curve that resulted from plotting the reaction velocity against the substrate concentration. From this curve, a double-reciprocal plot following Eq. 3 was constructed as depicted in Fig. 5. A weighted regression trendline was drawn through the points and K_m, calculated from the intercept on the abscissa, was estimated to be $2.16 \cdot 10^{-4}$ *M*. The K_i for EHNA was calculated according to Eq. 5 by comparison of K_m values in the absence [12] and in the presence of 12.5 n*M* EHNA and the result, $4.2 \cdot 10^{-9}$ *M* was consistent with previously reported values. Depending on the nature of the assay and on the origin of

Table 1
Injection parameters for EMMA of adenosine deaminase in the presence of EHNA

	V_i (kV)	t (s)	
Adenosine deaminase 14.4 n*M*	2.0	10	enzyme
Running buffer	2.0	10	intermediate buffer plug
Adenosine × μM	2.0	20	substrate
Running buffer	5.0	35	
Running buffer	0	300	zero potential amplification
Running buffer	5.0		electropherogram

Running buffer = 100 m*M* sodium phosphate buffer pH 7.5 containing 12.5 n*M* EHNA. Enzyme and substrate solutions were made in this buffer.

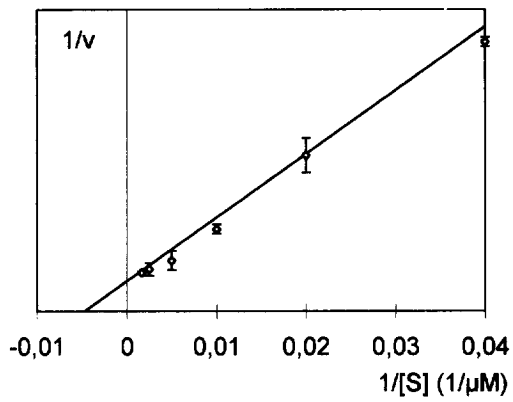

Fig. 5. Lineweaver–Burk plot for the enzymatic deamination of adenosine to inosine, inhibited by 12.5 n*M* EHNA. Error bars represent the estimated standard deviation on the mean of three analyses.

the enzyme, reported values range from 1.5 to 16·10^{-9} *M* [1].

4. Conclusion

Electrophoretically mediated micro analysis couples all advantages that are related to capillary electrophoresis to an assay of enzyme kinetics. The minimal sample and reagent requirements as well as the high separation efficiency of capillary zone electrophoresis are fully utilized. The experiments with adenosine deaminase and EHNA clearly show that the EMMA based determination of the enzymatic reaction constants K_m and K_i yields results that are consistent with the values reported in the literature. Since the K_i value of ADA inhibitors is one of the indicators of their possible biological relevance, this assay can be an interesting alternative for screening of ADA inhibitors.

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