AN INACTIVATOR FOR THE AFFINITY LABELLING OF ADENINE NUCLEOTIDE DEPENDENT ENZYMES

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1. Introduction

To enlighten the mechanism of enzyme action it is important to know the amino acids involved in catalysis. The amino acid specific reagents also attack amino acids outside of the active center. Therefore it is necessary to synthesize active site specific compounds, which are able to react there, forming a covalent bond. Woenckhaus and co-workers have described some highly specific inactivators for NAD-dependent dehydrogenases [1–4]. An analoge of 5′AMP, reacting specifically with SH-groups opposite the 6-position of the purine part, has been synthesized by Fasold and Hulla [5].

All these nucleotide analogs are only able to modify the binding site for the heterocyclic moieties. We have tried to synthesize an inactivator modifying the pyrophosphate or triphosphate binding sites in enzymes. In this part of the enzyme there is supposed to be a positive charged area to which the negative charged phosphate residue is fixed. Therefore the inactivator should also contain a negative charge in about the same distance to the sugar part as in ADP or ATP.

We have synthesized the adenosine-5′-ester of maleic acid, which is converted into the ester of dibromosuccinic acid by addition of bromine. This α-bromocarbonylic acid derivative is able to modify amino acid residues in the active center of NAD-dependent dehydrogenases and adenine nucleotide-dependent kinases.

2. Experimental

2.1. *Synthesis*

2′3′ Isopropylidene-adenosine (I), 10 mmole, and 50 mmole maleic anhydride were dissolved in 500 ml of dry dioxane and 1 ml of pyridine was added. The mixture was allowed to stand in the dark at room temperature for 4 days. The solvent was removed under reduced pressure at 30°C. The remainder, containing no more 2′3′ isopropylidene-adenosine when tested by high voltage electrophoresis, was dissolved in 30 ml of water by adding some ammonia and applied to an Amberlite CG50I (H^+ form) column (4 × 50 cm). The column was washed with water 180 ml/hr 2′3′ isopropylidene-adenosine-5′hydrogen maleate (II) appeared after about 3.6 ℓ in 5.5 ℓ solution. The solution was brought to pH 6 with 2 N NH_4OH and evaporated. By addition of isopropyl alcohol and ether a light yellow product precipitated. The purity of this product was checked by paper electrophoresis and thin-layer chromatography on cellulose covered sheets (solvent, isopropyl alcohol : 33% ammonia : water = 7:1:3 v/v). There was no impurity to detect.

To remove the protecting group 1.3 g of II were dissolved in 10 ml of 1 N H_2SO_4. The solution was kept in a water bath of 60°C for 10 min. The solution was chilled in ice and the pH adjusted to 4.5 with $Ba(OH)_2$. $BaSO_4$ was filtered of, washed with water and the filtrate applied to a Amberlite CG50I (H^+ form) column (2 × 50 cm). The column was washed with

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water. Adenosine-5′hydrogen maleate (III) was eluted after 600 ml in 1.8 ℓ solution. The fraction was lyophilized. The yield was 27%. III gave a single spot on thin-layer chromatography and on paper electrophoresis at pH 4.5.

$C_{14}H_{15}N_5O_7$ (mol.wt. 365.30)

calculated	46.03 C	4.14 H	19.17 N
found	45.87 C	4.29 H	19.03 N

Adenosine-5′(2,3-dibormohydrogensuccinate) (IV). Freshly prepared III (50 mg) was dissolved in 1 ml of water and after addition of bromine (0.15 mmoles) the solution was allowed to stand in the dark. The solution decolorized within about 6 hr. The solution was extracted with 5 × 10 ml of ether to remove HBr. By addition of acetone IV could be precipitated. IV Gave a single spot on paper electrophoresis at pH 4.5.

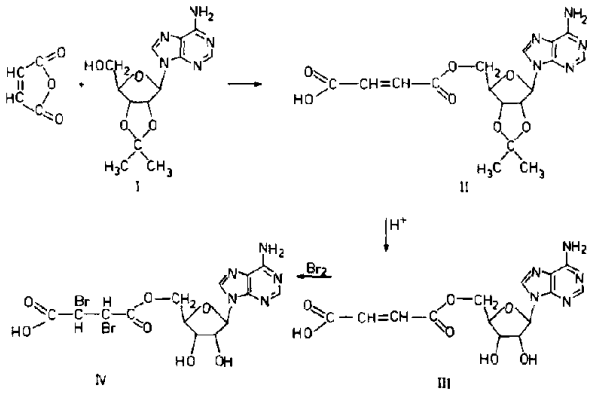
2.2. *Materials and methods*

AMP, ADP, ATP, glycerate-3-phosphate, NAD, NADH and phosphoenol pyruvate were obtained from Boehringer and Soehne, Mannheim, as were ADH_{yeast}, GAPDH from rabbit muscle and 3-phosphoglycerate kinase. The other enzymes were prepared in our laboratory with the indicated specific activities: ADH from horse liver 2.5 U/mg, pyruvate kinase from pig heart 290 U/mg, myokinase from pig heart 480 U/mg, LDH from pig heart 480 U/mg, cytoplasmic MDH from pig heart 710 U/mg and mitochondrial MDH from pig heart 1180 U/mg. The protein concentrations were quantitated using the biuret method. The enzyme activities were assayed at 25°C by measuring for ADH the oxidation of alcohol [6], for LDH the reduction of pyruvate [7] and for MDH the reduction of oxaloacetate [8]. GAPDH activity was determined by the method of Bücher with 3-phosphoglycerate kinase [7]. Pyruvate kinase activity was measured in the coupled test with LDH (10 U/ml) in triethanolamine buffer pH 7.6 containing 10 mM Mg^{2+}, 20 mM K^+, 1.5 mM ADP, 0.7 mM phosphoenolpyruvate and 0.2 mM NADH. Myokinase activity was assayed by coupling the pyruvate kinase reaction and the LDH reaction. The assay mixture contained 0.1 M triethanolamine buffer pH 7.6, 1.4 mM Mg^{2+}, 84 mM K^+, 2 mM AMP, 1 mM ATP, 0.4 mM phosphoenolpyruvate, 0.2 mM NADH, pyruvate kinase 10 U/ml and LDH 10 U/ml.

The inhibitor constants of adenosine-5′hydrogen-maleate were determined from Lineweaver and Burk plots. The inactivation reactions were accomplished at 37°C. Previous to inactivation, the enzymes were dialysed against the following buffers, dehydrogenases agains 0.5 M phosphate buffer pH 7.0, pyruvate kinase and myokinase against 0.1 M triethanolamine buffer pH 7.6 containing K^+ and Mg^{2+} in the same concentrations as in the appropriate test mixtures. The inactivator was added in aqueous solution, the same amount of water was added to a blank. The decrease of activity was measured against the blank.

3. Results and discussion

Reaction of 2′3′-isopropylidene-adenosine with maleic anhydride yields 2′3′-isopropylidene-adenosine-5′-hydrogenmaleate (scheme).



The protecting group can be removed by treatment with 1 N mineral acid liberating adenosine-5′-hydrogen maleate (III). In 2 N NaOH III is split into adenosine and maleic acid. III Is a competitive inhibitor against ADP in the pyruvate kinase system with a $K_I = 3 \times 10^{-2}$ M, against AMP in the myokinase system with a $K_I = 1.5 \times 10^{-2}$ M and in the GAPDH catalysed reaction against NAD with a $K_I = 2.2 \times 10^{-3}$ M (fig. 1).

Bromine in aqueous solution is added to the double bond of the acid residue, forming the ester of dibromosuccinic acid (IV). Under the conditions used there is no 8-bromoadenosine derivative formed. Adenosine is brominated under the same conditions only within 14

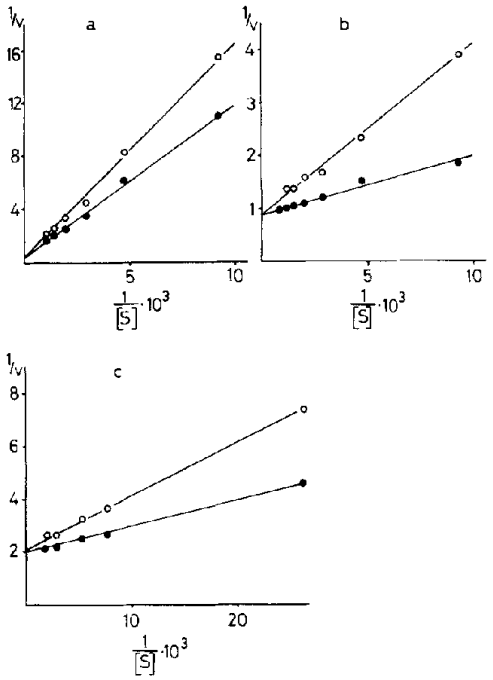


Fig. 1. Lineweaver and Burk plots for the action of: a) pyruvate kinase, III = 10^{-2} M, ADP varied $1-20 \times 10^{-4}$ M; b) myokinase, III = 10^{-2} M, AMP varied $1-21 \times 10^{-4}$ M; c) GAPDH, III = 1.2×10^{-3} M, NAD varied $0.4-8 \times 10^{-4}$ M in presence (o—o—o) and absence (•—•—•) of III.

days. Thereby the maximum of ultraviolet absorption is shifted 5 nm or red [9]. Such a shift does not appear during bromination of III. At pH 7.0 IV is stable at 37°C, above pH 7.5 hydrolytic decomposition occurs, at pH 8.0 the half-life is 2.5 hr.

In the presence of IV ADH_{yeast}, GAPDH, myokinase and pyruvate kinase lose their enzymatic activities (fig. 2). The fact that III is a competitive inhibitor for those enzymes which are inactivated by IV induced us to assume, that IV also attacks at the active site. This assumption is confirmed by the protection of natural substrates against inactivation. GAPDH is protected by NAD, myokinase by AMP and ATP. Pyruvate kinase is little protected by ADP whereas there is no protection by 10 mM ATP or phosphoenolpyruvate. An exception is ADH_{yeast} which is not protected by NAD. ADH from liver, LDH and both forms of MDH are not inactivated by IV. In short the synthesized adenosine-5′-(2,3-dibromohydrogensuccinate) is an active site directed inactivator of some adenine nucleotide dependent enzymes attacking the phosphate binding site.

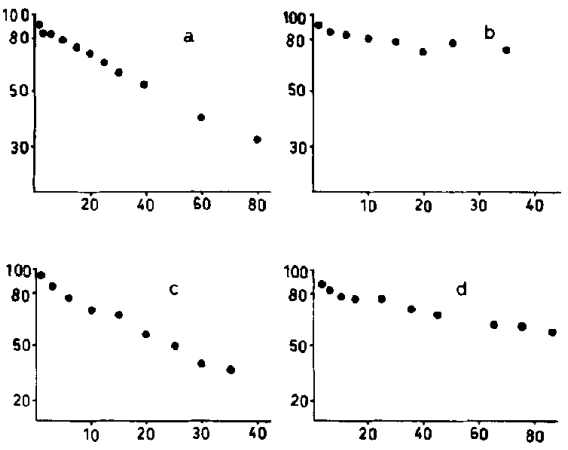


Fig. 2. Semi logarithmic plots of the decrease of enzymatic activity during incubation at 37°C with 4×10^{-3} M of IV: a) GAPDH (10 mg/ml); b) ADH_y (5 mg/ml); c) myokinase (0.1 mg/ml); c) pyruvate kinase (6 mg/ml). Abs.: time in min. Ord.: % Activity.

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