INACTIVATION OF ADH FROM YEAST AND GAPDH FROM RABBIT MUSCLE BY STRUCTURAL ANALOGUES OF NAD^+

C. WOENCKHAUS, R. JECK, E. SCHÄTTLE, G. DIETZ and G. JENTSCH

Gustav-Embden-Zentrum der Biologischen Chemie, Abt. f. Enzymologie, Universität Frankfurt, D-6000 Frankfurt, Theodor-Stern-Kai 7, W. Germany

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1. Introduction

[3-(4-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate and [3-(3-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate inactivate alcohol dehydrogenase from yeast and glyceraldehyle-3-phosphate dehydrogenase from rabbit muscle, forming a covalent (coenzyme—enzyme) bond [1, 2]. The suppression of the inactivation reaction in the presence of either NAD^+ or NADH as well as the optical behaviour of the inactivated enzymes reveal the attack of the inactivator at the functional coenzyme binding site.

2. Materials and methods

Enzymes and coenzymes. GAPDH 100 U/mg was prepared from rabbit muscle [3]. YADH, NAD^+ and NADH were purchased from Boehringer and Soehne, Mannheim; [methyl-^{14}C] 4-acetylpyridine was synthesized by modifying the method of Kartritzky [4]. 168 mg [2-^{14}C] ethyl acetate were dissolved in 5 ml tetrahydrofurane and added to a mixture containing 2 ml tetrahydrofurane, 65 mg sodium, 0.4 ml ethyl isonicotinate, and 0.09 ml ethanol. The mixture was refluxed for 17 hr. After cooling 4 ml of 6 N HCl were added and the whole refluxed for 4 hr. The cold solution was extracted with 2 × 50 ml ether. Potassium carbonate was added to the aqueous solution and the alkaline solution extracted with 4 × 30 ml ether. Ether was removed under reduced pressure from the dried extracts, yielding 122 mg [methyl-^{14}C] 4-acetylpyridine (2.1 mCi/mmole). 97.4 mg 3-(4-acetylpyridinio)-propyl phosphate were prepared as described previously [2], and condensed with 500 mg adenosine-5'-phosphoromorpholidate in freshly distilled *o*-chlorophenol [5]. The purification of the coenzyme analogue and the preparation of the inactivator were described in an earlier paper [2] (Yield: 84 mg, specific radioactivity 2.1 mCi/mmole). Bromoacetylpyridinio-propyl phosphates were prepared from the acetylpyridinio compounds by action of bromine in 3% aqueous hydrobromic acid:

$C_{10}H_{13}NBrO_5P \times HBr$ (mol. wt. 419.02).

calculated: 28.66C 3.37H 3.34N 38.14Br 7.39P.

found: 28.65C 3.37H 3.40N 38.08Br 7.47P.

For the preparation of 3-{3-[2-acetymido-2-carboxyethylmercapto)-acetyl]-pyridinio} propyl phosphate 2.99 g 3-(4-bromoacetylpyridinio)-propyl phosphate were dissolved in 4 ml water and 0.82 g *N*-acetylcysteine was added. After 3 hr standing at room temp. the solution was fractionized by column-chromatography on Dowex-50W X8 (200 – 400 mesh, H^+-form 2 × 30 cm) by elution with water.

The compound was collected after elution with 1.2 l in 600 ml solution. The solution was acidified with 2 ml acetic acid and evaporated. By addition of acetone 1.3 g of an amorphous yellow product precipitated:

$C_{15}H_{21}N_2O_8PS \times CH_3COOH \times H_2O$ (mol. wt. 498.45).

calculated: 40.96C 5.42H 5.62N 6.25P 6.43S.

found: 40.56C 5.24H 5.43N 6.89P 6.47S.

The same method was used to prepare the isomeric compound from 3-(3-bromoacetylpyridinio)-propyl phosphate:

found: 40.79C 5.65H 5.52N 6.02P 6.28S.

The oxidation of 3-{3-[(2-acetamido-2-carboxyethylmercapto)-acetyl]-pyridinio}-propylphosphate and its isomeric compound was performed at pH 8.0 and 25°C. The reaction mixtures were separated by paper electrophoresis at pH 1.9, 4.7 and 8.2. In all three cases one of the oxidation products could be identified by comparison with carboxymethyl-*N*-acetyl-cysteine which was prepared according to a method of Karrer [6]. For the modification of GAPDH and YADH the suspensions, containing 100 mg enzyme each, were centrifuged and the precipitates dissolved in 10 ml 0.2 M phosphate buffer pH 6.5. To 5 ml of each enzyme solution 4 mg (18 μCi) [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate or 4 mg (8.3 μCi) [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate were added. At 36°C the enzymatic activity of all enzyme solutions decreased to 10% within 30 min. Hydrogen peroxide oxidation and total hydrolysis of the modified proteins were performed as described previously [7]. Each amino acid mixture was dissolved in water and separated by paper electrophoresis. To investigate the incorporation of the inactivators in GAPDH and YADH 8 mg of each enzyme, dissolved in 1 ml of 0.2 M phosphate buffer pH 6.5 at 36°C, was treated with 0.5 – 5 mM radioactively labelled [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate or [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate. The decrease of enzymatic activity was measured and the inactivation reaction stopped by addition of 0.1 ml of 0.1 M cysteine solution pH 6.5. The proteins were purified, radioactivity and protein concentration determined [7]. The dissociation constants were evaluated according to Fahrney and Gold [8], the pH-dependency of the inactivation velocity was evaluated as previously described [2]. Activity of YADH (determined by the method of Vallee and Hoch) and activity of GAPDH (determined by the method of Ferdinand) were measured by the increase of absorbance at 366 nm. Protein concentration of YADH was quantitated using the Biuret method [9]; GAPDH concentrations calculated from dry weight and extinction [10].

For dissociation—reassociation experiments a solution of 9 mg inactivated GAPDH with 10% residual activity in 1 ml of 0.2 M phosphate buffer (pH 6.5) containing 0.5 mmole EDTA was diluted 10-fold, 100-fold and 1000-fold by the same buffer. For comparison, native enzyme was diluted in the same way and specific activity of both enzymes measured after 24 hr.

3. Results and discussion

At first the reactive coenzyme analogues form reversible dehydrogenase—inactivator complexes; in these the reactive group of the inactivator may react with a vicinal amino acid residue forming a covalent bond. The dissociation constant at pH 6.5 of the GAPDH complex with 3-(3-bromoacetylpyridinio)-propyl-adenosine pyrophosphate is 3 mM and the K_D with [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate is within the same range. The first-order reaction rate measured with the two inactivators is 0.6 min^{-1}. The inactivation rate of GAPDH with [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate is dependent on the concentration of protons (fig. 1). The acetylpyridinium ring is transformed into the dihydropyridine system by treatment with sodium dithionite [7]. This form of inactivator—enzyme compound shows the characteristic fluorescence of reversible binary coenzyme—enzyme complexes [7]. The preparation of radioactively labelled [methyl-^{14}C]-[3-(4-bromoacetylpyridinio)-

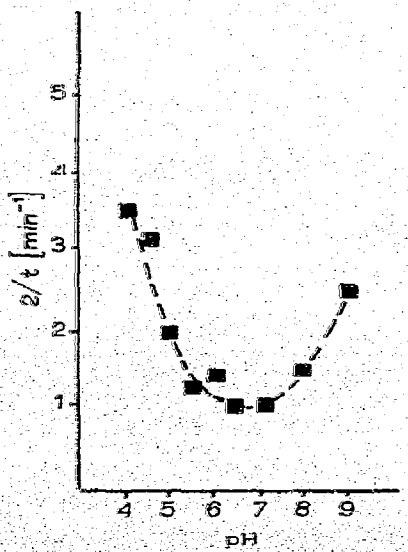


Fig. 1. pH-dependency of inactivation velocity of GAPDH. pH plotted versus reciprocal half-life of the inactivation reaction extrapolated from the initial velocity.

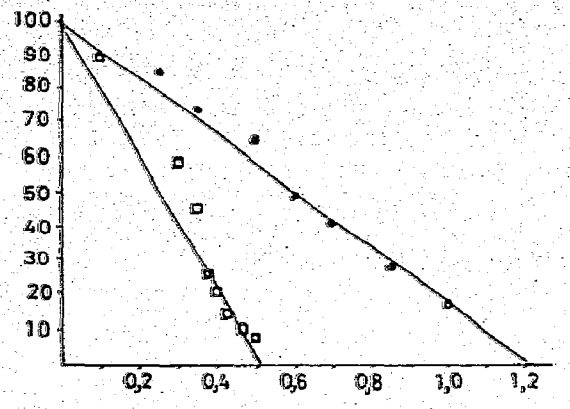
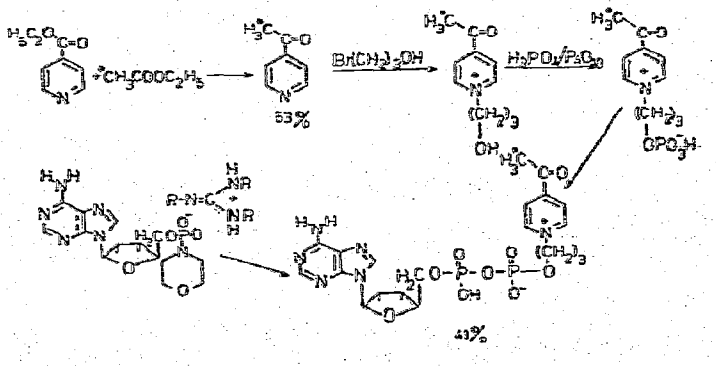


Fig. 2. Enzymatic activity as a function of incorporated [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate into 1 subunit of GAPDH (□) and YADH (●). Abs.: μmole inactivator/μmole subunit enzyme. Ord.: enzymatic activity [%].

propyl]-adenosine pyrophosphate was performed as shown in the following scheme:



[3-(3-Bromoacetylpyridinio)-propyl]-adenosine pyrophosphate was synthesized with [carbonyl-^{14}C]-3-acetylpyridine as the primary compound [7]. The use of the two radioactively labelled inactivators shows that the incorporation of 1 mole of the analogues in 1 mole subunit YADH (mol. wt. 37 500) leads to complete inactivation. GAPDH is completely inactivated after incorporation of 0.5 mole of either of the inactivators in 1 mole subunit (mol. wt. 37 500) (fig. 2).

There are only two of the four binding sites occupied by the inactivators but we were not able to recover any enzymatic activity by treatment of the enzyme–coenzyme compounds under conditions used for dissociation and reassociation of the native enzyme. In the electrophoresis on acetate sheets at pH 7.2 we found only one spot which was dyed with naphthalene black B and which contained the total radioactivity. In order to identify the alkylated amino acid it was necessary to split off the ionic parts of the coenzyme model, because the properties of amino acid–coenzyme compounds prepared from different amino acids are nearly the same. Treatment with hydrogen peroxides removes the dinucleotide part from the protein. The loss of radioactivity is 40% when [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate was used for inactivation, and is 20% when the enzymes were inactivated with the isomeric 4-bromoacetylpyridinio compound. Preferentially the cleavage takes place between the acetyl residue and the pyridinium ring. Model compounds prepared from *N*-acetylcysteine and 3-(4-bromoacetylpyridinio)-propyl phosphate or 3-(3-bromoacetylpyridinio)-propyl phosphate are oxidized by hydrogen peroxide forming S-carboxymethyl-*N*-acetylcysteine. The 4-substituted pyridinium system yields a 4-pyridone; oxidation of the isomeric compound probably leads to a hydroxypyridone system. YADH was treated with [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate; GAPDH, with both inactivators. After oxidation the proteins were hydrolysed.

Paper electrophoresis of the resulting amino acid mixtures at pH 1.9 and 6.5 shows only radioactively labelled compound with the same migration rate as S-carboxymethyl-cysteine. [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate inactivates YADH by alkylation of the imidazole moiety of a histidine residue [7], whereas the isomeric 4-bromoacetyl compound alkylates a sulfhydryl residue of this enzyme. The activation of the functional group in the YADH-NAD$^+$ complex involves both amino acid residues. The histidine should be neighboured to the nitrogen atom of the pyridinium ring and the sulfhydryl group to the carboxamide function. In the case of GAPDH both inactivators react with sulfhydryl residues.

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