SPECIFIC MODIFICATION OF 17 β-ESTRADIOL DEHYDROGENASE FROM HUMAN PLACENTA BY NICOTINAMIDE- [5-BROMOACETYL-4-METHYLIMIDAZOLE] DINUCLEOTIDE

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1. Introduction

The enzyme 17 β-estradiol dehydrogenase from human placenta is inactivated by substrate analogues [1–4] and the coenzyme analogue chloroacetyl-pyridinio-adenine dinucleotide [5,6]. Nicotinamide-[5-bromoacetyl-4-methylimidazole]-dinucleotide possesses a reactive bromoacetyl residue in the non functional part of the coenzyme analogue [7]. This compound reacts with nucleophilic groups of amino-acid residues, which are located in the coenzyme binding area [8].

2. Materials and methods

The preparation of the coenzyme analogue nicotinamide- [5-bromoacetyl-4-methylimidazole] dinucleotide [^{14}C] carbonyl-labelled in the nicotinamide carboxyl group has been reported before [7]. NAD^+ and $NADP^+$ were purchased from Boehringer Mannheim. The purification of 17 β-estradiol dehydrogenase (EC 1.1.1.62) and the enzyme assay have been described previously [9]. The inactivation reaction, pH-dependency, were carried out as follows.

The incorporation of the reactive coenzyme analogue was measured by using [^{14}C] carbonyl nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide with a specific radioactivity of 0.36 mCi/mmol. 10.2 mg of the inactivator were dissolved in 0.15 ml 0.6 M Tris–HCl buffer pH 7.2 and added to 5 ml solution of 2.9 mg enzyme in 0.03 M phosphate buffer pH 7.2 containing 20% glycerol. The extent of the inactivation at 37°C was measured using the enzymatic test [9]. The inactivation reactions were done with or without the substrate estradiol at a concentration of 1×10^{-4} M. At various inactivation levels, 1 ml aliquots were precipitated by addition of 4 ml 10% aquous trichloro acetic acid. This process was repeated ten times to remove the not covalently bound inactivator. The incorporated radioactivity was determined after solving the precipitates in 8 M urea buffer. For the identification of labelled amino acids 3.72 mg of 17 β-estradiol dehydrogenase in 0.03 M phosphate buffer pH 7.2, 20% glycerol were completely inactivated in presence of 0.1 mM estradiol by addition of nicotinamide-[5-bromoacetyl-4-methyl-imidazole] dinucleotide in 0.6 M Tris–HCl buffer, pH 7.2 (resulting concentration of the inactivator was 5.8 mM). After removing the excess of the inactivator by dialysis against 3 litres of 0.03 M phosphate buffer, pH 7.2, containing 20% glycerol, the carbonyl groups of the covalent enzyme coenzyme compound were reduced by treatment with 0.308 mg

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sodium borohydride with a specific radioactivity of 614 mCi/mmol, from CEA France. After standing for 36 h at room temperature the protein was precipitated by adding 10 ml 10% trichloroacetic acid and collected by centrifugation. The protein was dissolved in 5 ml 8 M urea which was adjusted to pH 8 by diluted ammonia and dialysed against water until the radioactivity of the protein did not further decrease. The sulphhydrylgroups were transferred into carboxymethyl derivatives by treatment with 1 mM iodoacetate [10] and the excess of iodoacetate removed by dialysis 5 h against 50 ml 8 M urea, pH 8.0. The labelled protein was hydrolysed completely after 21 h in 6 N HCl at 110°C. HCl and water were removed by freeze drying over potassium hydroxide. The labelled compounds were separated by an aminoacid analyser Technicon and identified by comparing the elution volume with that of the model compounds prepared by alkylating N-α acetyl aminoacids with 1-β-D-ribofuranosyl-[5-bromoacetyl-4-methylimidazole]-5′ phosphate, reduced with sodium borohydride and hydrolysed with 6 N HCl. After removing HCl, retention times on the aminoacid analyser were determined and compared with the retention times of the tritium-labelled fractions of the hydrolysed protein. Samples of stoichiometric analysis were digested by α-chymotrypsin. The digest was lyophilized and the chymotryptic peptides were separated by paper electrophoresis at pH 6.5 and pH 3.5. The labelled peptides were detected by autoradiography and scanning with a Panax scanner. The labelling was determined quantitatively after combustion of the protein on the electrophoresis paper in an Oximat Intertechnique, or after elution of the protein from the paper, in a Packard 3320 spectrometer. The scintillation solution was prepared according to Butler [11]. The dissociation constant of the enzyme inactivator complex was determined as described previously [7].

3. Results

The coenzyme analogue nicotinamide-[5-acetyl-4-methylimidazole] dinucleotide acts as hydrogen acceptor with 17 β-estradiol dehydrogenase. The K_M value is 7 mM and the turnover number 640 mol/min. With NAD^+ the K_M value is 0.02 mM and the turnover number 272. The coenzyme analogue nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide is active as a hydrogen acceptor in the oxidation of the substrate too. The reactive bromo-

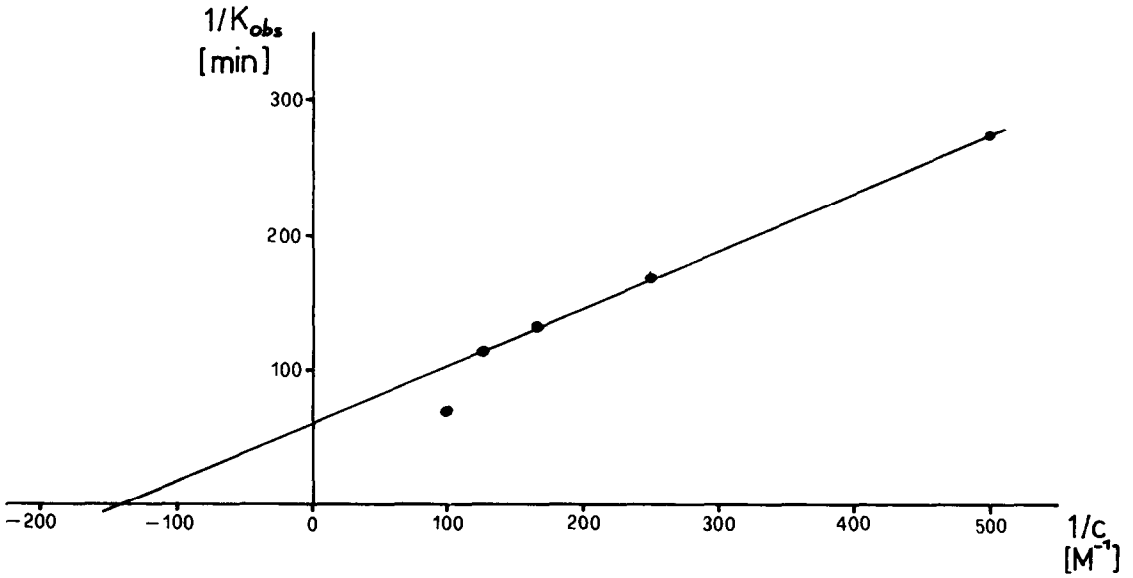

Fig.1. Double reciprocal plot $1/k_{obs}$ versus 1/c of the inactivation reaction. The intercept at the axis of abscisses corresponds to $-1/K_D$ and that of the ordinate to $1/k_2$.

acetyl group of the analogue inactivates the enzyme. The dissociation constant K_D of the reversible complex, which is formed at first, is 7 mM and in the same magnitude as the K_M of the non brominated analogue both determined at pH 7.0. (fig.1). The inactivation rate k_2 is 0.017 min^{-1} [7]. The incorporation of 2.2 mol ^{14}C-labelled inactivator into 1 mol dimer (mol. wt. 72 000) was observed (fig.2). In presence of 0.1 mM estradiol, the incorporation of 2.1 mole of the analogue into 1 mole enzyme dimer was observed. The presence of substrate effects a small increase of the inactivation velocity, but 0.05 mM $NADP^+$ completely protects the enzyme against inactivation by nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide (fig.3). The optical properties of the modified enzyme after reduction of the pyridinium system by sodium hyposulphite, are similar to that of the binary complex formed by dihydronicotinamide-[5-acetyl-4-methylimidazole] dinucleotide and the enzyme. After chymotryptic digestion of the enzyme completely inactivated by ^{14}C-labelled nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide 3 cysteine containing pep-

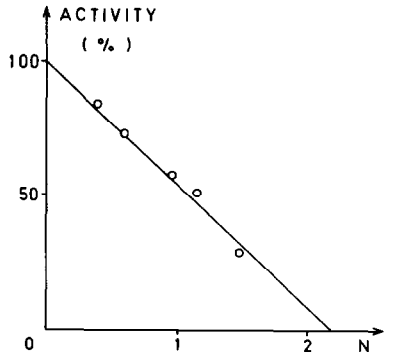

Fig.2. Plot of residual enzyme activity (%) against N (N= moles of ^{14}C-labelled inactivator covalently linked per mole of enzyme dimer).

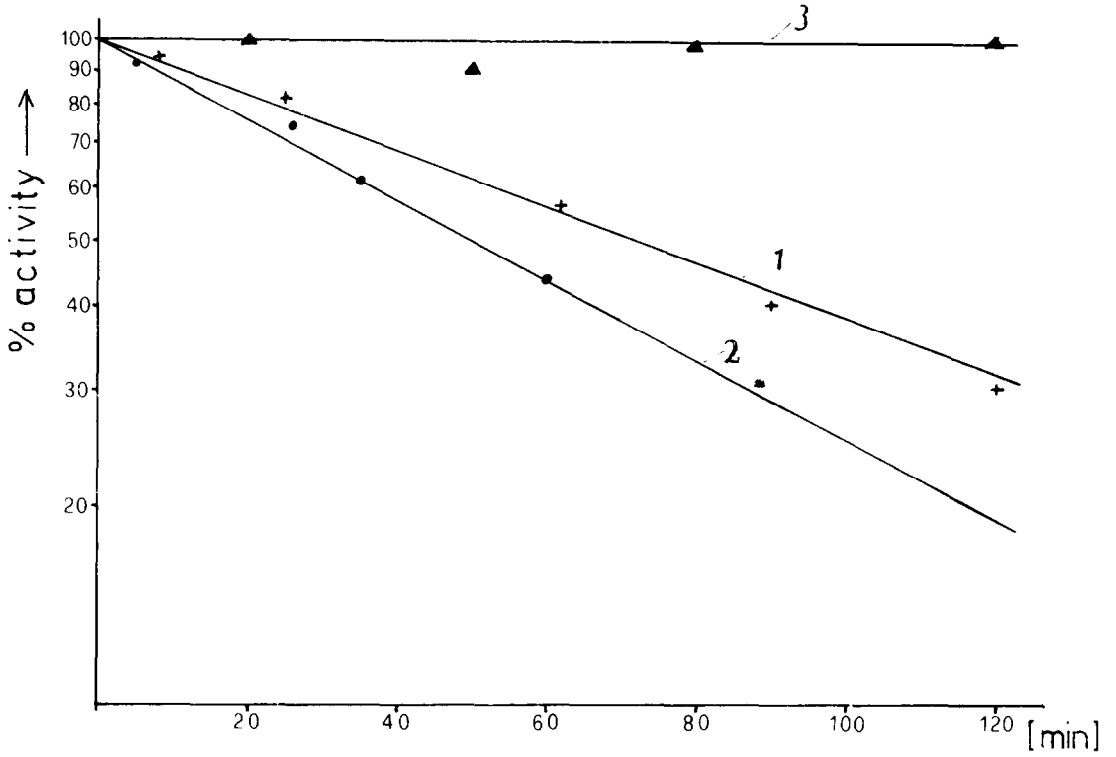

Fig.3. Inactivation rate of 17 β-estradiol dehydrogenase. (1) In presence of 5 mM nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide. (2) In presence of 5 mM nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide and 0.1 mM estradiol. (3) In presence of 5 mM nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide and 0.05 mM $NADP^+$.

tides showed changes in mobility in the electric field at pH 6.5 and pH 3.5. The three peptides are labelled to about the same extent. Acid hydrolysis of the inactivated and sodium [^{3}H] borohydride treated enzyme yields a tritium labelled compound, which has the same elution volume on the aminoacid analyser as a model compound formed by *N*-α-acetyl cysteine and 1-β-D-ribofuranosyl-[5-bromoacetyl-4-methylimidazole]-5′-phosphate and treated as described in Materials and methods. But the main radioactivity was recovered from the column together with ammonia.

4. Discussion

The alkylating coenzyme analogue nicotinamide-[5-bromoacetyl-4-methylimidazole] dinucleotide acts as coenzyme in assay with estradiol dehydrogenase, it combines with the enzyme like NAD^+ and alkylates aminoacid residues at the active center. These residues seem to be in the coenzyme binding site, since $NADP^+$ strongly protects against inactivation while the presence of estradiol caused a small acceleration of the inactivation rate. The incorporation of 1 mol inactivator into 1 mol enzyme subunit agrees with the number of active sites per subunit [1–5]. The optical properties after treatment with sodium hyposulphite are similar to that of the binary complex formed with dihydronicotinamide-[5-acetyl-4-methylimidazole] dinucleotide and the enzyme. The alkylated residue was determined as the sulphydryl group of cysteine. It is possible that more than one of these residues are located at the active site of the enzyme and are involved in the catalytic process or coenzyme binding. This possibility is indicated by the separation of three labelled peptides after enzymatical digestion. The stoichiometry of the inactivation, 1 mole inactivator/1 mole enzyme monomer, suggests that these cysteine residues are located at the active site possibly in such a way that the alkylation of one cysteine by the coenzyme analogue impairs the coenzyme binding and therefore the alkylation of the other residues.

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