AFFINITY LABELLING OF THE ACTIVE SITE OF THE 17β-OESTRADIOL DEHYDROGENASE OF HUMAN PLACENTAL CYTOSOL

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1. Introduction

The 17β-oestradiol dehydrogenase (EC 1.1.1.62) of the human placental cytosol [1–3] catalyses the reaction:

$$\text{Oestradiol} + \text{NAD(P)}^+ \rightleftharpoons \text{oestrone} + \text{NAD(P)H} + \text{H}^+$$

This enzyme is a dimer (34 000 × 2). In each monomer, 6 cysteines are present, of which two are protected by the coenzyme $NADP^+$ from the alkylating reagents inactivating the enzyme (*N*-ethyl maleimide, dithio-bis nitrobenzoic acid). The selective labelling of one of these residues has permitted the isolation of a hepadecapeptide which includes this residue and, thus, gives some initial information on the structure of the active site of this dehydrogenase (Nicolas [4]).

We have tried to obtain more information about this site, and especially about the substrate recognition site, by using an alkylating steroid: iodo-16α acetoxy-3 oestrone (IAE). IAE was chosen as a competitive inhibitor of the enzyme ($K_i = 1.2 \times 10^{-5}$ M); this inhibitor can bind the enzyme irreversibly by covalent binding. Here we are reporting the results obtained with this affinity label.

2. Experimental

2.1. *Synthesis of iodo-16α acetoxy-3 [6,7-^{3}H]oestrone*

This product was synthesized by Melle C. Reliaud in the laboratory, as follows: [6,7-^{3}H]Oestrone (450 mg; 1.2 mCi/mM) is refluxed for 1 hr in 20 ml of benzene and 4.5 ml of isopropenylacetate. 5 Drops of concentrated sulfuric acid are added and the mixture is distilled for 2 hr. The reaction products are chromatographed on a kieselgel column and 200 mg of diacetoxy-3,17-estratetraene-1,3,5,16 are eluted with a benzene–diethylether mixture (9:1). This product is dissolved in 20 ml of dioxane and stirred under nitrogen at 65° for 20 min. *N*-Iodosuccinimide (150 mg) is added and the mixture is left for 3 hr at 65° under nitrogen. After cooling, 200 mg of potassium iodide and 250 mg of sodium thiosulfate dissolved in a minimum of water are added. The crude products (225 mg) are extracted with chloroform. The purification on a kieselgel column, eluted with a pentane–benzene gradient, gives 50 mg of pure iodo-16α acetoxy-3 [6,7-^{3}H]oestrone. The chemical purity of the product is controlled by thin-layer chromatography, IR spectrophotometry and melting point determination (F = 142–145°).

The radiochemical purity, tested after thin-layer chromatography with a Panax scanner is more than 98%.

2.2. *Buffers and reagents used*

Buffer A: 0.07 M phosphate buffer, 20% glycerol, pH 7.2.

Buffer B: buffer A is brought to pH 8.8 with a 2 M Tris solution.

Buffer C: buffer B + [6-7-^{3}H] oestradiol (CEA) 10^{-5} M, (2400 cpm/ml).

Scintillation solution A; 400 mg PPO, 12 g POPOP in 4 ℓ toluene–Triton X-100 (2:1) mixture.

Scintillation solution B: 7 g butyl PBD (Intertech-

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nique), 20 g naphthalene (Merck), 700 ml dioxane, 300 ml toluene (Prolabo).

NADP, NADPH are from Boehringer Mannheim.

2.3. *Enzyme purification and protein determination*

The enzyme is purified by affinity chromatography according to [5] and rechromatographed on DEAE-cellulose just before the labelling. Its specific activity is 4 UI at the exit of the column (the enzyme is then in solution in buffer A).

The protein concentration is determined by amino acid analysis: 2 ml of enzyme (300–500 μg) are precipitated by 3 ml of 10% trichloroacetic acid (TCA). The precipitate is filtered on a Whatman GF/A glass filter, washed twice with 5 ml of TCA (10%) and once with 5 ml of ethanol. The precipitate and the glass filter are dried and introduced into a sealed tube and the hydrolysis is performed under vacuum during 24, 48 or 72 hr in 2 ml of 6 N hydrochloric acid. The hydrolysate is freeze dried and dissolved in 1 ml of 0.2 M citrate buffer pH 2.2 in order to analyse the amino acids (Multichrom Beckman).

2.4. *Inhibition of the enzyme by iodo-16α acetoxy-3 [6,7-^{3}H]oestrone*

The inhibition experiments are performed at pH 8.8 (buffer B) by addition of 40 or 100 μl of inhibitor solution (5×10^{-3} M in ethanol) to 10 ml of the enzyme solution (final conc. of the inhibitor: 2 or 5×10^{-5} M). We then incubate at 37° from 0 to 6 hr. We conjointly determine the kinetics of inactivation and the kinetics of irreversible binding of the inhibitor.

The binding of the inhibitor to the protein is measured by two different produres:

2.4.1. Procedure 1

1 ml of enzyme solution added to 1 ml of ethanol is precipitated at 0° by 3 ml of 10% TCA and washed according to 2.3. Then, the precipitate and the glass filter are dried and placed into a counting vial; the precipitate is dissolved with 1 ml of NCS (Nuclear Chicago) at 45° for 15 min, 15 ml of scintillation liquid A is added and the amount of radioactive inhibitor bound is determined by counting in a Packard 3320 spectrometer.

2.4.2. Procedure 2

The covalent labelling is stopped by addition of 10 μl of β-mercaptoethanol to 1 ml of the incubation medium described above (2.4). The mixture is dialysed 4 times with 1 ℓ of bidistilled water and once with 50 ml of 5% glycerol solution in bidistilled water. The dialysis bag is totally dried at 110° during 120 min. The amount of inhibitor is determined after combustion of the dialysis bag in an "Oxymat Intertechnique" apparatus, by counting in the scintillation solution B.

2.5. *Attempt to protect the enzyme from inhibitor*

We have studied the protective effect either of the coenzyme (NADP) or of the substrate (oestradiol) or of both from the enzyme inactivation and from the binding of the inhibitor. The concentration of each of these protective agents is 10^{-4} M.

2.6. *Enzyme–coenzyme binding*

Research for this enzyme–coenzyme binding was conducted, before and after inhibition, by fluorescence spectrometry (Velick [6], Jarabak [7], Mousseron [8]) and by measuring the fluorescence polarization factor by means of a spectrophotofluorimeter (Aminco Bowman) equipped with Glan polariser and analyser prisms. To 2 ml of native or inhibited enzyme (about 160 μg), we add 5 μl aliquots of 0.24 mM NADPH in the same buffer. The fluorescence polarization is determined at 1.8 μM NADPH concentration.

2.7. *Enzyme–substrate binding*

The binding difference between inhibited and native enzyme is studied by filtration on a Biogel P-60 column (25 × 1 cm) (Jarabak [7]). The column is equilibrated with buffer C. 1 ml of enzyme solution is placed in the same buffer on the column. The radioactivity is measured on a 50 μl aliquot of each fraction (1.5 ml) at the exit of the column, in 15 ml of scintillation liquid A.

3. Results

3.1. *Enzyme concentration*

The analysis of amino acids of an enzyme solution (A_{280} = 0.117, Acta III Beckman spectrophotometer) gave 160 mg of proteins/ml; the extinction coefficient of a freshly prepared enzyme solution is thus $\epsilon\%$ = 0.73. This enzyme solution was used for all affinity labelling described below.

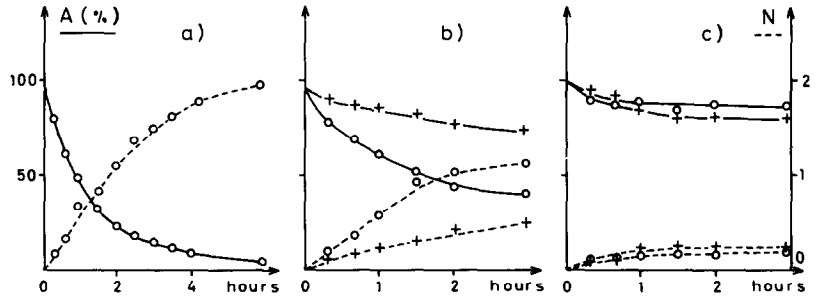

Fig. 1. Kinetics of enzyme inhibition by radioactive IAE (full lines ——) and of simultaneous irreversible binding of the inhibitor (dotted lines – – –) expressed by N (N = moles of radioactive IAE linked/mole of enzyme). The experimental conditions are: incubation medium: buffer B; pH 8.8; t° = 37°. a) Unprotected enzyme (○); IAE = 5×10^{-5} M; b) unprotected enzyme (○); enzyme protected by the substrate oestradiol (+); IAE = 2×10^{-5} M; oestradiol = 10^{-4} M; c) enzyme protected by the coenzyme NADP (+) or by NADP + oestradiol (○); IAE = 2×10^{-5} M; NADP = 10^{-4} M; oestradiol = 10^{-4} M.

3.2. *Preliminary studies of inhibition conditions*

After a 4 hr incubation of the enzyme (2×10^{-6} M) with 2×10^{-5} M IAE, the inactivation percentages at 25° and three different pH's (7.2, 8, 8.8) are, respectively, 3%, 5%, 30%; at 37° and pH 8.8 the inactivation percentage is 63%.

Consequently, the following experiments are all conducted at 37° and at pH 8.8. Also, it was observed that, at pH 7.2, IAE can be reduced enzymatically without detectable inhibition during the time course of the measurement: V_{max} = 68 moles of IAE reduced per mole of enzyme per min.

3.3. *Enzyme inactivation and irreversible binding of the inhibitor with and without protection*

Fig. 1 shows the inactivation and irreversible binding of IAE in the presence or in the absence of protection: the enzyme is quickly inactivated by the inhibitor in the absence of protection (fig. 1a). The binding velocity of IAE to enzyme is determined assuming that it is constant during the first 20 min: then for 2.3 nM of enzyme, 960 dpm are incorporated when the concentration of IAE = 2×10^{-5} M, and 1100 dpm when IAE = 5×10^{-5} M. This corresponds to, respectively, 0.008 and 0.009 M of inhibitor bound

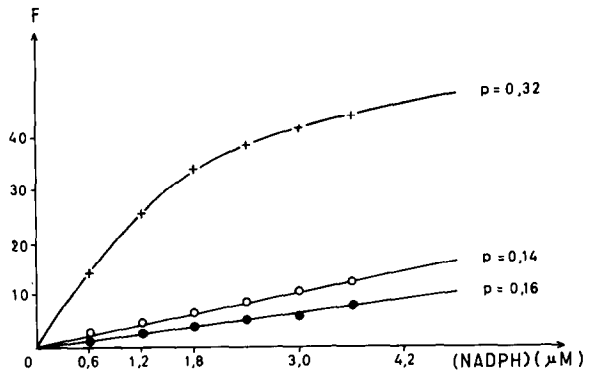

Fig. 2. Fluorescence of NADPH (excitation 340 nm, emission 450 nm). (○—○—○) Buffer B (pH 8.8); (+—+—+) buffer B plus native enzyme; (●—●—●) buffer B plus enzyme inhibited by IAE. p is the fluorescence polarisation coefficient for a NADPH concentration of 1.8×10^{-6} M.

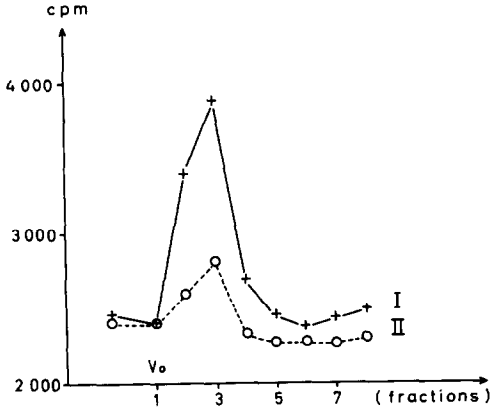

Fig. 3. Chromatography elution pattern of native enzyme (I) and of inactivated enzyme by non radioactive IAE (II) on a Biogel P.60 column equilibrated with tritiated oestradiol (2400 cpm/ml). The proteins are found in the fractions 2 and 3.

irreversibly per min per mole of enzyme. The substrate (fig. 1b) or the coenzyme or their association (fig. 1c) have an important protective effect against enzyme inactivation and against IAE irreversible binding.

3.4. *Enzyme–coenzyme binding*

Fig. 2 shows that the inhibited enzyme does not exalt the NADPH fluorescence (exaltation being characteristic of the formation of the enzyme–coenzyme complex), when compared in the same way to the native enzyme. Furthermore, the NADPH fluorescence polarisation in the presence of inhibited enzyme (p = 0.16) is very close to that of NADPH in buffer B (p = 0.14) and much smaller than that of NADPH in the presence of the native enzyme (p = 0.32).

These two results clearly indicate that the inhibited enzyme does not bind to the coenzyme anymore.

3.5. *Enzyme–substrate binding*

Fig. 3 represents the filtration results on the Biogel P-60 column equilibrated with tritiated oestradiol:

a) of a native enzyme sample (curve I);

b) of an enzyme sample inactivated by the non radioactive inhibitor (curve II).

The formation of the enzyme–substrate complex is evident in the case of native enzyme by an increase in the radioactivity eluted in the fractions containing the enzyme (fractions no. 2 and 3). There is a great decrease of binding in the case of inhibited enzyme.

4. Discussion

An inhibitor (I) is an enzyme affinity label when the irreversible inhibition follows the relationship (Baker [9]):

$$E + I \underset{\nu_{-1}}{\overset{\nu_1}{\rightleftharpoons}} E\cdots I \xrightarrow{\nu_2} EI$$

The following arguments show that this is the case with IAE and the 17β-oestradiol dehydrogenase:

a) The formation of an intermediary complex E···I is involved, since at a pH 7.2, the alkylation being impossible, IAE is a substrate of the enzyme. Furthermore, IAE is a competitive inhibitor in the oxidation of oestradiol to oestrone at pH 8.8 ($K_i = 1.2 \times 10^{-5}$).

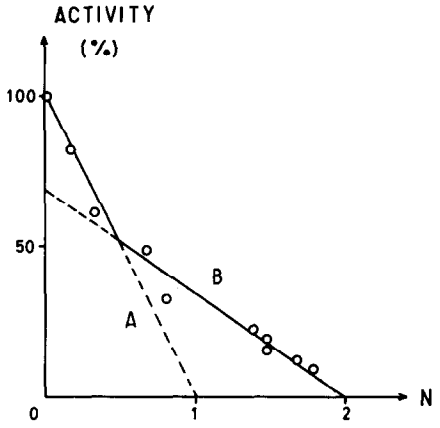

Fig. 4. A plot of residual enzyme activity (%) against N (N = moles of radioactive IAE linked/mole of enzyme).

b) The formation of this complex E···I is faster than the alkylation reaction since the minimum formation velocity limit of the Michaelis complex, measured by V_{max}, is quite superior to ν_2 (for 1 M of enzyme, $V_{max} = 68$ M × min^{-1} and $\nu_2 = 0.009$ M × min^{-1}).

c) The substrate exhibits a protective effect against IAE and the inactivated enzyme loses the ability of binding the substrate.

d) The kinetics of inactivation, at two different inhibitor concentrations, permit the exclusion of the eventuality of a non specific bimolecular reaction. With such a reaction we should have:

$E + I \rightarrow EI$ where $\nu' = k'(E)(I)$ and then, for $(IAE)_1 = 5 \times 10^{-5}$ M and $(IAE)_2 = 2 \times 10^{-5}$ M, the ratio $\frac{\nu'_1}{\nu'_2} = 2.5$.

In fact, the experimental alkylation velocities ratio for these two concentrations is 0.009/0.008 = 1.13. This value is incompatible with the preceding hypothesis but remains, on the contrary, compatible with the affinity labelling (Baker [9]): in this case $\nu_2 = k(E\cdots I) = k(E)(I)/(K_i + (I))$ and this relationship gives 1.25 as a theoretical ratio of these two alkylation velocities.

The affinity labelling shown by IAE possesses two particularities:

i) The complete inactivation of the enzyme is experi-

mentally carried out only after the binding of 2 inhibitor molecules to 1 enzyme molecule (fig. 4). However, it is observed that the stoichiometry of inactivation is, at first, 1/1 (curve A), then 2/1 (curve B). This result suggests that the blocking of one out of two binding sites is sufficient to inactivate the dimer, without preventing the binding of a second inhibitor molecule.

The determination of the binding site(s) of these inhibitor molecules is in progress by studying tryptic peptides.

ii) The experiments on protections show that the substrate or the coenzyme protects the enzyme from inactivation and from binding of the inhibitor: this seems to indicate that the amino acids alkylated by the steroid belong to a zone where the substrate recognition site covers up the coenzyme recognition site. This hypothesis is supported by the fact that alkylation binds the steroid in a region which is near to that where the catalysed reaction occurs.

Acknowledgements

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