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

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1. Introduction

The amino acid sequence of a tryptic peptide of the human placental 17 β-oestradiol dehydrogenase has been determined after labelling with [2-^{14}C]*N*-ethylmaleimide of an essential cysteine presumed to be in the neighbourhood of the coenzyme binding site [1].

We recently reported an affinity labelling directed towards the substrate binding site [2]. Now, we carry on the identification of essential amino acids of the active site of this enzyme by using other affinity labels: iodo [2'-^{14}C]acetoxy-3-derivatives of oestrone or oestradiol. These alkylating inhibitors do have close structural relationships with 3-alkyloxy or aryloxy-derivatives of oestradiol which are good substrates ($K_m = 1-2 \times 10^{-6}$ M; ref. [3]). Furthermore the alkylated amino acids can be easily identified after hydrolysis as [^{14}C]carboxymethylated derivatives. We report here the specific labelling of an histidyl residue presumed to be implicated in the substrate recognition.

2. Experimental

2.1. *Reagents*

The reagents were: buffer A, 0.07 M phosphate, 20% glycerol, pH 7.2; buffer B, 0.1 M ammonium bicarbonate, pH 8.0; buffer C, pyridine–acetic acid, pH 6.5; buffer D, pyridine–acetic acid, pH 3.5. Scintillation solution: 400 mg PPO, 12 g POPOP in 4 ℓ of a mixture toluene–Triton X-100 (2:1). NAD, NADP, NADPH are from Boehringer Mannheim.

2.2. *Synthesis of iodo[2'-^{14}C]acetoxy-3-oestrone and of iodo[2'-^{14}C]acetoxy-3-oestradiol*

48 mg of [2-^{14}C]iodoacetic acid (Amersham, specific activity 1 mCi/mM) dissolved in 4 ml of methylene chloride, were added to the oestrone solution (70 mg in 3 ml of methylene chloride) and cooled to 0°C. After adding 100 mg of dicyclohexylcarbodiimide (Fluka) dissolved in 2 ml of methylene chloride and 80 μl of pyridine, the reaction was allowed to proceed for 1 hr at 0°C and 1 hr at room temp. The reaction mixture was filtrated and the filtrate freeze-dried. After the addition of 2 ml of acetone, a second filtration removed the insoluble dicyclohexylurea. The product was purified by thin layer chromatography (TLC) on preparative plates Merck no. 5733 (benzene–ethyl-ether 8:2) and 30 mg of a colorless product were obtained after recrystallization in methanol. The chemical purity of the product was controlled by TLC, IR spectroscopy and micro-analysis.

The main IR absorption bands (KBr) were: ν(c=o); 1735 cm^{-1} (17 oxo) and 1755 cm^{-1} (3 ester); ν(c-o-c); 1250 cm^{-1}. Analysis; $C_{20}H_{23}O_3I$ – calculated C, 54.7; H, 5.2; I, 29.0; O, 10.9 – found C, 55.0; H, 5.3; I, 27.2; O, 12.

The melting point was 134–135°C. The radiochemical purity, controlled after thin layer chromatography with a Panax scanner was better than 97%.

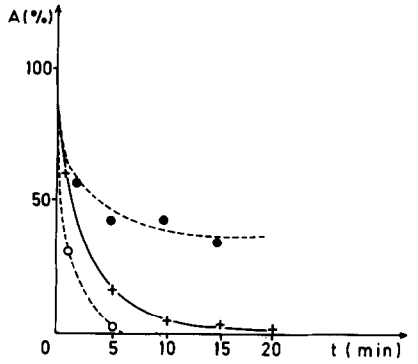

Fig. 1. Inactivation of the 17 β-oestradiol dehydrogenase by iodo [2'-^{14}C] acetoxy-3-oestrone (oxidized inhibitor). The enzyme was incubated in buffer A, at pH 7.2 and 37°C with: 10^{-4} M inhibitor (+—+—+); 10^{-4} M inhibitor and 2.4 × 10^{-4} M NADP (●---●---●); 10^{-4} M inhibitor and 2.4 × 10^{-4} M NADPH (○---○---○).

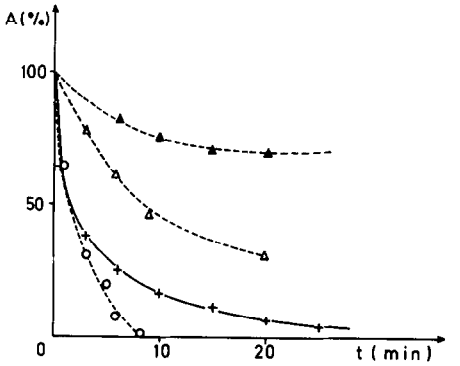

Fig. 2. Inactivation of the 17 β-oestradiol dehydrogenase by iodo [2'-^{14}C] acetoxy-3-oestradiol (reduced inhibitor). The enzyme was incubated in buffer A, at pH 7.2 and 37°C with: 10^{-4} M inhibitor (+—+—+); 10^{-4} M inhibitor and 1.2 × 10^{-4} M NADPH (△---△---△); 10^{-4}M inhibitor and 6 × 10^{-4} M NADPH (▲---▲---▲); 10^{-4} M inhibitor and 2.4 × 10^{-4} M NADP (○---○---○).

2.3. *Enzyme purification*

The 17 β-oestradiol dehydrogenase was purified according to [4] and an ultimate chromatography on DEAE-cellulose was processed just before its use. The protein concentration was determined by amino acid analysis [2] and the specific activity, measured as previously described [3] was 4 IU.

2.4. *Inhibition experiments*

The enzyme (3 × 10^{-6}M, expressed as the monomer) was incubated in buffer A at 37°C in the presence of 10^{-4} M inhibitor: this 30-times excess of inhibitor is necessary to get a 100% inhibition because of the instability of the inhibitor; this molecule is completely hydrolysed to iodoacetate and oestradiol in about 30 min in buffer A at 37°C.

The residual enzymatic activity was periodically measured as described in [3] but at pH 7.2 aliquots (1 ml) were removed for determination of the stoechiometry of the alkylation. After a 24 hr dialysis against 1 ℓ of bi-distilled water twice, the protein bound radioactivity was measured on a 100 μl aliquot in 15 ml of scintillation solution (Packard 3320 spectrometer).

The effect of 5 × 10^{-5} M substrate (oestradiol) and of 2.4 × 10^{-4} M coenzyme (NADP, NADPH) on the inactivation kinetics was studied in buffer A at 37°C.

2.5. *Binding of the substrate*

The ability of the inactivated enzyme to bind its substrate was determined by reference to the native enzyme using the method of gel filtration in the conditions described earlier [2].

2.6. *Enzymatic hydrolysis of the labelled enzyme and separation of the labelled peptide*

The samples of 100% inactivated enzyme were hydrolysed by chymotrypsin (Worthington, 1/20 W/W) in buffer B at 37°C for 4 hr. After freeze-drying the peptides were separated by high voltage electrophoresis at pH 6.5 (buffer C) and 3.5 (buffer D) on Whatman no. 3 MM paper. The radio-labelled peptides were identified by autoradiography (Eastman Kodak 'Regulix'); the corresponding zones were eluted by ammonia N and the radioactivity of an aliquot measured by scintillation counting.

2.7. *Identification of the labelled amino acid*

The labelled enzyme or the radioactive peptides were hydrolysed by 6 N HCl, under vacuum, at 110°C for 24 hr. Under these conditions the 3 ester function of the linked inhibitor is hydrolysed, the steroid is released and the radio-label remains linked to the amino acid as a [^{14}C]carboxymethyl residue.

The hydrolysate is freeze-dried, dissolved in 1 ml of 0.2 M citrate buffer pH 2.2 and analysed in a Multichrom Beckman amino acid analyser fitted with

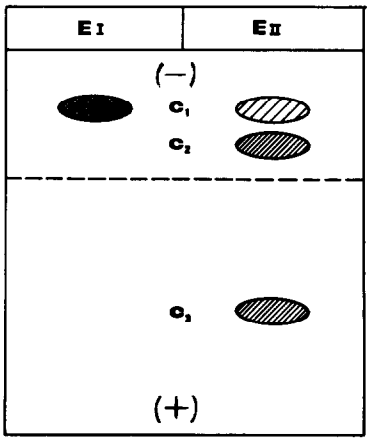

Fig. 3. Autoradiography of the chymotryptic peptides from enzyme inactivated by iodo [2'-^{14}C]acetoxy-3-oestrone in the presence of NADPH (E_I) and in the absence of coenzyme (E_{II}). The enzyme was inactivated and hydrolyzed as described in 2.4 and 2.6 and submitted to high voltage paper electrophoresis at pH 6.5 in buffer C on Whatman paper no. 3 MM. The radioactive peptides were revealed by autoradiography (cf. [11]).

a scale expansion. The amino acid residues are collected at the exit of the colorimeter for measurement of the radioactivity.

3. Results

3.1. *Inactivation of 17 β-oestradiol dehydrogenase by iodo[2'-^{14}C] acetoxy-3-oestrone ('oxidized inhibitor')*

A rapid inactivation is obtained when the enzyme is incubated with the inhibitor alone (fig. 1). In the presence of the oxidized coenzyme (NADP) an important decrease of the inactivation rate is observed, whereas, in the presence of the reduced coenzyme, the inactivation rate is higher.

The substrate (10^{-4} M oestradiol) exhibits the same protective effect as NADP (2.4×10^{-4} M).

3.2. *Inactivation of 17 β-oestradiol dehydrogenase by iodo[2'-^{14}C] acetoxy-3-oestradiol ('reduced inhibitor')*

Fig. 2 shows a rapid inactivation when the substrate and the coenzyme are omitted. The reduced coenzyme (NADPH) exerts a strong protective effect against the inhibitor, whereas, the oxidized coenzyme increases the inactivation rate. As in the case of the oxidized form of the inhibitor, the substrate exhibits the same protective effect as NADPH.

3.3. *Stoechiometry of the covalent binding of the oxidized inhibitor*

In the presence of NADPH, 100% inactivation is obtained in 5 min with the covalent binding of 0.9 to 1 M of inhibitor (oxidized form) per 1 M of enzyme monomer (mol. wt. 34 000 – [5–8]).

With the oxidized inhibitor alone, a complete inactivation is only observed in about 15 min after the covalent binding of more than 2 M of inhibitor per monomer.

3.4. *Identification of the labelled peptides and amino acids*

Fig. 3 shows the results of the separation by high voltage electrophoresis at pH 6.5 of the peptides obtained by chymotryptic digestion of two samples of inactivated enzyme.

A single radio peptide (C_1) can be identified when the enzyme is inactivated in the presence of the oxidized inhibitor and NADPH (E_I, fig. 3). After elution, acid hydrolysis and amino acid analysis, 90–95% of the radioactivity of this basic peptide C_1 was recovered in the peak of 3-carboxymethyl histidine.

In the presence of the oxidized inhibitor alone (E_{II}, fig. 3) three peptides are labelled (C_1, C_2, C_3) and the recovered radioactivity of the sample E_{II} is distributed as follows: 15% as carboxymethyl histidine in basic peptide C_1; 45% as carboxymethyl cysteine in the neutral peptide C_2 and 40% as carboxymethyl cysteine in the acid peptide C_3.

The labelled cysteinyl residue of C_3 is identical to the SH group of which the specific labelling by NEM results in a stoechiometric inactivation [1].

4. Discussion

4.1. *Influence of the coenzyme on the labelling*

The protective effects of NADP against inactivation by the oxidized inhibitor and of NADPH against the reduced inhibitor are in agreement with the results of Betz [9] which demonstrated the inability of the enzyme to form abortive ternary complexes

(here: iodoacetoxy-3-oestrone-enzyme-NADP or iodoacetoxy-3-oestradiol-enzyme-NADPH).

The formation of the evolutive ternary complex (iodoacetoxy-3-oestrone-enzyme-NADPH) results in the specific and stoechiometric alkylation of a single histidyl residue and in a higher inactivation rate than with the inhibitor alone.

The lack of specificity of the labelling occurring in the absence of coenzyme (2 Cys and 1 His are labelled) is not surprising since two cysteinyl residues of the 17 β-oestradiol dehydrogenase are relatively more reactive with hydrophobic reagents and since at least one of these two residues (acid peptide: C_3) is in the neighbourhood of the coenzyme recognition site as is shown by a selective labelling with *N*-ethylmaleimide [1].

Whether the selective histidine labelling obtained with iodoacetoxy-3-oestrone + NADPH results from a specific orientation of the inhibitor induced by the coenzyme in the evolutive ternary complex, or from the protection of the two cysteines by the coenzyme against the hydrophobic inhibitor, cannot yet be determined.

4.2. *Specificity of the labelling by iodoacetoxy-3-oestrone*

Some evidence of the labelling to occur at the substrate recognition site is as follows: The oestrone molecule directs the alkylating functional group (iodoacetoxy) towards the active site of the 17 β-oestradiol dehydrogenase, since a 10 min incubation in the presence of 10^{-4} M iodoacetoxy-3-oestrone and NADPH results in a complete inactivation, whereas, 24 hr and a 100-times higher concentration would be required to obtain the same result with iodoacetate or iodoacetamide; The substrate exhibits a good protective effect; NADPH induces the specific irreversible binding of 1 mole of oxidized inhibitor/mole of enzyme monomer. This stoechiometry and the irreversible binding are confirmed by the identification of a single histidyl residue in a single peptide. The inhibition thus obtained in the presence of NADPH indicates that the inhibitor is not binding at the coenzyme recognition site; The inactivated enzyme is unable to bind the substrate.

As demonstrated in other experiments [10], the 17 β-oestradiol dehydrogenase inactivated by iodoacetoxy-3-oestrone in the presence of NADPH can be reactivated by hydrolysis of the ester bond of the inhibitor. During this process the oestrone part of the inhibitor is released, leaving a 'super active' histidine carboxymethylated-enzyme. This phenomenon gives evidence for this labelling to occur at the substrate recognition site since reactivation results from the release of oestrone.

Thus, among the three residues (2 cys, 1 His) which have been labelled on native 17 β-oestradiol dehydrogenase (cf. sample E_{II}, fig. 3, peptides C_1, C_2, C_3), the histidyl residue of the peptide C_1 is constitutive of the oestradiol binding site and the essential cysteinyl residue of the peptide C_3 is probably in the neighbourhood of the NADP(H) binding site, since protection is by the coenzyme and not by the substrate.

Acknowledgements

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