(II)

ENHANCEMENT OF THE CATALYTIC ACTIVITY OF THE HUMAN PLACENTAL 17 β-OESTRADIOL DEHYDROGENASE BY CARBOXYMETHYLATION OF AN HISTIDYL RESIDUE OF THE ACTIVE SITE

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1. Introduction

The inactivation of the 17 β-oestradiol dehydrogenase by the active site directed inhibitor iodoacetoxy-3-oestrone in the presence of NADPH allowed the covalent specific labelling of an histidyl residue presumed to be located in the vicinity of the substrate recognition site [1].

An unexpected reactivation was observed when the inactivated enzyme was incubated for 48 hr at 37°C. This paper deals with this reactivation process: we show that it proceeds from the slow hydrolysis occurring at the ester bond of the covalently linked inhibitor. The subsequent release of the steroid leads to a 'superactive' histidyl carboxymethylated enzyme. Some kinetic properties of this modified enzyme are described.

2. Experimental

2.1. *Buffers and reagents*

The reagents were: buffer A, 0.03 M phosphate, 20% glycerol, pH 7.2; buffer B, 0.1 M phosphate pH 7.2, 3‰ bovine serum albumin (BSA), 10^{-4} M oestradiol; buffer C, 0.1 M pyrophosphate pH 9.5, 3% BSA; buffer D, 0.1 M pyrophosphate pH 9.5, 3% BSA, 10^{-4} M oestradiol. Dextran–charcoal suspension: 500 mg charcoal, 50 mg dextran T70 in 100 ml buffer A. Scintillation solutions: A) 400 mg PPO, 12 g POPOP in 4 litres of toluene–Triton X 100 mixture (2:1); B) 100 mg PPO, 3 g POPOP in 1 litre of toluene.

Charcoal: Norit A is obtained from Prolabo. Dextran T70 is from Pharmacia. NAD, NADP, NADPH are from Boehringer Mannheim. β-Mercaptoethanol is purchased from Fluka.

Iodoacetoxy 3-[6,7-^{3}H]oestrone was synthetized as described earlier for iodo [2'-^{14}C]acetoxy-3-oestrone [1] but using cold iodoacetic acid and [6,7-^{3}H]oestrone (specific activity 1 mCi/mM – CEA).

2.2. *Preparation of the alkylated 17 β-oestradiol dehydrogenase*

The enzyme (specific activity 4 IU/mg in buffer A was incubated until complete inactivation (about 20 min) at 37°C with 10^{-4} M radioactive iodoacetoxy-3-oestrone (iodoacetoxy-3 [6,7-^{3}H]oestrone or iodo [2'-^{14}C]acetoxy-3-oestrone).

Two samples were labelled in the same way; one in the presence of 2.4×10^{-4} M NADPH, the other in the absence of NADPH. On each sample, the reaction was stopped by addition of 10^{-3} M β-mercaptoethanol and the enzyme was precipitated by one volume of saturated ammonium sulfate solution. The precipitate was dissolved in 1 ml buffer A and incubated for 30 min with 1 ml of a dextran–charcoal suspension to remove the remaining excess of reagent After centrifugation, the supernatant was equilibrated with buffer A on a Sephadex G 25 column. The radioactivity of the eluate (alkylated enzyme) was determined on 50 or 100 μl aliquots by counting in 15 ml scintillation solution A (Packard 3320 spectrometer).

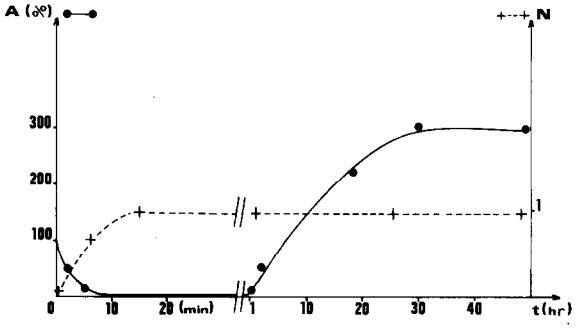


Fig. 1. Inactivation of the 17 β-oestradiol dehydrogenase by iodo [2'-^{14}C]acetoxy-3-oestrone and subsequent reactivation. The enzyme was inactivated by incubation for 20 min at 37°C, pH 7.2 in buffer A with 10^{-4} M inhibitor and 2.4 × 10^{-4} M NADPH. After elimination of the excess reagent as described in text (Section 2.2), the reactivation was obtained by incubation at 37°C, 48 hr in buffer A. (●—●—●) Enzymatic activity expressed as % of activity of native enzyme; (X---X---X) bound ^{14}C-radioactivity expressed as mole of carboxymethyl/enzyme monomer.

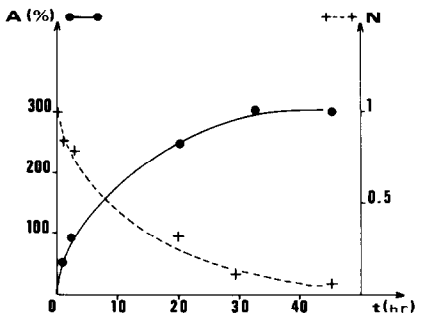


Fig. 2. Reactivation of 17 β-oestradiol dehydrogenase alkylated by iodoacetoxy-3-[6, 7-^{3}H]oestrone in the presence of NADPH. The enzyme was incubated for 48 hr at 37°C in buffer A. The released tritiated steroid was extracted with benzene and determined by scintillation counting. (For experimental details, see text (sections 2.2 and 2.3)): (●—●—●) Enzymatic activity expressed as % of native enzyme; (+---+---+) tritiated steroid bound to the enzyme during the reactivation process expressed by N (N = moles of tritiated steroid linked/enzyme monomer).

2.3. *Determination of the radioactivity released during enzyme reactivation*

The two samples previously obtained (see 2.2) were incubated for 48 hr at 37°C and the release of the steroid was determined on 1 ml aliquots. After extraction with 3 ml of benzene, the radioactivity was counted in 15 ml of scintillation solution B.

2.4. *Determination of enzymatic activity and Michaelis constants (K_m)*

During inactivation and reactivation experiments the enzymatic activity was determined spectrophotometrically as previously described [2] but at pH 7.2 in buffer B (at pH 9.5 the hydrolysis of the bound inhibitor would interfere with the determination of enzymatic activity). The kinetic data for the determination of the K_m of the substrates were determined in buffer C as follows: Light path cells, 5 cm; 1.2 × 10^{-4} M NAD; 10^{-6} M to 10^{-4} M oestradiol, final volume 9 ml. The kinetic data for the determination of the K_m of the cofactors were obtained in the same conditions but in buffer D with 10^{-6} M to 10^{-4} M NAD or NADP. The K_m was determined from double reciprocal plots according to Lineweaver and Burk.

3. Results

We described earlier [1] the inactivation of the 17 β-oestradiol dehydrogenase by iodo [2'-^{14}C]acetoxy-3-oestrone in the presence and in the absence of NADPH: in the presence of NADPH the inactivation occurred with the stoechiometric covalent labelling of a single histidyl residue, (His-labelled enzyme); in the absence of coenzyme, the inactivation occurred with a less specific labelling (15% His, 85% cys).

When the enzyme, inactivated in the presence of NADPH (His-labelled enzyme), is incubated for 48 hr

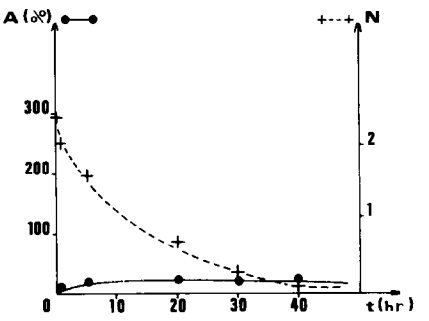
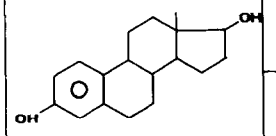
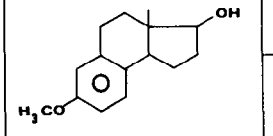


Fig. 3. Reactivation of 17 β-oestradiol dehydrogenase alkylated by iodoacetoxy-3-[6, 7-^{3}H]oestrone in the absence of enzyme. Same legend as in fig. 2.

Table 1

		Native	His-carboxymethylated (Superactive)
oestradiol 	Km	10^{-6}	5×10^{-5}
	Vm	100	300
 Methoxy-3 A-Des oestradiol	Km	1.5×10^{-5}	1.5×10^{-5}
	V*	600	600

V* is expressed as the ratio of methoxy-3-A Des oestradiol oxidation rate/oestradiol oxidation rate, at the same concentration.

at 37°C, a reactivation is observed which results in a 3-fold increase of the initial activity of the native enzyme (fig. 1). As the ^{14}C radioactivity covalently bound to the protein remains constant (one carboxymethyl-His/monomer) all over the reactivation process, we presumed the reactivation to be due to the hydrolysis of the ester bond of the inhibitor and to the subsequent release of the steroid from the active site. Indeed, when the enzyme has been inactivated by iodoacetoxy-3 [6,7-^{3}H]oestrone instead of 2'-^{14}C-labelled steroid, the tritiated steroid is released during the reactivation and both kinetics of reactivation and of steroid release can be compared (fig. 2 and fig. 3).

Table 2

		Native	His - carboxymethylated (Superactive)
NAD	Km	2×10^{-5}	5×10^{-4}
	Vm	100	300
NADP	Km	10^{-6}	5×10^{-6}
	Vm	75	300

Fig. 2 shows that when the inactivation has occurred in the presence of NADPH the *complete reactivation* is concomitant with the release of the whole bound steroid (1 mole/monomer). Fig. 3 shows that when the enzyme had been inactivated in the absence of NADPH, the whole bound steroid is released (more than 2 M of inhibitor/monomer) but only a 20% reactivation occurs.

The 300% reactivated enzyme 'superactive' is no longer modified by iodoacetoxy-3-oestrone or by 16-α-iodoacetoxy-3-oestrone, or by other affinity labels either in the presence or in the absence of NADPH. Neither inactivation nor alkylation could be obtained.

The K_m of oestradiol, methoxy-3-A Des oestradiol, NAD or NADP determined with the superactive enzyme are shown tables 1 and 2.

The modified enzyme exhibits higher K_m for oestradiol (50-fold), NAD (25-fold), NADP (5-fold) than the native enzyme. It should be noticed that neither the K_m nor the oxidation rate of the methoxy-3-A Des oestradiol are modified.

4. Discussion

The 17 β-oestradiol dehydrogenase can be rapidly inhibited by covalent labelling with iodoacetoxy-3-oestrone in the presence as well as in the absence of NADPH within 20 min. In both cases, the hydrolysis

of the phenolic ester of the inhibitor bound at His or at Cys residues can be achieved at 37°C in 48 hr. When the inactivation has been conducted in the presence of NADPH, *His* is the single residue labelled [1] and a maximum reactivation is obtained; when the inactivation has been conducted without NADPH only a very small reactivation occurs, and most of the enzyme molecules are labelled on *two Cys*; a few are presumed to be only labelled on His and to account for the small reactivation obtained in these conditions.

These observations indicate that the carboxymethylation of the cysteinyl residues leads to an irreversible inactivation, that the His-carboxymethylated enzyme is reactivated by the subsequent elimination of the steroid from the active site, and that the labelled histidyl residue is not essential for catalytic activity. However, this His residue should be located near the substrate binding site since the covalent binding of the acetoxy-3 steroid inhibits the enzyme and since after elimination of the steroid, the residual His-carboxymethylated enzyme exhibits a 3-fold activation and a dramatic decrease of the apparent affinity for oestradiol (50-fold increase in K_m). Moreover the lack of modification of the kinetic parameters of the methoxy-3-A Des oestradiol suggests that this His-carboxy methylation induces perturbations in the vicinity of the ring A of the substrate. The increase of the K_m of oestradiol and of NAD could explain the 3-fold increase of K_m by an enhancement of the steroid and (or) of the coenzyme exchange rates since it is known that the hydride transfer is not the rate limiting step in the reaction catalysed by the 17 β-oestradiol dehydrogenase [3].

Another consequence of this His-carboxymethylation is the complete lack of reactivity of the cysteines towards the iodoacetoxy-3-oestrone or other affinity labels. This implicates some interactions between the two cysteines and the histidine residues. As the cysteine residues are known to be located in the vicinity of the coenzyme binding site, such an interaction could explain the modification of the K_m of the coenzyme.

Some examples of an enhancement of enzymatic activity by chemical modifications have been described (thermolysine [4], alcohol dehydrogenase [5, 6]) but, to our knowledge, this phenomenon had never been observed after a preliminary inactivation by affinity labelling of a single residue.

Acknowledgements

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