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BINDING OF ESTRADIOL TO HORSERADISH PEROXIDASE AND HORSE HEART MICROPEROXIDASE

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SUMMARY

Horseradish peroxidase and horse heart microperoxidase can bind estradiol to human or bovine serum albumin in the presence of hydrogen peroxide. However, we have shown here that, in the absence of serum albumin, the hormone was fixed by the enzyme molecule itself. Evidence is presented that (a) the hormone is transformed into a water-soluble and dialysable derivative of estradiol ; (b) this new product is easily separated from the enzyme by gel filtration chromatography. It appears to have a high affinity for the chromatographic gel. The implications of the binding of an estradiol derivative to peroxidases are discussed.

INTRODUCTION

Mouse and rat alpha-fetoproteins are able to bind estrogens specifically (1) and it has been suggested that this property is associated with a physiological function of this protein. However, despite numerous attempts including physico-chemical modifications of the protein, we were unable to demonstrate a similar attachment of estrogens to human alpha-fetoprotein (Aussel et al., unpublished results).

In the course of experiments with crude human fetal extracts, we observed that tritiated estradiol was fixed on a high molecular weight component in the presence of hydrogen peroxide (2). Subsequent experiments revealed that this fixation was associated to the presence of a peroxidase which we purified and which, under certain conditions, bound E_2 on its own molecule (3).

As a result we investigated whether this reaction is specific to this fetal peroxidase or is common to others such as horseradish peroxidase or horse heart microperoxidase. Indeed some peroxidases have been shown to possess an enzymatic activity on phenols and especially on estrogens (4-5). Norymberski (6), using slightly different conditions, showed that HRPO catalysed the polymerisation of E_2.

Abbreviations : E_2 : Estradiol ; HRPO : Horseradish Peroxidase ; HSA : Human Serum Albumin ; BSA : Bovine Serum Albumin ; DCP : 2,4 - Dichlorophenol; MPO : Horse Heart Microperoxidase.

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In the present work we have tried to identify the binding of E_2 to HRPO and MPO molecules and to analyze the nature of this binding.

MATERIALS AND METHODS

Reagents. HRPO type VI (RZ value 3.42) and MPO type MP-II were purchased from Sigma Chemicals Co., St Louis, Missouri, USA. HSA (ORHA 20-21) was obtained from Behringwerke A.G., Marburg, West Germany and BSA from Koch-Light Laboratories Ltd., Colmbrook Bucks, England. E_2 was a gift from Roussel Uclaf, France : a 10^{-4} M solution in pure ethanol diluted 1/10th in 0.01M sodium phosphate buffer pH 7.2 was used. [2,4,6,7-^{3}H]E_2 (85 Ci/mmol) from the Radiochemical Centre, Amersham, England, was used : 20 µl of this solution were evaporated to dryness under air at room temperature and then dissolved in 10 ml of 0.01M sodium phosphate buffer pH 7.2. The distribution of tritium in this compound was approximatively 32 % at C-2, 15 % at C-4, 24 % at C-6 and 28 % at C-7 (Radiochemical batch analysis sheet). 2,4-Dichlorophenol and 30 % H_2O_2 were purchased from Merck, München, West Germany.

Incubation and measurement of steroid binding. The 5 ml reaction mixture in 0.01M sodium phosphate buffer pH 7.2, incubated with constant shaking for 1 hr. at 37°C, comprised : HRPO (1 or 10 µg/ml) or MPO (0.5 or 10 µg/ml), BSA or HSA (1 to 5 000 µg/ml), DCP (0.25 mM), E_2 (2 µM) and tritiated E_2 (2 nM), H_2O_2 (0.25 mM) used to initiate the reaction. All experiments were performed at least in triplicate. In control experiments, each component was omitted in turn. After incubation, a 1 ml aliquot of the mixture was dialysed overnight against 15 ml of the same phsophate buffer pH 7.2 at room temperature (Visking tubing, 8/32). Another 1 ml aliquot was extracted with 1 ml of ether by vigorous shaking for 30 sec. The yield of this extraction was 93 % for the first step, 5 % for the second extraction and 1 % for a third.

Gel filtration chromatography. After incubation, a 3 ml aliquot of the mixture was applied on a K 26/100 chromatography column filled with either Sephadex G-75 (Pharmacia Fine Chemical A.B., Uppsala, Sweden) or Ultrogel AcA 34 or Ultrogel AcA 54 (Industrie Biologique Française, Clichy, France) or Biogel P-150 (Bio-Rad Laboratories, Richmond, Ca , USA). Elution was performed with 0.01M sodium phosphate buffer pH 7 containing 0.05 M NaCl and monitored at 280 nm. Three ml fractions were collected and tested for serum albumin, peroxidase activity and radioactivity. From the radioactivity containing fractions, a 1 ml aliquot was dialysed overnight against 15 ml of 0.01M sodium phosphate buffer pH 7.2 at room temperature and another 1 ml aliquot was submitted to extraction with ether. The radioactivity of the dialysed mixture, the phosphate dialysis buffer, the ether soluble and the water-soluble fractions were determined. All the control mixtures were similarly treated.

Test for serum albumin. The method is a modified version of the rocket immunoelectrophoresis (7). A goat anti-normal human plasma antiserum (DiffuGen, Oxford Lab., Foster City, California, USA) was incorporated in the agarose gel. A 5 µl aliquot of each 3 ml chromatography fraction was transfered to the sample wells in the same order as they were collected.
After a 30 min. diffusion period, an electrophoresis in barbital buffer pH 8 (ionic strength : 0.02) was performed overnight with a voltage of 2 V/cm. The plates were stained by a Coomassie Brillant Blue R-250 solution.

Assay for peroxidase activity. Peroxidase activity was assayed by mixing 100 µl aliquots of the chromatographic fractions with 2.9 ml of the substrate solution. This latter solution contained : 5 ml molar phosphate buffer pH

6, 6 ml 0.3 % H_2O_2 in distilled water, 6 ml 1 % o-dianisidine, di-HCl (Sigma Chemicals Co., Saint Louis, Mo., USA) in pure methanol.
After 1 hr. incubation at 20° C, the reaction was stopped with one drop of 5 N HCl with vigorous mixing. Then, the absorbance at 400 nm was read on a Beckman Spectrophotometer 25.

RESULTS

In a reaction mixture containing HSA or BSA, H_2O_2 and $[^3H]E_2$, radioactivity was demonstrated in a non-dialysable, water-soluble product both with HRPO and MPO (Fig.1). The formation of this compound was enhanced by 35 % by addition of DCP. In the absence of peroxidase activity (no enzyme or no H_2O_2), the formation of this non-dialysable product was reduced by about 75 %. The formation of the non-dialysable, water-soluble E_2 (or derivative)-HSA complex does not depend greatly on the albumin concentration : even in the absence of albumin, this complex was formed in quite large quantities (Fig.1 D).
After gel filtration on Ultrogel AcA 34, radioactivity was found to be only partly associated with albumin and a second major peak was eluted after HRPO (Fig.2).

Further experiments showed that E_2 was associated with peroxidases. A reaction mixture with HRPO, H_2O_2 and $[^3H]E_2$ allowed the formation of a

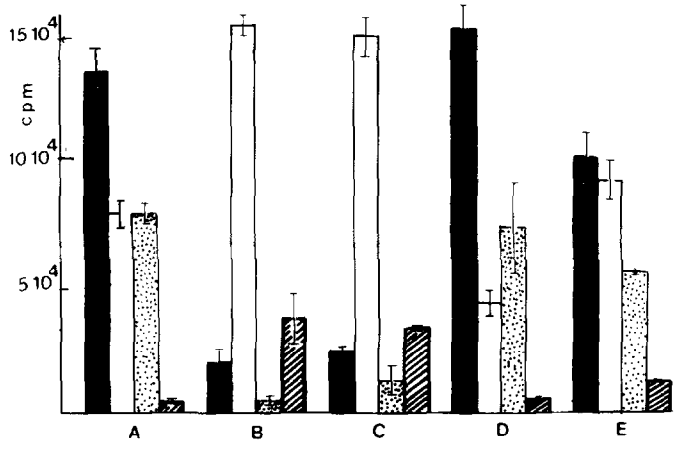


Fig. 1 - Total quantity in cpm of ^3H-labelled estradiol (80 Ci/mmol) in the different products of the reactions : A/HRPO (1 µg/ml), HSA (1 mg/ml), DCP (0.25 mM), E_2 (2 µM), $[^3H]E_2$ (2 nM), H_2O_2 (0.25 mM). 1 hr incubation, 37° C. All conditions as described in the text. - B/ same mixture as A but without H_2O_2. - C/ same mixture as A but without HRPO. - D/ same mixture as A but without HSA. - E/ same mixture as A but without DCP. Black bars : non dialysable products (dialysate volume : 1 ml). White bars : dialysable products (dialysis buffer volume : 15 ml). Dotted bars : water-soluble products (aqueous extract volume 1 ml). Streaked bars : ether soluble products (ether extract volume : 1 ml). Mean of 3 experiments ± 2 standard deviations.

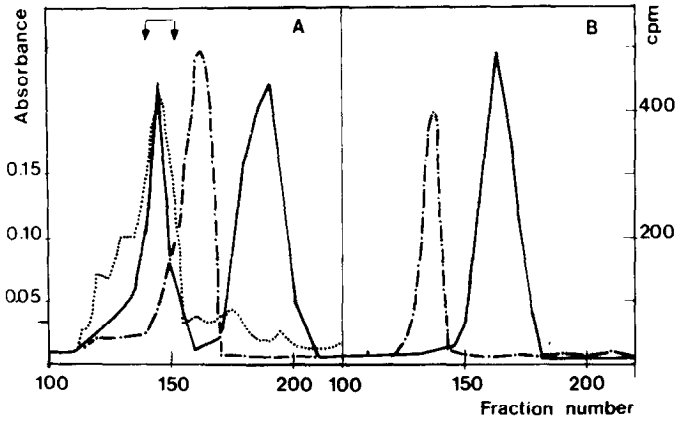


Fig. 2 - Elution diagram from an Ultrogel AcA 34 filtration chromatography column. Flow rate : 5 ml/hr. The 1 hr incubated reaction mixtures applied were - A/HRPO (10 µg/ml), HSA (1 mg/ml), DCP (0.25 mM), E_2 (2 µM), [^{3}H]E_2 (2 nM), H_2O_2 (0.25 mM). - B/ Control experiment : same mixture as A but without HSA. Three ml fractions were collected and measured for absorbance at 280 nm (...) absorbance at 400 nm after o-dianisidine reaction (.-.-.-) and radioactivity (———). HSA was detected by fused rockets immuno-electrophoresis in the fractions between the two arrows.

non-dialysable, water-soluble and tritiated product (Fig.3). The presence of DCP enhanced the incorporation of the radioactive label in the complex. In the absence of peroxidase activity (no enzyme or no H_2O_2), the formation of this complex was not demonstrated. The same results were found with MPO

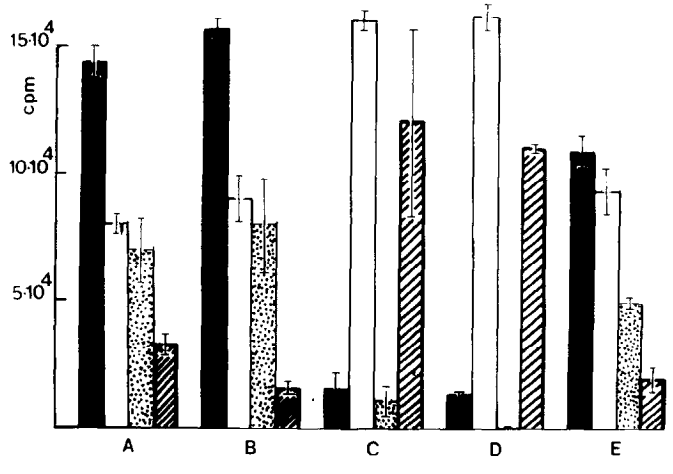


Fig. 3 - Total quantity in cpm of ^{3}H-labelled estradiol derivatives (80 Ci/mmol) in the different products of the reactions : A/HRPO (10 µg/ml), DCP (0.25 mM), E_2 (2 µM), ^{3}H-labelled E_2 (2 nM), H_2O_2 (0.25 nM). - B/MPO (10 µg/ml), DCP (0.25 mM), E_2 (2 µM), [^{3}H]E_2 (2 nM), H_2O_2 (0.25 mM). - C/ same mixture as B but without MPO. - D/ same mixture as B but without H_2O_2. - E/ same mixture as B but without DCP. Black bars : non dialysable products (dialysate volume : 1 ml). White bars : dialysable products (dialysis buffer volume : 15 ml). Dotted bars water-soluble products (aqueous extract volume : 1 ml). Streaked bars : ether soluble products (ether extract volume : 1 ml). Mean of 3 experiments ± 2 standard deviations.

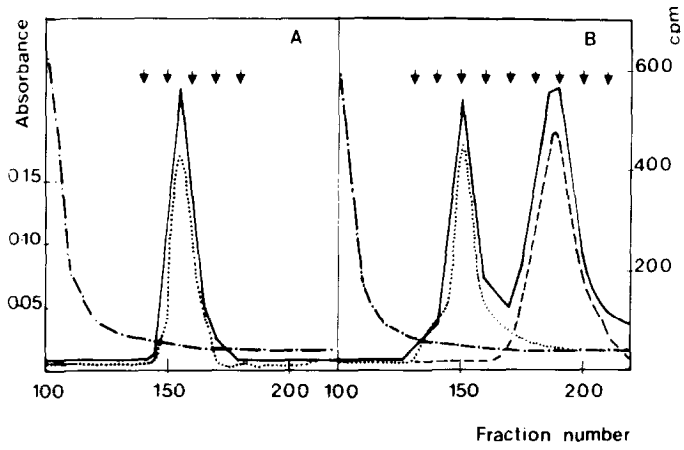


Fig. 4 - Elution diagram from an Ultrogel AcA 54 filtration chromatography column. Flow rate : 0,4 ml/min. The one-hour incubated reaction mixtures applied were - A/HRPO (10 µg/ml), DCP (0.25 mM), E_2 (2 µM), [^{3}H]E_2 (2 nM), H_2O_2 (0.25 mM). - B/ same reaction as A but with 500 µl of [^{3}H]E_2 (22 nM) added to the one hour incubated mixture just before chromatography. Three ml fractions were measured for absorbance at 400 nm after o-dianisidine reaction (.-.-.-) for total radioactivity (———), for radioactivity in the ether extracts (- - -) or in the aqueous extracts (.....). Dialysable fractions are indicated by arrows.

which was not dialysable within the equilibrium dialysis duration, in spite of its low molecular weight.

Surprisingly, gel filtration experiments did not reveal any association of E_2 radioactivity with HRPO activity (Fig.4 A). The radioactivity containing peak was composed of a dialysable, water-soluble product as was shown by equilibrium dialysis and ether extraction of the chromatography fractions. Furthermore, when tritiated E_2 was added to the 1 hr-incubated reaction mixture (cooled to 4 ° C) immediately before gel filtration, a second peak of radioactivity was observed in the same fraction range but was composed of a dialysable and ether-extractable product as expected for E_2 (Fig.4 B).

The same results and the same elution order were obtained whatever the nature of the gel used for chromatography : Sephadex G-75, Ultrogel Aca 34 and 54, Biogel P-150.

DISCUSSION

In recent experiments, we isolated a fraction with peroxidase activity from human fetuses obtained from legal abortions (10-12-week pregnancies) (2). This peroxidase was found in two immunologically identical forms and

was capable of binding E_2 in the presence of H_2O_2. Radiolabelled E_2, bound on the protein, does not compete with cold E_2, resists solvent extraction and protein denaturation suggesting a covalent bond between this estrogen and the enzyme itself (3).

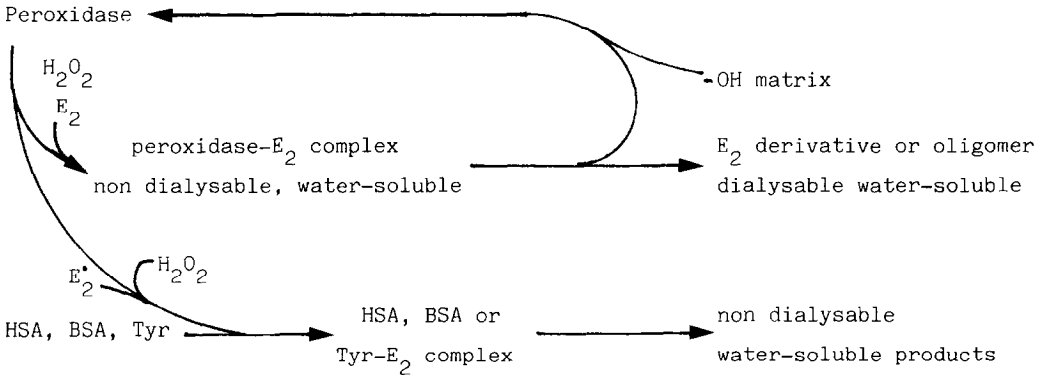
In another way, HRPO as well as a uterine peroxidase which is absent from the uterus of immature rats but can be induced by physiological doses of estrogens or gonadotropins, were shown to catalyze the binding of E_2 to albumin and to other tyrosine-containing proteins in the presence of H_2O_2 and thus to promote the formation of water-soluble products (5).
Jellinck and McNabb (8) showed it was likely that an interaction between tyrosine residues and the ring A of the steroid molecule at C-2 and C-4 would explain the formation of this water-soluble, E_2-containing product but no binding on the peroxidase has ever been described.

In their experiments, Lyttle and Jellinck (5) showed that the percentage of conversion or radio-labelled E_2 into water-soluble products was 56 % with the complete mixture (H_2O_2, E_2, BSA, DCP and peroxidase) and 36.9 % with the control mixture without BSA. Moreover, they claimed that the yield of water-soluble material was increased by the addition of BSA.

In the present study with HRPO, we were interested in this high background (36.9 %) and we observed that, even in an albumin-deprived mixture, MPO and HRPO incorporated E_2 in a non-dialysable water-soluble product. Moreover, we were unable to confirm the influence of albumin concentration which was negligible in our experiments.

Therefore, our present hypothesis is that peroxidases (HRPO and MPO) fix E_2 noncovalently and transform it into an oxidized derivative or oligomer. The E_2 (or E_2 derivative)-HRPO complex may be dissociated by certain types of hydroxyl radical-bearing matrix such as chromatographic gels
The results also show that the reaction of HRPO and MPO with E_2 is different from that observed with fetal peroxidase. In similar experiments with this fetal enzyme, precipitation of proteins by trichloro-acetic acid resulted in the precipitation of the radioactivity due to tritiated E_2. Exhaustive dialysis and competition with cold E_2 did not remove bound E_2. Finally, on chromatographic columns labelled E_2 was never dissociated from the enzyme (2). These facts suggested a covalent binding of E_2 to the fetal peroxidase. Conversely, HRPO and MPO do not bind E_2 covalently and in the presence of an E_2 acceptor in the reaction mixture (HSA, BSA, tyrosine) these peroxidases are able to transfer the steroid or its derivative to this new carrier. The pattern of the en-

zymatic activity of peroxidases upon E_2 proposed by Jellinck and Cleveland (11) may be completed as follows :



The physiological significance of an estrogen-peroxidases interaction of this type remains unknown and obviously requires further research.

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