Purification and Properties of Oestrogen-Induced Uterine Peroxidase

By TRUDY McNABB and P. H. JELLINCK

Department of Biochemistry, Queen's University, Kingston, Ont., Canada

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1. An enzyme that can be induced in rat uteri by oestrogens and that catalyses the oxidation of guaiacol and the metabolism and binding of $[4-^{14}\text{C}]$ oestradiol to protein in the presence of H_2O_2 was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and polyacrylamide-gel chromatography. 2. The molecular weight of this uterine peroxidase was estimated to be about 40000 and thus shown to differ from that of eosinophil peroxidase. 3. Cycloheximide, which blocks the increase in peroxidase activity brought about by oestrogen, was used to determine the half-life (about 4 h) of the induced uterine enzyme.

Lyttle & Jellinck (1972) have shown that an enzyme that catalyses the metabolism and binding of oestradiol to protein and other high-molecular-weight substances in the presence of H_2O_2 is absent from the uteri of immature rats and can be induced by physiological doses of oestrogen or pregnant mare-serum gonadotrophin. It has also been demonstrated that this peroxidase is of truly uterine origin (McNabb & Jellinck, 1974).

The present paper describes the purification of uterine peroxidase and the determination of its molecular weight and half-life. The relationship of this enzyme to other peroxidases found in mammalian tissues is discussed.

Experimental

Materials

Reagents. Cycloheximide, crystallized and freeze-dried bovine serum albumin, and lactoperoxidase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Eastman Kodak Co., Rochester, N.Y., U.S.A. supplied the cetyltrimethylammonium bromide. $[4-^{14}\text{C}]$ Oestradiol (40 mCi/mmol) from Schwarz BioResearch, Orangeburgh, N.Y., U.S.A. was shown by chromatography and radioautography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). The marker proteins for the molecular-weight determination were also obtained from Schwarz BioResearch. Bio-Gel P-150 was purchased from Bio-Rad Laboratories, Mississauga, Ont., Canada and the ultrafiltration membranes from Amicon Corp., Lexington, Mass., U.S.A. The diethyl ether used for extraction was free of peroxides; all other chemicals were the purest available commercially.

Animals. Female albino Holtzman rats (Canadian Breeding Laboratories, St. Constant, Quebec,

Canada) with free access to food (Purina Labena) and water were used. The immature (21–28-day-old) animals weighed 50–80 g and the mature ones 180–240 g. Oestradiol (20 µg/kg body wt.) dissolved in sesame oil was given in a single dose by subcutaneous injection, 18 h before killing the animals. Cycloheximide was injected intraperitoneally in 0.9% (w/v) NaCl.

Methods

Preparation of uterine extracts and incubation. The method was basically that of Klebanoff (1965). The animals, anaesthetized with CO_2 , were killed by cervical dislocation; the uteri were dissected free of adhering fat, blotted and weighed. The tissue was then cut into small pieces and homogenized in 5 ml of 0.1 M-sodium phosphate buffer, pH 7.0, in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to 11 ml with the phosphate buffer and centrifuged at 105000 g_{av} for 30 min in a Spinco model L centrifuge. The sediment was washed with the 0.1 M-phosphate buffer and rehomogenized in the appropriate amount of 1.2 M-NaCl to give a 5% (w/v) homogenate. It was centrifuged again at 105000 g_{av} for 30 min and portions of the supernatant fraction (1.2 M-NaCl extract) were incubated with constant shaking for 1 h at 38°C with $[4-^{14}\text{C}]$ oestradiol (1.84 µM), 2,4-dichlorophenol (0.25 mM), H_2O_2 (0.25 mM) and bovine serum albumin (10 mg) in 0.1 M-sodium phosphate buffer, pH 7.4; total vol. 4 ml. After incubation, the medium was extracted three times with equal volumes of peroxide-free ether and the combined organic phase was dried over anhydrous Na_2SO_4 .

The radioactivity in the aqueous and ethereal fractions was then determined by scintillation counting as described previously (Jellinck & Woo, 1967).

Partial purification of uterine peroxidase. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to a 1.2 M-NaCl extract of a

20% (w/v) uterine homogenate prepared from the uteri of 40 oestrogen-treated rats, to give a final concentration of 20% (w/v) at 5°C. The precipitate obtained after stirring for 90 min at this temperature was removed by centrifugation (1000g), resuspended in 2.5% (w/v) cetyltrimethylammonium bromide-1.2M-NaCl (4ml) and left for 20 min before dilution to 20 ml with water. Any insoluble material was removed by centrifuging at 150000g for 1 h and the peroxidase activity and the protein content of the clear supernatant fraction were determined. The enzyme was then further purified by chromatography on Bio-Gel P-150.

Chromatography and molecular-weight determination. A modification of the method of Desser *et al.* (1972) for purifying heterophil and eosinophil peroxidase was used. Bio-Gel P-150 (20g) wetted with methanol (50ml) was swollen in sodium acetate (25mM), acetic acid (25mM), NaCl (100mM), 0.02% cetyltrimethylammonium bromide, pH 4.6 (buffer A) for 24 h at 25°C before being packed in a glass column (2.5cm × 58cm) coated with dichlorodimethylsilane (Batlle, 1967). The solution to be applied was concentrated in an ultrafiltration cell (Amicon Corp.) by using a UM-10 membrane and dialysed overnight against buffer A. The void volume was determined with Blue Dextran and a constant flow rate of eluant (buffer A) of approximately 4 ml/h was maintained with a Mariotte flask. Cetyltrimethylammonium bromide (0.02–0.1%, w/v) did not inhibit the oxidation of oestradiol, guaiacol or NADH by uterine extracts. An estimate of the molecular weight was obtained by the method of Andrews (1965) by using γ -globulin, ovalbumin, myoglobin, bovine serum albumin and glutathione as markers. The concentration of these proteins was determined by light-extinction measurements at the appropriate wavelengths (Andrews, 1965) and that of glutathione by the nitroprusside method (Stadtman, 1957).

Determination of peroxidase activity. Uterine peroxidase activity was determined at 25°C by using guaiacol as substrate (Himmelhoch *et al.*, 1969). The

reaction mixture (3ml) contained guaiacol (13mM) and H₂O₂ (0.33mM) in 0.01M-sodium phosphate buffer (pH 7.0) and 0.05–0.2ml of the fraction containing peroxidase depending on its activity. The linear increase in absorbance at 470nm resulting from the oxidation of guaiacol was followed in a Unicam SP.800 recording spectrophotometer. One enzyme unit is defined as the amount of enzyme that oxidizes 1 μ mol of guaiacol/min based on an extinction coefficient of 26.6mM⁻¹·cm⁻¹ for tetraguaiacol (Maehly & Chance, 1954).

Determination of NADH oxidase activity. NADH oxidase activity was determined by the method of Temple *et al.* (1960) by measuring the decrease in absorbance of the coenzyme at 340nm. The incubation mixture consisted of NADH (0.1mM), MnCl₂ (0.5 μ M), oestradiol (10 μ M), 2,4-dichlorophenol (0.25mM) in 0.013M-sodium phosphate buffer, pH 7.7, and the solution to be tested; total vol. 3 ml. Values are expressed as μ mol of NADH oxidized/min based on an extinction coefficient of 6.3mM⁻¹·cm⁻¹.

Results

Purification of uterine peroxidase

The steps in the partial purification of uterine peroxidase are given in Table 1. A tenfold increase in specific activity of the enzyme preparation was achieved by extracting the microsomal fraction with 1.2M-NaCl followed by precipitation with 20% (w/v) (NH₄)₂SO₄. Desser *et al.* (1972) had shown that the cationic detergent cetyltrimethylammonium bromide helped in their purification of guinea-pig eosinophil peroxidase by preventing the tendency of the enzyme to bind irreversibly to a number of adsorbants, particularly to cellulose- or dextran-based materials. A modification of their procedure was therefore used to purify further the enzyme from uterine tissue on a polyacrylamide-based absorbent, Bio-Gel P-150, resulting in a further fivefold enrichment (Fig. 1). The ability of fractions to oxidize guaiacol, NADH or to

Table 1. *Partial purification of uterine peroxidase*

Uteri from 40 oestradiol-treated mature rats (160–200g) were fractionated as described in the text. Protein was determined by the method of Lowry *et al.* (1951). Units of peroxidase activity are given as μ mol of guaiacol oxidized/min. Percentage recovery was based on the activity in the homogenate.

Fraction	Total protein (mg)	Peroxidase activity (units)	Specific activity (units/mg)	Recovery (%)
Homogenate	360	19.4	0.05	100
1.2M-NaCl extract of microsomal fraction	72	12.7	0.18	65
Resuspended 20% (w/v) (NH ₄) ₂ SO ₄ precipitate	15	7.4	0.49	38
Peak fractions from Bio-Gel P-150	2	4.5	2.0	24

Fig. 1. Elution profile of uterine peroxidase on Bio-Gel P-150

The partially purified enzyme was concentrated by ultrafiltration and dialysed against buffer A. Fractions (4ml) were collected and tested for E_{280} ($\square$), ability to oxidize guaiacol ($\bullet$), NADH ($\blacksquare$) or to convert [^{14}C]oestradiol into water-soluble products ($\circ$). The methods are described in the text.

Fig. 2. Plot of elution volume (V_e)/void volume (V_0) against logarithm (molecular weight) for proteins and glutathione on a Bio-Gel P-150 column (2.5 cm $\times$ 58 cm) with buffer A, pH 4.6, as eluent

Experimental details are given in the text.

convert [^{14}C]oestradiol into water-soluble products coincided and the overall recovery was 24%.

The partially purified peroxidase had properties similar to those of the enzyme present in the crude uterine extract in catalyzing the interaction of oestradiol with various water-soluble acceptors and in its requirement for 2,4-dichlorophenol and H_2O_2 (Jellinck & Lyttle, 1973).

Determination of the molecular weight of uterine peroxidase

By using a Bio-Gel P-150 column, the molecular weight of uterine peroxidase was found to be close to

that of ovalbumin and was estimated to be about 40000. This value was obtained from the plot of the ratio of elution volume (V_e) to void volume (V_0) against logarithm (molecular weight) of reference peptides of known molecular weight (Fig. 2). Bio-Gel P-150, a copolymer of acrylamide and methylene bisacrylamide, has been shown to exhibit a relation similar to Sephadex between the molecular weight of proteins and their elution volume (Batlle, 1967).

Estimation of the half-life of uterine peroxidase

The method of Russell & Potyraj (1972) to determine the half-life of 5-adenosyl-L-methionine decarboxylase was adapted to evaluate the turnover of oestrogen-induced uterine peroxidase. Groups of three immature rats were given a single intraperitoneal injection of cycloheximide (3 mg in 0.3 ml of 0.9%, w/v, NaCl) 12, 14, 15 and 16 h after a single subcutaneous injection (20 $\mu\text{g}/\text{kg}$) of oestradiol in oil (0.2 ml). The animals were killed at 18 h after treatment with oestradiol, and various amounts of the uterine extract obtained with 1.2 M-NaCl were incubated with [^{14}C]oestradiol followed by extraction with ether. The relative activity of each sample was measured from the initial slope of the curve obtained by plotting the amount of oestradiol oxidized in 1 h against protein concentration. Under standard conditions the amount of oestradiol converted into water-soluble products was proportional to the concentration of protein present in the uterine extract within the range 0.1–0.3 mg/ml. Peroxidase activity was then plotted against time after treatment with cycloheximide to determine the half-life of the induced enzyme which was found to be

Fig. 3. Decline of oestrogen-induced uterine peroxidase activity after the administration of cycloheximide

Cycloheximide (3mg in 0.9% NaCl) was injected intraperitoneally into groups of three immature rats at the times indicated before they were killed. All animals received oestradiol (20 µg/kg) 18h before death. The uterine extract in 1.2M-NaCl was incubated for 1h with [14 C]oestradiol (1.84 µM), H_2O_2 (0.25 mM), bovine serum albumin (2.5 mg/ml) and dichlorophenol (0.25 mM). Other conditions are described in the text. Activity is expressed as nmol of [14 C]oestradiol converted into water-soluble products/h per mg of protein in the uterine extract.

about 4h (Fig. 3). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Discussion

It has been suggested that the presence of peroxidase might be characteristic of proliferating tissue because this enzyme is found principally in those organs that have a high rate of cell renewal (Neufeld *et al.*, 1958). More recently Anderson *et al.* (1975) have proposed that peroxidase was a specific marker enzyme for tissues dependent on oestrogen for growth. Our own studies (Lyttle & Jellinck, 1972; McNabb & Jellinck, 1974) have revealed the rapid induction of peroxidase in uterine tissue after treatment with oestrogen, supporting the earlier work of Brökelmann (1969).

Some of the biochemical characteristics of the peroxidase present in a uterine extract have been described (Lyttle & Jellinck, 1972; Jellinck & Lyttle, 1973) but attempts to purify the oestrogen-induced enzyme were hampered by its insolubility. It could be extracted from membrane fractions by 1.2M-NaCl, but removal of the salt by dialysis resulted in precipitation of the enzyme, making purification by standard techniques difficult. The need to maintain high salt concentration could, however, be overcome by using acetate buffer, pH 4.6, of low ionic strength containing the cetyltrimethylammonium bromide (Desser *et al.*, 1972). This cationic detergent proved

to be more effective than either sodium dodecyl sulphate or Triton X-100, and did not inhibit the enzyme at a concentration below 0.1% (w/v). Unfortunately it interfered with the zymogram staining procedure for peroxidase using 3,3'-diaminobenzidine (Graham & Karnovsky, 1966) after polyacrylamide-gel electrophoresis.

An acrylamide gel (Bio-Gel P-150) was used to estimate the molecular weight of uterine peroxidase by the method of Batlle (1967) because of the anomalous behaviour of glycoproteins in gel filtration on Sephadex (Andrews, 1965). The reference compounds were applied in 0.02% cetyltrimethylammonium bromide to minimize any effect due to this detergent. The value of 40000 daltons obtained for uterine peroxidase was the same as that reported for horseradish peroxidase (Maehly, 1955) and one-half that of lactoperoxidase (Polis & Shmukler, 1955; Morrison *et al.*, 1966). Molecular-weight determinations on Bio-Gel P-150 are fairly reliable and the absence of ionic groups from the inert matrix of polyacrylamide diminishes the undesirable adsorption effects to a minimum. The addition of detergent could not be avoided and could possibly introduce some inaccuracy in molecular-weight estimations including disaggregation of some oligomeric proteins. However, Desser *et al.* (1972) also used Bio-Gel P-150 and cetyltrimethylammonium bromide and obtained molecular weights of 75000 and 150000 for the two components of eosinophil peroxidase purified from guinea-pig bone marrow. Treatment with sodium dodecyl sulphate and mercaptoethanol produced a single component with a molecular weight of 65000. These results therefore support our previous findings (McNabb & Jellinck, 1974) that uterine peroxidase is distinct from the enzyme brought to the uterus by eosinophils after treatment with oestrogens. Martin *et al.* (1958) had also demonstrated that verdoperoxidase obtained from empyema fluid differed from the peroxidase in rat uterus in its rate of oxidation of pyrogallol and reduced cytochrome *c*.

Brökelmann & Fawcett (1969) showed histochemically that the increase in uterine peroxidase activity brought about by oestradiol could be prevented by the administration of acetoxycycloheximide, an inhibitor of protein synthesis. Our results (Lyttle & Jellinck, 1972) also indicated that the induction of this enzyme by oestrogen could be prevented by pre-treatment with cycloheximide. This inhibition was therefore used to determine the half-life of decline of oestrogen-induced uterine peroxidase. It was shown to be about 4h, which is higher than that of another oestrogen-induced uterine enzyme, *S*-adenosyl-L-methionine decarboxylase (Russell & Potyraj, 1972).

Studies on enzyme turnover rates based solely on the measurement of enzyme activity must be interpreted with caution since cycloheximide may be

blocking enzyme degradation as well as enzyme synthesis or may produce effects unrelated to protein synthesis (Schimke *et al.*, 1968). However, for certain liver enzymes, turnover rates calculated from the decrease in enzyme activity after treatment with inhibitors of protein synthesis were the same as when determined by changes in amount of isotopically labelled enzymes (Schimke, 1964; Schimke *et al.*, 1965; Kenney, 1967).

The physiological role of oestrogen-induced uterine peroxidase still remains to be determined, although it has been suggested that it may function to terminate oestrogen action in target tissues (Brökelmann, 1969; Lyttle & Jellinck, 1972) or act as a bacterial and possibly spermicidal agent (Smith & Klebanoff, 1970).

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