Comparative studies on oestrogen-induced rat uterus peroxidase and rat eosinophil peroxidase

Ragner L. OLSEN and Clive LITTLE

Institute of Medical Biology, University of Tromsø, P.O. Box 977, N-9001 Tromsø, Norway

(Received 16 July 1982/Accepted 31 August 1982)

Rat eosinophil peroxidase and rat uterine peroxidase II showed similar electrophoretic mobilities, molecular weights, specific activities and spectral properties and could be purified by essentially identical techniques. Antibodies raised against the uterine enzyme strongly inhibited the eosinophil enzyme. It is suggested that rat eosinophil peroxidase and rat uterine peroxidase II may well be one and the same enzyme.

The oestrogen-induced peroxidase in the rat uterus, first reported by Lucas *et al.* (1955), has attracted considerable interest. Although its physiological role is unclear, it has been suggested to be involved in oestrogen metabolism (Klebanoff, 1965; Brökelmann, 1969; Lyttle & Jellinck, 1972). More recently, several attempts have been made to use this oestrogen-induced activity as marker for tissues where growth is regulated by the hormone, but such studies have led to conflicting results (Anderson *et al.*, 1975; Strum & Becci, 1979; Collings & Savage, 1979; Penney *et al.*, 1980; Brightwell *et al.*, 1981; Levy *et al.*, 1981; Duffy & O'Connell, 1981; Holt *et al.*, 1981; King *et al.*, 1981). Uterine peroxidase activity can be fractionated into two very similar enzyme activities with different electrophoretic mobilities and molecular weights (peroxidase I, M_r 90 000; peroxidase II, M_r 40 000) (Olsen & Little, 1979). Peroxidase II has been purified to >95% homogeneity (Olsen & Little, 1981) and partially characterized (McNabb & Jellinck, 1975; Keeping *et al.*, 1981; Olsen *et al.*, 1982).

Two quite different views exist regarding the origin of the oestrogen-induced uterine peroxidase activity. One opinion is that it derives from the peroxidase-rich eosinophil leucocytes invading the uterus after oestrogen stimulation (Rytömaa & Teir, 1965; Klebanoff, 1965). On the other hand several workers suggest that it is produced in the endometrial cells after oestrogen stimulation (Brökelmann & Fawcett, 1969; McNabb & Jellinck, 1974).

Very recently we have shown that rat uterine peroxidase II and human and rat eosinophil peroxidase have very similar spectral properties (Olsen *et al.*, 1982). We now report a closer examination of these two enzymes and conclude that they are extremely similar if not identical.

Materials and methods

Isolation of enzymes

Female Wistar rats (160–180 g) were used as the source of the peroxidases. Uterine peroxidase was purified as described previously (Olsen & Little, 1981; Olsen *et al.*, 1982). After the gel-filtration on Ultrogel AcA 44 (LKB Products, Bromma, Sweden), the last step in the purification procedure, uterine peroxidase II was rechromatographed on the same gel-filtration column. Rat leucocytes were obtained from 20 rats by peritoneal lavage. The rats, deprived of food for 20 h, were given 8 ml of sterile 4% (w/v) gelatine in 0.9% (w/v) NaCl intra-peritoneally. The animals were killed by cervical dislocation 24 h later and the peritoneal cavity of each animal was flushed with 20 ml of saline (0.9% NaCl). The combined lavage (approx. 350 ml) was centrifuged (100 g for 10 min) and the pellet was homogenized in 10 ml of 0.1 M-potassium phosphate buffer, pH 7.4, with a hand-operated Dounce glass homogenizer. The homogenate was centrifuged for 15 min in an Eppendorf 5412 bench centrifuge. This washing procedure was repeated twice on the pellet. The peroxidase activity was solubilized from the final sediment by homogenization in 0.5 M-CaCl₂ using a hand-operated Dounce glass homogenizer. The supernatant (10 ml) obtained after centrifugation for 15 min in the Eppendorf centrifuge contained the leucocyte peroxidase activities. The peroxidase extraction was repeated and the supernatants were pooled. Both washing and extraction procedures were carried out at 5°C or on ice. The subsequent purification of the leucocyte peroxidases was performed using the same buffers and methods as for rat uterine peroxidase; first a concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden)

column, followed by a phenyl-Sepharose (Pharmacia) column, with a gel-filtration step at the end.

From the leucocytes, two peroxidase activities were obtained in the gel-filtration step. A high-molecular-weight form with a Soret absorption band at 430nm and an RZ value (ratio between the absorption at the Soret band and 280nm) of 0.45. As judged by the molecular weight and the absorption spectrum, this is myeloperoxidase. The low-molecular-weight peroxidase had a Soret absorption maximum at 412nm and an RZ value of 0.68 and is the eosinophil peroxidase. The third peroxidase used in the present study, uterine peroxidase II, had a Soret absorption maximum at 412nm and an RZ value of 0.83.

Immunological studies

A rabbit antiserum was raised against rat uterine peroxidase II. Multiple intradermal injections of highly purified peroxidase dissolved in 20mM-sodium acetate buffer, pH 5.6, and 25% glycerol were given on the back of the animal on day zero and after 3 and 6 weeks, the doses being 75 μ g, 50 μ g and 50 μ g of enzyme respectively. Only the primary dose and the first booster dose were dispersed in Freund's adjuvant (Behringwerke AG, Marburg, Germany) (complete and incomplete respectively). The animal was bled after 1 and 2 weeks and the immunoglobulin G fraction from the combined serum was isolated by precipitation with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ followed by chromatography on DEAE-32 cellulose (Whatman, Maidstone, Kent, U.K.) with 10mM-sodium phosphate buffer, pH 8.1, essentially as described by Fahey (1967). The fraction isolated migrated as a broad single protein band in analytical polyacrylamide gel electrophoresis at pH 9.1 (Davis, 1964) (gels not shown), and was the antibody preparation used in present study. A normal immunoglobulin G fraction from a non-immunized rabbit was isolated in exactly the same manner. The immunoglobulin concentrations were calculated by using an absorption coefficient at 280nm of 13.5/cm for a 1% solution (Stevenson & Dorrington, 1970).

The various peroxidases in 10mM-sodium acetate buffer, pH 5.6, containing 25% (w/v) glycerol, were incubated for 1 h at 37°C with an equal volume of different concentrations of immunoglobulin G in 10mM-sodium acetate buffer, pH 5.6, with 0.15M-NaCl. Peroxidase activity was determined in the supernatant after centrifugation in an Eppendorf 5412 bench centrifuge. Bovine serum albumin was used to obtain a constant concentration of protein (0.5 mg/ml) in all incubation samples.

Electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed using gel system 7 (Maurer, 1971) with

certain modifications; 10% gels were used and cetyltrimethylammonium bromide (0.05%, w/v) and glycerol (25%, w/v) were present both in the gels and electrode buffer. The pH of the latter was 4.6. Riboflavin was used as polymerization catalyst both in the stacking and separating gel. The peroxidase activity in the polyacrylamide gel was detected by staining as described in a previous report (Olsen & Little, 1979).

Peroxidase activity was measured and expressed in activity units as described previously (Olsen & Little, 1981). The RZ values of the enzyme were determined by using a Beckman model 25 spectrophotometer. Protein concentrations were calculated by the method of Schaffner & Weissman (1973) with bovine serum albumin as standard.

Results and discussion

The uterine peroxidase had a somewhat higher RZ value than did the eosinophil enzyme (0.83 as opposed to 0.68). This 18% difference could arise from differing degrees of purity of the enzymes and when their catalytic activities were expressed with respect to A_{412} , it was found that, for solutions of $A_{412} = 1.0$, the uterine enzyme had an activity of 5300 units/ml and the eosinophil enzyme 5870 units/ml, i.e. a difference of <10%. Myeloperoxidase purified from peritoneal lavage differed clearly from uterine peroxidase II in its visible absorption spectrum. In addition the myeloperoxidase had an activity of only 1970 units/ml in a solution of $A_{430} = 1.0$.

In gel filtration, myeloperoxidase has an apparent molecular weight greater than that of the eosinophil enzyme. In a column of Ultrogel AcA44, both the eosinophil and the uterine enzyme had an apparent M_r value of 45 000 (results not shown). Both of the latter enzymes required the presence of detergent to prevent aggregation.

The eosinophil and uterine peroxidase II were subjected to analytical polyacrylamide-gel electrophoresis and the gels were subsequently stained for peroxidase activity (Fig. 1). The eosinophil peroxidase and uterine peroxidase II were applied to gel (a) and to gel (b) respectively, whereas a mixture of equal volumes of the two enzymes was applied to gel (c). The results show that in this system these two enzymes have identical electrophoretic properties.

Different concentrations of purified rabbit antibodies against uterine peroxidase II were incubated for 1 h with the three different rat peroxidases and the mixtures were tested for enzyme activity (Fig. 2). Both uterine peroxidase II (Fig. 2b) and eosinophil peroxidase (Fig. 2a) were inhibited by the antibodies, whereas rat myeloperoxidase (Fig. 2c) was not inactivated at all. It is also apparent from Fig. 2 that at similar antibody/enzyme ratios, the eosino-

Fig. 1. Analytical polyacrylamide-gel electrophoresis of rat uterine peroxidase II and rat eosinophil peroxidase (a) Eosinophil peroxidase (4 μ g); (b) uterine peroxidase II (4 μ g); (c) uterine peroxidase II (2 μ g) + eosinophil peroxidase (2 μ g). The gels were stained for peroxidase activity and the position of the marker is indicated. Further experimental details are given in the Materials and methods section.

Fig. 2. Effect of antibodies against rat uterine peroxidase II on the activity of three peroxidases isolated from the rat

Each peroxidase was incubated at 37°C for 1 h with different concentrations of the purified antibody preparations and, after centrifugation, the peroxidase activity was measured in the supernatant. ●, Enzyme with control antibody; ▲, enzyme with antibodies against uterine peroxidase II. (a) Eosinophil peroxidase; (b) uterine peroxidase II; (c) myeloperoxidase. The concentrations of the various peroxidases were: eosinophil and uterine peroxidases, 1.3 μ g/ml; myeloperoxidase, 4.2 μ g/ml. Further experimental details are given in the Materials and methods section.

phil and the uterine enzyme are inhibited to very similar extents.

Experiments were also carried out in which the time-course inhibition was followed when the eosinophil and the uterine enzyme were incubated with the antibodies at the same peroxidase activity/immunoglobulin ratio. Under these conditions, the rates of inactivation were identical for the two enzymes (results not shown).

Rat uterine peroxidase II and rat eosinophil peroxidases are apparently very similar immunologically since they were equally sensitive to inhibition by antibodies against the former. The results in Fig. 2 indicate that rat myeloperoxidase is immunologically different from the eosinophil enzyme. Owing to the very limited amounts available of these enzymes, no immunodiffusion studies were possible.

Concluding remarks

It is very clear that the rat myeloperoxidase differs from the other two rat peroxidases studied.

The eosinophil and uterine enzymes, however, had a whole range of similar properties: spectral, electrophoretic, molecular weight, a tendency to aggregate in the absence of detergent, specific activity when expressed relative to the Soret absorption and immunological. Furthermore, the two enzymes could be purified by using essentially the same method, which means that they both bind to concanavalin A and to phenyl-Sepharose.

We suggest, therefore, that these two enzymes are probably identical, and hence that a substantial proportion of the oestrogen-induced peroxidase activity in the rat derives from invading eosinophils.

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