Partial purification and characterization of a peroxidase activity from human placenta

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Peroxidases can metabolize a variety of xenobiotics to reactive intermediates capable of binding to protein or DNA. The potential role of these enzymes in fetotoxicity has not been explored. In this study, the presence of peroxidase activity was observed in human term and pre-term placenta. Human term placental peroxidase activity (HTPP) was partially purified by concanavalin A affinity chromatography from CaCl_2 extracts of the particulate fraction. HTPP appears to be a membrane-bound glycoprotein. Arachidonic acid-dependent oxidation of guaiacol was not observed, suggesting that the peroxidase activity was not due to prostaglandin synthase. Moreover, HTPP preparations were devoid of catalase and spectrally dissimilar from human haemoglobin, cytochrome *P*-450, eosinophil peroxidase and myeloperoxidase, suggesting an endogenous origin. An M_r of approx. 119000 was determined for HTPP by gel filtration. Cathodic slab-PAGE of cetyltrimethylammonium bromide-solubilized HTPP yielded two peroxidase-staining bands.

INTRODUCTION

The placenta is important from a pharmacological and toxicological perspective, since it is the principal tissue controlling the access of drugs and other xenobiotics to the fetus. However, very limited information exists on placental activation and detoxification of xenobiotics (Juchau, 1980; Goodman *et al.*, 1982; Pelkonen & Pasanen, 1984). Activation of certain xenobiotics to ultimate teratogens and carcinogens in the placenta may potentially harm the fetus either directly, after circulatory translocation, or indirectly, by compromising placental function essential to the fetus.

In recent years, there has been increasing interest in the peroxidase-catalysed generation of biologically reactive intermediates, which have been linked to carcinogenesis and other forms of target-organ toxicities (Mason, 1982; Moldeus *et al.*, 1984). For example, diethylstilboestrol, a transplacental carcinogen in many animal species and in humans (Metzler, 1984), has been shown to be readily activated *in vitro* by horseradish peroxidase and animal uterine peroxidase (Metzler & McLachlan, 1978, 1979; Metzler & Epe, 1984) to protein- and DNA-binding intermediates. A peroxidase activity was reported previously in crude homogenates of human placenta (Matkovic *et al.*, 1975), and we believe that it may represent a pathway for the bioactivation of certain xenobiotics *in situ*. As a first step in understanding the potential of this pathway, we have partially purified and characterized the peroxidase activity from human term placenta, and the results of these experiments are presented in this paper.

MATERIALS AND METHODS

Chemicals

Guaiacol (*o*-methoxyphenol), 30% (v/v) H_2O_2 , 3,3'-dimethoxybenzidine, cetyltrimethylammonium bromide (CETAB), methyl α -D-mannoside, concanavalin A-Sepharose 4B (Con-A), catalase (bovine liver), human haemoglobin (type IV, twice crystallized), and lactoperoxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Riboflavin and Bio-Gel

P-200 were acquired from Bio-Rad Laboratories (Rockville Center, NY, U.S.A.).

Enzyme isolation

Human pre-term placentas (13–21 weeks gestation) were obtained from healthy women who chose legal abortion. Term placentas (38–40 weeks gestation) were procured after Caesarean delivery. Placentas from women with a known history of smoking, drug or alcohol abuse, as well as any physiological or pathological problem, were not used. The use of human placentas in the study *in vitro* was approved by The University of South Florida's Institutional Review Board governing the policies and procedures for research involving human subjects. Considering the availability and sample size, term placenta was employed as the enzyme source for purification. Human term placental peroxidase activity (HTPP) was isolated by the method described by Lyttle & DeSombre (1977) for rat uterine peroxidase. The placental villous tissue was teased and rinsed with 10 mM-Tris/HCl, pH 7.2 (buffer A), to remove excess blood. The washed tissue was homogenized (10–20%, w/v) in buffer A in a Waring blender. The homogenate was sonicated (30 s) and centrifuged at 39000 *g* (30 min). The resulting pellets were resuspended in buffer A to the original volume and re-centrifuged. The washed pellets were resuspended in buffer A + 0.5 M- CaCl_2 (buffer B) (10–20% original tissue w/v) with a glass/Teflon homogenizer and re-centrifuged at 39000 *g* (30 min). The resulting supernatant (crude enzyme extract) was assayed with guaiacol for peroxidase activity and served as the starting material for further purification.

Affinity chromatography

A modification of the procedure employed by DeSombre & Lyttle (1978) for rat mammary-tumour peroxidase was used for partial purification of HTPP. The crude enzyme extract was applied at a flow rate of approx. 0.5 ml/min to a Con-A column (11.0 cm $\times$ 1.9 cm), pre-equilibrated with 10 bed vol. of buffer B. After sample application the column was washed with 3 bed vol. of buffer B, until no protein (A_{280}) could be detected in the eluate. Peroxidase activity was eluted with buffer B + 0.5 M-methyl α -D-

Abbreviations used: HTPP, human term placental peroxidase; Con-A, concanavalin A; PGS, prostaglandin synthase; CETAB, cetyltrimethylammonium bromide.

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mannoside at a flow rate of approx. 4 ml/h. Fractions (2 ml) containing peroxidase activity within a distinct peak were pooled and concentrated to a small volume by ultrafiltration (PM-10 Diaflo membrane).

Subcellular distribution of HTPP activity

Differential centrifugation of the placental homogenate in buffer A was performed at 600 *g* (10 min), 2000 *g* (10 min), 10000 *g* (15 min) and 100000 *g* (1 h). The pellets were resuspended to the original volumes in buffer A, and re-centrifuged to obtain washed pellets. These were resuspended in minimal volumes of buffer A and assayed for marker enzymes for mitochondria, microsomes and cytosol, as well as for peroxidase activity and protein content.

Enzyme assays

Unless stated otherwise, peroxidase activity was assayed colorimetrically at 470 nm by published methods (Maehly & Chance, 1954; Himmelhoch *et al.*, 1969). The reaction mixture contained the indicated amount of enzyme (absent from the reference cuvette), guaiacol (13 mM) and H₂O₂ (0.33 mM) in 10 mM-Tris/HCl containing 0.5 M-CaCl₂, pH 7.2, in a 1 ml final volume. After a 2 min preincubation, the reaction was initiated by the addition of H₂O₂. All assays were performed at 37 °C in duplicate. In assaying arachidonic acid-dependent activity, H₂O₂ was replaced with 0.4 mM-arachidonic acid and 5 µM-haematin. Tetraguaiacol production is reported either as nmol/min per mg or as units of enzyme activity; 1 unit of enzyme activity is equivalent to an increase of 1 A₄₇₀ unit/min. Catalase activity in Con-A-purified HTPP was determined colorimetrically by the method of Chance & Maehly (1955).

Determination of *M_r* by gel filtration

The apparent *M_r* of Con-A-purified HTPP was determined by gel filtration on a Bio-Gel P-200 column. The column (1.6 cm × 80 cm) was equilibrated at 4 °C with 10 mM-Tris/HCl/0.5 M-CaCl₂, pH 7.2. The column was calibrated with thyroglobulin (*M_r* 670000) to mark the void volume and the following proteins as standards: γ-globulin (*M_r* 158000), bovine serum albumin (*M_r* 66000), horseradish peroxidase (*M_r* 40000), myoglobin (*M_r* 17000) and cytochrome *c* (*M_r* 12384). Non-enzymic proteins were detected by their A₂₈₀, and the peroxidase activity of horseradish peroxidase and Con-A-purified HTPP was detected by the guaiacol/H₂O₂ assay. Elution was performed with equilibration buffer at a flow rate of 6 ml/h.

Spectral analyses

The spectral properties of Con-A-purified HTPP were examined in the oxidized (Fe³⁺) and cyanide-bound (CN-Fe³⁺), dithionite-reduced (Fe²⁺) and CO-reduced states. Identical spectral analyses were performed with purified human haemoglobin. All spectra were performed in buffer B+0.5 M-methyl α-D-mannoside, pH 7.2, at room temperature, with an Aminco DW-2 spectrophotometer. Slit width was 3 nm and the scanning speed was 2.0 nm/s, except in determining the Soret maxima of the haem (Fe²⁺) groups, in which the scanning speed was 0.5 nm/s.

PAGE

Cathodic slab-PAGE of Con-A-purified HTPP was carried out by the method of Hames & Rickwood (1981), and modified from that of Olsen & Little (1979) for rat uterine peroxidase. The cathodic sample buffer was 0.1 M-acetic acid/KOH, pH 6.0, containing 0.05 % CETAB. The resolving gel, containing 7.5 % acrylamide/0.2 % NN'-methylenebisacrylamide, was prepared in 0.063 M-KOH/acetic acid, pH 4.3. The stacking gel, containing 3.75 % acrylamide/0.1 % NN'-methylenebisacrylamide, was pre-

pared in 0.125 M-KOH/acetic acid, pH 6.0. Both gels contained 20 % (w/v) glycerol and 0.05 % CETAB. Riboflavin 5-phosphate and NNN'-N'-tetramethylethylenediamine were used to polymerize the gels. The electrode buffer was 0.35 M-β-alanine/acetic acid (pH 4.5)/0.05 % CETAB. A bovine lactoperoxidase standard was included in each run. Electrophoresis was performed at 10 °C at a constant current of 25 mA/gel for 4.5 h under reverse polarity (gel slabs 0.15 cm × 12 cm). Methyl Green was used as the tracking dye. Peroxidase was detected with 3,3'-dimethoxybenzidine by a modification of the method of Thomas *et al.* (1976). Protein staining was performed in 0.1 % Coomassie Brilliant Blue R-250.

Protein determination

Protein was routinely determined by the method of Bradford (1976). When interfering substances were present, e.g. CETAB, the method of Peterson (1977) was substituted.

RESULTS

Measurable peroxidase activity was detected in all the membrane fractions of human term placenta sedimenting at 600, 2000, 10000 and 100000 *g*. However, in view of the significant cross-contamination (results not shown), the 39000 *g* pellets were used as the HTPP source in all the experiments. Similarly, owing to the presence of haemoglobin, peroxidase activity in the placental cytosol was not evaluated.

The results of several experiments indicated that > 95 % of the total HTPP activity present in the washed particulate fraction (39000 *g* pellets) was recovered in the initial CaCl₂ extract, making a second extraction step unnecessary. Routine isolation of HTPP therefore included a single extraction of the particulate fraction with buffer B.

Fig. 1. Affinity-chromatographic elution profile of HTPP from Con A-Sepharose 4B (10 ml bed volume)

Ca²⁺ extract was applied in buffer B (10 mM-Tris/HCl/0.5 M-CaCl₂, pH 7.2), washed with 3 bed vol. (30 ml) of buffer B, and eluted in buffer B+0.5 M-methyl α-D-mannoside (50 ml). Peroxidase activity (.....) in all HTPP fractions was determined by the standard guaiacol/H₂O₂ assay. Fractions (2 ml) were collected from the onset of column washing. Fractions 1–22 contained very low levels of peroxidase activity. Elution of HTPP with buffer B+0.5 M-methyl α-D-mannoside was begun at fraction 16. Protein was detected by A₂₈₀ (—). In a separate experiment, haemoglobin (1 mg) was chromatographed under identical conditions, and its elution was monitored at 410 nm (----).

Table 1. Comparison of Soret absorption maxima in the visible spectra of human peroxidases and haemoglobin

Spectral analyses for Con-A-purified HTPP and haemoglobin were conducted under identical conditions: Abbreviation: N.A., not available. References: ^a Olsen & Little (1983); Bolscher *et al.* (1984); Olsson *et al.* (1985); ^b Bolscher *et al.* (1984); ^c Olsen & Little (1983); ^d Desser *et al.* (1972); Wever *et al.* (1981); Olsen & Little (1983); Olsson *et al.* (1985); Carlson *et al.* (1985); ^e Desser *et al.* (1972); Wever *et al.* (1981); ^f Wever *et al.* (1981).

Enzyme	Maximum (nm)			
	Fe ³⁺	Fe ³⁺ -KCN	Fe ²⁺	Fe ²⁺ -CO
Con-A-purified HTPP	414	415	410	421
Myeloperoxidase	428–434 ^a	454 ^b	447–474 ^c	N.A.
Eosinophil peroxidase	410–415 ^d	N.A.	430–449 ^e	435 ^f
Human haemoglobin	408	422	400	420

Peroxidase specific activity in human pre-term placental Ca²⁺ extracts was 3.5 ± 0.8 nmol of tetraguaiacol formed/min per mg of protein (mean $\pm$ S.E.M.; $n = 7$ placentas from 13 to 21 weeks' gestation). This activity was both time- and protein-dependent. Boiled enzyme controls and assays without H₂O₂ were negative.

HTPP present in crude Ca²⁺ extracts was further purified by Con-A affinity chromatography, and a representative chromatographic profile is depicted in Fig. 1. Based on the A_{280} , two protein peaks were observed. The initial major peak (fractions 1–15), containing haemoglobin and other proteins, was eluted during sample application and subsequent washing with 3 bed vol. of equilibrating buffer (buffer B). Peroxidase was co-eluted in the second peak (fractions 22–50) only after transition to the eluting buffer containing 0.5 M-methyl α -D-mannoside (beginning at approx. fraction 16). The fact that HTPP remained firmly bound to the column matrix until application of the buffer containing 0.5 M-methyl α -D-mannoside suggests a glycoprotein nature of the enzyme.

After combination of the fractions within the distinct peroxidase activity peak, the overall percentage recovery and fold purification for the Con-A-purified preparations were approx. 90% and 20-fold respectively ($n = 12$ placentas). Peroxidase specific activity in the crude Ca²⁺ extract and in the Con-A-purified fraction was 23.2 ± 2.1 and 452.6 ± 46.7 nmol/min per mg of protein respectively (means $\pm$ S.E.M., $n = 12$). This activity was found to be both protein- and time-dependent. Control assays were negative.

Despite washing of teased placental tissue with a copious volume of buffer before homogenization, some haemoglobin contamination of the Ca²⁺ extract was observed. Purified human haemoglobin demonstrated measurable peroxidative activity (20 nmol/min per mg of haemoglobin) towards guaiacol in the presence of H₂O₂, under the same assay conditions as those used for HTPP. In view of these results, a separate experiment was performed in which pure human haemoglobin (1 mg) was chromatographed under identical conditions. The column effluent was monitored at 410 nm, and the results are included in Fig. 1 for comparative purposes. Haemoglobin did not bind to the affinity matrix and was eluted from the column in equilibration buffer lacking 0.5 M-methyl α -D-mannoside. Furthermore, no protein peak corresponding to haemoglobin was noted in the eluate which coincided with the HTPP peak.

Characterization of HTPP

No arachidonate-dependent oxidation of guaiacol was observed in the presence of HTPP in Ca²⁺ extract (0.2 guaiacol

Fig. 2. Cathodic slab PAGE of CETAB-solubilized Con-A-purified HTPP and lactoperoxidase

Gels were stained for haem (gel *a*) and protein (gel *b*) as described in the Materials and methods section. Lane assignments were as follows: 1, 10 μ g of lactoperoxidase; 2, 200 μ g of HTPP; 3, 400 μ g of HTPP; 4, 25 μ g of lactoperoxidase; 5, 100 μ g of HTPP; and 6, 25 μ g of HTPP.

unit), even with the inclusion of 5 μ M-haematin as a stimulator of prostaglandin synthase (PGS) activity. This suggests that PGS is not the source of HTPP in Ca²⁺ extracts. Similarly, no depletion of H₂O₂ was observed in the assays containing Con-A-purified HTPP (100–200 μ g of protein; $n = 2$), whereas the positive control depleted H₂O₂ at the rate of 2.5 μ mol/min per μ g of catalase. Therefore it was concluded that catalase is not a contaminant of the Con-A-purified HTPP preparations. The apparent M_r for Con-A-purified HTPP based on the calibration curve was 119380 ± 1690 (mean $\pm$ S.E.M.; $n = 3$). A comparison of the spectral data obtained for Con-A-purified HTPP with that of other human peroxidases, including a haemoglobin, is given in Table 1. The apparent Soret peak of the oxidized (Fe³⁺) HTPP is at approx. 414 nm. However, in the reduced (Fe²⁺) state, the Soret peak undergoes a blue-shift to 410 nm. In contrast, a red-shift of the Soret peak to 421 nm occurs in the CO-reduced state, whereas no significant change occurred upon addition of cyanide.

The results of cathodic PAGE of CETAB-solubilized Con-A-purified HTPP are depicted in Fig. 2. A major diffuse haem-staining band was observed (Fig. 2*a*, lanes 2 and 3), which co-migrated with a corresponding protein-staining band (Fig. 2*b*, lanes 5 and 6). The major band ($R_f = 0.32$, Fig. 2*a*) appeared to be a composite of at least several minor bands and migrated behind the lactoperoxidase standard (lane 1 in Fig. 2*a*; lane 4 in Fig. 2*b*). A second minor haem-staining band ($R_f = 0.44$, Fig. 2*a*) containing an undetectable amount of protein was present, which co-migrated with lactoperoxidase.

DISCUSSION

This study represents the first report on the isolation and partial purification of peroxidase activity that was previously described in human placental homogenates (Matkovics *et al.*, 1975).

Purification of membrane-bound peroxidases has frequently involved an extraction step using a buffer containing a high salt concentration (DeSombre & Lyttle, 1978; Olsen & Little, 1979, 1981, 1983; Keeping *et al.*, 1981). HTPP was efficiently extracted from placental particulates in the 0.5 M-CaCl₂ buffer in a single step.

Purification of crude HTPP extract by Con-A affinity chromatography allowed an average 20-fold increase in specific activity, with a 90% recovery, which is within the range observed in the purification of other tissue peroxidases by this technique (DeSombre & Lyttle, 1978; Olsen & Little, 1983). In addition, Con-A affinity purification appears to be effective in separating haemoglobin, a major contaminant of placental homogenates, from the protein fraction containing peroxidase activity, since haemoglobin is not bound to the affinity matrix employed. In addition, dissimilarities exist in the absorption maxima of the haemoglobin and Con-A-purified HTPP in the oxidized (Fe^{3+}), reduced (Fe^{2+}) and CN- Fe^{3+} states. Moreover, human haemoglobin has a reported M_r of 64 500, which is significantly less than that observed for Con-A-purified HTPP determined by gel filtration. Demonstration of the absence of significant haemoglobin contamination is significant in light of the criticism that reported endogenous peroxidase-mediated xenobiotic metabolism in placental tissue homogenates may be due to residual haemoglobin.

Since catalase is a common contaminant and can function as a peroxidase in the presence of certain hydrogen donors (Mason & Chignell, 1981), as well as hinder peroxidase reactions by removing H_2O_2 catalytically, its presence in the Con-A-purified HTPP preparations needed to be assessed. However, no catalase activity was observed in the Con-A-purified HTPP preparations. Moreover, under similar assay conditions, purified bovine liver catalase did not oxidize guaiacol. Furthermore, the observed estimated M_r for Con-A-purified HTPP of 119 380 corresponds to approx. 50% of that of the tetrameric catalase (Saunders, 1973), although this would be consistent with the dimeric form of catalase.

The CO-reduced spectrum of Con-A-purified HTPP possessed no absorbance maximum at 450 nm, suggesting the absence of substantial cytochrome *P*-450 contamination. This negative finding is consistent with the fact that cytochrome *P*-450-mediated aryl hydrocarbon hydroxylase activity, associated with many *P*-450-mediated xenobiotic oxidations in the placenta (Pelkonen & Pasanen, 1984), is extremely low or undetectable in human placenta unless maternal induction has occurred (Juchau, 1980; Gurtsoo *et al.*, 1983; Kaelin & Cummings, 1983, 1984). In addition, the isolation procedure for cytochrome *P*-450 requires detergent solubilization in deoxycholate, which was not employed in the HTPP isolation procedure. In fact, the extraction of HTPP in a Ca^{2+} buffer would not favour the purification of cytochrome *P*-450, even if it were abundantly present, since Ca^{2+} buffers have been utilized to precipitate cytochrome *P*-450 with the particulate fraction (Kamat & Narayan, 1972; Symms & Juchau, 1973; Hodgson & Juchau, 1977; Fisher & Moore, 1987), rather than to extract the enzyme. Finally, tetraguaiacol, the major product of peroxidase-catalysed oxidation of guaiacol, is not observed in the cytochrome-*P*-450-mediated reaction in the presence of either NADPH (Saeki *et al.*, 1982) or organic hydroperoxide (Hrycay & O'Brien, 1971). These data support the contention that Con-A-purified HTPP is devoid of substantial cytochrome *P*-450 contamination.

In the presence of an exogenous peroxide and a suitable hydrogen donor, PGS can function as a peroxidase, by-passing the usual pre-requisite for cyclo-oxygenase-mediated conversion of arachidonic acid into the endoperoxide. Since PGS activity has been shown to exist in human placenta (Keirse & Turnbull, 1976; Kinoshita *et al.*, 1977; Duchesne *et al.*, 1978), albeit at very low levels, and guaiacol has been identified as a substrate for ram-seminal-vesicular PGS in the presence of H_2O_2 (Ohki *et al.*, 1979), the observed peroxidase activity in HTPP preparations might theoretically be due in part to PGS. However, the fact that placental Ca^{2+} extracts did not catalyse the oxidation of guaiacol

in the presence of arachidonate, even with the inclusion of haematin, a necessary stimulator of PGS activity in reconstituted systems (Hemler *et al.*, 1976; Ogino *et al.*, 1978; Marnett, 1984; Eling *et al.*, 1985), suggests that PGS is not a source of the observed peroxidase activity. This contention is further supported by a recent study (Flammang *et al.*, 1989) in which neither PGS-supported benzidine oxidation nor PGS/antigen was detected in several human placental preparations.

The M_r values of eosinophil peroxidase and myeloperoxidase, estimated by gel filtration, are reported to be 77 000 and 118 000 respectively (Carlson *et al.*, 1985). Although the estimated M_r of Con-A-purified HTPP is much larger than that of eosinophil peroxidase, the value obtained by Carlson and associates for myeloperoxidase is practically identical with that associated with HTPP. With respect to spectral characteristics (Table 1), Con-A-purified HTPP exhibited a similar Soret absorption maximum in the oxidized (Fe^{3+}) state to that of eosinophil peroxidase at 415 nm; however, spectral dissimilarities were noted in the reduced (Fe^{2+}) and CO-reduced states. This suggests that Con-A-purified HTPP is probably not of eosinophilic origin. Similarly, the spectral characteristics of myeloperoxidase were different from those of Con-A-purified HTPP in the oxidized, CN-oxidized and reduced states. These data suggest that HTPP is not related to eosinophil peroxidase or myeloperoxidase. However, since the placental peroxidase has only been partially purified, the spectral data must be regarded as tentative.

Cathodic PAGE has been employed to characterize several membrane-bound peroxidases, which have pI values in the basic range, e.g. eosinophil peroxidase and myeloperoxidase (Bolscher *et al.*, 1984). Cathodic slab-PAGE of Con-A-purified HTPP (Fig. 2) revealed at least two basic haem-staining proteins: a minor band, which appeared to co-migrate with bovine lactoperoxidase; and a broad band, consisting of many smaller bands, which migrated well behind lactoperoxidase. Purified rat uterine peroxidase, a membrane-bound enzyme, also demonstrated a haem-staining protein, which co-migrated with lactoperoxidase in non-denaturing gels (Keeping *et al.*, 1981). It appears that one of the electrophoretic forms of HTPP and other animal membrane peroxidases share some physical characteristics with lactoperoxidase. The fact that the minor band did not stain with Coomassie Blue may be related to insufficient protein.

This study has confirmed the existence of an endogenous and unique protein in human placental membranes, which, on the basis of M_r value, electrophoretic or spectral properties, appears to be distinct from known human peroxidases, including contaminants such as haemoglobin and catalase.

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