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Differential modification of cyclo-oxygenase and peroxidase activities of prostaglandin endoperoxidase synthase by proteolytic digestion and hydroperoxides

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Prostaglandin endoperoxide synthase (PES, EC 1.14.99.1) catalyse the conversion of arachidonic acid into prostaglandin H_2. The enzyme is a 140 kDa homodimer which contains both a cyclo-oxygenase activity (converting arachidonate into prostaglandin G_2) and peroxidase activity (reducing prostaglandin G_2 to H_2). PES undergoes rapid self-inactivation during oxygenation of arachidonate to prostaglandin H_2 *in vitro*. The previously reported cDNA-derived amino acid sequence indicates numerous sites for trypsin or thrombin cleavage. Most of these sites must be inaccessible, since these enzymes cleave only at Arg^{253}. The enzyme appears to be a self-adherent and highly folded molecule, since after cleavage it retains its functional assembly and its homodimer size of 140 kDa, as well as its overall enzymic activity. Only under denaturing conditions (e.g. SDS/PAGE) can the proteolytic peptides be demonstrated: a 38 kDa *C*-terminal fragment containing the aspirin-derived-acetyl-binding ability, and a 33 kDa *N*-terminal fragment. In the present studies we investigated whether the two enzymic activities of PES can be differentially manipulated by proteolytic cleavage or by substrate (arachidonate) self-inactivation. The results indicated that, during arachidonate oxygenation by PES, the cyclo-oxygenase activity is selectively inactivated, whereas the peroxidase activity is essentially retained. By contrast, thrombin or trypsin cleavage of pure PES or microsomal PES (to yield the 38 and 33 kDa peptide fragments) inactivated the peroxidase, but not the cyclo-oxygenase. Taken together, these results suggest the presence of separate cyclo-oxygenase and peroxidase structural domains on the enzyme.

INTRODUCTION

Prostaglandin endoperoxide synthase (PES) (EC 1.14.99.1) is the first enzyme in the biosynthetic pathway for the conversion of arachidonic acid into prostaglandins and thromboxane. The enzyme possesses both cyclo-oxygenase (arachidonate-oxidizing) and peroxidase (hydroperoxide-reducing) activities and catalyses the overall conversion of arachidonic acid into prostaglandin endoperoxide H_2 (PGH_2). Purified PES (from sheep vesicular glands) is a homodimer of approximate molecular mass 140 kDa [1,2]; the enzymic activity is lost upon irreversible dissociation of the homodimer to the 70 kDa monomer [1]. Recently we [3] and others [4,5] have cloned and sequenced a cDNA clone encoding the PES enzyme. The monomeric protein contains 576 amino acid residues with a calculated molecular mass of 65.7 kDa. Trypsin cleaves the homodimer to produce two fragments of apparent molecular mass of 38 and 33 kDa, with the larger fragment containing the aspirin-binding site [1,6]. The trypsin-treated enzyme retains its molecular mass of 140 kDa and its enzymic capacity to convert arachidonic acid into PGH_2 if maintained in non-denaturing buffers [1]. However, after h.p.l.c. separation of the two peptide fragments, no enzymic activity was found to be associated with either of the two fragments or their mixture [1,6].

In the present study, we examined the relationship between modifications of PES, brought about by proteolytic cleavage or substrate-dependent self-inactivation, and changes in the cyclo-oxygenase and peroxidase activities.

EXPERIMENTAL

Preparation of sheep vesicular-gland microsomes and of purified PES

Sheep vesicular glands were collected at the slaughterhouse on ice, cleaned of fat and connective tissue and stored frozen at −70 °C. The glands were homogenized in 5 vol. of 50 mM-Tris buffer, pH 8.0, containing 0.1 M-KCl and 20 mM-diethyldiothio-carbamate. The homogenate was centrifuged at 9000 ***g*** for 10 min, and the resulting supernatant was centrifuged at 140 000 ***g*** for 45 min. The microsomal pellet was freeze-dried and kept at −20 °C.

Preparation of PES

Freeze-dried microsomes were solubilized [7] and the PES purified as described [8]. The final preparation was judged to be over 95% pure by SDS/PAGE and silver staining. The specific activity was 19000 units/mg, and after the addition of haematin (2 μM) it was 87000 units/mg.

Assay of peroxidase and combined cyclo-oxygenase plus peroxidase activities of PES

Spectrophotometric assay of the PES peroxidases reaction was done using either H_2O_2 or arachidonate as substrates, essentially as described previously [9]. Microsomes homogenized in 0.1 M-Tris buffer, pH 8.0 (1–3 mg of protein/ml) or PES in the same buffer were preincubated (in the spectrophotometer cuvette) with

Abbreviations used: PES, prostaglandin endoperoxide synthase; PGH_2, prostaglandin endoperoxide H_2; PGG_2, prostaglandin endoperoxide G_2; 15-HPETE, 15-L-hydroperoxy-5,8,11-*cis*,13-*trans*-eicosatetraenoic acid; TMPD, *NNN′N′*-tetramethyl-*p*-phenylendiamine dihydrochloride; DTT, dithiothreitol.

or without haematin (1 μM) for 1 min at 37 °C in a total volume of 0.4 ml. Freshly mixed solution (0.3 ml) containing *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) (200 μM) and either H_2O_2 (700 μM) or arachidonate (230 μM) was added and the change in the difference absorption at 611 nm between cuvettes with and without enzyme was recorded (Gilson, model Response instrument; see Fig. 1 below for format) for up to 6 min. The rate of peroxidase reaction was calculated using an absorption coefficient of 13500 $M^{-1} \cdot cm^{-1}$ for oxidized TMPD and a stoichiometric ratio of 2 mol of TMPD oxidized/mol of hydroperoxide reduced [9–11]. The relative content of native holoenzyme and of total enzyme (holoenzyme plus apoenzyme) was determined from assays in the absence or presence of 1 μM-haematin respectively.

In some experiments the combined activity of cyclo-oxygenase plus peroxidase was assayed by incubating microsomes or enzyme with arachidonic acid (100 μM) in the presence of 1 mM-adrenaline (epinephrine) and 1 mM-phenol. Incubations were done at 37 °C for 10 min, and the production of prostaglandin E_2 was determined by radioimmunoassay [12].

Substrate-dependent destruction of PES activities

The cyclo-oxygenase and peroxidase activities of PES were inactivated by incubating PES with arachidonic acid or 15-L-hydroperoxy-5,8,11-*cis*,13-*trans*-eicosatetraenoic acid (15-HPETE) as described previously [9]. PES, in 0.4 ml of 0.1 M-Tris buffer, pH 8.0, was preincubated with haematin (1 μM) for 1 min at 37 °C. Arachidonic acid (1 μM final concn.) or 15-HPETE (5 μM final concn.) was added as sodium salts, and the samples incubated for 2 min at 37 °C. A freshly mixed solution of TMPD and arachidonate or TMPD and H_2O_2 was added and the peroxidase activity determined as described above.

Proteolytic cleavage of PES

The purified enzyme (1–2 μg) was treated with either trypsin (0.1–0.2 μg) or thrombin (1–3 units) in 10–20 μl of 0.1 M-Tris buffer, pH 8.0. Incubations with trypsin were carried out for 2–30 min at room temperature and terminated by adding 1–2 μg of trypsin inhibitor (in 5–10 μl of the same buffer). Incubation with thrombin were carried out for 12–24 h at room temperature and terminated by freezing at -20 °C.

SDS/PAGE and protein staining

Samples were diluted with concentrated SDS/PAGE sample buffer containing 5% (v/v) β-mercaptoethanol (reducing), separated by SDS/PAGE (10% acrylamide) [13] and the proteins revealed by silver staining (Bio-Rad kit).

Materials

Haematin, dithiothreitol (DTT), diethyldithiocarbamate, trypsin (bovine pancreas or porcine pancreas type IX), trypsin inhibitor (from soybean), thrombin and TMPD were obtained from Sigma (St. Louis, MO, U.S.A.). 15-HPETE was biosynthesized as reported elsewhere [14]. Protein molecular-mass standards and silver-staining kit were purchased from Bio-Rad (Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

Cyclo-oxygenase and peroxidase activities of microsomal PES and of purified PES

Prostaglandin endoperoxide synthase possesses two enzymic activities: the cyclo-oxygenase (arachidonate-oxidizing, hydroperoxide-forming) and the peroxidase (hydroperoxide-reducing) [1,9,10]. The rate of each reaction can be determined independently: the cyclo-oxygenase activity by measuring oxygen consumption with an oxygen electrode during oxygenation of arachidonic acid to the prostaglandin endoperoxide G_2 (PGG_2), and the peroxidase activity by assaying the reduction of PGG_2 or other hydroperoxide substrates. Each of these two activities requires a co-substrate during the reaction. Thus the cyclo-oxygenase, which converts arachidonate into the endoperoxide PGG_2, requires molecular oxygen as co-substrate, whereas the peroxidase, which reduces PGG_2 to PGH_2, requires a reducing compound as co-substrate. Indeed, if the active enzyme (i.e. haem-containing holoenzyme) is incubated with either the cyclo-oxygenase substrate, arachidonate, or the peroxidase substrate, 15-HPETE, but in the absence of an efficient hydroperoxide reducer, both activities of the enzyme are very rapidly inactivated ([1] and Table 1). In contrast, when the enzyme is incubated in the presence of a suitable reducing compound, needed as a co-substrate in the peroxidase reaction [15], the combined action of both activities affords the conversion of arachidonic acid into the endoperoxide PGH_2. Coupling of hydroperoxide reduction (e.g., PGG_2 to PGH_2, 15-HPETE to 15-HETE, H_2O_2 to H_2O) to the oxidation of a suitable, non-coloured, co-substrate to a coloured product, permits spectrophotometric rate measurements of the PES reaction to be made. The format of the results obtained is shown in Fig. 1. By using this assay system and the same enzyme preparation, we observed that the cyclo-oxygenase-plus-per-

Table 1. Self-inactivation of both peroxidase and cyclo-oxygenase activities of PES by arachidonate and 15-HPETE

PES (2.8 μg) was preincubated (2 min, 37 °C) with either 15-HPETE (5 μM) or arachidonate (1 μM) in 0.3 ml of 0.1 μ-Tris buffer, pH 8.0, containing 1 μM-haematin. After preincubation, either H_2O_2 (300 μM) or arachidonate (100 μM) was added, the peroxidase rate recorded spectrophotometrically for 1 min and initial reaction rates calculated. Values are from a representative experiment (out of four experiments; S.E.M. values were less than 10%).

Inactivating substrate during preincubation	Substrate during incubation	Activity (nmol of hydroperoxide reduced /min per μg of protein)		Residual activity (% of original)
		Peroxidase	Cyclo-oxygenase plus peroxidase	
–	H_2O_2	2.01	–	100
–	Arachidonate	–	0.32	100
15-HPETE	H_2O_2	0.13	–	6.5
	Arachidonate	–	0.03	9.4
Arachidonate	H_2O_2	0.226	–	11.2
	Arachidonate	–	0.04	12.5

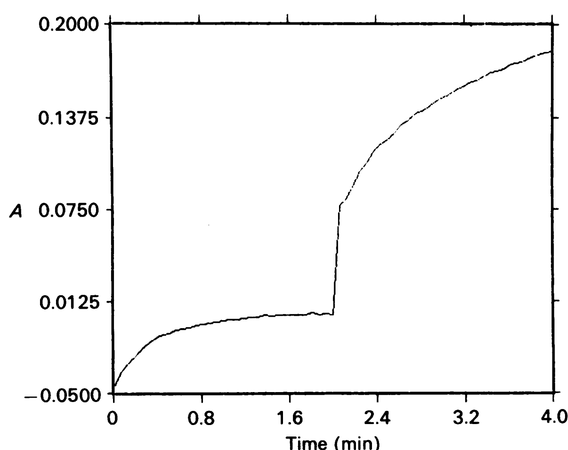

Fig. 1. Spectrophotometric rate determinations of combined cyclo-oxygenase-plus-peroxidase (arachidonate to PGH_2) and of peroxidase rate (H_2O_2 to H_2O)

A sheep seminal-vesicle microsomes suspension was prepared and preincubated with haematin as described in the legend to Table 3. At zero time a mixture of arachidonic acid (AA) and TMPD was added, and TMPD oxidation was monitored for 2 min (at which time the reaction rate was less than one-tenth the initial rate). H_2O_2 was then added and the peroxidase rate monitored for 2 min.

oxidase reaction rate is lower than the peroxidase-only reaction (Table 2). Therefore the observed peroxidase rate when assaying arachidonate oxygenation is in fact a measurement of the preceding rate-limiting cyclo-oxygenase reaction.

Both the cyclo-oxygenase and peroxidase reactions require haem for activity. The haem-free inactive apoenzyme can be reconstituted to produce fully active holoenzyme by the addition of haematin [15]. The sheep vesicular-gland microsomes we prepared consisted of approximately equal amounts of holoenzyme and apoenzyme (40–58 % apoenzyme; range for several preparations). After detergent solubilization of the microsomes with 1 % Tween 20 and purification of the enzyme to homogeneity, the PES preparations we obtained contained mostly apoenzyme (85–94 % apoenzyme; range for three enzyme batches). The haem-reconstituted purified PES exhibited a ratio for the activities of peroxidase over the combined cyclo-oxygenase plus peroxidase which was significantly higher than that for the microsomal enzyme (Table 2). This may result from a selective loss of the cyclo-oxygenase activity relative to the peroxidase activity during solubilization and purification of PES. In support of this is our observation that the combined cyclo-oxygenase-plus-peroxidase activity during the incubation of microsomes with arachidonic acid is largely (85–92 %) inactivated, whereas the peroxidase activity is only marginally decreased (Table 3), thus indicating that the cyclo-oxygenase is more susceptible to self-inactivation during substrate oxygenation. An alternative explanation, however, is that unidentified reducing compounds, which are present in the microsome preparations, and compete with TMPD for hydroperoxide reductase, are being removed during the purification, resulting in an apparent higher rate of TMPD oxidation.

Cleavage of PES by thrombin and trypsin

Kulmacz [11] and, more recently, Chen and co-workers [1] have reported on the specific tryptic cleavage of PES to two fragments with apparent molecular masses 38 and 33 kDa. In our initial attempts to obtain large proteolytic fragments of PES for further structural studies on the enzyme, we also employed trypsin (Sigma; from bovine pancreas). Our initial studies were, however, unsuccessful, as most of the PES was extensively degraded to small peptides (< 3 kDa). We next treated PES with thrombin and found this enzyme to produce a time-dependent and temperature-dependent formation of two fragments having molecular masses of approx. 38 kDa and 33 kDa (Fig. 2). We subsequently found that treatment with low concentrations of porcine trypsin (0.01–0.03 mg/ml) yields two fragments with the same apparent sizes (i.e. electrophoretic mobility on SDS/PAGE) as those for the fragments produced by thrombin treatment (Fig. 2).

Noteworthy is the fact that, although the enzyme contains numerous potential trypsin-sensitive cleavage sites and three potential thrombin-sensitive Pro-Arg sites [3], the proteolytic enzymes cleave the native homodimer enzyme only at one specific site, indicating that other potential sites are structurally shielded. Furthermore, Chen and co-workers [1] observed that, after tryptic cleavage of PES, the enzyme's apparent molecular mass of 140 kDa (as determined by non-denaturing gel filtration) is unchanged. These results were confirmed by Kulmacz & Wu [6] and indicate that, despite the single-site proteolytic cleavage, the enzyme possesses sufficient intramolecular and/or intermolecular (between the monomers) interaction to keep the overall dimeric structure intact. The proteolytic cleavage at Arg^{253} does, however, affect differentially the peroxidase and cyclo-oxygenase activities of the enzyme. In agreement with previous studies [1], trypsin treatment of purified PES for 10 min produced 75–85 % cleavage of the enzyme, with only minimal (5–10 %) decline in the combined rate of cyclo-oxygenase plus peroxidase (arachidonate-oxidizing) (Fig. 3). Similarly, we found that, after incubation of the enzyme with thrombin for 12 h at room temperature, 60–65 % of the combined cyclo-oxygenase-plus-peroxidase activity was retained, whereas 85–90 % of the protein was cleaved (results not shown). The tryptic cleavage did, however, cause a very sub-

Table 2. Enzymic activities of peroxidase (H_2O_2-reducing) and cyclo-oxygenase-plus-peroxidase (arachidonate-oxidizing) in sheep vesicular-gland microsomes and in purified PES

Sheep vesicular-gland microsomes suspension (125 μg of protein in 0.4 ml of 0.1 M-Tris buffer, pH 8.0) or purified PES (1 μg, in the same buffer) were preincubated with 1 μM-haematin for 1 min at 37 °C. A mixture of TMPD and either H_2O_2 or arachidonic acid (in 0.3 ml) was added, and the rate of TMPD oxidation measured spectrophotometrically. The reaction rate is linear for 12–15, s for which an initial reaction rate was calculated. For other details, see the Experimental section. Values are means ± S.E.M. for four experiments.

Enzyme preparation	Substrate	Initial rate of peroxidase (nmol of hydroperoxide reduced/min)	Ratio of activities (peroxidase/cyclo-oxygenase)
Microsomes	H_2O_2	2.89 ± 0.25	3.57
	Arachidonate	0.81 ± 0.06	
PES	H_2O_2	1.24 ± 0.13	4.96
	Arachidonate	0.25 ± 0.03	

Table 3. Preferential inactivation of cyclo-oxygenase activity during oxygenation of arachidonic acid by PES

Expt. 1: microsome suspension (125 μg in 0.4 ml of 0.1 M-Tris buffer, pH 8.0) was incubated for 1 min at 37 °C with haematin (final concn. 1 μM). TMPD solution (0.3 ml) was the added and the absorbance at 611 nm recorded for 2 min. H_2O_2 (0.3 ml, final concn. 0.3 mM) was then added and TMPD oxidation recorded for 2 additional min. Expt. 2: microsome suspension was similarly incubated with haematin. A 0.3 ml solution containing TMPD and arachidonic acid (final concentration 100 μM) was added (zero time) and the peroxidase reaction course monitored spectrophotometrically for 2 min. H_2O_2 solution was then added and the reaction monitored for 2 min. When additional arachidonate was added instead of H_2O_2 at the 2 min time point, no change in the reaction rate was seen. The spectrophotometric data obtained in such an experimental protocol is shown in Fig. 1.

			Cyclo-oxygenase-plus-peroxidase rate (nmol of hydroperoxide reduced/min)		Peroxidase rate after subsequent H_2O_2 addition (nmol of hydroperoxide reduced/min)		Residual peroxidase activity after preincubation with arachidonic acid (%)	
Expt.	Arachidonic acid during intial preincubation	Time...	0 min	2 min		Substrate...	Arachidonic acid	H_2O_2
1	–		–	–	1.33‡			
2	+		0.51*	0.06†	1.01‡ (0.95)		12§	79§

* Initial rate when arachidonate is added.

† Reaction rate at 2 min (just before addition of H_2O_2).

‡ Reaction rate after addition of H_2O_2; the value in parentheses is the net rate increase after subtraction of the residual arachidonate oxygenation rate at 2 min (0.06).

§ Data in the Table is from a single experiment (out of four such experiments; the S.E.M. values were less than 10%).

stantial loss (> 60%) of the peroxidase activity (H_2O_2-reducing) of purified PES (Fig. 3). In order to rule out the possibility that these results may pertain only to the solubilized enzyme and not to the native, microsome-associated, enzyme, we ran parallel experiments with native microsomes and obtained essentially the same results (Fig. 3). Taken together, these data indicate that, although the trypsin-cleaved enzyme retains its apparent homodimer molecular size of 140 kDa and also its combined cyclo-oxygenase-plus-peroxidase activity for the substrate arachidonate, the proteolytic cleavage at Arg^{253} causes a significant change in the active site of the peroxidase domain with an accompanying decline in peroxidase activity. These findings are in agreement with recent data of Marnett and co-workers [17], who observed, after 10 min trypsin treatment of PES, a selective loss (77%) of peroxidase with minimal loss of cyclo-oxygenase activity. In contrast, Kulmacz [18] recently reported results of similar experiments in which trypsin treatment of PES resulted in a time-dependent parallel destruction of both peroxidase and cyclo-oxygenase activities. He suggested that the discrepancy may be due to the fact that Marnett *et al.* [17] employed a PES preparation that was mostly apoenzyme. Our

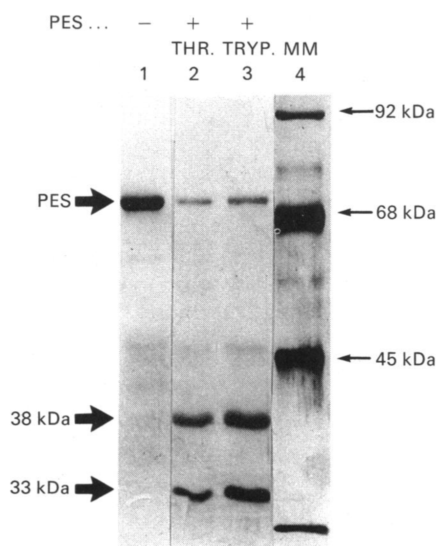

Fig. 2. Proteolytic cleavage of PES by trypsin and thrombin

PES (1 μg in 15 μl of 0.1 M-Tris buffer, pH 8.0) was incubated either with trypsin (TRYP.; 0.1 μg; for 10 min at room temperature; lane 3) and the incubation terminated by addition of trypsin inhibitor (1 μg) or with thrombin (THR.; 5 units; 8 h at 37 °C; lane 2). Reducing SDS/PAGE sample buffer was then added and the samples analysed by SDS/PAGE and silver staining. Protein molecular-mass markers (MM, lane 4) were phosphorylase *b* (92 kDa), BSA (68 kDa) and ovalbumin (45 kDa). Lane 1, untreated PES (control).

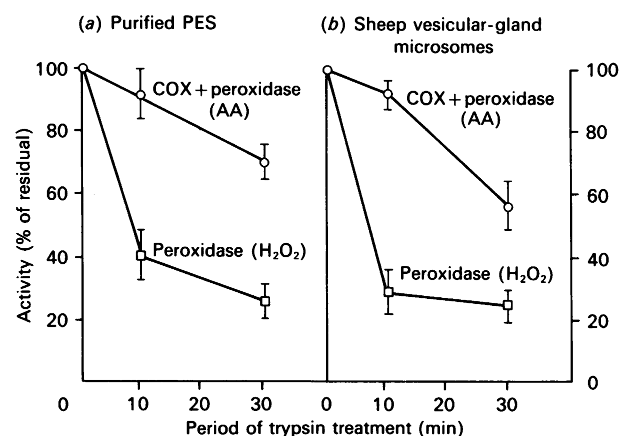

Fig. 3. Effect of trypsin cleavage on peroxidase and cyclo-oxygenase plus peroxidase activities of purified PES (*a*) and microsomal PES (*b*)

Purified PES (2.8 μg) or microsomes (125 μg of protein) were incubated for 10 min with trypsin as described in the legend to Fig. 2. After addition of trypsin inhibitor, the samples were diluted to 0.4 ml and the rates of peroxidase (H_2O_2-reducing) and cyclo-oxygenase (COX) plus peroxidase (assayed with arachidonic acid) were determined. Values are means ± S.D. for three experiments. To determine the extent of tryptic cleavage of microsomal PES, [^{3}H]aspirin-labelled microsomes were similarly treated with trypsin, solubilized with 1% Tween 20, and analysed by quantitative SDS/PAGE and fluorography (for details, see [16].

results show that the selective loss of peroxidase is also seen in intact microsomes (which contains mostly holoenzyme) and thus do not support such an explanation. A more likely explanation may be related to the type of trypsin employed. We and Marnett *et al.* [17] employed porcine trypsin type IX from Sigma. As discussed by us, the use of this type of trypsin may be critical to obtaining the single proteolytic cleavage at Arg^{253}. In summary, our data and those of Marnett *et al.* [17] suggest that the cyclo-oxygenase and peroxidase activities may reside in different domains of the enzyme and thus be amenable to a potential differential manipulation by selective drugs.

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