Vol. 180, No. 1, 1991
October 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 360-366

THE DUAL OXYGENASE AND PEROXIDASE ACTIVITIES OF PORPHOBILINOGEN OXYGENASE AND HORSERADISH PEROXIDASE: A STUDY USING THE REACTION WITH PHENYLHYDRAZINE

Marcelo Fernández and Rosalia B. Frydman

Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires,
Junin 956, Buenos Aires, Argentina

Received August 28, 1991

SUMMARY: Porphobilinogen oxygenase and horseradish peroxidase show dual oxygenase and peroxidase activities. By treating porphobilinogen oxygenase with phenylhydrazine in the presence of H_2O_2 both activities were inhibited. When horseradish peroxidase was treated in the same manner only the peroxidase activity was lost while its oxygenase activity toward porphobilinogen remained unchanged. The phenylhydrazine treatment alkylated the prosthetic heme group of porphobilinogen oxygenase and N-phenylheme as well as N-phenylprotoporphyrin IX were isolated from the treated hemoprotein. In horseradish peroxidase the modified heme was mainly 8-hydroxymethylheme. The apoproteins of the alkylated enzymes were isolated and recombined with hemin IX. The oxygenase and peroxidase activities of porphobilinogen oxygenase were entirely recovered in the reconstituted enzyme, while the reconstituted horseradish peroxidase regained 75% of its peroxidase activity.

Porphobilinogen oxygenase is a hemoprotein that catalyzes the oxidation of porphobilinogen (PBG) to oxopyrrolenines (1). Since PBG is one of the key intermediates in porphyrin metabolism its oxidation can be considered as a regulatory step in heme biosynthesis (2,3). Porphobilinogen oxygenase shares many structural and catalytic properties with horseradish peroxidase (HRP). Their electronic and magnetic spectra are very similar (1). Horseradish peroxidase also shows oxygenase activity toward PBG and it oxidizes PBG in the presence of a reducing agent (1). We report in this paper that porphobilinogen oxygenase also shows peroxidase activity and is able to oxidize phenols in the presence of H_2O_2. Both hemoproteins have therefore dual enzymatic activities; namely, they act as oxygenases in the presence of a reducing agent and oxygen, while they act as peroxidases in the presence of H_2O_2.

The heme prosthetic group of many hemoproteins alkylate by reaction with phenyl radicals released from phenylhydrazine in the presence of H_2O_2 or oxygen (4). Thus, N-phenylhemin is formed when hemoglobin (5), cytochrome P450 (6), or catalase (7) react with phenylhydrazine, while in horseradish peroxidase alkylation takes place on the δ–meso edge of the heme prosthetic group due to the shielding by the protein of the central part of the heme molecule (8). These regioselective alkylation reactions inhibit the catalytic activities of hemoproteins, and were used to correlate their activities with the topology and architecture of the heme pockets of the enzymes (4).

Despite their spectral and catalytic similarities, the phenylhydrazine treatment of porphobilinogen oxygenase and horseradish peroxidase reveals substantial differences among both

0006-291X/91 $1.50

hemoproteins. As we describe below, on alkylation with phenylhydrazine the oxygenase and peroxidase activities of porphobilinogen oxygenase are lost while only the peroxidase activity of horseradish peroxidase is affected. The alkylation products of the heme groups in both enzymes were also found to be different, suggesting that at variance with the topology found for horseradish peroxidase, the central iron group of porphobilinogen oxygenase is accessible to phenyl radicals.

MATERIALS AND METHODS

Materials. Porphobilinogen oxygenase was obtained from wheat germ and purified to homogeneity as described elsewhere (1). HRP (type VI), myoglobin, hydrogen peroxide, and pyrogallol were from Sigma. PBG was obtained by synthesis (9). Phenylhydrazine·HCl was from Aldrich and was recrystallized from ethanol. Solutions were prepared in 0.01 N HCl and used within 6 h. Buffers were prepared with glass-distilled deionized water and treated with Chelex 100. All the other reagents and solvents used were from Merck.

The concentrations of porphobilinogen oxygenase were determined using an $\varepsilon_{399} = 100{,}000$ M^{-1} cm^{-1}. For HRP an $\varepsilon_{402} = 95{,}000$ $M^{-1}cm^{-1}$ was used (10). Protein concentrations were estimated by the method of Bradford (11) using bovine serum albumin or myoglobin as standards. Hydrogen peroxide solutions were standardized by the peroxidase catalyzed oxidation of iodide to iodine, $\varepsilon_{353} = 23{,}000$ M^{-1} cm^{-1} (12).

Optical spectra were measured with a double beam Hitachi U-2000 spectrophotometer. Fluorescence determinations were performed using an Aminco Bowman spectrofluorometer. Field desorption mass spectra were obtained on a ZAB-VS-EQ spectrometer equipped with a BEQQ analyzer.

Enzyme assays. The oxygenase activities of both porphobilinogen oxygenase and HRP were assayed following described procedures (1). The standard incubation mixture contained, in a final volume of 100 µl: 10 µmol of phosphate buffer (pH 7.4), 18 nmol of porphobilinogen, 30 nmol of sodium dithionite and either porphobilinogen oxygenase or HRP (3 µg). Incubations were carried out at 37°C for 10 min. Blanks where the enzymes were added at the end of the incubations were run simultaneously. The reaction was stopped by addition of 0.7 ml of water and 0.8 ml of the modified Ehrlich's reagent (2% p-dimethylbenzaldehyde in glacial acetic acid/perchloric acid (84:16 v/v)). The absorbance at 552 nm was measured after addition of Hg^{2+} and the decrease in this absorbance was a measure of substrate disappearance (2). The peroxidase activity of both enzymes was measured in a final volume of 1 ml containing 50 mM phosphate buffer (pH 7.4), 5 mM pyrogallol, 0.6 mM H_2O_2 and enzyme (0.05 µg of HRP or 0.5 µg of porphobilinogen oxygenase). The reaction was started by addition of the enzyme and the increase in absorbance at 470 nm was recorded as a measure of the peroxidase activity.

Inactivation of porphobilinogen oxygenase and horseradish peroxidase by phenylhydrazine. The inactivation rates of the oxygenase and peroxidase activities were measured using a reaction mixture containing 20 µM enzyme, 0.2-1.0 mM phenylhydrazine and 0.40-2.0 mM H_2O_2 in a final volume of 2.0 ml. The reaction was initiated by addition of the H_2O_2. Aliquots (0.2 ml) were withdrawn from the mixture at 0, 1, 2, 5, 15 and 30 min and were filtered through PD-10 columns (Pharmacia). The eluted hemoproteins were assayed as described above for their oxygenase and peroxidase activities.

Reconstitution of the native and phenylhydrazine modified porphobilinogen oxygenase and horseradish peroxidase. The apoenzymes were prepared by the acid-butanone method of Teale (13). The apoenzymes (20 µM) were dialyzed against 20 mM phosphate buffer (pH 7.4). Hemin IX was dissolved in the minimum volume of 0.1 M NaOH and the solution was adjusted to pH 7.4 with phosphate buffer to about 1 mM. The concentration of heme in the solutions was determined spectrophotometrically by the pyridine-hemochrome method. The apoenzymes were mixed with the heme solution at a molar ratio of 2.0 and were allowed to stand for 2 h at 0-4 °C. The unbound heme was separated by a DEAE-Trisacryl column (0.6 x 10 cm), equilibrated with the above mentioned phosphate buffer. The hemoproteins were eluted with the same buffer. They were then dialyzed against 20 mM phosphate buffer (pH 5.9) and applied to a CM-Trisacryl column equilibrated with the same buffer (pH 5.9). HRP was eluted with this buffer, while porphobilinogen oxygenase was eluted with 100 mM NaCl in the same buffer.

Extraction and identification of modified hemes and their derivatives. A solution of porphobilinogen oxygenase (13 μmol), phenylhydrazine·HCl (650 mmol) and H_2O_2 (1.3 mmol) in 6 ml of 20 mM phosphate buffer, 0.1 mM EDTA (pH 7.4), was stirred during 15 min at 25°C. The mixture was filtered through a Sephadex G25 column using the same buffer. The hemoprotein containing fraction was adjusted to pH 2.8 with 0.2 N HCl and the aqueous solution was extracted twice with an equal volume of 2-butanone at 0 °C. The organic layers were pooled, washed with water, dried (Na_2SO_4), and evaporated to dryness in vacuum at 30 °C. The residue was dissolved in a minimum volume of methanol:acetic acid:water (60:10:40) and applied to an HPLC precolumn (Baker 10SPE octadecyl C18). Methanol:acetic acid (10:1) was used for elution. The heme containing fractions were concentrated at 20°C under vacuum and the residue was applied to HPTLC RP18 silica gel plates (Merck). The latter were developed using methanol:acetic acid:water (95:10:5). The heme bands were eluted with methanol. HRP was alkylated following the same procedure. The reaction products (modified hemes) were analyzed by HPLC as described elsewhere (8), as well as by the above mentioned method.

To isolate the iron-free modified protoporphyrin derivative resulting from the alkylation of porphobilinogen oxygenase the following procedure was used. A solution of the oxygenase (1.3 μmol) and phenylhydrazine·HCl (65 μmol) in 2.5 ml of 100 mM phosphate buffer, 0.1 mM EDTA (pH 7.4) was stirred in an open beaker for 2 h at 25 °C. The mixture was poured into 5% sulfuric acid in methanol (25 ml). The solution was kept at 4 °C during 24 h, chloroform (25 ml) was then added and the solution was washed with 5% sodium bicarbonate followed by a water wash. A solution of 5 mg of zinc acetate in methanol was added to the organic layer, the mixture was dried (Na_2SO_4), evaporated to dryness in vacuum and the residue, dissolved in a small volume of chloroform, was applied to TLC silica gel plates (Merck). The latter were developed with chloroform:acetone (5:1). The fluorescent porphyrin chelate band was eluted with acetone:methanol (10:1) and repurified using the same procedure. Myoglobin was alkylated following the same procedure and the dimethyl ester of the Zn N-phenylprotoporphyrin (5) was isolated as described above.

RESULTS

Effect of phenylhydrazine treatment on the oxygenase and peroxidase activities of porphobilinogen oxygenase and horseradish peroxidase Porphobilinogen oxygenase and horseradish peroxidase show oxygenase and peroxidase activities. By treatment of porphobilinogen oxygenase with phenylhydrazine in the presence of H_2O_2 both enzymatic activities were inhibited to the same extent in a concentration dependent manner (Fig. 1). Maximum inhibition was reached within the first minute (Fig. 2). The enzymatic activities were however, not completely inactivated even by a large excess of phenylhydrazine and H_2O_2 or by longer incubation times (Fig. 2). Maximum inhibition was about 85% in the presence of 50 equivalents of alkylating agent and 100 equivalents of H_2O_2. When the inactivated enzyme was filtered through Sephadex G-25 and then reincubated with the same ratio of reagents, a complete inactivation was obtained after 1 min (Fig. 2). When horseradish peroxidase was submitted to the same treatment, its peroxidase activity was lost after 1 min, and was phenylhydrazine concentration dependent (Figs. 1 and 2) (see also (8)). Its oxygenase activity however, remained unaffected by the treatment (Fig. 1), even at longer incubation times (Fig. 2). When a 65% inactivated horseradish peroxidase (Fig. 1) was filtered through a Sephadex G-25 column and then reincubated with phenylhydrazine plus H_2O_2, 90% of its peroxidase activity was lost after the first min. (Fig. 2). Its oxygenase activity remained unaffected. The oxygenase and peroxidase activities of porphobilinogen oxygenase and HRP were not affected by the Sephadex G-25 filtration in either the control or the partially inactivated enzymes.

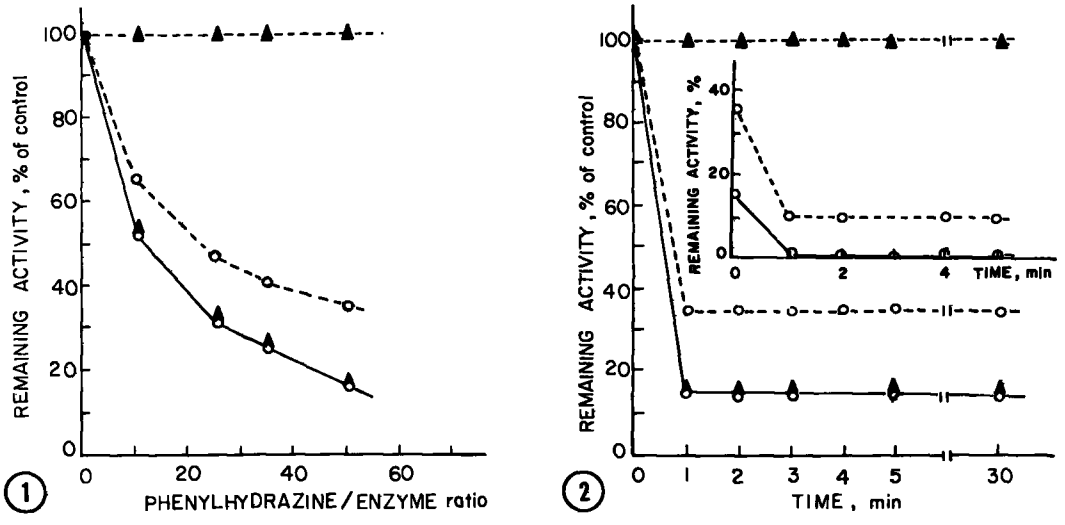

Figure 1. Inactivation of porphobilinogen oxygenase (—) and HRP (- -) as a function of the phenylhydrazine to enzyme ratio. The phenylhydrazine treatments and the activity assays were performed as described in Materials and Methods. Oxygenase activity (▲); peroxidase activity (o).

Figure 2. Inactivation of porphobilinogen oxygenase (—) and HRP (- -) as a function of time. The enzymes were treated for the indicated times with 50 equivalents of phenylhydrazine and 100 equivalents of H_2O_2 and the enzymatic activities were assayed as described in Materials and Methods. Oxygenase activity (▲); peroxidase activity (o). Inset: Effect of a second incubation with phenylhydrazine:H_2O_2 on the peroxidase activities of HRP (- -) and porphobilinogen oxygenase (—) after filtration through Sephadex G-25.

Incubation of porphobilinogen oxygenase with phenylhydrazine and H_2O_2 led to a decrease of about 40% in the absorbance of the Soret band which also shifted from 399 nm to 404 nm (Fig. 3A). The vis maxima at 636 nm and 502 nm of the native enzyme disappeared (Fig. 3A, inset). The fluorescence spectra of the phenylhydrazine-treated enzyme showed a decrease as well as a red shift in its tryptophan emission maximum as compared to the native enzyme (Fig. 3B). When horseradish peroxidase was treated in a similar manner its Soret absorbance decreased but without any shift in its maximum (Fig. 3C). The UV spectrum of the modified horseradish peroxidase showed an increase in the absorbance of the aromatic amino acids at 274 nm (Fig. 3C). An increase as well as a red shift of the tryptophan fluorescence in the treated enzyme was also observed which can be attributed to an exposure of tryptophan residues of a more hydrophilic character (Fig. 3D).

Characterization of the modified heme isolated from the phenylhydrazine treated porphobilinogen oxygenase. The alkylation of porphobilinogen oxygenase by phenylhydrazine in the presence of H_2O_2 gave a modified heme which accounted for almost 70% of the reaction products. No intact heme remained, while minor amounts of other modified hemes were also detected. The field desorption mass spectrum of the modified heme exhibited a molecule ion at m/e 693/694 corresponding to a phenylheme derivative. Its fragmentation pattern had a main peak at m/e 621 (M^+-72) corresponding to the loss of a propionate side chain (β cleavage), and a peak at m/e 499 (M^+-(2 x CH_2CO_2H + C_6H_5)). This fragment can be rationalized as arising from two benzylic-type cleavages of the propionate side chains, as well as from a cleavage of the covalent bond of the

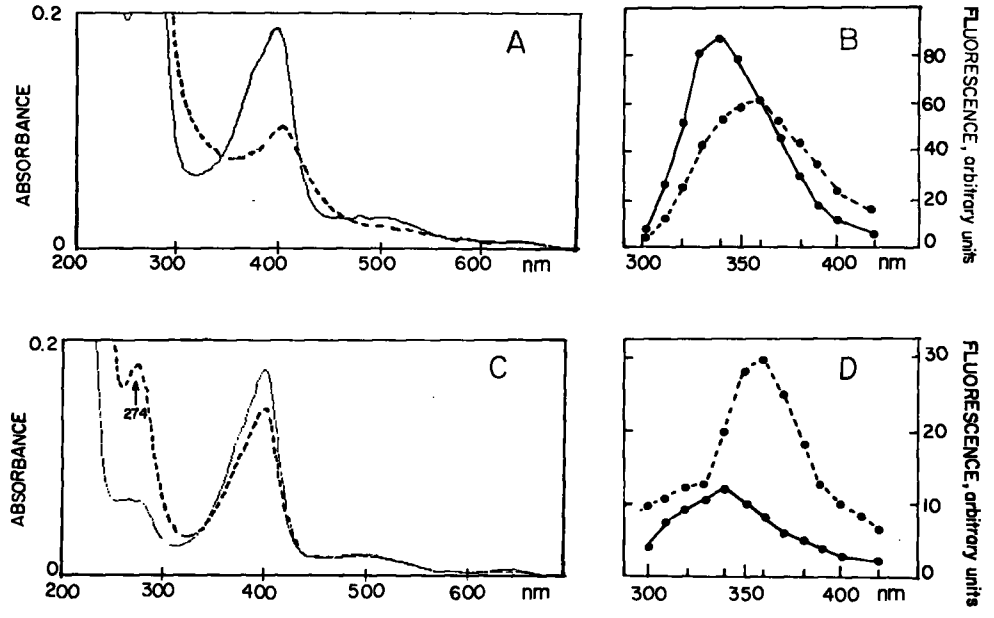

Figure 3. Electronic absorption (A and C) and fluorescence (B and D) spectra of native (——) and phenylhydrazine-treated (- -) porphobilinogen oxygenase (A and B) and HRP (C and D). The spectra of the native and treated enzymes were normalized to the same protein concentrations as measured by Bradford (11). The fluorescence spectra of the HRP was recorded at an intensity of 3x while that of porphobilinogen oxygenase was recorded at an intensity of 10x.

N-phenyl group with the electronegative atom. Such cleavages are known, while meso phenyl groups usually do not fragment (14). This heme derivative could not be detected among the alkylation products of horseradish peroxidase, where part of the heme remained intact, 8-hydroxymethylheme was the main reaction product, and δ–mesophenylheme was the minor product; as was reported by others (8). The alkylated porphobilinogen oxygenase was esterified and demetallated in acidic methanol and the porphyrin ester was converted into its zinc-complex. Its electronic absorption spectrum was identical to that of the zinc-complex of N-phenylprotoporphyrin IX dimethyl ester (Fig 4). The same compound was isolated from the alkylation of myoglobin with phenylhydrazine. The field desorption mass spectrum of the zinc free porphyrin ester gave a m/e 667 (M^+), thus confirming its structure. This product was absent when horseradish peroxidase was alkylated under identical conditions.

Enzymatic activities and fluorescence spectra of the phenylhydrazine treated enzymes after reconstitution with heme IX. To establish if the loss in the enzymatic activities of porphobilinogen oxygenase and horseradish peroxidase which result from the phenylhydrazine treatment should only be attributed to the alkylations of the heme moieties or are also a result of modifications of the apoproteins, the latter were isolated after the reaction with the alkylating agent. They were recombined with hemin IX, and the catalytic activities of the reconstituted enzymes were then assayed (Table I). In the case of porphobilinogen oxygenase, its oxygenase activity was entirely recovered after reconstitution of the native or the phenylhydrazine treated enzyme with hemin IX. The peroxidase activity of the treated enzyme was significantly higher than that of the native

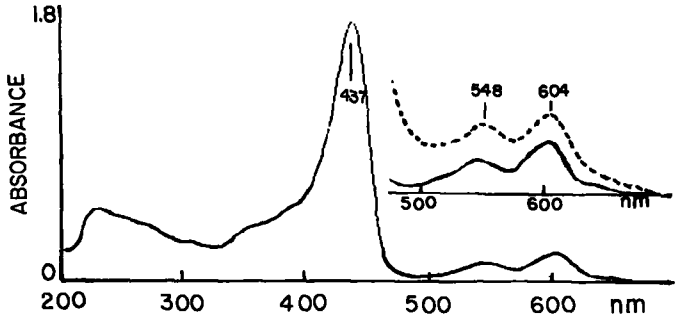

Figure 4. The purified green pigment from phenylhydrazine-treated porphobilinogen oxygenase after its complexation with divalent zinc. The inset shows the superimposed enlarged visible spectra of the purified green pigment (——) and the zinc chelate of dimethyl protoporphyrin IX isolated from treated myoglobin (- -).

reconstituted enzyme (Table I). Hence, the phenylhydrazine treatment did not affect the protein moiety of the enzyme. The spectrum of the reconstituted enzyme after alkylation still showed the 20 nm bathochromic shift found for the alkylated enzyme, although its fluorescence emission spectrum was not identical to that of the treated enzyme (Fig 3). Reconstitution of the apoenzyme of the treated horseradish peroxidase with hemin IX resulted in an enzyme which had recovered 75% of its peroxidase activity (Table I). Its fluorescence emission spectrum was essentially the same as that shown by the native enzyme (Fig. 3D).

DISCUSSION

Although porphobilinogen oxygenase and horseradish peroxidase have almost identical electronic and magnetic spectra (1) and although they show similar catalytic activities, the alkylation of their heme prosthetic groups with phenyl radicals released from phenylhydrazine show how differently the heme moieties are shielded by the protein pocket. The N-alkylation of heme in

Table I

Catalytic activities of the native and phenylhydrazine treated porphobilinogen oxygenase and HRP reconstituted with hemin IX

Enzyme		% Activity	
		Oxygenase	Peroxidase
PBG-O	Native	100	100
	Treated	100	180
HRP	Native	100	100
	Treated	100	75

Porphobilinogen oxygenase (PBG-O) and horseradish peroxidase (HRP), either native or treated with phenylhydrazine: H_2O_2 at a 1:50:100 ratio of enzymes to reagents, were reconstituted with hemin IX. Activities were assayed as described in Materials and Methods.

porphobilinogen oxygenase indicates that the phenyl radicals have free access to the central iron atom and can form the iron-phenyl intermediate which is the first step toward N-alkylation (7). The formation of this intermediate should therefore be responsible for the loss of the oxygenase and peroxidase activities of the enzyme. The recovery of both activities after reconstitution of the treated enzyme with hemin IX (Table I) suggests that the apoprotein is not inactivated by the phenyl radicals.

A different picture emerges from the alkylation of horseradish peroxidase. In this enzyme the access to the central iron is shielded by the protein (8) and the entry channel of the phenyl residues is only open at the δ–meso edge of the heme. We have confirmed that alkylation at this position leads to the loss of the peroxidase activity. Its oxygenase activity remains however unaffected (Figs. 1 and 2). These results suggest that the oxygenase activity of horseradish peroxidase is linked to the central iron atom. The peroxidase and oxygenase activities of this enzyme are therefore related to different regions of the heme moiety; the peroxidase activity requires of an unmodified central iron and of the δ–edge of the tetrapyrrole ring, while its oxygenase activity requires only the former. The protein moiety of the enzyme is apparently not affected by the phenyl radicals, but its interaction with the modified hemes seems to be altered (Figs. 3C and 3D).

ACKNOWLEDGMENTS: This work was made possible by Grant GM-11973 from the National Institutes of Health (PHS). Support from CONICET (Argentina) is also acknowledged.

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