Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 1-6

EFFECT OF HEME-APOPROTEIN INTERACTIONS ON THE ACTIVITY OF HORSERADISH AND WHEAT GERM PEROXIDASES

Marcelo Fernández, Irene Rezzano and Adriana Robinsohn

Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires,
Junin 956, Buenos Aires. Argentina

Received August 12, 1994

SUMMARY: The apoenzymes of horseradish and wheat germ peroxidases were reconstituted with synthetic hemins that differ from natural heme in the substitution pattern of side chains. Both enzymes show dual peroxidase and oxygenase activity, being the latter the oxidation of porphobilinogen in the presence of oxygen and a reducing agent. The oxygenase activity was almost unaffected in both enzymes reconstituted with synthetic hemes, while peroxidase activities were inhibited to different extents. According to the pattern of activity inhibition it was concluded that there is low flexibility of both apoproteins in the regions of the acid side chain contact which could be a general feature of peroxidases.

Horseradish peroxidase (HRP) is a hemoprotein that catalyzes the H_2O_2-dependent oxidation of a wide variety of substrates through a mechanism that involves one electron abstraction (1,2). It has been proposed that heme reactivity of HRP is controlled by protein structure, which supresses oxygen transfer activity of the ferryl species generated by H_2O_2, and promotes substrate interaction with the heme edge (3). It is generally accepted that the peroxide cleavage that generates the ferryl species is catalyzed by specific aminoacids at the active site (4). It is clear that heme-apoprotein interactions are essential to HRP activity.

We have described a porphobilinogen oxygenase in wheat germ that catalyzes the oxidation of pyrrols in the presence of oxygen and a reducing agent (5-8). This oxygenase turned out to be a hemoprotein of the peroxidase type and subsequently its oxygenase activity was also found in HRP (6-8). When acting as oxygenases, HRP and the wheat germ peroxidase (WGP)*, proceed through an intermediate which involves a radical located on the protein (7). Although the wheat germ enzyme is very similar to HRP, the topology of their active sites is different. In the former there is free access of the substrates to the heme iron, while it is restricted in HRP (8). Although there is no conclusive evidence yet as to the in vivo function of peroxidases acting as oxygenases and viceversa, the mechanism of pyrrols oxidation in vitro was useful to study the problem of the dual activities in these enzymes (7,8). To investigate the influence of the heme-protein interaction

* This enzyme was originally termed porphobilinogen oxygenase (5). Since we found that it is a peroxidase, we have chosen to term it wheat germ peroxidase.

0006-291X/94 $5.00

on the dual peroxidase and oxygenase activities of both enzymes, we have reconstituted the apoproteins with synthetic hemins which are isoelectronic isomers of natural heme IX **1** (Fig.1) but differ from it in the substitution pattern of the side chains.

MATERIALS AND METHODS

Wheat peroxidase was purified from wheat germ as the major cationic isozyme as described elsewhere (7). HRP (type VI), pyrogallol and hidrogen peroxide were obtained by synthesis (9).

The synthetic hemes were prepared by inserting iron into the corresponding porphyrins which were prepared following general procedures described elsewhere (10, 11).

Analytical methods

The concetrations of wheat peroxidase were determined using an ε399: 100,000 M^{-1} cm^{-1}; for HRP an ε402: 95,000 M^{-1} cm^{-1} was used (8). Heme concentrations were measured by the pyridine-hemochrome method (12).

Compound I of the reconstituted enzymes was obtained by adding one equivalent of H_2O_2 to each enzyme; Compound II was obtained by adding one equivalent of ferrocyanide to Compound I solutions.

Optical absortion spectra were measured with a double-beam Hitachi U-2000 Spectrophotometer.

Reconstitution of enzymes with synthetic hemes

Apoproteins were prepared by the acid/butanone method of Teale (13) and dialyzed against 20 mM potassium phosphate buffer pH 7,4. Hemes were recombined with the apoproteins at a molar ratio of 2:1 at pH 7,4. After 3 hs. at 0°, the reconstituted hemoproteins were purified from any unbound heme using a DEAE-Trisacryl column equilibrated with 20 mM potassium phosphate buffer pH 7,4. Hemoproteins were eluted with this buffer while unbound hemes were retained.

Enzyme assays

Peroxidase activities were determined by measuring the increase in absorbance at 470 nm due to the formation of purpurogallin, using pyrogallol 5 mM as substrate, 0,6 mM H_2O_2, 50 mM phosphate buffer pH 7,4, and enzyme (1nM HRP or 10 nM wheat peroxidase) in a final volume of 1 ml.

Oxygenase activities were assayed as described elsewhere (7) using 18 nmol of porphobilinogen as substrate, 30 nmol of sodium dithionite, 10 mmol of phosphate buffer pH 7,4 and either HRP or wheat peroxidase (1 μM) in a final volume of 100 μl. After 10 min. at 37°, remaining porphobilinogen was determined with Ehrlich's reagent.

RESULTS AND DISCUSSION

The hemins shown in Fig. 1 were used in the reconstitution studies. Reconstitution with the natural heme IX **1** was always performed as a control. Hemins **2** to **7** are isomers of heme IX where the sequence of acidic side chains has been changed; the position of methyl and vinyl groups also changed.

Both apoenzymes recombined with all the hemins and the synthetic enzymes exhibited electronic absortion spectra which were very similar to the native ones, although in the case of apo-HRP reconstituted with **5** and **6** the vis maxima were not well defined and the Soret band showed an inflexion (Fig. 2). The compulsory intermediates, compounds I and II, of the peroxidase catalytic cycle could not be detected when apo-HRP was recombined with **5** and **6**, but they were detected when apo-HRP was reconstituted with **3** and **4** (Fig. 3). In the case of WGP, compounds I and II were not detected with the native enzyme or with any of the synthetic enzymes.

The effect of the heme-apoprotein interactions on the peroxidase and oxygenase activities of both HRP and WGP was then analyzed. As can be seen in Table I their oxygenase activities were

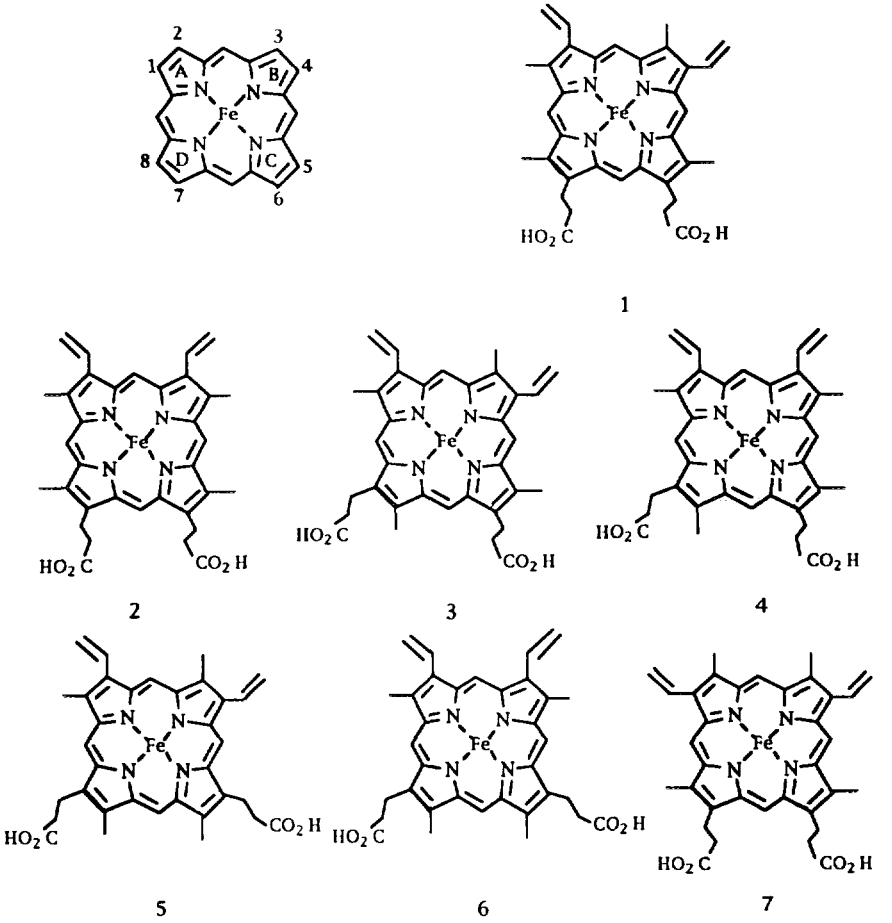

Figure 1. Hemins used in reconstitution studies.

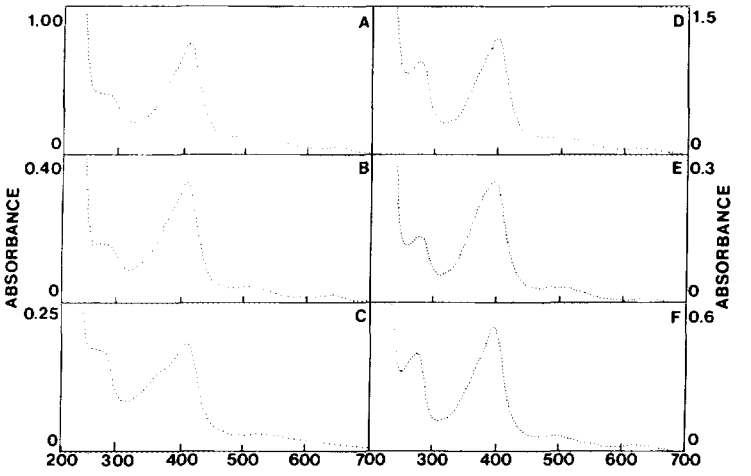

Figure 2. Optical absorption spectra of HRP reconstituted with A: heme **1**; B: heme **3**; C: heme **5** and of WGP reconstituted with D: heme **1**; E: heme **3**; F: heme **5**.

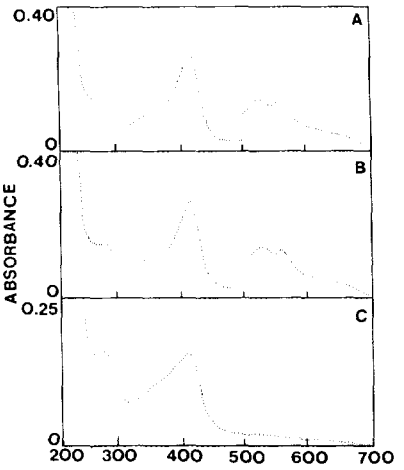

Figure 3. Spectra of compound II of HRP reconstituted with A: heme **1**; B: heme **3** and C: the resulting spectrum of treating HRP reconstituted with heme **5** with equimolecular amounts of H_2O_2 and ferrocyanide.

almost unaffected with the different heme prosthetic groups. On the other hand, their peroxidase activities were inhibited to different extents (Table II). When the acidic side chain was placed at C-8 instead of C-7, the peroxidase activities of both enzymes were reduced by more than 50 % (compare **1** with **3**). The inhibition due to the shift of the propionate to C-8 could be caused either by a steric hindrance imposed by the protein, resulting in an altered interaction between the heme and the aminoacids at the active site involved in the enzymatic activity, or by a perturbation of the

Table I: Oxygenase activities of HRP and wheat germ peroxidase (WGP) reconstituted with synthetic hemins

Hemins	Reconstituted enzyme (% of oxygenase activity)	
	HRP	WGP
1	100	100
2	91	87
3	81	88
4	96	100
5	88	98
6	69	95
7	100	94

The data represent the average of two independent determinations with different enzyme preparations. Consumption of 500 nmol/15 min. mg protein by the enzymes reconstituted with heme **1** was considered as 100 % of oxygenase activity.

Table II: Peroxidase activities of HRP and wheat germ peroxidase (WGP) reconstituted with synthetic hemins

Hemins	Reconstituted enzyme (% of peroxidase activity)	
	HRP	WGP
1	100	100
2	48	62
3	23	39
4	44	64
5	6	19
6	<1	5
7	89	92

The data represent the average of two independent determinations with different enzyme preparations. Formation of 9.8 x 10^4 and 2.3 x 10^3 nmol of purpurogallin/min . nmol of heme was considered as 100% of peroxidase activity of HRP and wheat germ peroxidase, reconstituted with heme 1, respectively.

substrate binding site (3,14). When the pattern of substituents of ring B was inverted with respect to **1** (as in **4**), no inhibition was observed as a consequence of the change in the propionate position (compare **2** with **4**, Table II). In the case of **4** heme could recombine rotated 180º (heme disorder (15)) since it is symmetrical with respect to side chains of rings A and B. The lack of inhibition, contradictory with what was expected for a propionate in C-8, could be then explained considering that heme recombines placing the propionate in the position corresponding to C-5, which could be a heme-protein contact less critical for activity. When propionates occupy positions C-5 and C-8 as in **5** and **6**, the inhibition of the peroxidase activities is almost complete. This points to the lack of flexibility of the active site of both enzymes in the region of rings C and D with respect to peroxidase activity. Swapping the vinyl and methyl residues of ring A does not affect peroxidase activity, which is nevertheless affected when the substituents of ring B are moved around (compare **1** with **2** and **7**) it has been shown that there is a steric clamping of the vinyls in HRP that places them in a near coplanar position with heme (16). This position would extend the conjugation of the heme π-electrons stabilizing the porphyrin cation radical in compound I. Our results suggest that at C-1 the vinyl could still be clamped, but that it may lose its in-plane location when at C-3, making the catalytic intermediate less stable. This falls in line with the finding that a 2-vinyl 4-deuteroheme adopts a 180° rotated orientation when reconstuted with apo-HRP, placing the vinyl at C-3 while 4-vinyl 2-deuteroheme recombines in the native position (16).

These recombination results suggest that the low flexibility of apoprotein in the region of the acid side chains-protein contacts is a general feature of the active site of peroxidases. The array of the methyl and vinyl residues at rings A and B is less critical but the highest activity is obtained

with the array present in heme IX. The C-3 methyl apoprotein contact seems to be less restricted than those of C-1, C-2 and C-4.

The inhibition of peroxidase activity was greater in HRP than in weat germ peroxidase, probably as a consequence of the different topology of their active sites (8), which could also account for their different specific activities, since wheat germ peroxidase shows only a 10% of HRP specific activity (7). So, rather than direct heme-apoprotein contacts, the difference between these two peroxidases would be in the protein machinery that promotes peroxide cleavage.

The oxygenase activity of the hemoproteins seems to be independent of the heme-apoprotein interaction, and probably depends more on the formation of a radical located at the protein (7).

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