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Oxidation of Porphyrinogens by Horseradish Peroxidase and Formation of a Green Pyrrole Pigment

J. M. Jacobs,[1,3] N. J. Jacobs,[1,*] C. B. Kuhn,[1] N. Gorman,[2,3] F. E. Dayan,[4] S. O. Duke,[4] J. F. Sinclair,[2,3] and P. R. Sinclair[2,3]

Departments of [1]Microbiology, [2]Biochemistry and Pharmacology/Toxicology, Dartmouth Medical School, Hanover, NH 03755; [3]VA Medical Center, White River Junction, VT 05009; and [4]United States Department of Agriculture, A.R.S., Southern Weed Science Laboratory, Stoneville, MS 38776

When humans or plants are exposed to certain chemicals which interfere with heme biosynthetic enzymes, porphyrinogen intermediates accumulate and are oxidized to cytotoxic porphyrins. Here we have investigated the role of peroxidases in porphyrinogen oxidation. Horseradish peroxidase (HRP) rapidly oxidizes uroporphyrinogen to uroporphyrin and this is inhibited by ascorbic acid. HRP also oxidizes deuteroporphyrinogen (a synthetic porphyrin similar to protoporphyrinogen), but the yield of porphyrin is lower than with uroporphyrinogen as substrate. This low yield is in part due to a rapid, HRP-dependent conversion of deuteroporphyrin (but not uroporphyrin) to a green compound with spectral characteristics of a chlorin with a large peak at 638 nm. This reaction requires addition of a sulfhydryl reductant such as glutathione and is inhibited by ascorbic acid. These findings suggest that cellular peroxidases and ascorbic acid levels may play a role in modifying the phototoxic tetrapyrroles which accumulate in plants and humans after certain environmental exposures.

Exposure of humans or plants to certain chemicals which interfere with heme biosynthetic enzymes can result in accumulation of excess porphyrin intermediates (see references in 1, 2). For instance, treatment of plants with photobleaching herbicides which inhibit protoporphyrinogen oxidase can result in transient protoporphyrinogen accumulation with subsequent oxidation to the phototoxic protoporphyrin (1). In mammals, some chemically-induced uroporphyrias result from oxidation of uroporphyrinogen and inhibition of uroporphyrinogen decarboxylase leading to accumulation of large amounts of the phototoxic porphyrin, uroporphyrin (2). We are investigating the mechanisms by which these excess porphyrinogens are oxidized, either by chemical oxidation or by oxidative enzymes. We examined a possible role for peroxidases in this reaction based on an earlier report that some porphyrins were formed when certain porphyrinogens, including protoporphyrinogen and uroporphyrinogen, were incubated with HRP (3). In the present study, we report that HRP oxidizes uroporphyrinogen to uroporphyrin, but has a more complex effect on deuteroporphyrinogen oxidation. We chose deuteroporphyrinogen for use in this study since deuteroporphyrin is a dicarboxylic acid porphyrin similar to the naturally occurring protoporphyrin, and has often been used as a synthetic enzymatic substrate instead of protoporphyrin, because of its greater stability.

MATERIALS AND METHODS

The initial rate of uroporphyrinogen oxidation was followed by direct fluorometric measurement for 3-4 minutes as described previously (4). The reaction mixture (0.25 ml) contained 0.25M sucrose, 50 mM HEPES pH 7.4, 1 mM EDTA, 5 μM uroporphyrinogen, 0.02 mg HRP/ml (type VI Sigma Chemical Company St. Louis, MO) and where indicated, 0.1 mM hydrogen peroxide, 50 μM deferoxamine mesylate (95% pure), or 0.03 mg catalase /ml from

* To whom correspondence should be addressed: Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755-3842.

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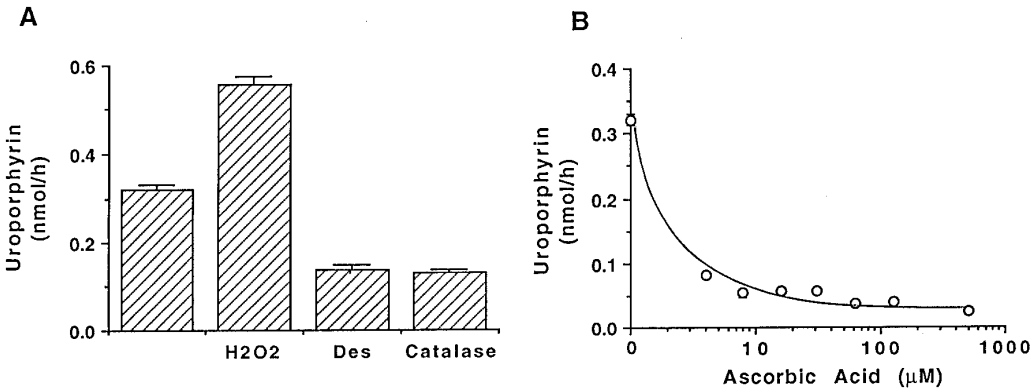

FIG. 1. (A) Uroporphyrinogen oxidation catalyzed by horseradish peroxidase. Uroporphyrinogen oxidation was determined under conditions described in Materials and Methods, with additions as indicated. (Des= deferoxamine). Uroporphyrin formation, expressed as nmoles/ml/h was assayed in duplicate with the means and ranges indicated. (B) The effect of ascorbic acid on HRP-mediated uroporphyrinogen oxidation.

bovine liver (thymol free, Sigma). Uroporphyrin formation, expressed as nmoles/ml/h was assayed in duplicate with means and ranges as indicated.

A second assay, described previously (5), was utilized to compare the initial kinetics of deuteroporphyrinogen and uroporphyrinogen oxidation by HRP. The reaction mixture (5ml total volume) was incubated at room temperature and contained 0.1M TRIS buffer pH 7.5, 1 mM EDTA, 0.02 mg HRP/ml (type II Sigma), and 39 μM deuteroporphyrinogen, or 18 μM uroporphyrinogen, with reduced glutathione (5 mM) added to minimize porphyrinogen autooxidation. Fluorometric excitation was at 395 nm, and emission was measured at 620 nm (deuteroporphyrin) or 615 nm (uroporphyrin). Total recovery of both porphyrinogens and porphyrins was quantitated as described (6).

To follow the conversion of deuteroporphyrin to the green pigment (see Results), we periodically scanned the reaction mixture, which was as described above, except that deuteroporphyrin was substituted for deuteroporphyrinogen (Shimadzu UV-2101 PC Spectrophotometer). The initial kinetics of green pigment formation were also followed directly in this reaction mixture by monitoring the linear period of increase in absorbancy at 638 nm following the addition of deuteroporphyrin as substrate.

RESULTS

We found that uroporphyrinogen was rapidly oxidized to uroporphyrin by catalytic quantities of HRP (60 nmole uroporphyrin formed/min/mg protein) (Fig. 1A). Addition of H_2O_2 was not required but was stimulatory. Catalase caused 50% inhibition, as did the ferric iron chelator, deferoxamine (Fig. 1A). These effects suggest that although peroxide addition was not required for uroporphyrinogen oxidation by HRP, reactive oxygen species and iron catalyzed reactions may be involved. There was a slow rate of uroporphyrinogen autooxidation in the absence of HRP but this was always less than 5% of the enzymatic rates even in the presence of added H_2O_2. Although peroxidase activity did not require added H_2O_2, it seems likely that this slow rate of uroporphyrinogen auto-oxidation could generate sufficient peroxides to initiate peroxidase action (7).

Ascorbic acid caused marked inhibition of this HRP-mediated uroporphyrinogen oxidation at the lowest dose tested (4 μM) (Fig. 1B). The mechanism of inhibition by ascorbate is unknown but may involve a reaction between ascorbic acid and HRP (7). Ascorbate may reduce the ferryl heme in HRP or reduce some reactive oxygen species produced by HRP.

In preliminary experiments, we compared the oxidation of deuteroporphyrinogen to that of uroporphyrinogen. The effect of HRP on deuteroporphyrinogen appears to be complex. However, we did find that deuteroporphyrinogen was oxidized, with HRP-dependent porphyrin formation in the initial 30 min time period occurring at a rate (5.1 nmoles/ml/hr) similar to that obtained for uroporphyrinogen oxidation (1.8 nmoles/ml/hr). This experiment utilized the

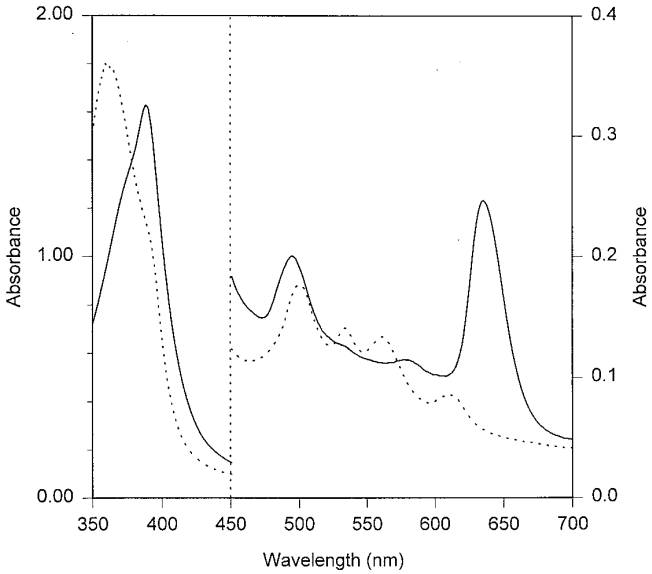

FIG. 2. Absorption spectra of 20μM deuteroporphyrin (dotted line) and the green pigment (solid line) formed after 120 minutes in the presence of HRP and glutathione under conditions as described in Materials and Methods.

second assay for following porphyrin formation described in Materials and Methods, which allowed for measurement of initial kinetics as well as total porphyrin recovery. The initial rates of porphyrinogen oxidation were higher under these assay conditions than in the assay shown in figure 1, probably due to the higher substrate concentrations used (see Materials and Methods). Glutathione (5 mM) was included in this reaction mixture to minimize porphyrinogen auto-oxidation. The initial rate of deuteroporphyrinogen auto-oxidation was less than 5% of the initial rate of enzymatic oxidation.

Although HRP oxidized deuteroporphyrinogen to deuteroporphyrin in the initial time period, the porphyrin recovery in the later time intervals was much lower than during HRP-mediated oxidation of uroporphyrinogen. For instance, after 60 min incubation, the concentration of deuteroporphyrin formed from deuteroporphyrinogen was 4.0 μM, but had decreased to 2.4 μM after 180 min. During this same period, the concentration of uroporphyrin formed from uroporphyrinogen increased from 4.0 μM to 8.7 μM. Apparently, HRP oxidizes some deutero-porphyrinogen to deuteroporphyrin, but also converts large amounts to other products which remain to be identified. This breakdown of deuteroporphyrinogen is HRP dependent. The yield of deuteroporphyrin in control tubes allowed to slowly auto-oxidize in air for 24 h in the absence of HRP was much greater (80%) than was the yield after this period of incubation in the presence of HRP (less than 10%).

Spectrophotometric examination of the reaction mixture suggested that this poor yield of deuteroporphyrin was at least partially due to an HRP-dependent conversion of some of the deuteroporphyrinogen to a green compound with a spectrum that differs from that of deuteroporphyrin with a prominent and unique absorption peak at 638 nm. Further studies indicated that this unique pigment is probably derived from deuteroporphyrin rather than deuteroporphyrinogen, since incubation of the pink colored deuteroporphyrin with HRP in the presence of glutathione caused the rapid appearance of even larger amounts of this green pigment. After a 2 h incubation at room temperature, the spectrum of deuteroporphyrin was changed to that of the green pigment (Fig. 2). The spectral characteristics of this green pigment are strongly suggestive of a chlorin, a hydroporphyrin (8, 9). Similar spectra have been reported

for oxygenated chlorins formed in low yields during the chemical oxidation of porphyrins or porphyrinogens (10, 11). Studies are underway to identify the chemical structure of this pigment.

The initial kinetics of green pigment formation in the reaction mixture were followed as indicated in Materials and Methods. In the presence of 1 mM glutathione, we observed an absorbancy increase at 638 nm of 8×10^{-4} absorbancy units per min. Further kinetic studies showed that the optimal concentration of glutathione was 1 mM and that the rate with 1 mM dithiothreitol was 10 to 15 percent of this rate. The rate with β mercapto ethanol was even slower. No green compound was formed in the absence of a sulfhydryl reductant. Hydrogen peroxide could not replace this sulfhydryl requirement. The initial rate of HRP and glutathione-dependent conversion of deuteroporphyrin to this unique pigment was completely inhibited by 4 μM ascorbic acid. Of interest, this is the same concentration of ascorbic acid required for maximal inhibition of the HRP catalyzed oxidation of uroporphyrinogen to uroporphyrin (see above). Uroporphyrin was not converted to this green pigment in the presence of HRP and glutathione.

DISCUSSION

Our findings on HRP-induced uroporphyrinogen oxidation may have implications for the accumulation of uroporphyrin in certain of the chemically induced porphyria diseases of humans. In some chemically induced porphyrias, cytochrome P450 isoenzymes play a prominent role in uroporphyrinogen oxidation with subsequent uroporphyrin accumulation (4, 12, 13). However, peroxidase activity may be an additional intracellular mechanism for converting uroporphyrinogen to tissue damaging uroporphyrin in other porphyrias. Our findings also suggest that ascorbic acid could play a role in preventing uroporphyrin accumulation in these porphyrias. A role for dietary ascorbic acid in the prevention of cytochrome P450 mediated-uroporphyria has been demonstrated in ascorbic acid requiring rats (14).

Our observation that HRP converted deuteroporphyrin to a green chlorin raised the question of whether HRP could also chemically modify protoporphyrin, which accumulates when plants are treated with photobleaching herbicides. Deuteroporphyrin is very similar to protoporphyrin, but with H atoms instead of vinyl groups at positions 2 and 4 of the porphyrin ring. If further studies indicate that HRP does modify protoporphyrin, this would have implications for herbicide action. For instance, plant peroxidases may convert accumulated protoporphyrin and protoporphyrinogen to products with altered chemical properties affecting their photoreactivity and cellular location. Cellular ascorbic acid could modify herbicide toxicity by inhibiting this peroxidase action.

Further studies on the effect of HRP on protoporphyrin, protoporphyrinogen, and a variety of other natural and synthetic porphyrins will also have implications for the specificity of the heme containing active site of HRP for porphyrin substrates. To date, our results indicate that although HRP can oxidize the octacarboxylic uroporphyrinogen and the dicarboxylic deuteroporphyrinogen to their respective porphyrins, only the dicarboxylic porphyrin, deuteroporphyrin, can be further metabolized to the unusual chlorin compound by HRP.

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