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# Glutathione-Dependent Oxidative Modification of Protoporphyrin and Other Dicarboxylic Porphyrins by Mammalian and Plant Peroxidases

N. J. Jacobs,*[,1] H. G. Kruszyna,* J. S. L. Hier,* F. E. Dayan,†
S. O. Duke,† F. Pont,‡ and F. P. Montforts‡

**Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755; †USDA/ARS Natural Products Utilization Research Unit, P.O. Box 8048, University, Mississippi 38677; and ‡Institut für Organische Chemie, FB2, Universität Bremen, Leobener Str. NW2, D-28359 Bremen, Germany*

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**Protoporphyrin, an intermediate in heme and chlorophyll biosynthesis, can accumulate in human and plant tissues under certain pathological conditions and is a photosensitizer used in cancer phototherapy. We previously showed that protoporphyrin and the related non-natural dicarboxylic porphyrin deuteroporphyrin are rapidly oxidized by horseradish peroxidase in the presence of some thiols, especially glutathione. This study reports that bovine lactoperoxidase, but not leucocyte myeloperoxidase, can also catalyze this reaction and that Tween and ascorbic acid are inhibitors. Exogenous hydrogen peroxide is not required and cannot replace glutathione. Deuteroporphyrin was oxidized to a unique green chlorin product with two oxygen functions added directly to the characteristic reduced pyrrole ring of the chlorin. Spectroscopic and chromatographic results suggest that protoporphyrin was oxidized not to a green chlorin, but to a much more polar red porphyrin modified by oxidative addition to the two vinyl side chains. Two related nonnatural dicarboxylic porphyrins, with ethyl or hydroxyethyl instead of vinyl side chains, are not substrates or products for this enzymatic conversion.** 

Excess accumulation of the biosynthetic intermediate protoporphyrin can lead to extensive tissue damage upon exposure to light since protoporpyhyrin is a potent photosensitizing agent, giving rise to membrane-destroying oxygen radicals or singlet oxygen. For instance, in the human porphyria disease porphyria variegata, a genetic deficiency in a heme biosynthetic enzyme, protoporphyrinogen oxidase, leads to protoporphyrin accumulation and light-dependent skin photosensitivity (1). In plants, protoporphyrin accumulation and light dependent tissue damage occur upon treatment with certain photobleaching herbicides which inhibit protoporphyrinogen oxidase (2). In one form of photodynamic therapy of cancer, excess protoporphyrin is synthesized by enzymes in tumor tissue following application of excess amounts of the heme precursor 5-aminolevulinate. Laser light or even ordinary light can then be applied to produce a tissue specific photodynamic destruction of malignant tissue (3).

These considerations have increased interest in the fate of accumulated protoporphyrin in animal and plant tissue. We recently found that horseradish peroxidase (HRP) in the presence of glutathione (GSH) could oxidize the non-natural porphyrin deuteroporphyrin IX, which is closely related to protoporphyrin IX (4,5). The product was not a red porphyrin, but a unique green chlorin (4,5,6). One of the pyrrole rings had been modified by addition of an hydroxy and an oxo group (6), thus giving the characteristic reduced pyrrole ring of the chlorin as shown in Fig. 1. This reaction did not require exogenous peroxide and rapidly consumed oxygen only if GSH, HRP and porphyrin were all present (5). We suggested that the actual substrate for this oxidation was probably a reduced porphyrin radical formed in the reaction rather than the porphyrin itself (5). More recently, we reported that protoporphyrin was also rapidly metabolized by HRP and GSH or other thiols (7). In this previous study, we reported that although the enzymatic parameters were similar to the deuteroporphyrin conversion, the products of protoporphyrin oxidation were not green chlorins (7). In this present study, we further characterize the protoporphyrin oxidation product(s) and the enzymatic reaction, including the ability of other porphyrins to

[1] Corresponding author. Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755-3842. Fax: 603-650-1318. E-mail: nicholas.j.jacobs@dartmouth.edu.



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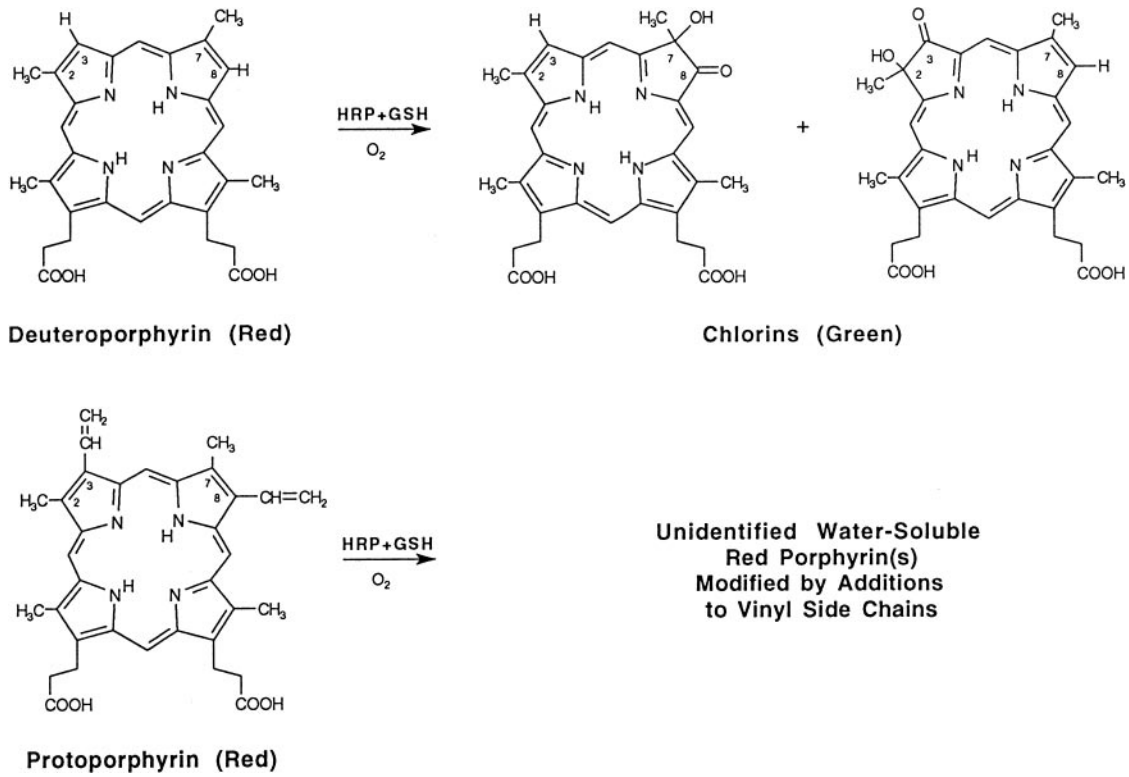


**FIG. 1.** Oxidation of deuteroporphyrin to green chlorins and of protoporphyrin to unidentified porphyrins by Horseradish Peroxidase (HRP) in the presence of glutathione (GSH).

serve as substrates. Of most importance for human medicine, we now report that peroxidative enzymes present in mammalian cells can also carry out these GSH-dependent oxidative conversions of protoporphyrin and deuteroporphyrin.

## MATERIALS AND METHODS

The conversion of deuteroporphyrin to chlorin or protoporphyrin to water-soluble products was followed by directly scanning the spectrum (4,5) of the reaction mixture in cuvettes or by following the initial rate of absorbance increase at 635 nm for deuteroporphyrin oxidation (5) or 395 nm for protoporphyrin oxidation. (Protoporphyrin's conversion to a water soluble porphyrin causes a 395 nm peak to be visualized in aqueous solution as mentioned in Results.) The standard reaction mixture contained Tris buffer, pH 7.5 (50 mM), EDTA (1 mM), GSH (5 mM), and either deuteroporphyrin, protoporphyrin, mesoporphyrin, or hematoporphyrin (40 $\mu$M), and either HRP, lactoperoxidase (LPO) or myeloperoxidase (MPO) as indicated. The level of peroxidase enzymes was expressed as activity levels (peroxidase units) using either purpurogallin (for HRP and LPO) or guaicol (for MPO) as substrate.

The protoporphyrin conversion to water soluble products was also followed qualitatively by reversed phase TLC (RPTLC). Approximately 10 $\mu$l of each reaction mixture was spotted onto the plate developed with 60/40 MeOH/0.1 M ammonium acetate pH 4.5 for 20-60 minutes unless indicated otherwise. The separated fluorescent spots were viewed using a long wavelength UV lamp. (Protoporphyrin remains at the origin and the fluorescent product(s) of protoporphyrin metabolism move near the solvent front in this system.)

To obtain the spectral characteristics of chromatographically separated protoporphyrin derivatives, protoporphyrin was allowed to react with HRP and GSH for three hours. Four 60 $\mu$l volumes were streaked at the origin of two 5 cm $\times$ 20 cm RPTLC plates. The plates were run in 60/40 MeOH/$H_2O$ for 30 min. While the gels were still damp the fluorescent products near the solvent front were scraped off with the gel. The gel was rinsed with portions of 50/50 MeOH/$H_2O$ and centrifuged at 9000 g for 3 min. The supernatant was collected. The same procedure was repeated with protoporphyrin alone in buffer, and this supernatant was collected. Both supernatants were made up to a total volume of 950 $\mu$l and divided in half. To one half, an equal volume of 10% Tween 20 was added to obtain the neutral spectrum of protoporphyrin in the presence of detergent. An equal volume of HCl (2.5 N) was added to the other half to obtain the acidic spectrum of protoporphyrin. (Protoporphyrin itself is not soluble unless dissolved in detergent or acid.) The spectra of protoporphyrin were multiplied approximately 3.6 fold for comparative purposes. The reaction products were also purified on $C_{18}$ RP chromatography columns. The reaction mixture (50 ml) was adjusted to pH 4.5 with acetic acid, 11 ml of methanol was added and applied to a silica-based $C_{18}$ column (2 $\times$ 18 cm) equilabrated with 0.1 M ammonium acetate pH 4.5 containing 20% methanol. All fluorescent porphyrins were absorbed and the column washed with several column volumes of the equilabrating solvent. The fluorescent reaction products were eluted with 55% methanol:45% ammonium acetate with the unconverted protoporphyrin remaining at the top of the column. (The reaction products were not eluted with 40% methanol.) The eluted reaction product was concentrated by dilution with $H_2O$ to 20% methanol and absorbance to a small SPE Whatman $C_{18}$ column (VWR Scientific) and subsequent elution with a small volume of methanol.

*Materials.* HRP type II (activity 150-200 units/mg protein); LPO-from bovine milk (activity 80-150 units/mg protein); MPO (activity

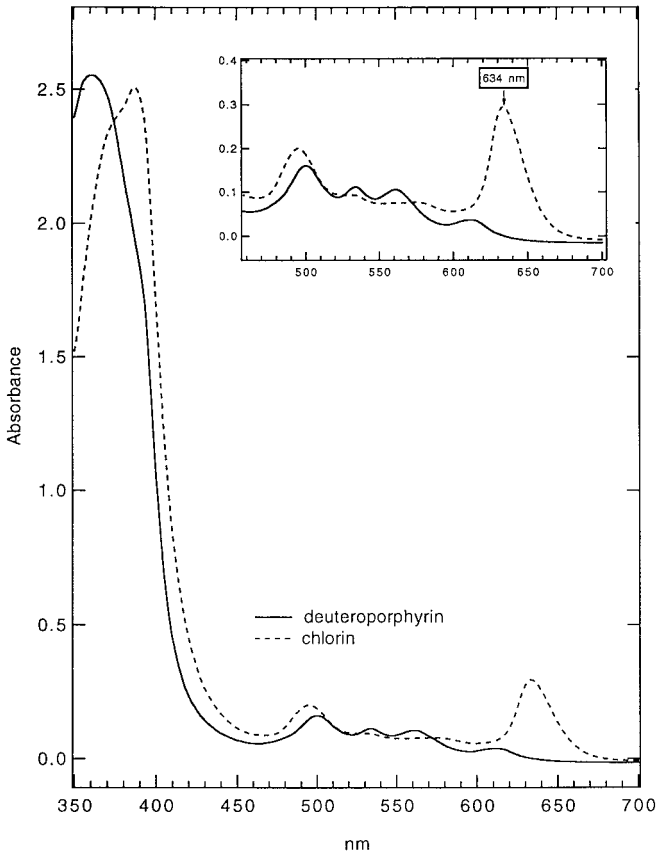


**FIG. 2.** Lactoperoxidase (LPO) catalyzed conversion of deuteroporphyrin to chlorin in the presence of GSH. Deuteroporphyrin (38 $\mu$M) was incubated for 2 hours with bovine LPO (30 $\mu$g/ml or 3.7 units/ml) and GSH (5 mM) at pH 7.5 as described in Methods.

1015 units/mg protein) were from Sigma Chemicals Co. (St. Louis, MO). Whatman $KC_{18}H/KC_{18}F$ Reversed Phase TLC plates were from VWR Scientific. Protoporphyrin IX (>97%), deuteroporphyrin IX dihydrochloride (>97%), mesoporphyrin IX dihydrochloride (>97%), were from Porphyrin Products (Logan, UT), and hematoporphyrin IX dihydrochloride (of undetermined purity) was from Sigma Chemical Co. Porphyrins were dissolved and other chemicals obtained as previously described (4). Silica bonded with $C_{18}$ for reversed phase separation (Whatman LRP-2 ODS) was from Whatman, Inc. (Clifton, NJ).

## RESULTS

### *Oxidative Conversion of Deuteroporphyrin and Protoporphyrin by Mammalian and Plant Peroxidases*

After 2 hours incubation in the presence of 5 mM GSH, bovine lactoperoxidase (LPO) (30 $\mu$g/ml or 3.7 peroxidase units/ml) converted deuteroporphyrin to the unique green chlorin with its prominent peak at 634 nm (Fig. 2). This spectral shift is characteristic of the conversion of porphyrins to chlorins in which one of the pyrrole rings becomes reduced. The initial rate of chlorin formation, measured as absorbance increase at 634 nm, was 37% of the rate in the presence of an equivalent level of HRP (5.8 units of HRP or LPO/ml). Leucocyte myeloperoxidase at a higher level (6.7 units/ml) was inactive, even when the reaction was supplemented with 15 mM hydrogen peroxide.

We previously reported that protoporphyrin is also rapidly metabolized by HRP in the presence of GSH (7). In this previous study, protoporphyrin disappearance, as determined by HPLC, was used to follow the reaction. The oxidation products were only characterized by HPLC as more polar compounds with porphyrin-like spectra. We now report that this reaction can also be conveniently followed directly in the reaction mixture by measuring the absorbency increase at the Soret peak (395 nm) of the product formed since protoporphyrin itself is relatively insoluble at neutral pH and does not exhibit its Soret peak until after it is enzymatically converted to its polar metabolites (Fig. 3). By following the rate of increase of this peak, we determined that the rate of protoporphyrin conversion with LPO was lower than the rate with

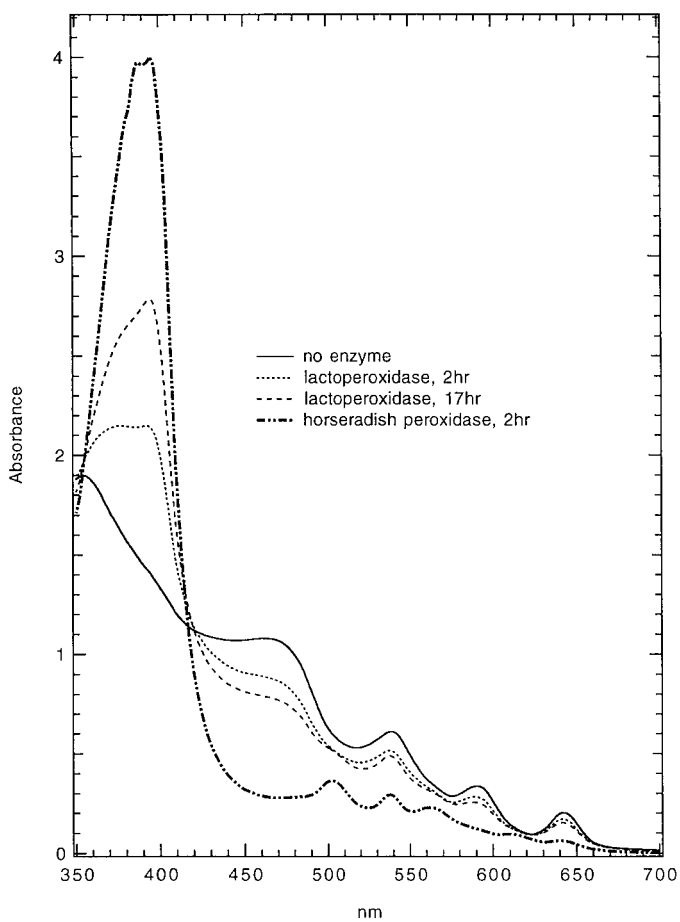


**FIG. 3.** GSH dependent conversion of protoporphyrin to water-soluble porphyrin(s) catalyzed by LPO and HRP. Protoporphyrin (40 $\mu$M) was incubated with GSH (5 mM) and either LPO or HRP (both at 5.8 units/ml) under conditions described in Methods for the time periods indicated.

HRP (range of 14-27% of the HRP rate in two experiments) at equivalent levels of each enzyme (5.8 peroxidase units/ml). Ascorbic acid (10 $\mu$M), when included in the reaction mixture, caused 100% inhibition of the LPO conversion as we previously reported for the HRP conversion (5). Leucocyte myeloperoxidase (3.7 units/ml) when compared to HRP (2.9 units/ml) in the presence of glutathione showed no activity in this conversion. This conversion also did not occur when HRP was reacted with hydrogen peroxide in the abscence of glutathione under exactly the same conditions previously described to peroxidize a wide variety of porphyrins by a glutathione independent but peroxide-dependent reaction (8).

We also found that adding low concentrations (0.1%) of the detergent Tween 20 to the enzymatic reaction mixture completely inhibited the HRP catalyzed conversion. For this experiment, protoporphyrin conversion to the polar product(s) was followed by RPTLC (see below). This inhibitory action of Tween has implications for the reaction mechanism and is also noteworthy since many enzymatic assays for protoporphyrin formation from porphyrinogen precursors include Tween as a solubilizing agent in the reaction mixture (9).

*Spectroscopic and Chromatographic Analysis of the Product Formed from Protoporphyrin*

We previously showed that protoporphyrin was oxidized by HRP and GSH with appearance of a more water soluble product as determined by HPLC (7). However, this previous study did not report the exact spectra of the product(s) other than that they were not chlorins (7). The present study further characterizes these products. On $C_{18}$ RPTLC, developed with 60% methanol and 40% ammonium acetate (see Methods), protoporphyrin itself remained at the origin but the product(s) of the HRP and LPO conversion moved as a predominant red fluorescent band and several lighter bands near the solvent front. This indicates conversion to much more polar porphyrin-like product(s) as we previously showed by HPLC analysis of the same reaction mixture (7).

To further characterize the product(s) of the HRP plus GSH conversion, we eluted the fluorescent band(s) near the solvent front from a heavily loaded TLC plate (see Methods). The eluate was diluted into either Tween detergent or 1.5 N HCI to solubilize protoporphyrin, as explained in Methods. The absorption spectra were recorded as compared to unreacted protoporphyrin. The results (Fig. 4) indicated that the product(s) have a pronounced Soret peak and 4 visible peaks under neutral conditions and a Soret peak and 2 visible peaks in the acidic medium. This is typical of a porphyrin very similar to protoporphyrin except with a characteristic shift of all peaks to a slightly lower wavelength. This indicates that the reaction product is a porphyrin very similar to protoporphyrin but where the 2 vinyl groups at positions 3 and 8 of the porphyrin macrocycle (see Fig. 1) have been saturated. This shift of absorption peaks to lower wavelength is further illustrated in Table 1, where the absorption peaks (in acidic medium) of two other dicarboxylic porphyrins with saturated side chains are compared to protoporphyrin and to the reaction product separated from protoporphyrin on a reversed phase chromatography column (see Methods). Mesoporphyrin and hematoporphyrin are non-natural porphyrins identical to protoporphyrin except that the vinyl groups at positions 3 and 8 are replaced with ethyl or hydroxyethyl groups. To confirm this probable saturation of vinyl groups during the enzymatic oxidation, we also tested these other two dicarboxylic porphyrins as enzymatic substrates. After 4 hour incubation with HRP and glutathione, followed by RPTLC chromatography, neither mesoporphyrin nor hematoporphyrin were modified to more polar products. They remained at the origin of the chromatogram along with protoporphyrin itself, indicating that the conversion to a water soluble porphyin derivative did not occur if the vinyl groups were already saturated.

## DISCUSSION

The observation that mammalian as well as plant peroxidases rapidly catalyse this GSH-dependent reaction extends the significance of this mechanism for protoporphyrin detoxification from the protoporphyrin accumulation which occurs during herbicide treatment of plants (2) to the protoporphyrin accumulation which occurs in human porphyria variegata (1), erythrocytic protoporphyria (10), and in the photodynamic therapy of tumors (3,11). Rapid conversion of accumulated protoporphyrin to more water soluble porphyrins by mammalian peroxidases could markedly affect its tissue distribution, excretion, and even its efficiency as a photodynamic agent.

The structural elucidation of the product(s) formed from this GSH dependent oxidation of protoporphyrin is currently under investigation. HPLC (7) and RPTLC patterns suggest formation of several porphyrins, all of which are much more polar than either protoporphyrin or the more soluble dicarboxylic porphyrin, hematoporphyrin. Our spectroscopic studies on these water soluble products, which have been chromatographically separated from the protoporphyrin substrate, clearly indicate the classical four banded spectrum of a neutral porphyrin. There is no shift to a chlorin-like spectrum in which reduction of one pyrrole group results in a markedly increased band above 600 nm such as occur with other GSH-independent oxidations of protoporphyrin (8) including photoprotoporphyrin formation (12). Pont et al. (6) have shown that when deuteropor-

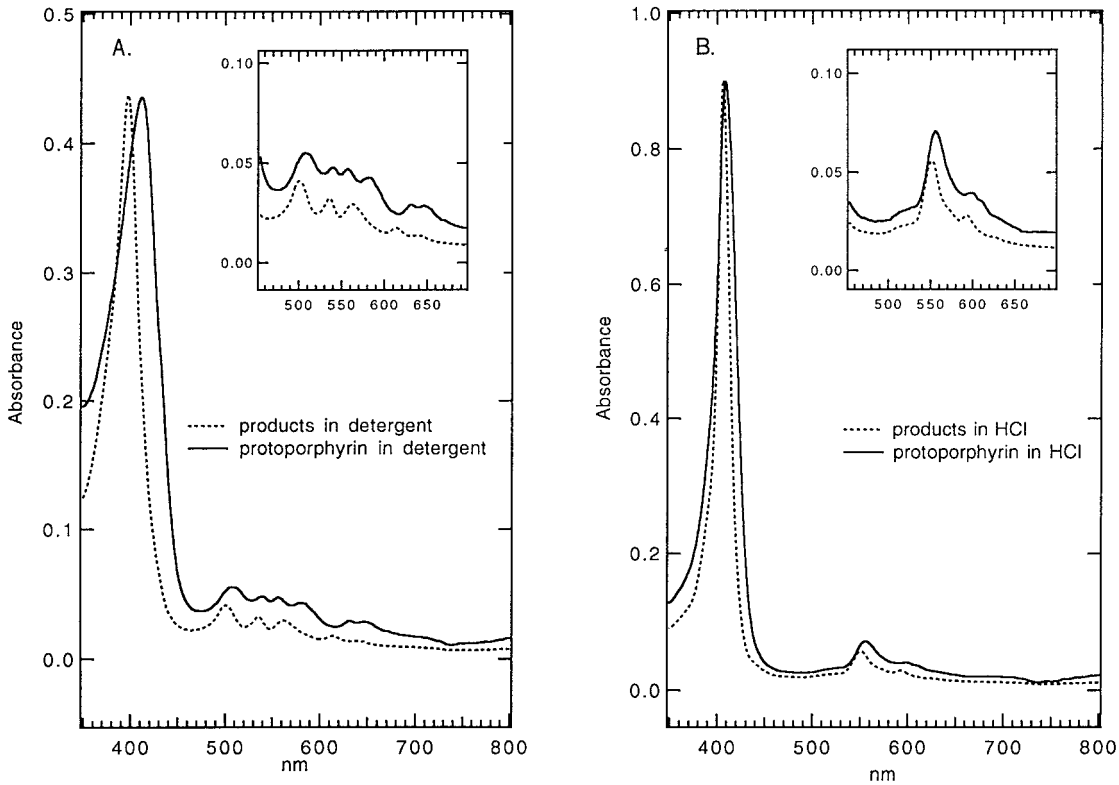


**FIG. 4.** Spectral comparison of protoporphyrin and the chromatographically separated water soluble porphyrin product formed from protoporphyrin by the action of HRP and GSH. Protoporphyrin (40 $\mu$M) was reacted with HRP and GSH for 3 hours, chromatographed on RPTLC, and subsequently eluted as described in Methods, along with a control of unconverted protoporphyrin incubated without HRP. The eluates were diluted into an equal volume of either (A) 10% Tween 20 or (B) 2.5 N HCl to solubilize the unconverted protoporphyrin to compare to the spectra of the soluble product (see Methods).

phyrin is the porphyrin substrate utilized, this same GSH-dependent enzymatic reaction does produce a chlorin which contains two oxygen (one hydroxy and one oxo) functions added directly to the reduced pyrrole ring as shown in Fig. 1. These additions directly onto the unsaturated pyrrole ring occured only in porphyrins with positions 3 and 8 of the macrocycle occupied only with H atoms (6). In the case of protoporphyrin, these two positions are occupied by two highly accessible unsaturated vinyl groups. In analogy to the deuteroporphyrin conversion, we suggest that similar oxygen functions are probably added to these vinyl side chains in the case of protoporphyrin. Three of our observations support this suggestion. First, the spectrum of the product shows a slight shift of all porphyrin peaks to slightly lower wavelengths, as is typical of porphyrins where the vinyl side chains are saturated such as in mesoporphyrin or hematoporphyrin. Second, mesoporphyrin, which has saturated ethyl rather than unsaturated vinyl groups at positions 3 and 8, is not a substrate for this reaction. Third, if two oxygen functions (either two hydroxyls or one hydroxy and one oxo function) were added to each of the two vinyl groups, this would account for the marked increase in water solubility. One possibility is that the new protoporphyrin derivative may be similar to the diglycol derivative of protoporphyrin in which two hydroxyl groups have been added to each of the two vinyl side chains. This porphyrin has been synthesized chemically by osmium tetroxide oxidation of protoporphyrin IX dimethylester

**TABLE 1**

UV-Vis Absorption Maxima for Various Dicarboxylic Porphyrins and the Product of Enzymatic Protoporphyrin Oxidation

| Porphyrin | Soret | ~550 nm | ~590 nm |
|---|---|---|---|
| Protoporphyrin IX | 407 | 554 | 598 |
| Mesoporphyrin IX | 400 | 545 | 589 |
| Hematoporphyrin IX | 402 | 549 | 591 |
| Reaction product | 405 | 551 | 593 |

*Note.* Known porphyrins were diluted in 1 N HCI at 2.5 $\mu$M to obtain the Soret peak and at 25 $\mu$M for the two visible peaks. The chromatographically separated reaction product (which is free from protoporphyrin) was prepared by diluting 10 $\mu$l (for the Soret peak) or 100 $\mu$l (for the 2 visible peaks) of the $C_{18}$ concentrated eluant (see Methods) up to 1 ml in 1 N HCI. All porphyrins exhibited similarly shaped spectra to that shown in Fig. 4B.

(13) and was described as markedly more polar than protoporphyrin itself. However, another possibility is that GSH is also added to the vinyl group through a sulfhydryl linkage during this oxidative conversion.

To gain further insight into these porphyrin conversions by HRP in the presence of GSH, we have attempted the conversion of deuteroporphyrin to an oxygenated chlorin by chemical rather than enymatic peroxidation. We did observe oxygenated chlorin formation from deuteroporphyrin in lower yield following a reaction in which deuteroporphyrin was reacted with $H_2O_2$, imidazol as cocatalyst, and meso-tetrakis-(pentafluorophenyl) porphyrin-iron (III)-chloride as catalyst (14). However, the chlorin formed was not the hydroxy-oxo chlorin obtained from the enzymatic reaction shown in Fig. 1, but a dihydrydroxy chlorin where a hydroxy group replaced the oxo functions at positions 3 and 8. Apparently, the unique feature of the enzymatic chlorin formation is the introduction of the oxo function at the unsubstituted positions 3 and 8 of deuteroporphyrin. One possibility is that the dihydroxy chlorin which is the product of the chemical reaction is a transient intermediate in the enzymatic conversion, and that the secondary alcohol function at positions 3 and 8 of the postulated dihydroxychlorin intermediate is converted to an oxo function perhaps by an enzyme catalyzed peroxidative reaction in the presence of endogenously generated hydrogen peroxide. The inability of other porphyrins, such as mesoporphyrin and hematoporphyrin with substituents at the 3 and 8 positions, to be substrates for this enzymatic conversion (6) suggests that the presence of any substituent at positions 3 and 8 of the porphyrin macrocycle itself would preclude a subsequent peroxidative reaction at these sites to form an oxo function.

This previously unknown ability of mammalian peroxidases, such as LPO, to transform protoporphyrin into much more polar metabolites in the presence of GSH may have a significant impact in human medicine. For example, studying the effect of thiols on *in vivo* porphyrin degradation by peroxidases may lead to new treatments for the abnormal accumulation of protoporphyrin occurring in patients with porphyria variegata (1,15,16) and erythrocytic protoporphyria. As well, high dosage of ascorbic acid or other peroxidase inhibitors could be used to enhance photodynamic therapy treatments by reducing endogenous peroxidase-dependent protoporphyrin degradation.

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