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The Protoporphyrin–Apoperoxidase Complex. Photooxidation Studies†

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ABSTRACT: Protoporphyrin IX, a photosensitizer, was found to combine in 1:1 stoichiometry with apoisozyme C of horseradish peroxidase. The complex at pH 9 and 25° was irradiated with visible light. After photooxidation to an uptake of 2 mol of O_2/mol of apoenzyme, the visible absorption spectrum remained unchanged, indicating that the complex was still intact. The porphyrin-free photooxidized apoenzyme was found to be homogeneous on disc gel electrophoresis at pH 4.5, but was less cationic than the native molecule. Spectrophotometric titrations revealed that the ability of the apoenzyme to recombine with hemin was altered markedly after photooxidation. This effect was accompanied by a lowered enzymatic activity, which reached only 25% of the normal value in the presence of 100-fold molar excess of hemin. Amino acid analysis of the 2-O_2 photoproduct indicated that only one histidyl residue had been lost. Tryptic peptide maps of native and photooxidized apoenzyme were prepared. Of the five Pauly-positive peptides observed on maps of the unphotooxidized protein, one (peptide I) was conspicuous by its absence on maps of the 2-O_2 photoproduct. Peptide I was isolated, subjected to amino acid analysis, and found to be a pentadecapeptide containing one histidyl residue. Our results suggest that the selectively photooxidized histidine in protoporphyrin–apoperoxidase is actually located in the heme binding site of peroxidase itself.

The iron-free derivative of heme, protoporphyrin IX, is a potent photosensitizing agent that acts *via* the formation of singlet molecular oxygen (Dalton *et al.*, 1972). We have been exploring the possibility of using proto[1] as a specific photosensitizer for probing the active sites of hemoproteins in which heme can be replaced by proto. The rationale behind this approach is the following: photooxidizable residues lying close to the porphyrin ring should be identifiable on the basis of their greater susceptibility to attack by excited oxygen than residues far removed from the porphyrin binding site. Experiments to test this idea were carried out on the model complex proto–apomyoglobin by Breslow *et al.* (1967), and more recently by Mauk and Girotti (1973b). One particular residue in the myoglobin sequence, His-93, was found to be unusually photoreactive (Mauk and Girotti, 1973b). This observation is consistent with the fact that His-93 is known to coordinate directly with heme iron in crystalline myoglobin (Kendrew, 1962). The high degree of selective modification observed with proto–apomyoglobin indicated that the technique described might be applicable to other hemoproteins.

We have recently undertaken photooxidation studies on the proto–horseradish apoperoxidase complex with the object of probing the enzyme's active site. Although horseradish peroxidase has been the subject of extensive kinetic studies in the past, until recently relatively little work has been done on the amino acid sequence or the conformation of this heme-containing glycoprotein (Welinder *et al.*, 1972; Welinder and

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‡ This material will be submitted in a thesis to The Medical College of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of this work has appeared (Mauk and Girotti, 1973a).

[1] Abbreviations used are: proto, protoporphyrin IX; apoMb, sperm-whale apomyoglobin; Tos-PheCH$_2$Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; HRP, horseradish peroxidase (isozyme C unless specified otherwise); apoHRP, apoperoxidase; apoHRP(1-O_2), apoHRP(2-O_2), apoperoxidase photooxidized to an uptake of 1 and 2 molar equiv of oxygen, respectively, in the presence of 1 molar equiv of proto; unphotooxidized apoHRP, native apoenzyme that was never in contact with proto.

Smillie, 1972; Shih *et al.*, 1971). Structural work has been impeded somewhat by the fact that HRP exists in the form of at least seven isozymes, which have similar molecular weights, but different amino acid and carbohydrate contents (Shannon *et al.*, 1966). As a result of the limited structural information available on HRP, particularly that pertaining to the heme environment in the active site, the mechanism of action of the enzyme has not yet been elucidated. In the present paper we report that photooxidation of the proto–apoHRP (isozyme C) complex occurs in a highly selective manner, a single histidyl residue being destroyed in the early stages of the reaction. From our results we conclude that the photolabile histidine is situated in the porphyrin binding site of proto–apoHRP and, furthermore, that this residue is associated with the heme group in HRP itself.

Experimental Section

Materials. Crude HRP (type I) was obtained from Sigma Chemical Co. and was purified as described below. Recrystallized hemin and protoporphyrin IX dimethyl ester were purchased from Schwarz-Mann. Proto was prepared from the dimethyl ester by the procedure described previously (Mauk and Girotti, 1973b). Methylene Blue (zinc free) was supplied by Matheson Coleman and Bell and Tos-PheCH_2Cl-trypsin by Worthington Biochemical Corp. All other chemicals were reagent grade and solutions were prepared with glass-redistilled water.

Separation of Isozymes. The chromatographic procedure of Paul (1958), as modified by Shannon *et al.* (1966), was used for separating the HRP isozymes. The different isozymes were identified on the basis of their column elution volumes and disc electrophoretic properties (see below). The reported method (Shannon *et al.*, 1966) was altered slightly to allow purification on a larger scale. In a typical separation 2 g of crude HRP was loaded onto a column of carboxymethyl cellulose (5 × 120 cm) previously equilibrated at 4° with 5 mM sodium acetate buffer (pH 4.4). The A isozymes together with other acidic proteins were eluted first with the equilibrating buffer at a flow rate of 100 ml/hr. A linear gradient, formed with 3.5 l. each of 5 and 100 mM acetate buffer (pH 4.4), was then used to separate isozymes B and C.[2] Fractions were monitored for hemoprotein at 403 nm and for total protein at 280 nm. The RZ ratio ($A_{403}:A_{275}$ at pH 5.8) across each peak was used as a preliminary criterion of purity (Shannon *et al.*, 1966). Fractions under each peroxidase peak were pooled, concentrated at 4° by means of a 50-ml Bio-Fiber device (Bio-Rad Laboratories), and then dialyzed exhaustively against distilled water. The concentrated materials were then lyophilized and stored at −15°. Because of the relatively high yield of isozyme C (approximately 250 mg from a single separation) and its high state of purity (see Results), this component was used exclusively in the studies reported here.

Protein Determinations. Isozyme C concentrations were determined by measuring absorbance at 401 nm. There is a degree of uncertainty about the extinction coefficient of the enzyme at this wavelength; reported values range from 90 to 110 mM^{-1} cm^{-1} (Paul, 1958; Shannon *et al.*, 1966; Tamura *et al.*, 1972). We used a value of 95 mM^{-1} cm^{-1} at pH 5.8 (Shannon *et al.*, 1966). Determinations of isozyme C (as well as its apoenzyme) were also made by the Folin–Lowry method (Lowry *et al.*, 1951). In this case the hemoprotein was used for construction of a standard curve. The small contribution of hematin to the standard curve absorbance readings (Boctor, 1972) was subtracted for determinations of the apoenzyme.

Enzymatic Activity. Peroxidase activity was measured by following at 460 nm the oxidation of *o*-dianisidine in the presence of excess H_2O_2 (Worthington Enzyme Manual, 1972). All assays were carried out at 25° with a Gilford Model 240 spectrophotometer and Model 6040 recorder. One unit of peroxidase activity is that amount of enzyme decomposing 1 μmol of H_2O_2 per min at 25°.

Preparation of Apoenzyme and Recombination Studies. Apoperoxidase was prepared from isozyme C by the 2-butanone extraction procedure of Teale (1959) as modified by Breslow (1964). Stock solutions of apoHRP (10–20 mg/ml in water) were stored at 4° and used within 2 weeks. The ability of apoHRP to recombine with hemin or to combine with proto was tested by spectrophotometric titration at 4°, as described previously for apoMb (Mauk and Girotti, 1973b). Titrations with proto were conducted in the dark.

Photooxidation Procedure. Photooxidation reactions of the proto–apoHRP complex were carried out on a Warburg apparatus essentially as reported earlier for proto–apoMb (Mauk and Girotti, 1973b). A stock solution of proto–apoHRP was prepared in the following manner. ApoHRP was incubated in the dark with an equimolar amount of proto for 48 hr at 4° in 0.12 M borate buffer (pH 9.1). Complex formation was usually complete within this time interval (see Results). Each Warburg vessel was filled with 3.0 ml of the stock solution (20–30 mg of protein). A series of 30-W spot lights positioned below the reaction vessels was used for irradiation, and reactions were monitored by following oxygen consumption at 25°. An uptake of 1 mol of O_2/mol of apoHRP required approximately 10 min with the lighting intensities used.

Methylene Blue sensitized photooxidation of HRP was followed manometrically under conditions similar to those used for the proto complex. The Methylene Blue concentration in each vessel was 0.05 mg/ml.

Polyacrylamide Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out at room temperature in precision-bore glass tubes (Buchler Instruments) according to the procedure of Reisfeld *et al.* (1962). The acrylamide monomer concentration was 7% (w/v). Electrophoresis was performed at 2.5 mA/tube with a β-alanine–acetate buffer (pH 4.5). The gels were stained with Amido Black 10B.

Tryptic Hydrolysis and Peptide Mapping. Tryptic peptides of native and photooxidized apoHRP were prepared as follows: ApoHRP (*ca.* 2 mg/ml in water) was denatured in boiling water for 20 min and allowed to cool. The solution was transferred to a thermostated beaker (38°), and the pH was adjusted to 8.5 with 0.5 M ammonium bicarbonate buffer. Tos-PheCH_2Cl-trypsin (1.25% by weight of the apoHRP) was added, the beaker stoppered, and the solution stirred for 24 hr. The pH was then lowered to 6.4, and the solution was placed in a boiling water bath for 15 min to stop any further proteolytic action. All the tryptic peptides were soluble after this treatment.

Peptide maps were prepared by the procedure reported previously (Mauk and Girotti, 1973b). Peptide solutions were standardized by the Folin–Lowry method (Lowry *et al.*, 1951); samples of 1.7–2.5 mg were used for each map. Maps

[2] The chromatographic pattern of one lot of HRP (121C-9520) indicated the presence of isozymes B and C in approximately equal amounts, whereas the pattern of another lot (72C-9500) showed the complete absence of the B component.

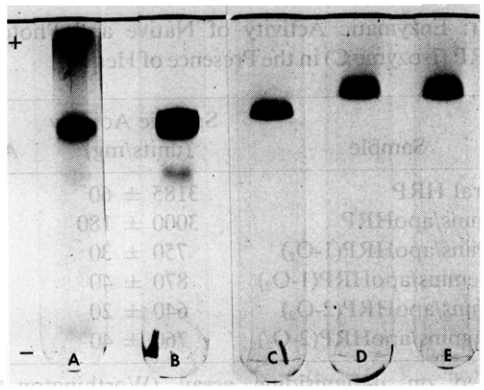

FIGURE 1: Polyacrylamide gel electrophoresis patterns of crude HRP (A) and HRP, isozyme C (B); sample load: 150 μg/gel. Patterns of unphotooxidized apoHRP (C), apoHRP(1-O_2) (D), and apoHRP(2-O_2) (E); sample load: 50 μg/gel. Migration is toward the cathode. The gels are shown aligned at their origins.

were sprayed either with ninhydrin (0.2% in ethanol) or with the Pauly reagent (Fraenkel-Conrat and Singer, 1956). Detection and removal of a particular tryptic peptide from maps of native apoHRP were done as described earlier (Mauk and Girotti, 1973b). In order to reduce background contamination, papers from which a peptide was to be eluted were washed with water and dried before the maps were prepared.

Amino acid analyses were performed according to the procedure of Moore and Stein (1963). Samples of apoHRP before and after photooxidation were hydrolyzed in sealed evacuated tubes with 5.7 N HCl for 24 hr at 110°. The same procedure was followed for an isolated tryptic peptide of peroxidase. The methionine content of photooxidized apoHRP was determined after hydrolysis in 3.75 N NaOH for 16 hr at 110° (Neumann, 1967). Alkaline hydrolysis was necessary for this amino acid because methionine sulfoxide, the product of methionine photooxidation, reverts to methionine during acid hydrolysis (Ray and Koshland, 1960). Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (1946), modified by the use of 6 M guanidine hydrochloride in 0.1 N NaOH as the solvent system instead of 0.1 N NaOH alone.

Results

Isolation of Isozyme C. Isozyme C of HRP was isolated as described in the Experimental Section. The identity and degree of purity of the isozyme were established in different ways. When rechromatographed on carboxymethyl cellulose, a sample of the isolated material emerged in a single peak; the elution volume was unchanged and corresponded closely to that reported for isozyme C by other workers (Shannon *et al.*, 1966). In contrast to the starting material, the purified isozyme was found to be essentially homogeneous on disc gel electrophoresis at pH 4.5 (Figure 1); one major band (electrophoretic mobility 3.8 mm hr^{-1}) is evident. Although crude peroxidase contained several anionic components on disc electrophoresis at pH 8.3 (not shown), no such components were observed in the purified enzyme. The RZ value of our preparation (3.2–3.4), its specific activity (3180 ± 60 units/mg of enzyme), and its amino acid composition were all in good agreement with published data for isozyme C (Paul, 1958; Shannon *et al.*, 1966; Kay *et al.*, 1967).

Interaction of apoHRP with Hemin and with Proto. The binding capacity of apoHRP (isozyme C) for hemin or proto was determined by spectrophotometric titration at pH 9.

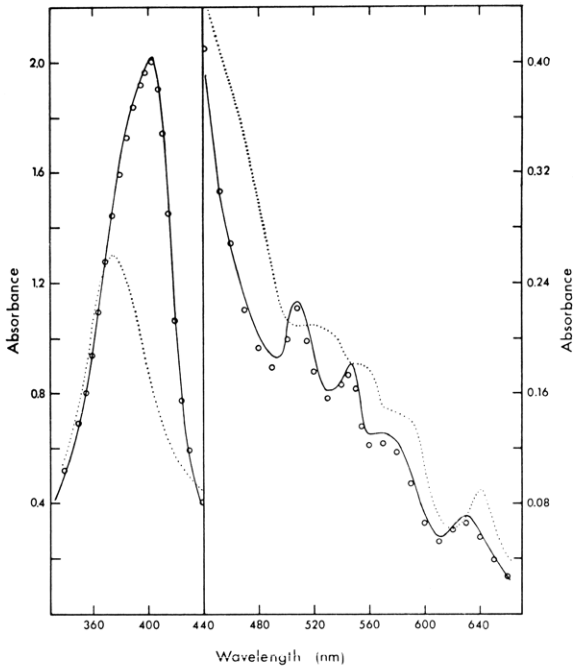

FIGURE 2: Visible spectra of unphotooxidized proto–apoHRP complex, 22.8 μM (—); proto–apoHRP photooxidized to 2-O_2, 22.8 μM (○); and free proto, 22.8 μM (· · · ·). Solutions were buffered at pH 9.2 with 0.32 M sodium borate.

In each case the titration curves (Soret absorbance *vs.* porphyrin equivalents) exhibited a characteristic break at or very close to the expected 1:1 point. The λ_{max} and extinction coefficient of the Soret peak of 1:1 hemin–apoHRP were found to be identical with values obtained with the natural hemoprotein (Shannon *et al.*, 1966). During the titration experiments we noticed that the rate of formation of either the hemin or the proto complex of apoHRP is considerably lower than that of apoMb under the same conditions (Mauk and Girotti, 1973b). A related observation was made by Theorell and Maehly (1950), *i.e.*, that at pH 7 full recombination of apoHRP and hemin requires more than 30 min. We have also noticed that proto combines with apoHRP more slowly than does hemin. It is interesting that Asakura and Yonetani (1969) reported a similar finding with cytochrome *c* peroxidase. On the basis of spectral evidence they concluded that the relatively slow interaction of proto and cytochrome *c* apoperoxidase consists of at least two steps, a fast reaction in which the porphyrin becomes attached to the apoenzyme and a slow reaction involving conformational changes in the protein. It is possible that the binding of proto by horseradish apoperoxidase follows a similar scheme.

The spectral characteristics of the proto–apoHRP complex prior to irradiation are shown in Figure 2. To ensure complete complex formation, equimolar apoHRP and proto were incubated in the dark for 48 hr before the spectrum was recorded. The Soret band occurs at 403 nm (ϵ 90 mM^{-1} cm^{-1}); additional peaks are seen at 508, 547, 570, and 630 nm.

Photooxidation of Proto–ApoHRP. The proto–apoHRP complex was irradiated on a Warburg apparatus (see Experimental Section) and photooxidation was allowed to proceed to the following stages of oxygen consumption: 1 mol of O_2/mol of apoHRP and 2 mol of O_2/mol of apoHRP. A dark control (the complex shielded from the light) was not prepared. That any reaction could have occurred in the dark was ruled out, however, on the basis of previous observations with proto–apoMb (Mauk and Girotti, 1973b). Samples of

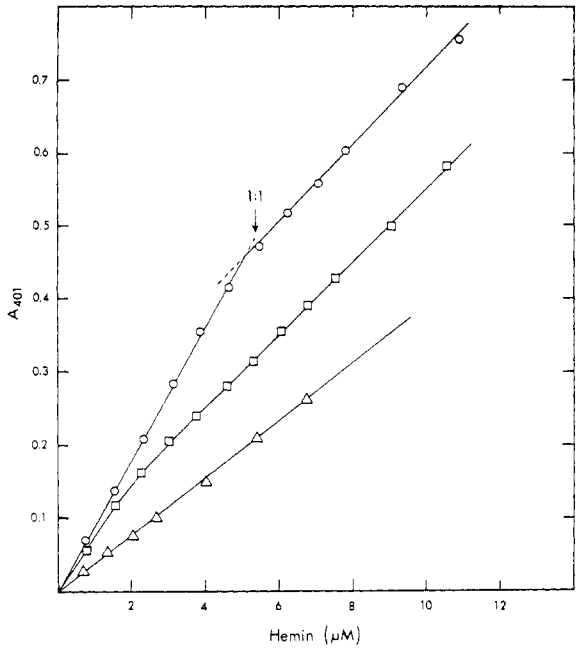

FIGURE 3: Spectrophotometric titration of apoHRP with hemin before and after proto-sensitized photooxidation. The curves represent native apoHRP (○); apoHRP photooxidized to 2-O_2 (□); and protein-free blank (△). ApoHRP concentrations were 5.4 μM, in 0.3 M borate buffer (pH 9.2). Expected equivalence point is shown by arrow.

each of the photooxidized preparations were removed immediately for spectral analysis. As can be seen in Figure 2, no significant changes in Soret or longer wavelength bands of the complex are evident after photooxidation to 2-O_2, and the spectrum is still clearly distinct from that of free proto.[3] The same observation was made on the 1-O_2 sample (data not shown). The fact that proto remained associated with apoHRP during photooxidation to 2-O_2 argues in favor of the idea that the reaction occurred in a specific or site-directed manner, rather than at random over the protein surface.

After removing proto with methyl ethyl ketone, we examined the photooxidized apoenzymes by disc gel electrophoresis at pH 4.5. The patterns produced by unphotooxidized apoHRP, apoHRP (1-O_2), and apoHRP(2-O_2) (all electrophorized simultaneously) are shown in Figure 1. The unphotooxidized sample represents apoenzyme that was never mixed with proto. As can be seen, this material migrates as a single band; its electrophoretic mobility (7.3 mm hr^{-1}) is nearly twice that observed for the holoenzyme under the same conditions. In the case of apoHRP(1-O_2) and apoHRP(2-O_2) one major band (with very faint trailing) is still seen, but the mobility is now 5.5–5.6 mm hr^{-1} for each sample. A similar change to less cationic character was observed in apoMb after proto-sensitized photooxidation (Mauk and Girotti, 1973b). It is significant that (1) no trace of unmodified apoHRP can be detected in the electrophoretic patterns of the 1- and 2-O_2 material, and (2) the homogeneity of the protein is effectively preserved during photooxidation. The latter observation strengthens the suggestion made earlier in conjunction with the spectral data (Figure 2), *i.e.*, that the photooxidation of proto–apoHRP occurred in a highly selective manner.

TABLE I: Enzymatic Activity of Native and Photooxidized ApoHRP (Isozyme C) in the Presence of Hemin.

Sample	Specific Activity (units/mg)[a]	% of Activity
Natural HRP	3185 ± 60	100
2 hemins/apoHRP	3000 ± 180	94
2 hemins/apoHRP(1-O_2)	750 ± 30	23
100 hemins/apoHRP(1-O_2)	870 ± 40	27
2 hemins/apoHRP(2-O_2)	640 ± 20	20
100 hemins/apoHRP(2-O_2)	760 ± 40	24

[a] Based on *o*-dianisidine assay (Worthington Enzyme Manual, 1972). Each value is an average from at least five assays; standard deviations are shown.

We were interested in determining whether photooxidized apoHRP could be reconstituted to the active enzyme. The ability of the modified apoenzyme to recombine with hemin was assessed by spectrophotometric titration as shown in Figure 3. The plot of the data for unphotooxidized apoHRP shows the expected break close to the theoretical 1:1 point. However, in the case of apoHRP(2-O_2), and to a lesser extent apoHRP(1-O_2) (not shown), immediate curvature in the plot is observed, and no distinct equivalence point can be detected. Thus although the proto–apoperoxidase complex was found to be intact after photooxidation (Figure 2), the binding of hemin by the apoenzyme after removal of proto appeared to be altered. It is possible that conformational aberrations in the proto-free photooxidized apoenzyme hindered its normal recombination with hemin.

Upon completing the spectral titrations (Figure 3), we tested the protein samples for enzymatic activity, each sample being in the presence of twofold molar excess of hemin. The results are shown in Table I. It can be seen that the reconstituted unphotooxidized enzyme (sample 2) has almost the same specific activity as natural isozyme C. ApoHRP(1-O_2) in the presence of two hemins, however, exhibited only 23% of the normal activity, and the value was slightly lower in the case of apoHRP(2-O_2). Upon incubating each of the photooxidized samples with 50-fold more hemin for 18 hr, we found that their respective activities increased by only a small amount. These increases cannot be attributed to the intrinsic activity of hemin, since free hemin over the concentration range studied had no significant peroxidatic activity. On the basis of the electrophoretic and spectral titration properties of photooxidized apoHRP (Figures 1 and 3), it is clear that the residual specific activity (~20%) must not be due to the presence of unmodified material, but rather to some altered binding of hemin by the modified apoenzyme.

The effect of proto-sensitized photooxidation on the amino acid content of apoHRP is shown in Table II.[4] As can be seen histidine is the only type of residue affected by photooxidation to the 2-O_2 stage. Approximately 0.9 of a histidyl residue is missing in apoHRP(2-O_2). The data indicate that the loss of a single histidine was nearly complete at the 1-O_2 stage, relatively little further loss occurring between 1- and

[3] In preliminary studies we found that irradiation of proto–apoHRP would eventually lead to spectral changes. When the complex (at pH 9) was exposed to fluorescent room lighting for 18 hr in a 3-ml quartz cuvet, the Soret absorbance decreased by 20% relative to a control sample kept in the dark. No shift in λ_{max} was seen. Evidently proto binding was altered during prolonged irradiation, and partial dissociation of the complex may have taken place.

[4] We have not examined the carbohydrate portion of photooxidized apoHRP for any possible modification.

TABLE II: Amino Acid Content of Native and Photooxidized ApoHRP (Isozyme C).[a]

Amino Acid	Residues/40,000 g of Protein			
	Native[b]	Expected[c]	1-O_2[b]	2-O_2[b]
Histidine	3.00	3	2.31	2.13
Methionine[d]	ND	3	ND	3.07
Tyrosine	4.60	5	4.63	4.64
Tryptophan[e]	0.90	1	0.80	1.07

[a] Only the potentially photooxidizable amino acids are shown. All others were unchanged after photooxidation. [b] Based on glycine = 17.0. [c] From Shannon *et al.* (1966) and Strickland *et al.* (1968). [d] Determined by alkaline hydrolysis (Neumann, 1967). Values for native and 1-O_2 material were not determined (ND). [e] Determined spectrophotometrically by the procedure of Goodwin and Morton (1946), modified as described in the Experimental Section.

2-O_2. The observed reaction beyond 1-O_2 is attributed for the most part to further oxidation of the initial photoproduct of histidine. This interpretation is supported by the fact that the photooxidation of free histidine (proto-sensitized at pH 9 and 25°) is a biphasic reaction consisting of (1) a fast step in which 1 molar equiv of oxygen is consumed and Pauly reactivity is lost, and (2) a slower step of additional oxygen consumption (M. R. Mauk and A. W. Girotti, unpublished observation). Evidently the different photoproducts of the histidyl residue in apoHRP had the same charge characteristics under the conditions of disc electrophoresis, since the 1-O_2 and 2-O_2 material had virtually indistinguishable electrophoretic patterns (Figure 1).

In an attempt to locate the photolabile histidyl residue in the primary structure of peroxidase, we prepared peptide maps of photooxidized apoHRP, and compared them with maps of the unphotooxidized apoenzyme. A typical map of the tryptic peptides of native apoHRP is shown (Figure 4A) along with an identically prepared map of the apoHRP(2-O_2) peptides (Figure 4B). The maps were sprayed simultaneously with Pauly's reagent to detect the histidine- and tyrosine-containing peptides. Approximately five spots are seen on the map of the native material. The spot designated by I is noteworthy since its Pauly color is barely visible on the apoHRP-(2-O_2) map. In the case of apoHRP(1-O_2) the color intensity of spot I was reduced, but to a lesser extent than in apo-HRP(2-O_2). None of the other Pauly-positive peptides appear to have been affected by photooxidation to 2-O_2, and no new Pauly spots are evident. The migration characteristics of peptide I were obviously different after photooxidation, since the spot corresponding to this peptide was absent on maps of apoHRP(2-O_2) sprayed with ninhydrin. However, we have not been able to identify any anomalous peptide(s) on such maps. The results of our mapping experiments are consistent with our other observations, and clearly indicate that one particular histidyl residue in the proto–apoHRP complex is subject to selective photooxidation.

On maps of unphotooxidized apoHRP that were developed with ninhydrin, peptide I was found to be well separated from neighboring peptides. It appeared feasible, therefore, that this peptide could be isolated from such maps in sufficient purity for amino acid analysis. Accordingly the area of peptide I was cut out from four identical maps and eluted

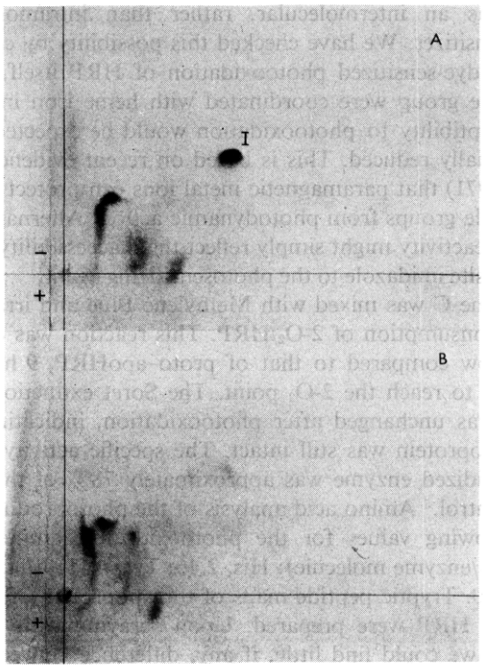

FIGURE 4: Tryptic peptide maps of native apoHRP (A) and apoHRP (2-O_2) (B). The maps were each prepared with 1.75 mg of tryptic peptides and were sprayed simultaneously with Pauly's reagent. Peptide spot I is discussed in the text.

with water. The recovered peptide was found to have the amino acid composition shown in Table III. Except for the discrepancy of one glutamate residue, the composition of peptide I agrees closely with that of a previously described tryptic peptide (T-8) from HRP (Welinder *et al.*, 1972). The single histidyl residue of T8 was shown to lie within the sequence: Ser-Ser-Asp-Leu-Val-Ala-Leu-Ser-Gly-Gly-His-Thr-Phe-Gly-Lys.

Methylene Blue Sensitized Photooxidation. The rapid photooxidation of the residue in question might not be attributable necessarily to its proximity to the porphyrin ring, but possibly to its high degree of exposure on the protein's outer surface. If the latter were true, bound proto could be

TABLE III: Amino Acid Composition of Peptide I.[a]

Amino Acid	Found[b]	Expected[c]
Lysine	1.12	1
Histidine	0.69	1
Aspartic acid	1.57	1
Threonine	1.14	1
Serine	2.33	3
Glutamic acid	1.09	0
Proline	0.33	0
Glycine	3.00	3
Alanine	1.14	1
Valine	0.90	1
Isoleucine	0.39	0
Leucine	1.60	2
Phenylalanine	0.71	1

[a] See Figure 4A. [b] Based on glycine = 3.0. Residues of content less than 0.1 have been omitted. Values were obtained after a single 24 hr acid hydrolysis. [c] Based on composition of peptide T8 (Welinder *et al.*, 1972).

acting as an intermolecular, rather than intramolecular, photosensitizer. We have checked this possibility by carrying out the dye-sensitized photooxidation of HRP itself. If the imidazole group were coordinated with heme iron in HRP, its susceptibility to photooxidation would be expected to be substantially reduced. This is based on recent evidence (Jori *et al.*, 1971) that paramagnetic metal ions can protect photooxidizable groups from photodynamic action. Alternatively a loss of reactivity might simply reflect the inaccessibility of the binding site imidazole to the photosensitizing agent.

Isozyme C was mixed with Methylene Blue and irradiated to the consumption of 2-O_2/HRP. This reaction was exceedingly slow compared to that of proto-apoHRP, 9 hr being required to reach the 2-O_2 point. The Soret extinction coefficient was unchanged after photooxidation, indicating that the hemoprotein was still intact. The specific activity of the photooxidized enzyme was approximately 78% of that of a dark control.[5] Amino acid analysis of the photoproduct gave the following values for the photooxidizable amino acids (residues/enzyme molecule): His, 2.46; Tyr, 4.11; Met, N.D.; Trp, 0.80. Tryptic peptide maps of the apoprotein of photooxidized HRP were prepared. Upon spraying with Pauly's reagent, we could find little, if any, difference between such maps and those of unphotooxidized apoHRP (see Figure 4A). Presumably the observed loss of histidine and tyrosine was randomly distributed and could not be detected on our maps. Significantly there was no visible loss of peptide I. That the histidyl residue of peptide I is resistant to photooxidation in HRP but highly reactive in proto-apoHRP is compelling evidence that this particular residue is situated in the proto and protohemin binding site of the apoenzyme.

Discussion

It has long been recognized that the catalytic activity of horseradish peroxidase cannot be attributed solely to its prosthetic group, which is found in other types of hemoproteins, but rather to the manner in which this prosthetic group interacts with the apoenzyme. The nature of this interaction and its role in the mechanism of action of the enzyme have been the subject of many investigations. On the basis of titrimetric and spectrophotometric data, Theorell (1943) concluded that heme iron in HRP is coordinated with a carboxylate group on the apoprotein. However, this type of interaction was later considered unlikely (Theorell, 1947), and various other possibilities were advanced (Nicholls, 1962). Recent physicochemical evidence has provided new insight as to the nature of the heme–apoHRP linkage. For example, from potentiometric titration experiments on isozyme B of HRP, Phelps *et al.* (1971) found that one histidyl residue in the enzyme is unavailable for titration. This residue, however, titrated normally in apoHRP, suggesting to these workers that its imidazole group is heme-linked in the holoenzyme, possibly occupying the fifth coordination position of iron. A tyrosyl residue was implicated as an additional heme-linked group. An alternate interpretation of the results of Phelps *et al.* (1971) is apparent, *viz.*, that the abnormally titrating residues in HRP may not be necessarily associated with the heme moiety, but merely "buried" in the interior of the holoenzyme. Nevertheless independent physical studies, primarily spectroscopic, have recently given support to their conclusions. For example, absorption difference spectra of liganded forms of model hemoproteins (*e.g.*, low spin cyanometMb *vs.* high spin free metMb) have been shown to contain a characteristic band at ~240 nm, which has been ascribed to a transition associated with the iron–imidazole bond (Brill and Sandberg, 1968). The observation of such a band in the difference spectrum of low spin compounds of HRP (*e.g.*, compound II *vs.* free HRP) has suggested the occurrence of a similar imidazole–iron interaction in HRP (Brill and Sandberg, 1968; Sandberg and Balegh, 1973). On the other hand, electron spin resonance data for HRP and low spin compounds of HRP (Peisach and Blumberg, 1972) have indicated that the iron ligand in HRP must have an electronegativity differing from that of the proximal histidine in hemoglobin, myoglobin, or cytochrome *c;* however, Blumberg (1971) has suggested that this ligand might possibly be the imidazolyl anion.

In addition to the physical methods described, a number of chemical approaches has been used to probe the active site of HRP. Shih *et al.* (1971), for example, found that on treating HRP with the imidazole reagent diazonium-1*H*-tetrazole, no loss of histidine ensued. In the case of apoHRP, however, all three histidines were modified, indicating that these residues had become "unmasked" upon removal of the prosthetic group. From these results Shih *et al.* (1971) speculated that at least one of the unreactive residues in HRP might be heme-linked.

Recent studies by Schonbaum *et al.* (1971) have revealed that HRP is reversibly inhibited by acetic anhydride. The uv spectral properties of the transient acetyl derivative of the enzyme suggested that modification had occurred at a functionally important histidyl residue. Since the absorption changes in the Soret region were relatively small after acetylation, Schonbaum *et al.* (1971) assigned the modified group to the distal (*i.e.*, sixth coordination position) side of the heme ring. This same group of workers has recently announced (Welinder *et al.*, 1972; Welinder, 1973) that their determination of the primary structure of HRP is essentially complete. On the basis of sequence homologies among HRP and members of the globin family, they have suggested that one particular HRP-tryptic peptide (T9), which contains two histidyl residues, may be positioned distally to heme iron.

From several independent investigations, therefore, the prevailing view has been that at least one of the three histidyl residues in HRP is located in the immediate environment of the heme group. This view is substantiated by the photooxidation studies we have carried out. Our conclusion that the selectively photooxidizable histidyl residue in proto-apoHRP is an active site residue in HRP itself is based not only on the obvious structural similarity of proto and hemin, but on previous evidence that proto can compete with hemin for a common binding site on the apoenzyme. The existence of a common site was demonstrated by Maehly (1961), who found that compound II of HRP cannot be generated when hemin and a suitable peroxide are added to a solution containing 1:1 proto-apoHRP. Recent studies with synthetic horseradish peroxidases prepared with various modified hemins (Tamura *et al.*, 1972) and with the manganic–proto complex (Schonbaum, 1973) have offered additional evidence for the existence of a single porphyrin binding site.

It is possible that the photosensitive imidazole group in proto-apoHRP is not only situated in the heme binding site of natural HRP, but is actually heme-linked, *i.e.*, proximal to the iron atom. This consideration is based on our identification of tryptic peptide I (Figure 4A), which contains the

[5] HRP incubated with Methylene Blue in the dark for 9 hr had the same specific activity as natural HRP (Table I). Similarly HRP irradiated 9 hr in the absence of dye showed no loss of activity.

photolabile histidine. Peptide 1 is essentially identical with peptide T8 of Welinder *et al.* (1972), and clearly distinct from their T9, in which the remaining two histidines of HRP are located. As mentioned above, peptide T9, the two histidines of which are separated by a single phenylalanyl residue, is believed to occupy the distal region in HRP (Welinder, 1973). If apoHRP does, in fact, interact with its prosthetic group in myoglobin- or hemoglobin-like fashion, *i.e.*, with proximally and distally placed histidyl residues, our data indicate that only one of these groups (presumably the proximal) is destroyed during photooxidation of proto–apoHRP to 2-O_2. The apparent lack of photoreactivity of a second histidine might be attributable to the relatively large distance separating this residue and the porphyrin ring. With regard to this point, we are currently attempting to probe the active site of peroxidase more extensively by photooxidizing the proto–apoHRP complex beyond the 2-O_2 stage.

Acknowledgments

We are grateful to Dr. Beatrice Kassell for the use of the amino acid analyzer and to Mr. Peter Ward for assisting us in the analyses.

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