SENSITIZED PHOTOOXIDATION OF LOW SPIN HORSERADISH PEROXIDASE

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Received October 18,1976

Summary: Horseradish peroxidase differs from most enzymes in that it is almost completely resistant to photodynamic action due to the paramagnetic ferric ion in the prosthetic group, heme. Chelation of horseradish peroxidase at the sixth coordination position of the iron with a cyanide or hydroxyl group converts it to a low spin diamagnetic state. Upon illumination with visible light with eosin Y, flavin mononucleotide or methylene blue as sensitizer, the low spin enzyme lost both peroxidative and oxidative activities with the same quantum yields. Several amino acid residues, including one histidine and one tyrosine were destroyed in the low spin enzyme after 60 min of illumination with eosin Y as sensitizer.

Peroxidases are unusually resistant to a variety of inactivating agents such as heat (1-3), ionizing radiation (2,4,5) and ultraviolet radiation (2). They are also unusually resistant to photodynamic inactivation (dye-sensitized photooxidation) (6-10). Most hemoproteins have the heme iron in a high spin state and are not directly sensitive to visible light (11-12). However, it was shown recently that changing the high spin heme iron to a low spin state endowed horse and sperm-whale myoglobin with marked sensitivity to light (11). In this communication we report an examination of the sensitivity to photodynamic treatment of HRP** in which the sixth coordination position of the paramagnetic heme iron (high spin state) is coordinated with cyanide or hydroxyl groups to yield diamagnetic heme iron (low spin state). Such low spin derivatives were sensitive to light in the presence of EOY, FMN or MEB as sensitizers.

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**Abbreviations used: HRP, horseradish peroxidase; HRP-OH, hydroxide-complexed horseradish peroxidase; HRP-CN, cyanide-complexed horseradish peroxidase; apoHRP, horseradish apoperoxidase; EOY, eosin Y; FMN, flavin mononucleotide; MEB, methylene blue.

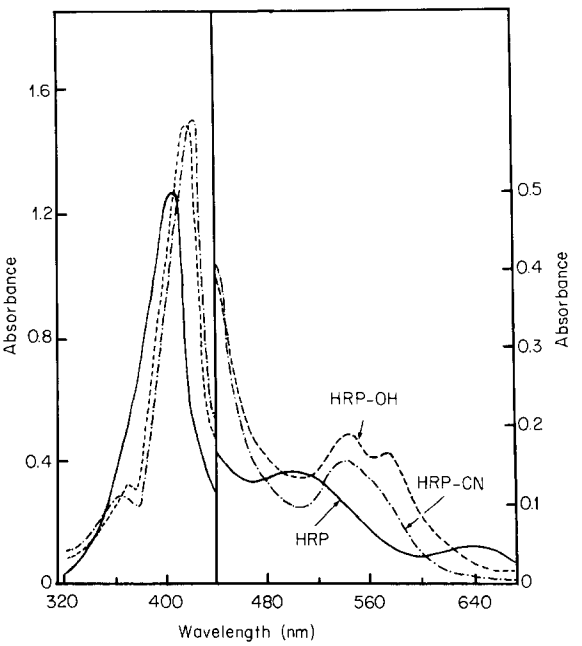

Fig. 1. The near-ultraviolet and visible absorption spectra of 12.6 μM HRP in 0.5 M sodium phosphate buffer, pH 8; of HRP-CN in a solution 20 mM in NaCN and 0.5 M in Na_2HPO_4 and of HRP-OH in 0.85 M NaOH, pH 12.4.

MATERIALS AND METHODS

Horseradish peroxidase (EC 1.11.1.7, Sigma Chemical Co., Type II, lot 20-C-2820, R.Z. 1.37) was further purified on CM-and DEAE-cellulose columns (13) and the isoenzyme C fraction with R.Z. 3.2 was isolated as described elsewhere (10). HRP-CN was prepared by dissolving the enzyme (1 mg/ml) in a solution 20 mM in NaCN and 0.5 M in Na_2HPO_4. HRP-OH was prepared by dissolving the protein (1 mg/ml) in 0.85 M NaOH (pH 12.4).

Reaction mixtures for photooxidation contained 1.0 ml peroxidase solution, 0.5 ml dye solution (1×10^{-4} M EOY or MEB, or 1×10^{-3} M FMN) and 0.5 ml of 0.5 M pH 8 sodium phosphate buffer (for the HRP-CN experiments) or 0.5 ml of 0.85 M NaOH (for the HRP-OH experiments). The reaction mixtures were illuminated in air with stirring at 25°C in a plexiglass cell. Illumination was provided by a 500 watt projector; Baird-Atomic multilayer interference filters were used to give narrow wavelength bands corresponding to the absorption peak of the sensitizer used (437 nm for FMN, 517 nm for EOY, 675 nm for MEB). At intervals during illumination, samples were removed for enzyme assay. Light measurements and calculation of the quantum yields of inactivation (defined as the number of enzyme molecules inactivated per number of photons absorbed, extrapolated to zero time of illumination) were performed as described earlier (14).

For activity measurements, 100 μl samples of the HRP-CN system were removed and mixed with 1.0 ml of 28 mM silver nitrate. The resulting precipitate was removed by centrifugation and filtration, after which 100 μl aliquots of the mixture were used for the enzyme assay. For the HRP-OH system, 100 μl samples of the reaction mixture were mixed with 1.0 ml of 0.5 M sodium phos-

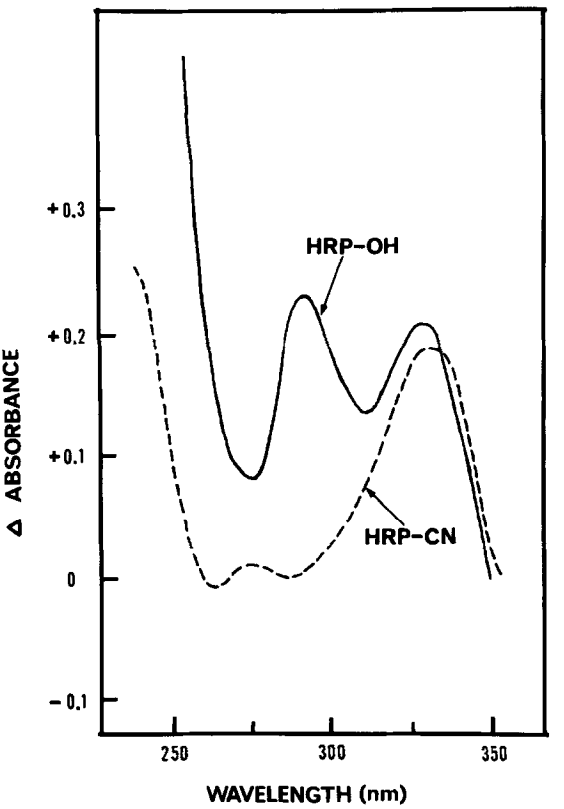

Fig. 2. The ultraviolet difference spectra of 32 μM HRP-CN in 0.5 M Na_2HPO_4 (front cell of Cary Model 14M spectrophotometer) against an equimolar solution of HRP in 0.5 M Na_2HPO_4 (rear cell) and 32 μM HRP-OH in 0.85 M NaOH (front cell) against an equimolar solution of HRP in distilled water (rear cell).

phate buffer, pH 6.0; 50 μl aliquots of this solution were then used for the assay. The peroxidative activity of the HRP preparations was assayed with o-dianisidine coupled with hydrogen peroxide as substrate (15) while the oxidative activity was measured with indoleacetic acid as substrate by a slight modification of the method of Siegel and Galston (16). The photometric assays and spectral measurements were performed with a Cary Model 14M spectrophotometer. Amino acid analyses were carried out with a Beckman Model 120C amino acid analyzer on enzyme samples hydrolyzed in acid or in base as described before (10).

RESULTS AND DISCUSSION

Spectral properties of HRP-CN and HRP-OH - Fig. 1 shows the near ultraviolet and visible absorption spectra of HRP, HRP-CN and HRP-OH. The Soret band shifts from 403 nm for the native enzyme to 416 nm and 423 nm for HRP-OH

and HRP-CN, respectively; this is characteristic for the shift from a high spin ferriheme protein to a low spin ferriheme protein (17). Small peaks around 357 nm and 366 nm for HRP-CN and HRP-OH were also formed. Further the two absorption bands of native HRP at 498 nm and 640 nm disappear and are replaced by absorption bands at 545 nm and 572 nm in HRP-OH and at 538 nm and 568 nm (appears as a shoulder) in HRP-CN. These latter spectral alterations are also due to the change in spin state.

The ultraviolet difference spectra for the HRP-CN and HRP-OH versus native HRP are given in Fig. 2. Both complexes show the appearance of a new peak at approximately 325 nm; nothing is known as to the cause or significance of this peak. The HRP-OH also exhibits a new peak at approximately 280 nm; this peak may result from a conformational change (unfolding) in the complex resulting in an altered environment for some of the aromatic amino acid residues

Illumination of HRP-CN and HRP-OH in the absence of added sensitizers - Horse and whale ferric myoglobin are insensitive to visible light in the absence of added sensitizing dyes. Ligation of the heme with O_2, CO or cyanide renders these proteins very sensitive to light with the low spin heme acting as an efficient endogeneous photodynamic sensitizer (11). In contrast, the low spin HRP-CN and HRP-OH used in our study were insensitive to light. No photodynamic destruction of amino acid residues was observed even after 6 hr of illumination at a distance of 6 cm from a glass-filtered General Electric A-H6 1000 watt high pressure mercury arc which has a very high output in the spectral regions absorbed by hemes.

Photodynamic inactivation of HRP-CN and HRP-OH with added sensitizers - Both the peroxidative and oxidative activities of HRP-CN and HRP-OH are rapidly lost on illumination in the presence of EOY, FMN or MEB. The inactivation is first order in enzyme concentration. This is in sharp contrast to native HRP, which is almost completely insensitive to photodynamic treatment (8-10). Quantum yields for the photodynamic inactivation of HRP-CN are presented in Table I. The values for the loss of peroxidative and oxida-

Table 1. Quantum Yields for the Photodynamic Inactivation of the Peroxidative and Oxidative Activities of HRP-CN

Sensitizer	EOY	FMN	MEB
Peroxidative Activity	1.80×10^{-3}	1.52×10^{-3}	1.75×10^{-3}
Oxidative Activity	1.79×10^{-3}	1.53×10^{-3}	1.75×10^{-3}

Table 2. Quantum Yields for the Photodynamic Inactivation of the Peroxidative and Oxidative Activities of HRP-OH

Sensitizer	EOY	FMN	MEB
Peroxidative Activity	5.21×10^{-3}	0.95×10^{-3}	1.55×10^{-3}
Oxidative Activity	5.20×10^{-3}	0.96×10^{-3}	1.54×10^{-3}

tive activities are essentially identical and do not differ much with different dyes. For comparison, the quantum yields for the photodynamic destruction of apoHRP under similar conditions are 1.2×10^{-3}, 0.35×10^{-3} and 1.2×10^{-3} with EOY, FMN and MEB, respectively (18).

Table 2 gives similar data for HRP-OH. The peroxidative and oxidative activities, again, are lost with the same quantum yields. In contrast to the HRP-CN results, however, the yields depend on the sensitizing dye with EOY being the most efficient. This may result from the high pH (12.4) of the reaction mixture. EOY typically shows a progressive increase in sensitizing efficiency with increasing pH up to very high values; FMN and MEB initially show an increasing efficiency with increasing pH, but this tends to level off and efficiency actually decreases at higher values with FMN (7,14).

The marked increase in the photodynamic sensitivity of low spin HRP described above fits in well with what is known about the behavior of paramagnetic species in photodynamic systems. We have shown earlier that heme (high spin iron) is an efficient photodynamic protective agent for certain of the photooxidizable amino acid residues in HRP (18-19). This might be expected since a variety of paramagnetic metal ions have been shown to protect substrates against photodynamic treatment by quenching excited states involved in the reaction sequence (20-23). In contrast, diamagnetic metal ions showed no quenching of excited photosensitizers (24) and do not act as photodynamic protective agents (10,23).

The question arises as to whether the photodynamic sensitivity of HRP-CN and HRP-OH results simply from the change in spin state of the heme iron. It is possible, of course, that the complexes are altered in other ways which would increase the sensitivity to photodynamic treatment. For example, we have shown that HRP unfolded in guanidine hydrochloride becomes sensitive to photodynamic treatment (18-19); presumably this results from the exposure of photooxidizable amino acid residues which were buried in the native enzyme. The present studies cannot give a completely unambiguous answer to this question, although the absence of change in the ultraviolet spectrum of the HRP-CN in the region corresponding to the aromatic amino acids (Fig. 2) suggests that there is no significant unfolding in this complex. There may be some unfolding of the HRP-OH as shown by the ultraviolet difference spectrum in Fig. 2.

Amino acid residue destruction in photodynamically-treated low spin HRP - As shown in Table 3, 60 min illumination of HRP-CN in the presence of EOY results in the destruction of one histidine residue and one tyrosine residue per molecule. This probably results from the lack of photodynamic protective function by the low spin heme since the ultraviolet difference spectrum (Fig. 2) does not indicate major conformational changes in the cyanide derivative which could expose sensitive residues buried in the native enzyme. Since at least

Table 3. Amino Acid Analyses of Low Spin HRP After 60 Min of Photodynamic Treatment with EOY

Photosensitive Amino Acid	Number of Residues per Molecule		
	Dark Control	HRP-CN	HRP-OH
Histidine	3.1	2.2	1.4
Methionine	3.2	2.7	1.8
Tryptophan	1.1	0.9	0.7
Tyrosine	4.9	4.0	0.6

one histidine residue (10,18,25) and one tyrosine residue (25-26) are present near the heme in HRP, these might be expected to be the only residues destroyed by treatment of HRP-CN.

When HRP-OH is photooxidized under the same conditions, more than one histidine residue, one methionine residue and almost all of the tyrosine residues are destroyed (Table 3). This more extensive destruction of amino acid residues as compared to HRP-CN may result from a change of the protein conformation of HRP-OH leading to the exposure of more photosensitive amino acid residues. A conformational change in the HRP-OH has been reported (27), and the present study shows an increased absorption at 280 nm (Fig. 2) which suggests that more tyrosine residues are exposed to solvent. This could account for the increased number of tyrosine residues photooxidized in HRP-OH. It is also possible that the increased photooxidation of tyrosine residues might result from the high pH of the HRP-OH system, since tyrosine residues in some other proteins are photooxidized more rapidly at higher pH values (28).

ACKNOWLEDGMENT. This work was supported by the U.S. Energy Research and Development Administration under Contract No. E(11-1)-875.

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