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Mechanism-Based Inactivation of Horseradish Peroxidase by Sodium Azide. Formation of *meso*-Azidoprotoporphyrin IX†

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ABSTRACT: Catalytic turnover of sodium azide by horseradish peroxidase, which produces the azidyl radical, results in inactivation of the enzyme with K_I = 1.47 mM and k_{inact} = 0.69 min^{-1}. Inactivation of 80% of the enzyme requires approximately 60 equiv each of NaN_3 and H_2O_2. The enzyme is completely inactivated by higher concentrations of these two agents. *meso*-Azidoheme as well as some residual heme are obtained when the prosthetic group of the partially inactivated enzyme is isolated and characterized. Reconstitution of horseradish peroxidase with *meso*-azidoheme yields an enzyme without detectable catalytic activity even though reconstitution with heme itself gives fully active enzyme. The finding that catalytically generated nitrogen radicals add to the meso carbon of heme shows that biological meso additions are not restricted to carbon radicals. The analogous addition of oxygen radicals may trigger the normal and/or pathological degradation of heme.

The azide anion functions in different situations as a substrate, reversible inhibitor, or irreversible inhibitor of catalytic hemoproteins like catalase and horseradish peroxidase. In the case of catalase, coordination of the azide anion to the prosthetic heme[1] iron atom inhibits the catalytic dismutation of H_2O_2 (Deisseroth & Dounce, 1970). If H_2O_2 is present, however, the azide anion is also oxidized by catalase to nitrous oxide, nitric oxide, and nitrogen gas (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Keilin & Nicholls, 1958; Rein et al., 1968). Spin trapping studies suggest that these azide metabolites are formed by reaction of the catalytically generated azidyl radical with molecular oxygen (Kalyanaraman et al., 1985). In contrast, azide does not bind at neutral pH to the prosthetic heme iron atom of horseradish peroxidase (Morishima et al., 1977). Nevertheless, horseradish peroxidase is at least as vulnerable as catalase to inhibition by this agent because it is inactivated rather than simply inhibited by azide. Brill and Weinryb reported in 1967 that horseradish peroxidase is inactivated by a H_2O_2-independent reaction (but see below) of the azide anion with a methionine residue of the protein. Spin trapping studies have shown that horseradish peroxidase, like catalase, oxidizes the azide anion to the azidyl radical (Kalyanaraman et al., 1985). The one-electron oxidation of azide appears to be a general peroxidatic reaction because it is also catalyzed by chloroperoxidase, myeloperoxidase, and lactoperoxidase (Kalyanaraman et al., 1985).

We recently demonstrated that horseradish peroxidase is inactivated during the catalytic turnover of phenyl- and al-

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the iron oxidation and ligation states; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase.

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kylhydrazines (Ator & Ortiz de Montellano, 1987; Ator et al., 1987). Inactivation by phenylhydrazine is due primarily to reaction of a reactive metabolite with the protein whereas inactivation by the alkyl hydrazines is due primarily to addition of the hydrazine-derived alkyl radical to the δ meso position of the prosthetic heme group. Evidence that carbon-free radicals are actually involved in these inactivation reactions is provided by the observation of phenyl radical spin adducts in incubations of horseradish peroxidase with phenylhydrazine and a spin trap (Sinha, 1983). Catalytic oxidation of nitromethane and cyclopropanone hydrate, presumably to the nitromethyl and 3-oxopropyl radicals, respectively, has also been shown to result in meso alkylation of the prosthetic heme group (Porter & Bright, 1983; Wiseman et al., 1982).

Meso alkylation of the prosthetic heme group by *carbon* radicals suggests that heteroatomic radicals, including the azidyl radical, may also add to the prosthetic group. The reactions of heteroatomic radicals with heme are of particular interest because of their relevance to the normal and pathological degradation of heme. We report here that sodium azide is, indeed, a mechanism-based inactivator of horseradish peroxidase and show that it reacts primarily with the prosthetic heme group rather than with the protein matrix.

Experimental Procedures

Materials. Horseradish peroxidase (type VI), bovine liver catalase, hydrogen peroxide, guaiacol, sodium ascorbate, and biliverdin were obtained from Sigma. Pyridine-d_5 was purchased from Aldrich. Buffers were prepared with glass-distilled deionized water and were passed through a column of Chelex 100 (Bio-Rad) before use. All peroxidase experiments were performed at 25 °C in 50 mM sodium phosphate buffer (pH 7.0). Experiments were run a minimum of 2 times.

Analytical Methods. Absorption spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using $E_{402} = 95\,000$ M^{-1} cm^{-1} (Mauk & Girotti, 1974). High-pressure liquid chromatography was carried out with two Beckman Model 110A pumps, a Model 420 controller, and a Hewlett-Packard 1040A diode array detector. ^1H NMR spectra were obtained on a General Electric 500-MHz instrument in deuteriochloroform or pyridine-d_5. Chemical shifts are reported in parts per million relative to tetramethylsilane. Mass spectra were obtained on a Kratos MS-50 instrument operating in the liquid matrix secondary ion mode.

Rate of Inactivation of HRP by Sodium Azide. The rate of inactivation of HRP was measured at approximately 25 °C in 1-mL incubations containing the enzyme (1 μM), H_2O_2 (0.8 mM), and 0.15–0.6 mM sodium azide. At specified time intervals, 10-μL aliquots of the incubation mixtures were transferred to cuvettes containing 1.0 mL of an assay mixture composed of 5 mM guaiacol and 0.6 mM H_2O_2 in 50 mM sodium phosphate (pH 7.0) buffer. The peroxidase activity was measured by the increase in the absorbance at 470 nm. In some experiments, the inactivated enzyme was passed through a Sephadex G-25 column (20 × 1.5 cm) with sodium phosphate buffer (pH 7.0) and was incubated for several hours at 30 °C in the absence of peroxide or sodium azide to see if activity could be recovered. In some instances, concentrations of sodium azide and H_2O_2 equal to those used in the initial incubation were added to the enzyme after passage through the G-25 column and the time dependence of further activity loss was monitored as described above.

Peroxide Dependence of the Inactivation Reaction. The peroxide dependence of the inactivation reaction was examined by incubating HRP (2.5 μM) and sodium azide (125 μM) for 1 h at 25 °C with concentrations of H_2O_2 that ranged from 0 to 200 μM. The incubations were then assayed for residual catalytic activity as described above.

Partition Ratio. Solutions (1-mL volume) of horseradish peroxidase (10 μM), H_2O_2 (1 mM), and sodium azide (0–0.6 mM) were incubated for 10 min at 25 °C. The remaining activity was determined as a function of time by withdrawing 10-μL aliquots at appropriate times and assaying their guaiacol peroxidase activity as already described.

Chromophore Alterations. The chromophore changes in incubations analogous to those described above were monitored as a function of time in incubations containing horseradish peroxidase (10 μM), sodium azide (1.0 mM), and H_2O_2 (1.0 mM). The chromophore change in the absence of H_2O_2 was also recorded.

Qualitative Analysis of Heme Modification. Standard 1-mL incubations of HRP (10 μM), H_2O_2 (1 mM), and sodium azide (0 to 1 mM) were incubated for 1 h at 25 °C. Aliquots taken from the incubations were assayed for remaining peroxidatic activity as already described. Excess H_2O_2 in the primary incubations was then destroyed by adding 2 μL of a 5.6 mg/mL solution of bovine liver catalase followed 3 min later by 2 μL of 50 mM sodium ascorbate. The resulting mixtures were acidified with acetic acid (0.3 mL), and the prosthetic groups were extracted with two aliquots (1 mL each) of diethyl ether. The extraction mixtures were spun on a tabletop centrifuge to separate the layers. The combined organic layers were washed with water and were evaporated to dryness under a stream of nitrogen. The residues were analyzed by high-pressure liquid chromatography on a 4.6 × 250 mm Whatman Partisil 5 ODS-3 reverse-phase column. The column was eluted at a flow rate of 1 mL/min with a linear gradient rising from 0% to 100% of solvent B in solvent A over a period of 15 min, where solvent A consists of 6:4:1 (v/v/v) methanol/water/acetic acid and solvent B of 10:1 methanol/acetic acid. The eluent was monitored at 400 nm.

Isolation and Characterization of the Prosthetic Group from Azide-Inactivated HRP. A mixture containing HRP (50 μM), sodium azide (2.5 mM), and H_2O_2 (5.0 mM) in 60 mL of 50 mM sodium phosphate (pH 7.0) buffer was incubated at room temperature for 15 min before the excess peroxide was destroyed by adding 15 μL of a 5.6 mg/mL solution of catalase followed, 5 min later, by 0.5 mL of 50 mM sodium ascorbate. The ascorbate returns the enzyme to the ferric state. Glacial acetic acid (25 mL) was then added, and the prosthetic group was extracted with two 50-mL portions of diethyl ether. The combined ether extracts were washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness on a rotary evaporator. The residue was fractionated by high-pressure liquid chromatography on a 9 × 250 mm Whatman Partisil 10 ODS-3 column eluted with 68:32:10 (v/v/v) methanol/water/acetic acid at a flow rate of 4.0 mL/min. The eluent was monitored at 400 nm. A part of the fraction containing the material of interest was analyzed by LSIMS mass spectrometry, but the bulk of the material was converted to the chloroiron(III) form by partitioning between diethyl ether and a solution of 0.1 N DCl in NaCl-saturated D_2O. The chloroiron(III) derivative was dissolved in pyridine-d_5, reduced by adding a 10-fold excess of $SnCl_2$ relative to the estimated amount of prosthetic group, and examined by ^1H NMR spectroscopy on a General Electric 500-MHz instrument. The chemical shifts of the peaks were determined with reference to the chemical shift of pyridine at 8.70 ppm.

Demetalation of the Modified Heme Group. The prosthetic group isolated from inactivated HRP as described above was

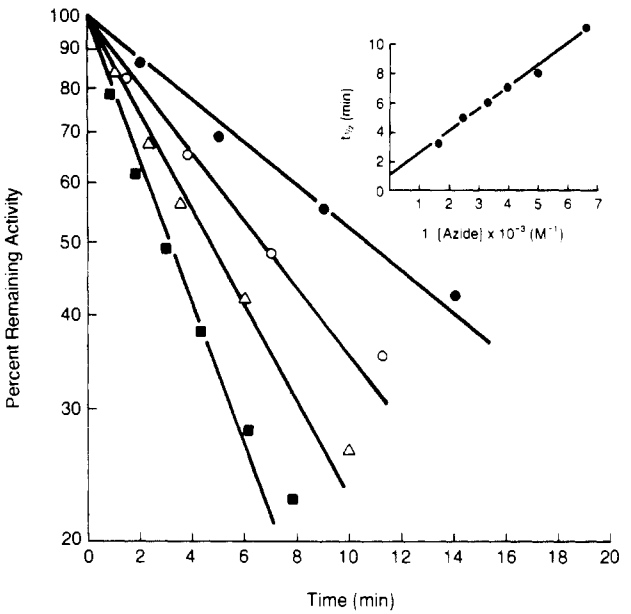

FIGURE 1: Time-dependent inactivation of horseradish peroxidase by sodium azide at pH 7.0. The concentrations of sodium azide are 0.15 (●), 0.25 (○), 0.40 (△), and 0.60 mM (■). The inset shows the time required to inactivate half of the enzyme ($t_{1/2}$) versus the reciprocal of the inhibitor concentration for an expanded range of concentrations.

esterified with trimethyloxonium tetrafluoroborate (Dean et al., 1976) before it was demetalated by the procedure of Smith and Fuhrhop (1975). The spectroscopic changes indicated that the prosthetic group decomposed in the presence of the reducing agents required to remove the iron. Unsuccessful attempts were made to purify the demetalated prosthetic group by HPLC on a 4.6 × 250 mm Partisil 5 PAC column eluted with 1:1 (v/v) tetrahydrofuran/hexane at a flow rate of 1 mL/min (column monitored at 422 nm).

Reconstitution of Horseradish Peroxidase with meso-Azidoheme. HRP (10 mg) was dissolved in 1 mL of water, and 5 drops of 0.1 M HCl were added. The heme was completely extracted by shaking with three 1-mL aliquots of butanone. The apoprotein in the aqueous phase was dialyzed in 2 L of water for 2 h and then with 2 L of 50 mM borate buffer (pH 9.2) for a further 2 h. The apoprotein thus obtained exhibited no heme absorbance at 404 nm. *meso*-Azidoheme, isolated from 15 mg of HRP after reaction with sodium azide and H_2O_2, was purified by HPLC as already described. *meso*-Azidoheme was dissolved in pH 9.2 buffer containing 1% KOH, and the mixture was added dropwise to the stirred apoprotein solution. The final mixture was eluted through a Sephadex G-25 column to remove excess heme. The activity of the reconstituted enzyme and its spectroscopic properties were determined as described for HRP itself.

RESULTS

Inactivation of HRP by Sodium Azide. Incubation of HRP with a fixed concentration of H_2O_2 and increasing concentrations of sodium azide results in pseudo-first-order concentration- and time-dependent inactivation of the enzyme (Figure 1). A replot of the half-life of the enzyme ($t_{1/2}$) at each sodium azide concentration versus the reciprocal of the azide concentration yields a straight line (Figure 1, inset). The value for the binding constant calculated from this plot (K_I = 1.47 mM) indicates that azide binds reversibly to HRP with only moderate affinity prior to the inactivation step (Walsh, 1977). Likewise, the inactivation rate constant calculated from the plot (k_{inact} = 0.69 min^{-1}) indicates that the enzyme is not inactivated very rapidly.

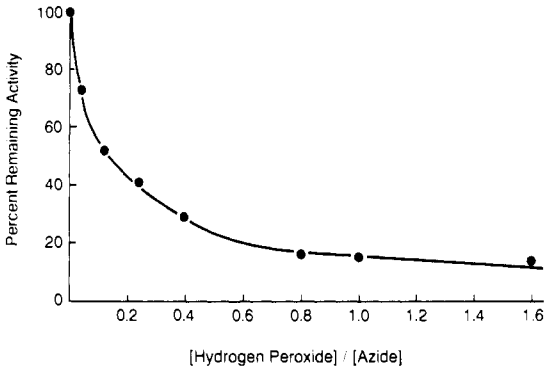

FIGURE 2: Dependence of the inactivation of horseradish peroxidase on the H_2O_2:sodium azide ratio. The details of the incubations are given under Experimental Procedures.

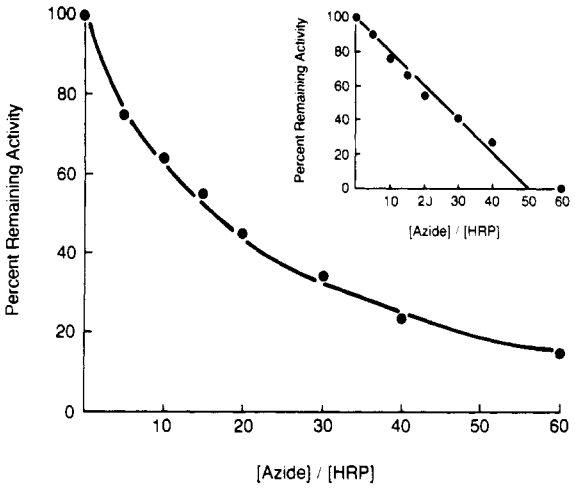

FIGURE 3: Determination of the partition ratio for the inactivation of horseradish peroxidase by sodium azide. The inset shows the percent remaining activity corrected for the fact that 20% of the activity remains when maximum inactivation is achieved with 60:1 azide:HRP ratio. The corrected values were obtained by subtracting the observed rate constant corresponding to the residual 20% activity from all the other rate constants and assuming that 100% activity corresponds to the corrected value in the absence of azide. It is therefore a plot of the percent of the *vulnerable* activity that remains versus the [azide]/[HRP] ratio.

The relationship between the extent of enzyme inactivation and the concentration of H_2O_2 was determined in incubations in which the concentration of sodium azide was held constant and the concentration of H_2O_2 was varied (Figure 2). The results indicate that optimal inactivation is achieved with a 1:1 ratio of H_2O_2 and sodium azide. The extent of enzyme inactivation is shown by experiments in which the azide:HRP ratio was increased in the presence of a fixed excess concentration of H_2O_2 to be a nonlinear function of the sodium azide concentration (Figure 3). Approximately 80% of the catalytic activity is lost in incubations with 60 equiv of sodium azide and 95% in incubations with 600 equiv of sodium azide. Control incubations show that the enzyme is not inactivated in the presence of 125 μM sodium azide but no peroxide, or 125 μM peroxide but no azide. No catalytic activity is regained if the incubation mixtures are passed through a Sephadex G-25 column to remove the azide and peroxide, but reincubation of the filtered enzyme with sodium azide and H_2O_2 results in further loss of catalytic activity (not shown).

Spectroscopic Changes. Addition of 1 mM sodium azide to 10 μM HRP at pH 7.0 causes no changes in the Soret band at 402 nm or in the rest of the spectrum of the resting enzyme (not shown). If H_2O_2 is then added to the mixture, however,

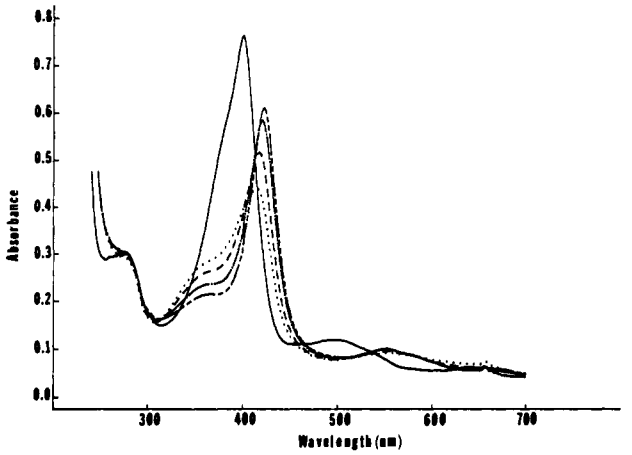

FIGURE 4: Spectroscopic changes accompanying the reaction of HRP with H_2O_2 (1 mM) and sodium azide (1 mM): spectrum of the intact enzyme (—) and after 15% (···), 25% (---), 50% (-·-), and 80% (----) inactivation. The final Soret maximum is at 424 nm.

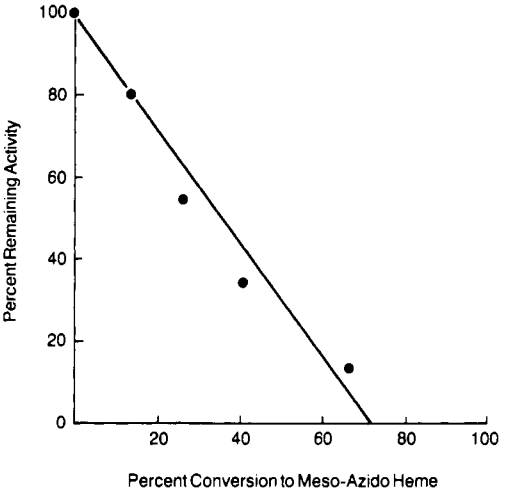

FIGURE 5: Correlation of the percent remaining activity with the percent conversion of the prosthetic heme group to the *meso*-azidoheme adduct.

the Soret band immediately broadens, decreases in intensity, and shifts from 402 to 404 nm (not shown). These changes indicate that compound I is immediately formed. Compound I then gives way, over a period of several minutes, to a species with the spectrum characteristic of compound II. This is indicated by a gradual shift of the Soret band to approximately 424 nm with a parallel increase in the intensity of the band (Figure 4). Enzyme inactivation parallels the increase in the 424-nm absorption, so that inactivation levels off as the 424-nm absorption reaches its maximum. This reflects the fact that the inactive enzyme reacts with H_2O_2 to give a compound II species without catalytic activity (see below). Finally, after prolonged incubation, the spectrum of compound II is replaced by a new spectrum with a Soret maximum at 404 nm. This is the final spectrum of the inactivated enzyme. The Soret absorbance of the inactivated enzyme in a typical incubation is lower than that of the starting enzyme due to partial degradation of the prosthetic heme group. The same spectroscopic changes are observed if partially inactivated enzyme is reincubated with azide and H_2O_2. At no stage in the reaction sequence is an absorbance peak detected in the 800-nm region. This is similar to the reaction of HRP with phenylhydrazine, which also does not exhibit a transient absorbance at approximately 800 nm, but is different from the reactions of HRP with alkylhydrazines, which do exhibit fairly long lived intermediates with absorbance maxima in that region (Ator et al., 1987). The absorption in the 800-nm region has been attributed to the isoporphyrin intermediate expected to intervene between addition of a radical to the meso position and rearomatization due to loss of the corresponding meso proton. The spectroscopic data suggest that the isoporphyrin intermediate is not generated in the reactions with the azidyl or phenyl radicals or, more probably, is too unstable to be detected by static spectroscopic methods.

Reaction of the Inactivated Enzyme with H_2O_2. Addition of an equimolar concentration of H_2O_2 to azide-inactivated enzyme after removal of excess azide by passage through a G-25 column causes a shift in the Soret band from 404 to 424 nm and the appearance of a typical compound II spectrum. The heme chromophore is gradually lost in prolonged incubations. Attempts to isolate the prosthetic heme group of inactivated enzyme after reincubation with H_2O_2 show that most of the prosthetic group is degraded to products that are not detected by the usual workup and chromatographic procedures. The trace of heme-derived products that is detected coelutes with an authentic sample of biliverdin and has an absorption spectrum indistinguishable from that of biliverdin. It appears that reaction of azide-inactivated enzyme with H_2O_2 degrades most of the prosthetic group to unidentified heme fragments and only a minor fraction to biliverdin-like products.

Correlation of Changes in the Prosthetic Group with Inactivation of the Enzyme. Extraction of the prosthetic group from enzyme that has been inactivated to different extents and analysis of the extracted material by high-pressure liquid chromatography indicate that a single heme-derived product (retention time, analytical HPLC, 15.8 min; retention time, preparative HPLC, 8.5 min) is formed at the expense of the original prosthetic heme group (retention time, analytical HPLC, 13.7 min; retention time, preparative HPLC, 6.8 min). A plot of percent conversion of the recovered prosthetic group to the modified product versus the percent of remaining catalytic activity indicates that approximately 75% of the prosthetic heme group is modified when 80% of the activity is lost (Figure 5). Conversely, the heme group is intact in approximately 25% of the enzyme. If the assumption is made that modified heme has the same extinction coefficient as heme itself (see below), these extraction experiments account for something in the order of 90% of the original prosthetic group. The correlation of inactivation with heme modification is likely to be even better than indicated because the modified prosthetic heme of inactive enzyme is degraded more rapidly by reaction with H_2O_2 than is the prosthetic heme group of the intact enzyme. Formation of the *meso*-azido adduct thus accounts well for inactivation of the enzyme.

Structure of the Modified Prosthetic Heme Group. The modified prosthetic group from large-scale incubations has been isolated and has been purified by high-pressure liquid chromatography. The electronic absorption spectrum of the ferric prosthetic group (λ_{max} 402 nm) is very similar to that of iron protoporphyrin IX itself. The absorption spectrum of the prosthetic group after carboxyl group esterification and demetalation supports the idea that the basic porphyrin chromophore has not been greatly modified, although the reducing agent required for demetalation of the modified heme causes partial decomposition of the sample. The spectrum of the dimethyl esterified free base has maxima (relative intensity) at 422 (100.0), 524 (16.7), 562 (10.8), and 598 nm (8.3) with a superimposed maximum at 656. The peak at 656 nm is shown by HPLC to be due to a decomposition product distinct from the free base porphyrin. The mass spectrum of the iron porphyrin exhibits a weak molecular ion at m/z 656,

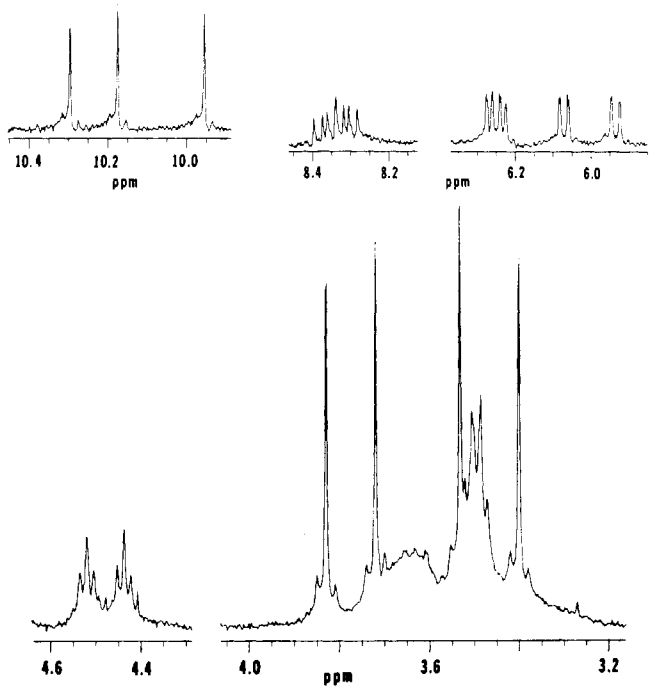

FIGURE 6: ^1H NMR (500 MHz) spectrum of the modified prosthetic group isolated from azide-inactivated HRP. The pertinent regions of the NMR spectrum are presented.

Table I: Correlation of Chemical Shift Values (in ppm) for Proton Signals in the Dipyridyl Complexes of Ferrous Heme and *meso*-Azido Ferrous Heme

proton	heme[a,b]	*meso*-azidoheme
meso	10.283 (γ)	10.294 (s)
	10.236	10.173 (s)
	10.069	9.954 (s)
	9.906 (δ)	
methyls	3.623 (1 or 8)	3.828 (s)
	3.611 (3 or 5)	3.719 (s)
	3.468 (1 or 8)	3.531 (s)
	3.445 (3 or 5)	3.399 (s)
$-CH_2CH_2CO_2H$	4.467	4.518 (t, J = 7.5 Hz)
	4.461	4.435 (t, J = 7.5 Hz)
$-CH_2CH_2CO_2H$	3.504	3.504 (t, J = 7.5 Hz)
	3.500	3.484 (t, J = 7.5 Hz)
$-CH=CH_2$	8.510	8.367 (dd, J = 17.5, 11.5 Hz)
	8.451	8.311 (dd, J = 17.5, 11.5 Hz)
$-CH=CH_2$ (cis)	6.299	6.255 (dd, J = 17.5, 1.5 Hz)
	6.269	6.241 (dd, J = 17.5, 1.5 Hz)
$-CH=CH_2$	5.960	6.069 (dd, J = 11.5, 1.5 Hz)
(trans)	5.947	5.932 (dd, J = 11.5, 1.5 Hz)

[a] The chemical shift values obtained in this study for heme more or less correspond to those reported by O'Keefe (1974). The peak assignments given by O'Keefe for the individual meso and methyl signals on the basis of correlation with model compounds are incorrect, however. The δ meso and 1- and 8-methyl proton assignments given here are based on the finding that irradiation of the protons at 3.623 and 3.468 ppm causes a nuclear Overhauser enhancement of the meso signal at 9.906 ppm. The γ,δ meso assignment is similarly based on the fact that irradiation of the methylene protons at 4.5 ppm causes a nuclear Overhauser enhancement of the meso proton signal at 10.283 ppm. [b] The heme signals exhibit approximately the same multiplicities and coupling constants as those shown for *meso*-azidoheme: s, singlet; t, triplet; dd, doublet of doublets. The specific assignments are shown in parentheses.

the position expected for a heme–azide adduct, and a strong peak at m/z 630, a peak readily explained by loss of N_2 and acquisition of two protons (not shown). The NMR spectrum of the modified heme (Figure 6, Table I) is similar to those of δ meso-alkylated hemes except that no protons are present for a meso alkyl group (Ator et al., 1987). Of particular importance is the fact that only three meso proton signals are present in the NMR spectrum (Figure 6, Table I). Identification of the product as a *meso*-azido adduct of heme is confirmed by the presence of two sets of doublets of doublets due to the two internal vinyl protons, four doublets of doublets due to the four external vinyl protons, two triplets due to the four internal methylene protons of the two propionic acid side chains, four singlets due to the protoporphyrin IX methyl groups, and two triplets due to the external methylene protons of the propionic acid side chains. The *only* signal not present is thus that of one meso proton. The mass spectrum argues for simple addition of an azide moiety and the electronic absorbance spectrum for only a slight perturbation of the porphyrin conjugation. These results, in conjunction with the demonstration by NMR that the protoporphyrin IX framework is intact except for loss of a meso proton, unambiguously identify the prosthetic group as a *meso*-azidoheme adduct.

Four isomers are possible for a meso-substituted heme. The meso adducts obtained with phenylhydrazine and the alkylhydrazines have been shown by detailed nuclear Overhauser experiments to involve exclusive addition of the hydrazine-derived moiety to the δ meso position (Ator & Ortiz de Montellano, 1987; Ator et al., 1987). It has only been possible to obtain limited nuclear Overhauser data here because the *meso*-azidoheme adduct decomposes when it is reduced to the ferrous state for NMR analysis. We have been able to show that irradiation of the internal propionic acid methylene protons at 4.4–4.5 ppm cleanly enhances the meso proton signal at 10.294 ppm, and vice versa. The γ meso position between the two propionic acid side chains is thus clearly not the site of azide substitution. We have not been able to carry out the more complicated nuclear Overhauser experiments required to differentiate between α, β, and δ meso substitution. The fact that the chemical shifts of the two internal vinyl protons differ by less than 0.06 ppm, however, indicates that the azide group is not located at the α or β meso positions because the chemical shift of the internal vinyl proton vicinal to the azide should be substantially different from that of the second internal vinyl proton. For example, the chemical shifts of the methylene protons of the ethyls vicinal to the substituent in meso-substituted octaethylporphyrins differ from those of the other methylene protons by 0.32 ppm (*meso*-cyano) (Inhoffen et al., 1966), 0.22–0.40 ppm (*meso*-nitro) (Cavaleiro et al., 1986), and 0.23–0.39 ppm [*meso*-(benzoyloxy)] (Jackson et al., 1987). Similarly, the chemical shifts of the two internal vinyl protons in α-(benzoyloxy)protoporphyrin IX differ by 0.39 ppm and those in β-(benzoyloxy)protoporphyrin IX by 0.26 ppm (Jackson et al., 1987). Similar arguments can be made for the terminal vinyl protons cis to the porphyrin ring, which only differ in the azide adduct by 0.035 ppm. Indirect support for the proposal that the azide moiety is at the δ meso position is provided by the fact that the alkyl- and arylhydrazines react exclusively with the δ meso position. It thus appears that reaction of HRP with sodium azide results in addition of the azide moiety exclusively to the δ meso position of the prosthetic heme group.

HRP Reconstituted with meso-Azidoheme. Replacement of the heme group of horseradish peroxidase with *meso*-azidoheme isolated from a separate incubation of HRP with sodium azide and H_2O_2 yields an enzyme with spectroscopic properties very similar to those of azide-inactivated HRP. The A_{280}/A_{404} ratios of enzyme reconstituted with heme and with *meso*-azidoheme are essentially the same, which suggests that the extinction coefficients of the two prosthetic groups are comparable. The *meso*-azidoheme-reconstituted enzyme has

no detectable catalytic activity when assayed by the standard guaiacol oxidation assay even though it is more unstable to incubation with azide and H_2O_2 than native HRP. Addition of sodium azide and H_2O_2 to the reconstituted enzyme immediately yielded a species with a Soret maximum at 424 nm. This absorbance band was gradually replaced by a band at 404 nm if low concentrations of azide and H_2O_2 were used. With high concentrations of these reagents, the Soret maximum does not shift but rather slowly disappears. Thus, only 30% of the initial absorbance remained after incubation of the enzyme for 20 min with 1 mM sodium azide and 1.2 mM H_2O_2.

Discussion

The interaction of azide with HRP under catalytic turnover conditions was shown by Brill and Weinryb (1967) to inactivate the enzyme and by Kalyanaraman et al. (1985) to generate the azidyl radical, although Brill and Weinryb concluded that catalytic turnover was not required for inactivation. We show here that catalytic turnover *is* actually essential and that the enzyme is inactivated by addition, probably of the catalytically generated azidyl radical, to one of the meso positions of the prosthetic group. Inactivation of HRP by azide is thus a pseudo-first-order, irreversible process with K_I = 1.47 mM and k_{inact} = 0.69 min^{-1} (Figure 1). The inactivation, as required of a catalytically driven process, depends on the concentration of H_2O_2 (Figure 2). Inactivation of 80% of the enzyme is achieved with approximately 60 equiv of azide and H_2O_2, although substantially higher azide:HRP ratios are required to achieve complete inactivation of the enzyme (Figure 3). The inactivation by azide, like that by phenylhydrazine (Ator & Ortiz de Montellano, 1987), may be inhibited by the accumulation of an unidentified product that can be removed by gel filtration. The residual activity decreases as the initial azide and peroxide concentrations are increased, but complete inactivation requires gel filtration and reincubation with azide and peroxide rather than simple addition of more azide and peroxide to the initial incubation. Brill and Weinryb presumably did not detect the essential role of peroxide in their experiments due to inactivation of the enzyme by the 1 mM sodium azide still present when H_2O_2 was added to assay residual catalytic activity.

Brill and Weinryb (1967) postulated that a methionine residue is modified in the reaction of HRP with azide even though their amino acid analyses only provided evidence for loss of a proline. The present results clearly show, however, that inactivation is due to addition of azide to the prosthetic heme group. The loss of approximately 80% of the catalytic activity thus correlates well with addition of azide to approximately 75% of the prosthetic heme groups, particularly if allowance is made for the fact that the percent of modified prosthetic groups may be slightly underestimated due to more rapid degradation of *meso*-azidoheme than heme. Conversely, the 20% residual catalytic activity obtained with 60 equiv of sodium azide is fully explained by the fraction of the enzyme that retains its heme group intact under those conditions.

The reactions of heme with the azidyl, phenyl, phenylethyl, ethyl, and methyl radicals are comparable in that the radicals add to only one of the four possible meso positions to give the corresponding meso-substituted prosthetic group. The carbon radicals have been unambiguously shown to react only with the δ meso position. The data on the regiospecificity of the azidyl radical are less definitive but nevertheless suggestive of reaction with the δ meso position. Adducts from reaction of the radicals with the other meso positions have not been detected and, if formed, must be minor products. Reaction

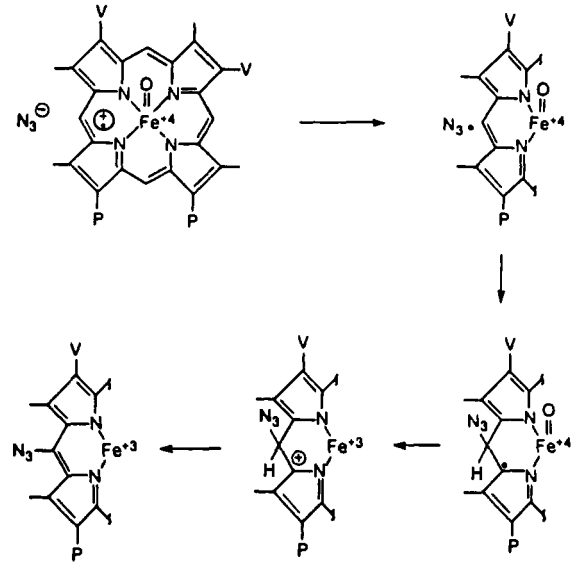

FIGURE 7: Mechanism proposed for addition of azide to the δ meso carbon of the prosthetic heme group of horseradish peroxidase.

of the prosthetic group of HRP with the phenyl radical, however, does yield a second product identified as the 8-hydroxymethyl derivative of heme. This product is not formed with the alkyl or azidyl radicals, presumably because they are more nucleophilic and less reactive. These two properties favor addition to the meso carbon over hydrogen atom abstraction.

The strong parallels between the reactions of HRP with sodium azide and the alkylhydrazines suggest that both proceed by similar mechanisms (Figure 7). The first step is therefore likely to be oxidation of the azide anion to the azidyl radical with concomitant reduction of compound I to compound II. Spin trapping studies have specifically demonstrated that the azidyl radical is formed in incubations of sodium azide with HRP (Kalyanaraman et al., 1985). Our work with the hydrazines suggests that electrons are transferred from substrates to the heme edge in the vicinity of the δ meso carbon (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano, 1987). The azidyl radical therefore probably adds to the δ meso position to give an isoporphyrin that, by loss of the meso proton, yields *meso*-azidoheme. The isoporphyrin intermediate is too unstable to detect spectroscopically, however, even though the isoporphyrins formed by addition of *alkyl* radicals are readily detected. The azide reaction is more like that with phenylhydrazine, which also does not give a detectable isoporphyrin intermediate. The factors that determine the stabilities of isoporphyrin intermediates are not known and are under investigation.

Both catalase and HRP oxidize azide to the azidyl radical (Kalyanaraman et al., 1985), but only HRP is inactivated by meso addition of the azidyl radical to the prosthetic heme group. Catalase and HRP likewise oxidize nitroalkanes to nitroalkyl radicals, but only HRP is inactivated by meso addition of nitroalkyl radicals to the prosthetic heme group (Porter & Bright, 1983). Catalase is inactivated by the nitroalkyl compounds but apparently by a reaction with the protein rather than with the heme group (Porter & Bright, 1987). Finally, reaction of phenylhydrazine with catalase results in formation of a phenyl–iron σ complex rather than, as with horseradish peroxidase, in meso phenylation of the heme. These differences are consistent with the active site structures proposed for the two enzymes. The crystal structure of catalase shows that the active site is reached via a narrow channel that delivers incoming substrates directly to the iron (Fita & Rossman, 1985). The meso heme positions are pro-

tected by protein residues in the catalase active site and are therefore not readily accessible to catalytically generated species. The phenylhydrazine-derived free radical is therefore trapped by the iron to give the observed phenyl–iron complex. In contrast, the meso regiospecificity of prosthetic heme alkylation by alkyl- and arylhydrazines has led us to propose that the ferryl oxygen in HRP compounds I and II is physically sequestered so that substrates interact with an exposed heme edge rather than directly with the ferryl oxygen (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano, 1987). Reaction of phenylhydrazine with HRP thus yields a meso heme adduct but not an iron–phenyl complex. By analogy, the azidyl radical should add, as observed, to the exposed meso carbon of HRP but not to the protected meso carbons of catalase.

Smith et al. (1979) reported that reaction of azide with the radical cation derived from zinc or magnesium etioporphyrin yields a product with the electronic absorption properties expected for a *meso*-azidoporphyrin. The product was too unstable to isolate, however, and was therefore not characterized. The azido adduct described here thus appears to be the first well-characterized *meso*-azidoporphyrin. Ferric *meso*-azidoprotoporphyrin IX, in contrast to the porphyrins described by Smith et al., is reasonably stable in the absence of reducing agents. In the presence of reducing agents it decomposes rapidly to unidentified products, a trace of which have biliverdin-like properties.

The first step in the degradation of heme to biliverdin either by heme oxygenase or by coupled oxidation is introduction of a hydroxyl group at the meso position that is cleaved (Schmid & McDonagh, 1979). The mechanism of this hydroxylation reaction is not known. Nevertheless, the observation that a catalytically generated nitrogen radical adds to the meso position suggests that the hydroxyl is analogously introduced by addition of a catalytically generated oxygen radical. Further work will be required to determine if the reactivity of the azidyl radical has a parallel among oxygen radicals.

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