Interaction of Peroxidases with Aromatic Peracids and Alkyl Peroxides*

PRODUCT ANALYSIS*

(Received for publication, June 1, 1971)

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SUMMARY

Two limiting structures may be assigned to the primary derivative of peroxidases (Compound I) demanding either (a) total or (b) partial retention of peroxide components. It is now shown that (a) interaction of horseradish peroxidase with m-nitroperbenzoic acid, in 15 mM phosphate, pH 6.2, 25°, leads to the stoichiometric formation of the enzyme-peroxide derivative, m-nitrobenzoate and 1 proton; and (b) in 5 mM phosphate, pH 6.6, 10°, the conversion of horseradish peroxidase into Compound I with ethyl hydrogen peroxide, is attended by the release of ethanol, but without concomitant prototropic changes. The results establish that horseradish peroxidase (H-peroxidase) Compound I is not an enzyme peroxide complex, but a derivative in which the active site is oxidized.

The optical spectra of H-peroxidase and Compound I in 5 mM phosphate, pH 6.6, 25°, were re-examined and extended to include previously undefined features in the near infrared absorption region. The spectrum of Compound I is devoid of bands typical of isoporphyrins, suggesting that conversion of the enzyme into Compound I does not involve covalent modification of the prosthetic group.

Reactions mediated by peroxidases involve the intermediary formation of an enzyme derivative known as Compound I.1 Theorell (2) defined it as a "... primary, addition compound, formed by equivalent amounts of hydrogen peroxide and peroxidase ..." Expressed in these terms, the formation of Compound I resembles other peroxidase-ligand interactions (Equations 1 and 2).

Enzyme + Ligand ↔ Enzyme-Ligand complex

(E) L (E=L) (1)

H-peroxidase + H₂O₂ ↔ H-peroxidase·H₂O₂ (2)

Kinetically and stoichiometrically, the above scheme is viable (2-6); but the optical spectrum of Compound I (7-11), its magnetic susceptibility (12, 13), the lack of a discernible EPR signature (14, 15), and a distinctive Mossbauer spectrum (11) are unlike those of other, high or low spin, peroxidase-ligand complexes (16, 17). Moreover, George's discovery (8, 18, 19) that Compound I can be formed with nonperoxidatic oxidants created further doubts as to the enzyme-substrate nature of this derivative and led to several hypotheses of Compound I formation. These can be summarized in three stoichiometric mechanisms, representing (a) electron (or hydrogen) transfer (8) (Equation 3); (b) retention of oxygen atom (15, 20) (Equation 4); and (c) incorporation of oxidant components into the enzyme matrix (21) (Equation 5).

2H⁺ + E + ROOH + (E.ROOH) ↔ E⁺ + ROH + H₂O (3)

E + ROOH ↔ (E·ROOH) ↔ E⁺ + ROH (4)

E < OH OR (5)

Which of the above schemes corresponds to the actual reaction pathway can be decided only by total analysis of the reaction products. These objectives are partly met in the following experiments.

EXPERIMENTAL PROCEDURE

Materials

Electrophoretically purified H-peroxidase (Lot HP0FF 8 AF, A₄₅₀·₄₃₀ = 3.25) was obtained from Worthington as a lyophilized powder. The isoenzyme composition of this preparation has not been defined but appears to represent predominantly Isoenzyme C (22).

Cytochrome c peroxidase (A₄₅₀·₄₃₀ = 0.9) was prepared according to Yonetani (23).

Bonniesen's procedure (24), as modified by Nagahusa (25), was adopted in the preparation of crystalline horse erythrocyte catalase (A₄₃₀·₄₃₀ = 1.32).
from the molar absorptivities of alkaline permanganate. Scientific Co. solutions were made in water distilled from lithium and Masher's method (27), but without use of methanol in the reaction medium.

Boehringer Mannheim Corporation was the source of yeast alcohol dehydrogenase, while nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH), Grade III, were Sigma products.

Fig. 1. A, spontaneous reduction of Compound I to Compound II in 1 m phosphate buffer, pH 6.0, 25°C. (H-peroxidase) 83.9 X 10^{-4} M, (H2O) 90 X 10^{-3} M. At 566 nm, Compound I and Compound II are isosbestic, showing that in the initial stages of Compound I reduction free enzyme is not formed. Scan, 25 A per s. B, illustrating biphasic course of Compound I reduction; first, mainly to Compound II and then to free enzyme. At 631 ± 1 nm, Compound II and H-peroxidase are isosbestic.

Boehringer Mannheim Corporation was the source of yeast alcohol dehydrogenase, while nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH), Grade III, were Sigma products.

m-Nitroperbenzoic acid was synthesized in this laboratory, courtesy of Dr. J. Wodak, according to Silbert, Siegel, and Swern (26) or purchased from Raylo Chemicals, Ltd., Edmonton, Alberta.

Ethyl hydrogen peroxide was prepared, essentially, by Williams and Mosher's method (27), but without use of methanol in the reaction medium.

Other, highest purity reagents were obtained from Fisher Scientific Co. All solutions were made in water distilled from alkaline permanganate.

Assays

Hemoproteins—Concentrations of peroxidases were calculated from the molar absorptivities (m^{-1} cm^{-1}): ε_{403} 102 X 10^4 for H-peroxidase and ε_{408} 95 X 10^4 for Ce-peroxidase which, in turn, were based on hemin assays (28).

Hydrogen Peroxide—Hydrogen peroxide was titrated with potassium permanganate, standardized against oxalic acid.

m-Nitroperbenzoic acid, m.p. 88° (uncorrected), literature 90° (29), was estimated iodometrically (30). On this basis, its purity was 98%.

Ethyl Hydrogen Peroxide—Ethyl hydrogen peroxide was determined enzymically by two procedures; both agreed within ±4%. The first method was based on titration of Ce-peroxidase with peroxide (31). The second procedure was based on catalase-mediated oxidation of ethanol to acetaldehyde in the presence of peroxides (32, 33):

Catalase + EtOOH → Compound I

Assays were carried out in 0.1 M phosphate, pH 7, 25°C, using 5 X 10^{-3} M catalase, 5 X 10^{-4} M ethanol, and 10 to 40 X 10^{-4} M ethyl hydrogen peroxide. Acetaldehyde was estimated at 340 nm by measuring (yields) alcohol dehydrogenase catalyzed oxidation of NADH, taking ΔA_{522} 6.22 X 10^{5} M^{-1} cm^{-1}.

Purity of EtOOH—For analytical studies (Table III), it was essential to determine the amount of ethanol entrained in ethyl hydroperoxide. EtOOH was accordingly reduced to EtOH either with borohydride at pH 9 or enzymically in the presence of peroxidase and ferrocyanide at pH 7 [Fe(CN)₄²⁻:EtOOH ~ 3] and, following static, low temperature, low pressure evaporation of the sample ("Method"), the distillate was assayed for ethanol (34, 35). Any excess of ethanol above the amount expected from ethyl hydroperoxide represents impurity entrained in the peroxide sample; on a molar basis, the latter was equal to 12% (EtOOH:EtOH ≈ 1:0.14). The above method was validated by experiments with standardized ethanol solution.

Methods

Titration of H-peroxidase with m-NPA—Solutions of m-NPA, 3 to 5 mM, were prepared by dissolving a weighed amount of peracid in tertiary butanol and diluting it 100-fold in water. The aqueous peracid solution was kept at 0°C and used immediately in enzyme titrations. The latter were carried out in 0.02 M phosphate buffers, pH 6.15 ± 0.05, either at 5°C or 25°C. Small aliquots of peracid solution (1 to 5 μl) were transferred to 2.0 ml of peroxidases (4 to 8 μM) with a Hamilton syringe.

Preliminary Treatment of H-peroxidase with H₂O₂—In experiments aimed at product analysis of the H-peroxidase-EtOOH reaction, it was essential to decrease the rate of spontaneous reduction of Compound I since assays require 10 to 15 min. Such stabilization of Compound I (Fig. 1) was achieved by pretreating the enzyme (2.0 ml of 0.2 mM H-peroxidase in 5 mM phosphate, pH 6.6) with equimolar amounts of hydrogen peroxide (6).

Static Distillation—The apparatus consisted of two 15-ml test tubes joined by a Y-shaped connector attached, through a 4-mm pump stopcock, to a two-stage, high vacuum Precision pump.

Product Analysis of H-peroxidase-EtOOH Reaction—H-peroxidase solution, 2 ml of ~0.1 mM, in 0.005 M phosphate, pH 6.65, was placed in a distillation flask, chilled to 0°C, and treated with 50 μl of 3.3 mM EtOOH. The solution, now containing 82 μM Compound I (165 nmoles), was immediately frozen at -190°C; the reaction vessel was attached to a static distillation assembly and, after evacuation to approximately 0.05 mm Hg, the system was isolated from the pump. Compound I was then melted at 9 ± 2°C and static distillation began by immersing the receiver flask in liquid nitrogen. After collecting at least 0.7 ml (2 to 3 min), distillation was interrupted and the sample (Distillate, Table III) was set aside for ethanol assays. Enzyme, 0.1 ml, from the remaining solution (Residue, Table III) was diluted in...
Spectrophotometric Measurements—Spectrophotometric measurements were carried out at controlled temperatures, using a Cary model 14 recording spectrophotometer.

Potentiometry—The pH of the reaction medium was measured with a Radiometer model 25 pH meter equipped with a Radiometer type GK 2021 C combined calomel glass electrode. The same assembly was used in potentiometric titrations of H-peroxidase with H+ or m-NPA. The measurements were carried out in a closed, thermostated, reaction vessel, the solution being continuously stirred by a Teflon-coated magnet. All additions were made through a septum protected port.

RESULTS

Spectra of H-peroxidase and Compound I—As used in the present experiments (Fig. 2), these spectra include the previously unexplored region between 700 to 1300 nm. From 370 to 700 nm, the spectra are similar to those given by Chance (10), while the extinction coefficients are in close agreement with the data reported by Blumberg et al. (9). Note also that the spectrum of Compound I is independent of the nature of the oxidant. Chance (7) and, more recently, Moss et al. (11) observed this identity, using H₂O₂ and monoalkyl hydrogen peroxides; and, as Fig. 2 indicates, the same is true for the compound given by H-peroxidase with m-nitroperbenzoic acid.²

² In addition, identical results were obtained with p-nitro, m-chloro, and p-methylperbenzoic acids.

Stoichiometry of Enzyme-Peracid Reaction—The stoichiometry of the enzyme-peracid reaction is 1:1 (Fig. 4). The titrations were made in 0.015 M phosphate buffer, pH 6.1 at 5°. Absorbance changes were recorded within 5 s of mixing the reagents and conversion of H-peroxidase into Compound I computed, using \( \Delta \varepsilon = 38 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \), at 411 nm.

Hydrogen peroxide, which might be formed hydrolytically, e.g. Equation 6,

\[
- \text{RCOO}^- + \text{H}^+ + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2 + \text{RCO}_2^-
\]

is not an intermediate in the above reaction since the rate constant for the formation of Compound I is greater with m-nitroperbenzoic acid \( (k = 2.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) \) than with hydrogen peroxide \( (k = 1.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) \). Yet, the spectroscopic analyses of the peroxidase-peracid reaction (Table I) suggest that the experimentally observed \( \Delta A_x \) is equivalent to the sum of absorbances contributed by Compound I and the m-nitrobenzoate anion. The calculations were based on the following relationship,

\[
\Delta A_{x,tot} = (\text{RCO}_2\text{H}) \Delta A_x + \varepsilon \lambda
\]

where \( \Delta A_x \) denotes the difference in molar absorptivities between the enzyme and its primary hydrogen peroxide compound at \( \lambda \) nm (Fig. 3) (cf. to Brill and Sandberg (36)); \( \varepsilon \) is the extinction coefficient of m-nitrobenzoate at the same wave length (Fig. 5) and \( (\text{RCO}_2\text{H}) \) is the molarity of m-nitrobenzoic acid.

The validity of Equation 7 was tested between 225 and 275 nm, according to the procedure outlined in Fig. 6. Approximately 4.8 \( \mu \text{M} \) H-peroxidase was titrated with the peracid, each addition being equivalent to 1.5 \( \mu \text{M} \) oxidant. At 235 nm, for example, \( \Delta A_{x,tot} \) was estimated as \( 1.5 \times 10^{-4} (7.3 \times 10^8 + 5.4 \times 10^9) \) or 0.019 \( \text{cm}^{-1} \), while the observed values were 0.021, 0.019, and 0.019 \( \text{cm}^{-1} \). On further addition of peracid, when little unreacted peroxidase remained in the solution, the absorb-
Interaction of horseradish peroxidase with m-nitroperbenzoic acid (H-peroxidase) T 4.8 μM; pH 6.2; 0.015 M phosphate, 25°C.

<table>
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<tr>
<th>λ (nm)</th>
<th>ΔA (observed)</th>
<th>ΔA (calc.)</th>
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<tr>
<td>275</td>
<td>1.2</td>
<td>0.011</td>
</tr>
<tr>
<td>270</td>
<td>-1.2</td>
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<tr>
<td>257</td>
<td>0</td>
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<tr>
<td>240</td>
<td>5.2</td>
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<td>230</td>
<td>8.3</td>
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</tr>
<tr>
<td>225</td>
<td>9.2</td>
<td>0.022</td>
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</table>

*Within 5 s of peracid addition.*

**Fig. 4.** Titration of horseradish peroxidase (8.3 × 10⁻⁶ M) with m-nitroperbenzoic acid, 0.015 M phosphate, pH 6.1, 5°C; λ 411 nm. Each point represents a single experiment using the same H-peroxidase solutions.

**Fig. 5.** Spectrum of m-nitroperbenzoic acid (—) and m-nitrobenzoate (—); 0.05 M phosphate, pH 5.5, 25°C.

**Proton Release in H-peroxidase-m-NPA Reaction**—Fig. 7 illustrates the changes in pH attending the reaction of H-peroxidase with m-NPA. H-peroxidase solution, 0.1 mM, at pH 6.64, 25°C, was first treated with 15 μM H₂O₂ and then, twice, with 22 μM m-NPA. In the reaction with hydrogen peroxide, no release, or uptake, of protons was detected but the expected amount of proton changes became biphasic, showing (a) an increase (≈0.016) attributable mainly to peracid itself (ΔAₚₚₚ = 1.5 × 10⁻⁶ × 10.8 × 10⁶ = 0.0152, where 10.8 × 10⁶ = Δₚ₀ₐ for a 0.05 M solution) followed by (b) a decrease caused by reduction of m-NPA to m-nitrobenzoate: ΔA = 0.008 = (Δₚₚₚ - 0.005) (m-NPA) (m-NPA) = (10.8 - 5.4) 10⁶ × 1.5 × 10⁻⁶. However, this secondary side reaction is nearly a 1000-fold slower than Compound I formation and does not complicate analysis of the data when enzyme is in excess over peracid.

**Fig. 3.** Difference spectrums, H-peroxidase Compound I versus H-peroxidase (—); (H-peroxidase) 4.7 × 10⁻⁶ M; (H₂O₂) 0 × 10⁻⁶ M. Scan, 25 A per s. Points represent titration of enzyme with H₂O₂ at indicated wave length. (H₂O₂) varied from 0.5 to 6 × 10⁻⁶ M; 0.05 M phosphate, pH 6.2, 25°C.

Reduction of Compound I by ferrocyanide, also shown in Fig. 7, occurred with the uptake of 1 mole of protons per mole of reductant. The spectrophotometric assays of Compound I formation and not complex analysis of the data when enzyme is in excess over peracid.
the preformed Compound I (50 μM) resulted each time in the uptake of 43 ± 3 μM H⁺. The amount of ferrocyanide required for complete reduction of Compound I was 114 ± 4 μM eq (cf. theoretical 118 μM), in agreement with Equation 8.

$$\text{Compound I} + 2[\text{Fe(CN)}_6]^{3-} + 2 \text{H}^+ \rightarrow \text{H-peroxidase} + 2[\text{Fe(CN)}_6]^{4-} \quad (8)$$

**Product Analysis of H-peroxidase-EtOOH Reaction**—The results, shown in Table III, indicate that formation of Compound I is attended by the release of ethanol. In evaluating the data, note that:

1. In all experiments (H-peroxidase) > (EtOOH), ensuring that no unreacted peroxide remained in the reaction mixture.
2. Ethanol, 94 ± 2%, is recoverable by static distillation; this was demonstrated using standardized 10 to 50 μM ethanol solutions.
3. The ratio of total recovered ethanol (from the distillate and

<table>
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<tr>
<th>Experiment</th>
<th>Method</th>
<th>Initial conditions</th>
<th>Residue</th>
<th>Distillate</th>
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<td>Partial distillation; distillate 45% v/v ...</td>
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<td>165</td>
<td>130 31 87 102</td>
</tr>
<tr>
<td>4</td>
<td>Extensive distillation; distillate 85% v/v ...</td>
<td>200</td>
<td>165</td>
<td>52 89 0 183</td>
</tr>
<tr>
<td>8</td>
<td>Distillation to dryness after reduction with 5-fold molar excess of ferrocyanide ...</td>
<td>200</td>
<td>165</td>
<td>0 0 0 185</td>
</tr>
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</table>

*Average of four assays.*

*Initial volume (v₁) = 2.65 ml.*

**TABLE II**

<table>
<thead>
<tr>
<th>pH before addition of peroxide</th>
<th>H₂O₂ × 10⁶</th>
<th>m-NPA × 10⁻⁸</th>
<th>pH after formation of Compound I</th>
<th>Compound I × 10⁻³</th>
<th>ΔH⁺(H₂O) × 10⁻³</th>
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<tr>
<td>6.64</td>
<td>15</td>
<td>m</td>
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<td>m</td>
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<tr>
<td>6.55</td>
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<td>44</td>
<td>m</td>
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<td>38</td>
<td>44</td>
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FIG. 6. Titration of H-peroxidase (4.8 × 10⁻⁴ M) with m-nitroperbenzoic acid; 0.015 M phosphate, pH 6.2, 25°C; λ 235 nm.

FIG. 7. Effect of oxidants (H₂O₂ and m-NPA) and ferrocyanide on proton balance in the presence of 10⁻⁴ M H-peroxidase, 25°C. H₂O₂ causes no change in the hydronium ion concentration (pH_initial 6.64), but with m-nitroperbenzoic acid 1 proton is released per mole of Compound I formed (pH_f 6.64). Reduction of Compound I by ferrocyanide results in the uptake of 2 protons (Note, downward deflection of the trace indicates proton release, cf. to calibration with HCl). m-NPBA = m-nitroperbenzoic acid.
are relatively slow (38). Typically, the rate constants are seldom greater than 10^{-3} M^{-1} s^{-1}, so that the reaction half-time at an aliphatic carbon atom, whether by SN1 or SN2 mechanism, alkyl hydroperoxides. In addition, nucleophilic substitutions at according, there is no precedent for S1 solvolysis of primary tert,ary peroxides, such as diphenyl ethyl peroxide (37) which

An alternative, but less likely, mechanism allowed by our observations is shown in Equation 10. The envisaged oxidation-reduction reorganization of the initial enzyme-peroxide complex through heterolysis of the O−O bond would be particularly plausible if the reaction is subject to general acid-general base catalysis, either by water, or by a system YH contributed by the apoprotein. This suggestion follows the established mechanisms of peroxide heterolysis. Thus, extending Bateman and Hargrave’s concepts (42) to the peroxidase reactions, an activated complex between the enzyme peroxide and general acid (YH) could be (Equation 11).

$$E + ROO \rightarrow E(ROO)$$

$$E(ROO) \rightarrow E(RO) + OH$$

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Without the participation of YH, scission of the oxygen-oxygen bond entails electrostatically unfavorable charge separation, particularly if the microscopic dielectric constant near the active site is low. This can be avoided by a concerted proton transfer in the transition state but demands rigid orientation of the reactants. Hence, the entropy of activation for the H-peroxidase-peroxide reaction should be quite negative. Preliminary experiments indicate that this is so.

The above model also suggests that disruption of the hydrogen bonded lattice should greatly decrease or abolish enzyme activity. Inhibition of H-peroxidase following selective and reversible acetylation of the enzyme (43), at a residue situated near the coenzyme, is consonant with this interpretation. The observed rate constants for Compound I formation (k_{app}) with m-NPA, H_{2}O, and EtOOH, are of the same order of magnitude, 1.2 ± 0.8 × 10^{-7} M^{-1} s^{-1} (6), in spite of large differences in the basicity of the leaving group (p_{Ka}: m-nitrobenzoic acid, 3.45, water, 15.7, ethanol, 18). This implies that interaction of H-peroxidase with the substrate does not proceed by an unassisted nucleophilic displacement on the outer peroxide oxygen. Whenever such reactions occur, as for instance in the epoxidation of trans-stilbene by substituted perbenzoic acids, a good correlation is observed between the rate of reaction and the basicity of the leaving group (44). Equally, no relationship is apparent between k_{app}'s and O−O bond dissociation energies for H_{2}O (51 kcal per mole); EtOOH (43 kcal per mole) and perbenzoic acids (~35 kcal per mole).

Seission of EtOOH and release of EtOH were observed also with Ce-peroxidase and wheat germ peroxidase (unpublished results).
Accordingly, we must assume that the rate-limiting step is virtually independent of the polarizability of the peroxide O—O bond or the nature of the leaving group. The remaining possibilities are therefore (a) association of H-peroxidase with the peroxide; (b) ligand interchange at the sixth coordination position; and (c) rearrangement of iron-protoporphyrin associated with the enzyme-peroxide oxidation-reduction reaction. In all cases, the dissociation constant of any initial enzyme-substrate complex must be very large since no deviation from second order kinetics has yet been observed, even at high concentrations of peroxide (6).

Any structure assigned to Compound I must also explain its physical properties such as absorption spectrum (7, 9), magnetic susceptibility (12), EPR (15), and Mössbauer spectra (11). The magnetic susceptibility of horseradish Compound I is 6600 × 10⁻⁴ e.m.u. (12), consistent with the presence of three unpaired electrons. Such a distribution of electrons can be expressed in terms of (a) FeIV; (b) a radical combined with FeIV; and (c) a diradical in conjunction with low spin FeII. None of the three possibilities reflects fully the nature of Compound I but, as implied by Winfield (45) and emphasized by Hamilton (46), all could contribute to its resonance form.

The chief objection to the proposals implicating a free radical moiety stems from the absence of a distinct EPR signature for Compound I at g ~ 2 (14, 15). This difficulty led Peisach et al. (15) to propose the incorporation of an oxygen atom, most probably in the protoporphyrin. Nonetheless, such arguments are not necessarily conclusive (47, 48), since in Compound I the spin relaxation times may be so short that no resonance absorption can be discerned. It was, therefore, argued (46) that among structures contributing to Compound I resonance hybrid, Structure B (Equation 12) should be taken into consideration.

\[
\begin{align*}
\text{Fe}^{III} & \text{OH}_2 \quad + \text{H}_2\text{O} \\
\text{Fe}^{IV} & \text{O}^{-} \quad + \text{H}_2\text{O} \\
\text{Fe}^{III} & \text{OH}_2 \quad + \text{H}_2\text{O} \\
\text{Fe}^{IV} & \text{O}^{-} \quad + \text{H}_2\text{O} \\
\text{Fe}^{III} & \text{OH}_2 \quad + \text{H}_2\text{O} \\
\text{Fe}^{IV} & \text{O}^{-} \quad + \text{H}_2\text{O} \\
\end{align*}
\]

(\(X^{++}\) denotes a radical moiety, for instance, a porphyrin \(\pi\)-cation radical).

According to Dolphin et al. (49), the spectroscopic data accord with the expected properties of such a porphyrin \(\pi\)-cation radical. Also, stabilization of a free radical in highly conjugated porphyrins has been demonstrated on reversible one electron oxidation of magnesium and cobaltic octaethyl porphyrins (50, 51) and zinc and magnesium tetraphenylporphyrins (50). In all cases, the optical spectra of one electron oxidation products share features also found in Compound I. These are (a) a decrease of \(\pi - \pi^*\) transitions associated with the Soret band and (b) the appearance of bands in the 620 to 670 nm region. Alternative interpretations of Compound I spectrum, such as an Fe(III)-peroxide complex in which the haem is bent (52) or in which the RO— component of the substrate is covalently bound at the methane bridge of the prosthetic group (21), are difficult to reconcile with our results showing release of the ROH group and the absence of infrared bands in the 500 to 900 nm region typical of porphyrin addition compounds (53).

According to Moss et al. (11), the presence of Fe(IV) in the oxidized enzyme Compound I is consistent with the information given by the Mössbauer spectra. Oxidation of the ferric ion to the Fe(IV) state would be unlikely if metal ion-bridged interaction were ionic but, in compounds with a substantial covalent character, definition of the oxidation state becomes less certain as evident in coordination compounds with delocalized ground states, such as metal dithienes (54, 55) and metal-nitric oxide complexes (56).

It seems unrealistic, therefore, to interpret all Compound I properties solely in terms of the interplay of electronic effects within the prosthetic group. The influence of the environment, particularly that of the trans-ligands, must also be taken into account. Ideally, such ligands should be polarizable, thus contributing to charge delocalization, but the process must not be so efficient as to cause their oxidation. An imidazole group at the fifth position, proposed by Brill and Sandberg (36), could fulfill such a function and, as the sixth ligand, the oxygen anion meets these requirements as shown by its complexes with higher oxidation state cations in TiO₂⁻, CrO₄⁻, MnO₄⁻, FeO₄⁻, or OsO₄. The ability of oxygen to stabilize such higher oxidation states is due both to its sigma-type coordination and \(\pi\)-donor bonding. The stability of Compound I would be equally enhanced if at least one oxygen were coordinated to the prosthetic group, as in Structure B (Equation 12). Solvation of such a complex to give a dihydroxy, heptacoordinated derivative

\[
\begin{align*}
X^{++} & \text{Fe}^\text{IV} \quad \text{OH} \\
\end{align*}
\]

is another possibility. It is not precluded by any current criteria of Compound I structure and offers an insight into the possible pathways of Compound I interaction with hydrogen donors through ligand intercalation at the sixth coordination position.

Acknowledgments—We thank Professor B. Chance for his frequent hospitality and unfailing cooperation, and Mrs. P. Fackre for her excellent assistance.

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