In the electrochemistry of plastocyanin, $MgCl_2$ was required to realize the direct electrode reaction.[7] Consequently, the present study is the first direct electrochemistry of blue copper proteins at an untreated glassy-carbon electrode in the absence of mediator, promoter, or additive. This success may be due to the fact that the present three blue copper proteins carry a large overall positive charge (The p*I* values of pseudoazurin, plantacyanin, and stellacyanin are 8.9, 10.6, and 9.9, respectively).[9a,12,13] Very recently, Armstrong et al.[24] proposed a microscopic model for the electrochemistry of cytochrome *c*, plastocyanin, and ferredoxin at an edge- and basal-plane graphite electrode. The new model assumes that electron transfer occurs at arrays of oxygen-containing functional sites of microscopic dimensions. Oxygen-containing sites generated by surface abrasion (polishing) may facilitate the access of basic blue copper proteins, although the total charge on the glassy-carbon electrode is supposed to be positive under the present experimental conditions.[25] The combined effect of a local protein structure change by deprotonation from several amino acid residues and an electronic state change in the cupric and cuprous ions would result in the present diverse pH dependence for the redox behavior. The primary factor will differ for each protein and pH. Flash photolysis of the three blue copper proteins is under investigation.

Acknowledgment. We are grateful to the ministry of Education, Science, and Culture of Japan for a Grant-in-Aid (No. 01540505). We are partly indebted to Dr. Takamitsu Kohzuma of Osaka University for the preparation of pseudoazurin.

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Iron Porphyrin Models of Peroxidase Enzymes: Catalytic Activity, Regeneration, and Oxidative Degradation of Mesoferriheme

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The reaction of the iron(III) complex of mesoporphyrin IX, mesoferriheme (mfh), with two-electron oxidants, such as sodium hypochlorite and *m*-chloroperoxobenzoic acid, produces a spectroscopically distinct oxidized heme species viewed as a functional analogue of the reaction intermediate obtained by oxidation of hemoprotein peroxidase enzymes. Unlike the peroxidase reaction product, which is the result of a 1:1 stoichiometric interaction between enzyme and oxidant, however, the oxidized mfh species displays a stoichiometric equivalence of 2 mol of heme Fe(III)/mol of two-electron oxidant. This is consistent with previously reported results on the oxidation of deuteroferriheme (dfh) and is attributed to a two-electron oxidation of monomeric heme followed by comproportionation with the free-heme Fe(III) monomer to form a dinuclear oxidation product. Second-order rate constants for the oxidation of mfh are comparable to those previously reported for dfh. Thus, although substitution of ethyl groups for hydrogen in the 2- and 4-positions of the porphyrin ring system has been shown to increase the degree of heme dimerization, it has a negligible effect on the rate of intermediate formation. Such substitution, however, does appear to increase the susceptibility of the porphyrin ring to oxidative degradation. Studies of the heme and oxidant concentration dependence of degradation suggest that porphyrin ring cleavage results from attack of the oxidant on the initially formed two-electron-oxidation product depicted as $Fe^{V}O$ and that degradation may be hindered by the scavenging action of the heme dimer on $Fe^{V}O$ with consequent comproportionation. The oxidized mfh species displays peroxidatic activity through biphasic oxidation of phenol and also undergoes biphasic in situ reduction, regenerating free mesoferriheme through processes thought to involve consecutive one-electron reductions of dinuclear analogues of enzyme compounds I and II. The formation of such dinuclear species probably relates to the tendencies of both mfh and dfh to undergo aggregation in contrast to coproferriheme, which has been reported to exist predominantly in monomeric form and to produce mononuclear oxidation products.

Introduction

Inasmuch as the catalytic activity of hemoprotein peroxidase enzymes involves redox chemistry of an iron(III) porphyrin IX or ferriheme prosthetic group,[1] a number of protein-free hemes have been studied as peroxidase models. In the absence of protein, however, ferrihemes undergo dimerization in aqueous solution[2-5] with a consequent decrease in peroxidase-like activity. The extent of dimerization is pH and concentration dependent[3] and also varies markedly with the nature of the substituents in the 2- and 4-positions of the porphyrin ring, decreasing in the series proto- > meso- > deutero- > coproferriheme[6] (figure 1). Since the reactivity of deuteroferriheme (dfh) is qualitatively similar to that of protoferriheme (pfh), and since it is readily prepared by treatment of pfh with molten resorcinol,[7] the relatively low degree of dimerization of dfh has commanded its widespread use as a peroxidase model system.[8-20]

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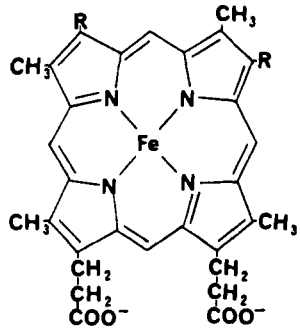

Figure 1. Iron(III)-porphyrin IX complexes (ferrihemes): R = $-CH=CH_2$, protoferriheme; H, deuteroferriheme; $-CH_2CH_3$, mesoferriheme; $-CH_2CH_2COOH$, coproferriheme.

Kinetic and mechanism studies of Jones and co-workers have shown that, as in the case of peroxidase enzyme systems, deuteroferriheme reacts with two-electron-oxidizing peroxo substrates, such as substituted peroxobenzoic acids, to form a reaction intermediate that subsequently serves as an oxidant of a variety of species, such as aromatic amines[17] and phenolate[16] and halide ions.[12] However, unlike the enzyme system in which intermediate formation is the result of a 1/1 molar interaction of peroxidase with oxidant, dfh oxidation exhibits an equivalence of 2 mol of monomeric heme Fe(III) to 1 mol of each of a number of two-electron oxidants, which include substituted peroxobenzoic acids,[11] iodosobenzene and its diacetate,[19] and hypochlorite ion.[20] A corresponding 4/1 stoichiometry is found for the reaction of monomeric heme and the four-electron-oxidizing ClO_2^- ion[15] in which the byproduct of initial oxidation is the two-electron oxidant OCl^-. This stoichiometry has been speculatively attributed to initial two-electron oxidation of heme to an analogue of peroxidase compound I followed by comproportionation with monomeric iron(III), as shown in Scheme I, where ROOH denotes a two-electron oxidant and where $Fe^{V}O$ and $Fe^{IV}{}_2O$ are intended to denote species in oxidation states above that of free heme without implication as to specific sites in which electron loss has occurred.

Scheme I

$$Fe^{III} + ROOH \rightarrow Fe^{V}O + ROH \quad (1)$$

$$Fe^{V}O + Fe^{III} \rightarrow Fe^{IV}{}_2O \quad (2)$$

Bretscher and Jones also have reported the formation of a mononuclear compound I analogue via the peroxoacid oxidation of coproferriheme under conditions in which the heme is presumed to exist predominantly in monomeric form.[21]

Studies on mesoferriheme (mfh) as a model system to date have been directed toward its dimerization equilibria,[5] oxidation by H_2O_2,[22] and the subsequent catalase-like (catalatic) decomposition of H_2O_2.[23] The rate of mfh oxidation by H_2O_2 was found to be comparable to that observed for monomeric deuteroferriheme;[22] however, due to oxidative decomposition of H_2O_2, studies of the stoichiometry of intermediate formation as well as the reactivity of the mfh-derived intermediate with respect to both its kinetic stability and peroxidase-like activity have been limited. For these reasons, we have extended our investigations of ferriheme model systems to the oxidation of mfh by hypochlorite, which, unlike H_2O_2, is not subject to peroxidatic oxidation. Also included are investigations of porphyrin ring degradation that accompanies heme oxidation, the in situ regeneration of heme from the oxidized reaction intermediate, and the peroxidatic activity of the intermediate species toward the oxidation of phenol.

Experimental Section

Materials. Triply recrystallized hemin was obtained from Nutritional Biochemical Corp. Sodium hypochlorite was obtained as a 5% solution in aqueous NaOH from Mallinckrodt and used to prepare stock solutions approximately 10^{-3} M in NaOCl that were then diluted to the desired concentrations for reaction with mesoferriheme. Hypochlorite concentrations were determined by iodometric analysis. Buffer components were of reagent grade, and NaCl was used to adjust each solution to the desired ionic strength. All solutions were prepared by using deionized water that was passed through a Barnsted mixed-ion-exchange column. The conductance, measured as NaCl, was less than 0.01 ppm.

Mesoferriheme. Mesoferriheme chloride was obtained by the reduction of hemin with H_2 in alkaline solution in the presence of 10% Pd on charcoal, according to the method of Davies.[24] In a typical preparation, 2 g of hemin was dissolved in 225 mL of CH_3OH and 25 mL of 0.5 N KOH. A total of 2 g of palladinized charcoal was added under an atmosphere of dry N_2, following which the mixture was boiled under reflux while H_2 was bubbled through the solution. The reduction was monitored by taking samples of the mixture, separating the charcoal by filtration, converting heme species to their respective pyridine hemochromes after reduction with $Na_2S_2O_4$, and scanning the spectrum of the solution from 370 to 610 nm. Hydrogenation was continued (~8 h) until only the mfh hemochrome bands at 518 and 547 nm were apparent (pfh derivative at 520 and 557 nm). Following the removal of charcoal by filtration, 100 mL of 1 M HCl was added to precipitate mfh. After being washed with H_2O and dried over NaOH, the crude mfh chloride was dissolved in 50 mL of $CHCl_3$ containing 2 g of quinine and this solution added dropwise to 75 mL of glacial acetic acid that had been saturated with NaCl and heated to >90°. Chloroform was removed by entrainment in a stream of N_2. Crystalline mesoferriheme was precipitated from the cooled solution, collected by filtration, sequentially washed with 50% acetic acid, water, and a mixture of EtOH and Et_2O, and dried over NaOH. Yield = 65%. The purity was in excess of 99%, as indicated by spectral analysis of the pyridine hemochrome derivative ($\epsilon = 2.74 \times 10^4$ M^{-1} cm^{-1}).

Apparatus and Methods. Stoichiometric and kinetic studies were based on optical density changes in the Soret region (389 nm), where the absorbance difference between heme and the oxidized species is relatively large. Measurements were carried out on a Dionex D-110 stopped-flow spectrophotometer in conjunction with a Tektronix Model 5310N and/or Model 2221 digital storage oscilloscope with HC100 digital plotter. Conventional ultraviolet and visible spectra were obtained with a Perkin-Elmer 552 spectrophotometer.

Three methods were used to measure the rate of reaction of mfh with NaOCl: a pseudo-first-order treatment employing excess oxidant, a second-order treatment in which initial concentrations of mfh and NaOCl were near stoichiometric equivalence, and initial-rate measurements in which absorbance changes were obtained over the first 1–5% reaction.

A. Pseudo-First-Order Treatment. Oxidation of mfh employing 10–20-fold excess NaOCl yields, over 2 half-lives, a linear plot of $\ln (A_t - A_\infty)$ vs time, where A_t denotes the absorption at time t and A_∞, the absorption corresponding to complete oxidation of mesoferriheme. The resulting pseudo-first-order rate constant, k_{obs} ($=-d[mfh]/([mfh]\,dt)$), obtained from the slope of the line is interpreted, in terms of Scheme I, as $k_{obs} = 2k_m\alpha[NaOCl]_0$, where k_m denotes the second-order rate constant for reaction of NaOCl with monomeric mfh and α represents the fraction of total mesoferriheme existing as a monomer depicted as Fe(III). Thus, $\alpha = [Fe(III)]/[mfh]_0$ where $[mfh]_0$ denotes the stoichiometric concentration of heme *calculated* as monomeric iron(III). The factor of 2 arises due to the rapid disappearance of a second Fe(III) unit following the rate-limiting attack of NaOCl on monomeric mfh. The usefulness of this method is diminished at $[OCl^-]/[mfh]$ ratios greater than 50 since, under such conditions, rates are too fast to be conveniently measured on a stopped-flow time scale. Also, high concentrations of oxidant tend to promote the oxidative cleavage of the porphyrin ring system with accompanying loss of optical density, thereby obscuring the absorbance change resulting from formation of the oxidized heme species.

B. Second-Order Treatment. Initial concentrations of mfh and NaOCl are denoted below as a and b, respectively, and the reaction variable, x, defined as $dx/dt = -d[NaOCl]/dt = -^1/_2 d[mfh]/dt$. A plot of $\ln [[a(b-x)]/[b(a-2x)]]$ vs time is linear with slope equal to $k_m\alpha$. This approach normally avoids problems arising from immeasurably fast reactions associated with the pseudo-first-order treatment but also may

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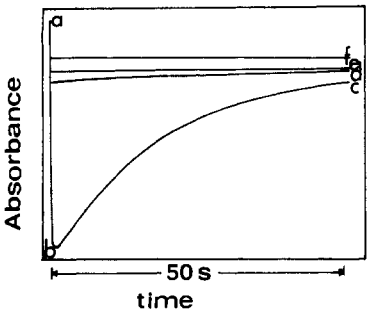

Figure 2. Absorbance–time profile for mesoferriheme–NaOCl with $[mfh]_0 = 9.14 \times 10^{-6}$ M, $[NaOCl]_0 = 9.08 \times 10^{-6}$ M, pH = 6.8, $I = 0.1$ M, and $T = 25$ °C: a, initial absorbance on mixing, A_0; b, absorbance of mfh oxidation product; c, absorbance level 50 s after formation of oxidized species; d, absorbance level 100 s after formation of oxidized species; e, absorbance level 150 s after formation of oxidized species; f, final absorbance level, A_∞. $\Delta A_{max} = a - b = 0.4$.

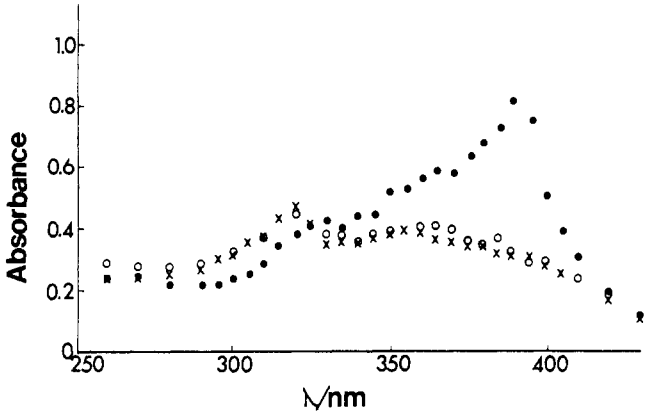

Figure 3. Absorption spectra of mesoferriheme and of the peroxidatically active intermediate obtained by oxidation with NaOCl and *m*-chloroperoxobenzoic acid at $T = 25$ °C, pH = 6.88, and $I = 0.1$ M: ●, 9.32×10^{-6} M mfh; ○, solution resulting from $[mfh]_0 = 9.32 \times 10^{-6}$ M + $[m\text{-CPBA}]_0 = 9.18 \times 10^{-6}$ M; ×, solution resulting from $[mfh]_0 = 9.32 \times 10^{-6}$ M + $[NaOCl]_0 = 13.9 \times 10^{-6}$ M.

be complicated by accompanying porphyrin ring cleavage.

C. **Initial Rate Treatment.** Disadvantages of the previous methods are avoided by measuring the rate in the early stages of reaction, where ring degradation has not yet become significant and where the absorbance change is essentially linear with time. Here, the term $(\Delta A_t/\Delta t)/\Delta A_{max}$ equates to $(\Delta[mfh]/\Delta t)/\Delta[mfh]_0$ and varies with hypochlorite concentration. The terms ΔA_t and ΔA_{max} are defined respectively as $A_0 - A_t$ and $A_0 - A_\infty$.

Studies of the peroxidatic activity of mesoferriheme were carried out by following absorbance changes accompanying the stopped-flow mixing of NaOCl with a mixture of mesoferriheme and phenol. Normally, the $[mfh]_0/[NaOCl]_0$ ratio was set near the stoichiometric ratio of 2.0 and the phenol concentration maintained in 3–15-fold excess relative to $[mfh]_0$.

Results and Discussion

The Mesoferriheme–Hypochlorite Reaction Sequence. As seen in Figure 2, the addition of NaOCl to mfh produces an initial decrease in absorbance in the Soret region corresponding to heme oxidation to a peroxidatically active species. This is followed by slower regeneration of the heme absorbance, signifying in situ reduction back to free mfh. Such regeneration is nearly always less than complete; i.e., the final (recovered) absorbance level is normally lower than that corresponding to the initial mfh concentration. This difference is taken as a measure of the degree of heme destruction occurring via porphyrin ring cleavage, a process known to accompany the oxidation of other model systems. Thus, at high H_2O_2 concentrations dfh has been shown to be degraded to biliverdin derivatives that are transparent in the Soret region.[25] Similar effects are observed in the reaction of dfh with other oxidants,[20] and extensive degradation of coproferriheme by

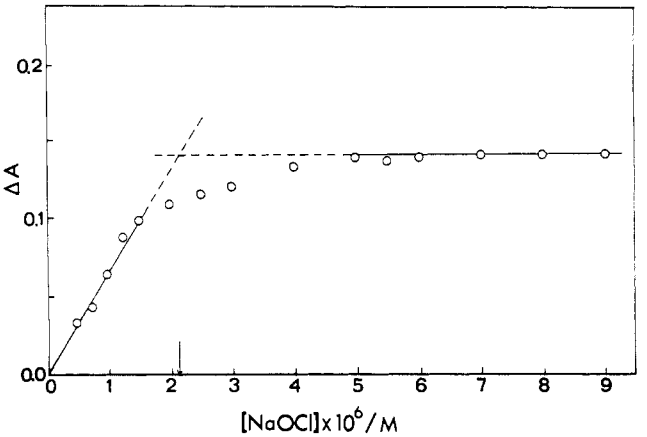

Figure 4. Stopped-flow spectrophotometric titration of mesoferriheme with NaOCl with $[mfh]_0 = 4.34 \times 10^{-6}$ M, $\lambda = 389$ nm, pH = 8.38, $I = 0.1$ M, and $T = 25$ °C. $[Fe(III)]/[OCl^-] = 2.02/1$.

Table I. Variation of Stopped-Flow Titration Data with pH ($I = 0.1$ M; $\lambda = 389$ nm: $T = 25$ °C)

pH[a]	[mfh]/[OCl⁻] (calc)	pH[a]	[mfh]/[OCl⁻] (calc)
10.25	1.20/1	8.69	1.85/1
9.64	1.54/1	8.42	2.07/1
8.83	1.85/1	8.38	2.02/1

[a] $H_2PO_4^-$–HPO_4^{2-} buffer at pH < 8.5; HCO_3^-–CO_3^{2-} at pH > 8.5.

m-chloroperoxobenzoic acid has also been reported.[26] A pattern of reaction formally similar to that shown in Figure 2 has also been shown to occur upon addition of peroxo substrates to coproferriheme[21] and in the reaction of a variety of oxygen-donor species with deuteroferriheme.[15,19,20]

Spectrum of the Oxidized Heme Species. In Figure 3, the mesoferriheme spectrum, obtained via the stopped-flow mixing of mfh with water, with water as a reference, is shown as a series of points at 5-nm intervals. The spectrum of the oxidized mfh species is obtained by subtracting from, or adding to, the mfh–H_2O spectrum the maximum absorbance decrement or increment resulting from the stopped-flow mixing of heme with a stoichiometric excess of oxidant. Within experimental error, the same solution spectrum is obtained upon reaction of mfh with *m*-chloroperoxobenzoic acid (*m*-CPBA) and with NaOCl. This is consistent with a mechanistic model of oxidation involving O atom transfer from oxidant to the heme iron(III) center, as depicted in (1).

Stoichiometry of Intermediate Formation. The equivalence of 2 mol of mesoferriheme (calculated as monomeric mfh)/mol of hypochlorite is shown in Figure 4, which depicts the "titration" of heme wherein the absorbance decrement at 389 nm is obtained as a function of NaOCl concentration for the stopped-flow mixing of NaOCl solutions with solutions containing a fixed concentration of mfh. The "apparent" [Fe(III)]/[oxidant] ratio decreases above pH 9 (Table I). This effect is not presently understood. The optical spectrum of the oxidized mfh species is not pH dependent in this region, which implies no pH dependence on the stoichiometric composition of the mfh oxidation product. As noted below, however, degradation of mfh is dependent on the initial concentration of hypochlorite. It also appears to be enhanced in highly alkaline solutions. Thus, it is possible that the lower [Fe(III)]/[oxidant] ratios calculated at pH > 9 are due to disproportionately high plateau ΔA values obtained as a consequence of an added absorbance decrement resulting from accompanying porphyrin ring degradation.

Kinetics of Intermediate Formation. Since the "initial-rate" method minimizes complications due to side reactions, it was largely the method of choice for the determination of the rate of mfh oxidation. Under circumstances in which such side reactions

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Table II. Rate of Mesoferriheme Oxidation as a Function of pH and mfh Concentration (I = 0.1 M; T = 25 °C; λ = 389 nm)[a]

pH	10^6[mfh]$_0$, M	α[b]	$10^{-6}k_{obs}$,[c] M^{-1} s^{-1}	$10^{-6}k_m$,[d] M^{-1} s^{-1}
6.92[e]	5.16	0.334	2.48	3.72
	6.45	0.305	2.28	3.74
	10.70	0.247	1.94	3.93
	12.90	0.228	1.65	3.62
7.49[e]	6.45	0.173	0.91	2.6
	12.90	0.126	0.60	2.4
8.60[f]	6.45	0.047	0.23	2.5
8.88[f]	4.34	0.146	0.23	2.5
			0.18	1.9[g]
			0.11	2.4[h]
9.63[f]	6.45	0.016	0.20	6.2[i]
10.22[f]	6.45	0.0082	0.16	9.8[i]

[a] Initial rate treatment. [b] α = [monomer]/[mfh]$_0$. Values calculated as functions of [mfh]$_0$ and pH.[3-6] [c] k_{obs} = −d[mfh]/(dt [mfh][NaOCl]). [d] k_m = $^{-1}/_2$d[monomer]/(dt [monomer][NaOCl]) = $k_{obs}/2\alpha$. [e] Phosphate buffer. [f] Carbonate buffer. [g] Pseudo-first-order treatment; [NaOCl] = 1.5 × 10^{-5} M. [h] Second-order treatment. [i] May contain a contribution of mfh dimer.

could be neglected, e.g., relatively low oxidant concentrations, however, each method presented a profile of mfh oxidation involving a first-order dependence on both *monomeric* mfh and NaOCl with self-consistent second-order constants. For the initial-rate treatment, the term $(\Delta A_t/\Delta t)/\Delta A_{max}$ was found to be linear in [NaOCl] at low concentrations of hypochlorite. At high oxidant concentrations, the system displayed saturation kinetics, suggesting the mechanistic modification shown in Scheme II, where "FeOCl$^-$" denotes a reversibly formed mfh monomer–oxidant precursor to the enzyme compound I analogue (Fe^VO). Here, k_m, the second-order rate constant for reaction of monomeric heme with oxidant, is obtained in the region where $(\Delta A_t/\Delta t)/\Delta A_{max}$ is linear in [NaOCl]. The slope of this line is equal to $2k_m\alpha$. With reference to Scheme II, $k_m = k_3/K_m$ with $K_m = (k_{-1} + k_3)/k_1$.

Scheme II

$$Fe^{III} + OCl^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FeOCl^- \quad \text{(fast)} \qquad (3)$$

$$FeOCl^- \xrightarrow{k_3} Fe^VO + Cl^- \quad \text{(slow)} \qquad (4)$$

$$Fe^VO + Fe^{III} \rightarrow Fe^{IV}OFe^{IV} \quad \text{(fast)} \qquad (5)$$

Table II presents k_m values obtained by the initial-rate method at 25 °C in the range pH = 6.9–10.2. Measurements were made at hypochlorite concentrations below saturation level, i.e., in the region in which a first-order dependence on NaOCl is observed. Also included for comparison are k_m values obtained by each of the three kinetic methods at [mfh]$_0$ = 4.34 μM and pH = 8.9. It is noteworthy that the second-order rate constant for hypochlorite oxidation of monomeric mfh is analogous to that obtained in the dhf/NaOCl system in magnitude (~3 × 10^6 M^{-1} s^{-1}) and in being pH independent in the range 6.9–8.9. As observed in dfh oxidation,[19] an enhancement of rate occurs in carbonate buffer at pH > 10, and this may reflect a contribution of heme dimer to the oxidation process. It appears then that the insertion of ethyl groups into the 2- and 4-positions of the porphyrin while increasing the heme dimerization constant (0.034 for dfh and 0.069 for mfh at 25 °C) has little if any effect on the oxidative reactivity of heme monomer with hypochlorite. Below pH = 9, k_m is essentially pH independent, which argues for indiscrimination between hypochlorite ion and its conjugate acid, HOCl, as the oxidant species (for HOCl pK_a = 7.5).[27]

Peroxidatic Activity in the Mesoferriheme/Phenol System and the Spontaneous Regeneration of Mesoferriheme. The absorbance increase corresponding to in situ heme regeneration is biphasic and can be interpreted in terms of two pseudo-first-order processes.

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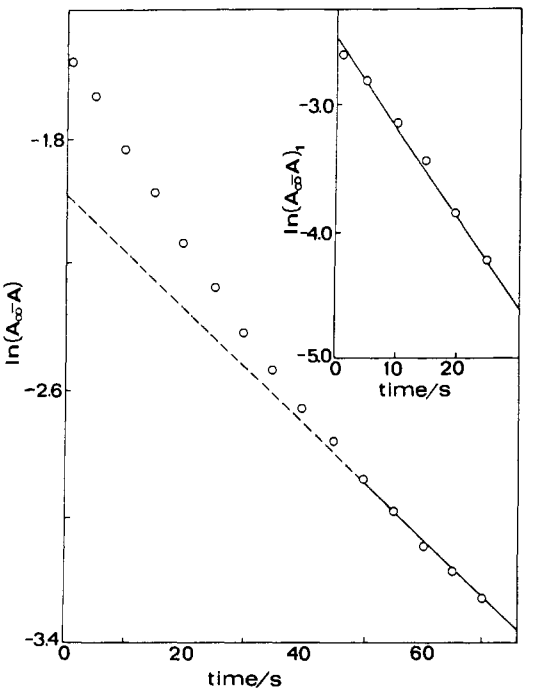

Figure 5. Spontaneous or in situ regeneration of mesoferriheme from the product of hypochlorite oxidation with [mfh] = 4.51 × 10^{-6} M, [NaOCl]$_0$ = 2.30 × 10^{-6} M, λ = 389 nm, pH = 6.92, T = 25 °C, and I = 0.1 M. A_∞ = final absorbance. A = absorbance at time, t. $(A_\infty - A)$ measures total absorbance change due to both processes. $(A_\infty - A)_1$ measures absorbance change due solely to faster process. k_1^r = 7.6 × 10^{-2} s^{-1}; k_2^r = 1.9 × 10^{-2} s^{-1}.

Table III. Variation of the Rate of Mesoferriheme Regeneration with Phenol Concentration ([mfh]$_0$ = 9.95 × 10^{-6} M; [NaOCl] = 4.98 × 10^{-6} M, λ = 389 nm; I = 0.1 M; pH = 6.88; T = 25 °C)

10^6[phenol], M	k_1^{obs}, s^{-1}	k_2^{obs}, s^{-1}	% D[a]
0	0.13	0.03	10
31.0	0.51	0.103	10
46.5	0.86	0.189	9
62.0	1.06	0.227	14
77.5	1.14	0.215	12
124.0	1.12	0.245	10
141.0	1.14	0.246	12
155.0	1.20	0.261	9

[a] Percent degradation taken as $100[(A_0 - A_\infty)/A_0]$.

Thus, a plot of ln $(A_\infty - A)$ vs time shows curvature in the early stages and is linear at larger reaction times (Figure 5). The slope of the linear portion of the graph, denoted k_2^r, is taken as the first-order rate constant for the slower process. Extrapolation toward t_0 allows the contribution of this slower process to the total absorbance change to be determined in the early stages of reaction, in which both processes occur at significant rates. The contribution of the faster process in this region is then determined by difference. A typical rate profile for the faster process is shown (inset) from which k_1^r, the corresponding rate constant, is derived. In the range pH = 7–9, values of k_1^r ~ 0.08–0.14 s^{-1} and k_2^r = 0.02–0.03 s^{-1} were obtained. Corresponding values for deuteroferriheme regeneration have been reported[31] as k_1^r = 0.05–0.12 s^{-1} and k_2^r = 0.006–0.012 s^{-1}.

The stopped-flow mixing of NaOCl with mfh–phenol mixtures also produces the oxidized mfh species, but the subsequent regeneration of optical density is accelerated due to the reducing action of phenol.[16,28] Biphasic kinetics are also observed, and similar treatment of the data yields respective first-order rate constants denoted k_1^{obs} and k_2^{obs}, whose values at different phenol concentrations at pH = 6.9 and 25 °C are shown in Table III.

(28) The oxidation of phenols by HRP compound I is presumed to involve formation of phenoxy radicals: Reference 16. Shiga, T.; Imaizumi, K. *Arch. Biochem. Biophys.* **1975**, *167*, 469.

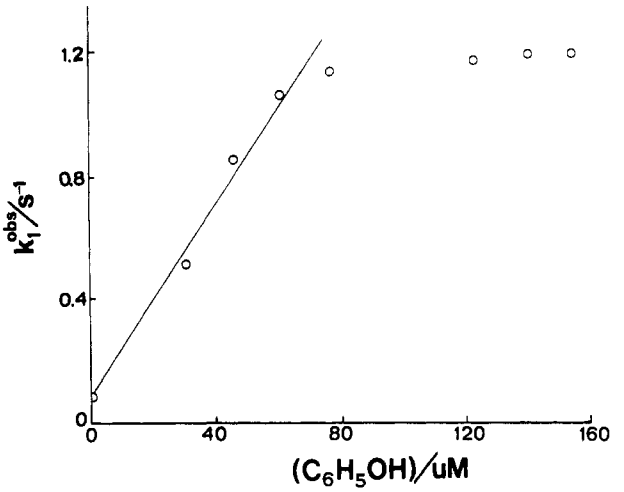

Figure 6. Variation of the k_1^{obs} contribution to mesoferriheme regeneration with phenol concentration. $[mfh]_0 = 9.99 \times 10^{-6}$ M, $[NaOCl]_0 = 4.98 \times 10^{-6}$ M, $\lambda = 389$ nm, pH = 6.88, $T = 25$ °C, and $I = 0.1$ M. $k_1^{ph} = 1.6 \times 10^4$ M^{-1} s^{-1}.

Both processes are subject to saturation kinetics, and the variation of k_1^{obs} with [phenol] at given initial concentrations of mfh and NaOCl is shown in Figure 6. In all cases, incomplete recovery of the original mfh spectrum is observed, the process being accompanied, typically, by about 10–12% porphyrin ring degradation.

The spontaneous, or in situ, reaction makes a small contribution to mfh regeneration in the presence of phenol. Consequently, a plot of k_1^{obs} (or k_2^{obs}) vs [phenol], such as that shown in Figure 6, should have a nonzero intercept equal to k_1^r (or k_2^r). Since the in situ contribution is relatively small, its determination by extrapolation to zero phenol concentration is subject to considerable error; however, since k_1^r and k_2^r can be determined directly, their corresponding values are introduced as points at zero phenol concentration. Accordingly, at relatively low phenol concentrations, where k_1^{obs} and k_2^{obs} increase linearly, equations of the form $k_{1(2)}^{obs} = k_{1(2)}^r + k_{1(2)}^{ph}[phenol]$ emerge, from which the correspondingly second-order rate constants for biphasic phenol induced regeneration, i.e., peroxidatic reduction of the oxidized species, are obtained. At pH = 6.88 and 25 °C, $k_1^{ph} = 1.6 \times 10^4$ M^{-1} s^{-1} and $k_2^{ph} = 2.7 \times 10^3$ M^{-1} s^{-1}.

Spontaneous, or in situ, regeneration of enzyme and enzyme models from peroxidatically active oxidation products have been reported previously. A biphasic regeneration of chloroperoxidase, following its oxidation by peroxoacid has been interpreted by Hager et al.[29] as involving parallel first-order reductions of two forms of oxidized enzyme reaction intermediate. On the other hand, in recent investigations of the regeneration of coproferriheme, following its oxidation by *m*-chloroperoxobenzoic acid, Bretscher and Jones[30] proposed series reactions involving sequential reduction of cph analogous of enzyme compounds I and II.

It has been demonstrated[31] that such kinetic studies alone do not allow a distinction between series first-order and parallel first-order mechanisms, each of which gives rise to an exponential variation of optical density with time of the form $A_\infty - A = \beta e^{-k_1 t} + \gamma e^{-k_2 t}$. However, recent studies of the deuteroferriheme system show a temperature *dependence* of the rate of dfh oxidation, implying a measurable activation energy for formation of the corresponding oxidized species, but a temperature *independence* of the resulting absorption spectrum.[31] This argues against the formation of more than one form of oxidized species and in favor of the series model for regeneration of dfh from a single heme oxidation product.

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(30) Bretscher, K. R.; Jones, P. *J. Chem. Soc., Dalton Trans.* **1988**, 2273.
(31) Rodriguez, R. E.; Woo, F. S.; Huckaby, D. A.; Kelly, H. C. *Inorg. Chem.* **1990**, *29*, 1434.

Table IV. Mesoferriheme and Hypochlorite Concentration Dependence of Heme Degradation (pH = 6.88, $T = 25$ °C)

10^6[NaOCl], M	$10^6[mfh]_0$,[a] M	10^6[dimer],[b] M	[NaOCl]/([NaOCl] + [dimer])	% D
1.98	3.98	1.23	0.617	20.5
2.49	4.98	1.61	0.607	18.7
4.98	9.95	3.66	0.576	9.45
7.45	14.93	5.80	0.562	4.70
3.98	3.98	1.23	0.764	40.1
4.98	4.98	1.61	0.755	34.9
9.95	9.95	3.66	0.731	27.7
14.93	14.93	5.80	0.720	22.0

$[mfh]_0 = 9.95 \times 10^{-6}$ M

10^6[NaOCl], M	% D	10^6[NaOCl], M	% D
9.74	22.8, 20.9[c]	41.0	82.5
20.0	50.6, 27.3[c]	51.3	90.0
30.8	76.2, 36.1[c]	61.5	93.9

[a] Stoichiometric concentration of mfh calculated as monomeric Fe(III). [b] [dimer] = [monomer](1 - α)/2α; α = [monomer]/$[mfh]_0$.[3-6] [c] In presence of 1.4×10^{-4} M phenol.

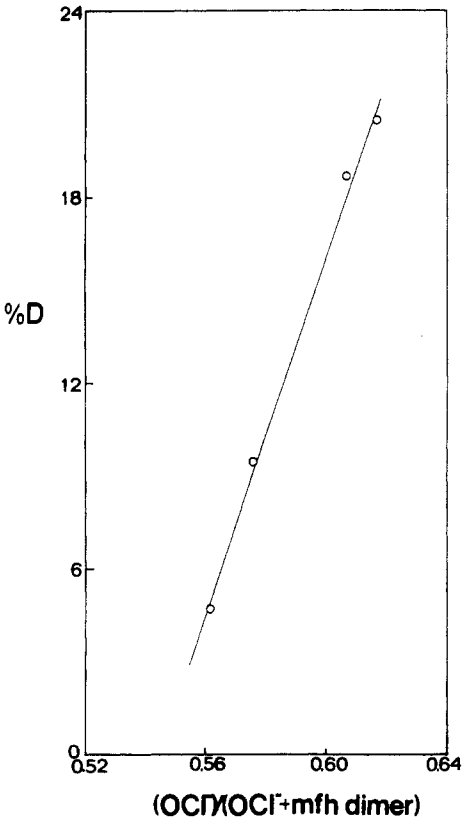

Figure 7. Percentage mesoferriheme degradation as a function of relative concentrations of hypochlorite and mesoferriheme dimer. $[mfh]_0/[NaOCl]_0 = 2/1$, pH = 6.88, $T = 25$ °C, and $I = 0.1$ M.

Accordingly, we propose analogous series pathways both for in situ mesoferriheme regeneration, where the nature of the reductant is unknown,[32] and for the reaction involving peroxidatic reduction by phenol. Although much remains unknown of the *intimate* mechanism, a *stoichiometric* pathway involving consecutive one-electron reductions of the dinuclear mfh analogues of enzyme compounds I and II is suggested:

$$\underset{\text{cpd I analogue}}{Fe^{IV}{}_2O} \rightarrow \underset{\text{cpd II analogue}}{Fe^{IV}OFe^{III}} \rightarrow \underset{\text{dimer}}{Fe^{III}{}_2O} \xrightleftharpoons[\text{ferriheme}]{} \underset{\text{monomer}}{2Fe^{III}} \quad (6)$$

This is analogous to, and consistent with, the scheme proposed by Bretscher and Jones for the spontaneous regeneration of co-

(32) Bretscher and Jones suggest coproferriheme regeneration may involve reductive action of solvent water on cfh compound I.[30]

proferriheme from analogues of compounds I and II, which in that model system are presumed to exist as mononuclear iron species.[30]

Mesoferriheme Degradation. Table IV shows the extent of heme degradation reported as % *D* as a function of heme and oxidant concentrations under various conditions. Values of % *D* are estimated from a comparison of the final recovered absorbance with the initial absorbance from stopped-flow traces, such as those shown in Figure 2.[33] Degradation increases with increasing concentrations of NaOCl in the presence of a fixed mesoferriheme concentration and decreases with increasing total (heme plus oxidant) concentration at a fixed $[mfh]_0/[OCl^-]_0$ ratio. Addition of phenol reduces the extent of degradation at relatively high hypochlorite concentrations but has a negligible effect at $[mfh]_0/[OCl^-]_0$ ratios of 1/1 or 2/1. The fact that, through peroxidatic action, phenol facilitates the regeneration of free mfh from its oxidation product suggests the species susceptible to degradation is an oxidized form of mfh rather than free heme itself, and, inasmuch as residual degradation is apparent even at low NaOCl concentrations, it appears that degradation may be signficant within the time frame of the oxidation process. The reduction in % *D* with increasing $[mfh]/[OCl^-]$ ratio also argues against degradative attack of hypochlorite on free mfh. It may be indicative, however, of competition between mfh and its oxidized form for OCl^- wherein high concentrations of free heme favor consumption of OCl^- in the *formation* of oxidized species thereby reducing the probability of degradative attack *upon* the oxidized species.[34]

Higher mfh concentrations should also facilitate comproportionation. This raises speculation that $Fe^{V}O$ may be the species most susceptible to porphyrin ring cleavage. The effect of total reactant (heme plus oxidant) concentration on % *D* at fixed $[mfh]/[OCl^-]$ ratios is not well understood; however, it is well established that the degree of heme dimerization increases with heme concentration. If dimeric heme provides a more effective "trap" for $Fe^{V}O$ than monomeric heme, e.g., via (7), then the

$$Fe^{V}O + Fe^{III}OFe^{III} \rightarrow Fe^{IV}OFe^{IV} + Fe^{III} \quad (7)$$

degree of degradation would be expected to be reduced at higher concentrations of total reactant. Figure 7 shows a linear relationship between % *D* and the ratio $[OCl^-]_0/([OCl^-]_0 + [\text{heme dimer}])$ for a series of experiments at $[mfh]_0/[OCl^-]_0 = 2/1$. Although conjectural, this is consistent with a model of competitive second-order reactions in which bimolecular attack of $Fe^{V}O$ by OCl^- and the heme dimer occurs with approximately the same probability, i.e., with similar second-order rate constants, in which case degradation would depend on the ability of oxidant to compete with free dimeric heme for $Fe^{V}O$, the mononuclear model analogue of compound I.

Molecular detail is not available with regard to the heme degradation process, but it is likely that ring fragmentation produces reducing ligands, in which case higher rates of in situ regeneration would be expected when the extent of degradation is high. At present, there are insufficient data to draw much correlation; however, it is clear that degradation of mfh exceeds that for dfh under comparable conditions, and this may be partially reflected in the relative k_2' terms for in situ regeneration. Thus, a contribution of reductant emanating from the degradation process may explain, in part, why k_2' for mfh is 20–50 times greater than the corresponding term for dfh. In terms of (6), this would be speculatively attributed to the action of the reductant on the respective dinuclear model compound II analogues.

Acknowledgment. This research was supported by the Robert A. Welch Foundation (Grant P-0162) and the TCU Research Fund.

(33) A more accurate A_0 value is obtained from an initial rate profile established by using a rapid oscilloscope scan time as described by Bretscher.[26]

(34) A thorough study of heme destruction in the coproferriheme system is described by Bretscher, who concludes "...only the cfh intermediate(s) is susceptible to destructive attack."[26]

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New Hexaaza Macrocyclic Binucleating Ligands. Oxygen Insertion with a Dicopper(I) Schiff Base Macrocyclic Complex

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Direct 2:2 condensation of isophthalaldehyde with diethylenetriamine produces a hexaaza 24-membered macrocyclic tetra Schiff base in good yield. The crystal structure of an isomeric form of the Schiff base is reported. Crystal data for $N_6C_{24}H_{30}\cdot{}^1/_2MeOH$: $C2/c$, a = 17.283 (2) Å, b = 16.930 (2) Å, c = 17.953 (3) Å, β = 118.73 (2)°, V = 4607 (2) Å^3, Z = 8, $R(F)$ = 0.059 for 1538 observations ($I > 3\sigma(I)$) and 289 variables. The amber binuclear Cu(I) complex of the tetraaza macrocyclic Schiff base combines with a molar equivalent of dioxygen to form a green binuclear Cu(II) complex, which contains a bridging hydroxide ion and, by oxygen insertion, a bridging phenolate donor. These results provide the first macrocyclic tyrosinase model. Hydrogenation of the Schiff base provides the corresponding saturated hexaaza macrocyclic ligand. The protonation constants of the reduced macrocycle, as well as the binding constants of its mononuclear and binuclear Cu(II) complexes, have been determined potentiometrically.

Introduction

In a recent communication[1] the synthesis of a 24-membered macrocyclic tetra Schiff base, **1** (Scheme I), by a 2:2 dipodal condensation of *m*-phthaldehyde and diethylenetriamine was reported. The binuclear Cu(I) complex of this ligand, **2** (Scheme I), was found to react with dioxygen to give the corresponding binuclear Cu(II) complex, **3**, with hydroxide and ligand-derived phenoxide bridging donor groups, thus providing the first macrocyclic tyrosinase model system.

Tyrosinase and dopamine-β-hydroxylase, which insert oxygen into organic substrates, contain dinuclear Cu(I) active sites and presumably bind dioxygen as a bridging peroxo ligand between the metal centers. Karlin et al.[2] reported the first tyrosinase model, which consists of a *m*-xylyl binucleating ligand that provides two pyridine nitrogens and one aliphatic nitrogen donor to each Cu(I)

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