Contribution from the Department of Chemistry,
Texas Christian University, Fort Worth, Texas 76129

Iron Porphyrin Models of Peroxidase Enzyme Intermediates: Hypochlorite Oxidation of Deuteroferriheme

Ricardo E. Rodriguez† and Henry C. Kelly*

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The iron(III) complex of deuteroporphyrin IX, deuteroferriheme (dfh), undergoes oxidation by hypochlorite ion to produce a reactive "intermediate state" that is functionally analogous to catalytically active intermediates obtained via the peroxide oxidation of selected peroxidase enzymes. This intermediate state exhibits the same optical spectrum as that obtained through dfh oxidation by other O atom oxidants such as substituted peroxybenzoic acids, chlorite ion, and iodosobenzene and its diacetate derivative, and as is the case with such oxidants, a stoichiometric equivalence of 2 mol of heme iron(III)/mol of two-electron oxidant is observed through stopped-flow spectrophotometric titration experiments. At low [OCl^-], formation of the intermediate state is first-order in both hypochlorite and monomeric dfh. Saturation kinetics are displayed at high oxidant concentrations. The rate is independent of pH in the range 6.9-8.5 but increases markedly in carbonate buffer from pH 8.5 to 10.6, an effect tentatively attributed to a contribution of dfh dimer enhanced by carbonate ion, which may be functioning as a general base. Reciprocal plots suggest rate acceleration in regions of relatively high basicity to be reflected primarily in the Michaelis constant, K_m, and determined more by the binding of dfh to oxidant than by the conversion of heme-oxidant complex to the intermediate state. Oxidation at high hypochlorite concentrations is accompanied by less extensive porphyrin ring degradation than is observed with comparable concentrations of H_2O_2 or *m*-chloroperoxybenzoic acid, and under such conditions, formation of a 1/1 heme iron(III)-OCl^- product is indicated. It is proposed that this may be analogous to the 1/1 enzyme-oxidant product obtained in oxidation of selected peroxidases and to the proposed intermediate obtained by peroxyacid oxidation of monomeric coproferriheme.

Introduction

Peroxidases are hemoprotein enzymes that catalyze the peroxide oxidation of selected substrates in organisms. To a degree, their action can be mimicked by protein-free ferrihemes, and the scope of catalysis and mechanistic detail of action of such models has been investigated by many workers.[1-15] Kinetic and spectrophotometric studies suggest a model of peroxidatic activity involving oxidation of the monomeric form of ferriheme to one or more reactive species, i.e., an "intermediate state" that, through subsequent reactions with reducing substrates, is converted back to free heme. Specific studies on the iron(III) complex of deuteroporphyrin IX, deuteroferriheme (dfh), have shown that solutions obtained by treating this model with various oxidants exhibit virtually identical spectra and similar kinetic parameters for the subsequent peroxidatic oxidation of substrates such as hydroquinone,[2] iodide ion,[5,10] and selected anilines[16] and phenolate ions.[6] Such oxidants include hydrogen peroxide, substituted peroxybenzoic acids, chlorite ion, and iodosobenzene and its diacetate derivative. Except for hydrogen peroxide, which also rapidly reduces the intermediate state, thereby providing a catalase-like or "catalatic" mechanism for H_2O_2 oxidation,[1,3,9] a stoichiometric equivalence of 2 mol of monomeric heme Fe(III)/mol of two-electron oxidant has been demonstrated by quantitative spectrophotometric "titration" of dfh with oxidant in the Soret region of the heme spectrum. This has been interpreted in terms of a two-electron oxidation involving O atom transfer from oxidant to heme iron to give a species that may be viewed as a peroxidase-compound I analogue,[17] followed by a rapid comproportionation reaction, as shown in Scheme I, where the

Scheme I

$$\mathrm{Fe(III) + ROOH} \xrightarrow{k_{ox}} \mathrm{Fe^{V}O + ROH} \quad (1)$$

$$\mathrm{Fe^{V}O + Fe(III)} \xrightarrow{k_{com}} \mathrm{Fe^{IV}{}_2O} \quad (2)$$

$Fe^{V}O$ and $Fe^{IV}O$ notations are used solely for "bookkeeping" purposes to depict electron loss and stoichiometry; the location of oxidation sites remains largely conjectural. Thus, spectral studies of oxidation products of cobalt(II) porphyrins as well as of heme systems suggest the product depicted as "$Fe^{V}O$" to be an Fe(IV) π-cation-radical species.[18,19] On the other hand, Sawyer et al.[20] find the oxidation product of the perchlorate of [tetrakis(2,6-dichlorophenyl)porphinato]iron(III) to display reactivity and properties characteristic of horseradish peroxidase compound I and, on the basis of spectral, magnetic, and electrochemical studies, propose a structure involving oxygen bonded to an iron(II)-porphyrin radical species.

The 2/1 stoichiometry exhibited by the dfh model system contrasts to that observed in peroxidase systems where two-electron oxidation to compound I species is not accompanied by comproportionation. Formation of dinuclear $Fe^{IV}{}_2O$ is speculative and is based on observed stoichiometry and analogy to iron(III) porphyrins, which are known to form oxo-bridged dimers.[21-23] In

† Present address: National Cancer Institute, NIH, Frederick, MD 21701.

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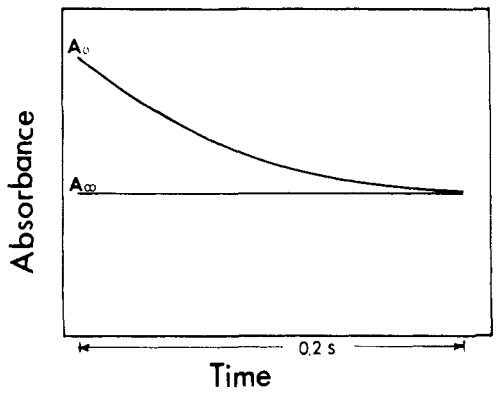

Figure 1. Oscilloscopic trace for the stopped-flow spectrophotometric study of the reaction of deuteroferriheme with NaOCl (pH = 7.5 ($H_2PO_4^-$, HPO_4^{2-} buffer); μ = 0.1 M; λ = 384 nm, $[dfh]_0 = 7.74 \times 10^{-6}$ M; $[NaOCl]_0 = 2.08 \times 10^{-5}$ M; $A_0 - A_\infty = 0.195$).

this sense, it should be noted that coproferriheme, which exists predominantly in monomeric form under solution conditions in which dfh is extensively dimerized, has been reported to display a 1/1 stoichiometric relationship upon oxidation with *m*-chloroperoxybenzoic acid.[15] Our present focus on the hypochlorite–heme system is on the stoichiometry and kinetics of formation of this intermediate state and the corresponding pH dependence of dfh oxidation. Studies are prompted, in part, by results of earlier investigations of dfh oxidation by chlorite ion,[10] in which the observed molar relationship, [heme Fe(III)]/[ClO_2^-] = 4/1, was interpreted as the result of consecutive oxidations by ClO_2^- and (its consequent reduction product) OCl^- of two heme iron(III) species, with action of OCl^- being the more rapid, followed by comproportionation, as depicted in Scheme I.

Experimental Section

Deuteroferriheme was prepared by a resorcinol melt treatment of triply recrystallized hemin, which was obtained from Nutritional Biochemicals Corp. dfh was recovered by using extraction and recrystallization procedures described by Falk[24] and characterized as the pyridine hemochrome, which was analyzed spectrally and found to be of purity exceeding 99% ($\epsilon = 2.4 \times 10^4$ M^{-1} cm^{-1}; λ_{max} = 544 nm). Sodium hypochlorite was obtained from Mallinckrodt as a 5% solution in aqueous NaOH. Solutions of NaOCl to be used in stopped-flow experiments were freshly prepared by dilution of a stock solution that had been adjusted to between 0.5 and 5.0 mM NaOCl and analyzed iodometrically. *m*-Chloroperoxybenzoic acid was obtained from Pfalz and Bauer. Stock solutions 1–2 mM in peroxyacid were prepared and analyzed iodometrically.

All solutions were prepared by using deionized water that was subsequently passed through a Barnstead mixed-bed ion-exchange column. The conductivity, measured as NaCl, was <0.01 ppm. Carbonate, phosphate, and borate buffer solutions were prepared by using reagent grade components. Ionic strengths of solutions were adjusted by using NaCl.

Stopped-flow studies were carried out by using a Durrum-Gibson D-110 spectrophotometer in conjunction with a Tektronix oscilloscope. Other spectral measurements involved use of Gilford Model 250 and Perkin-Elmer 552 UV–visible spectrophotometers.

Results and Discussion

Treatment of dfh with excess hypochlorite produces pseudo-first-order oxidation to the peroxidatically active "intermediate state" evidenced by a decrease in absorbance in the Soret region (384 nm) of the dfh spectrum, as shown in Figure 1. Although not represented in the figure, a slow recovery of the original heme absorbance is observed when the solution is allowed to stand following attainment of a minimum absorbance level. This has been previously described for dfh oxidation by *m*-chloroperoxybenzoic acid and iodosobenzene.[12]

This recovery of the dfh optical density is sufficiently slow that the stoichiometric equivalence of oxidant to heme may be determined through stopped-flow spectrophotometric "titration" of

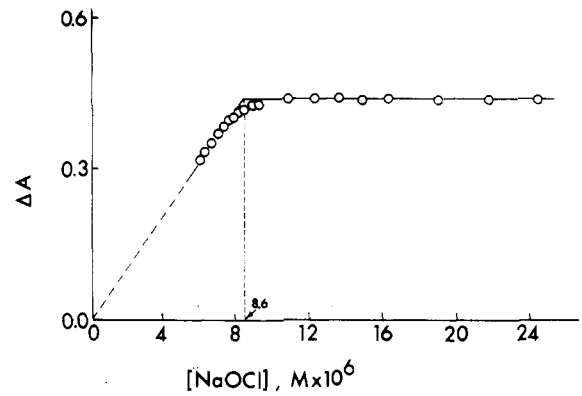

Figure 2. Stopped-flow spectrophotometric "titration" of deuteroferriheme with NaOCl ($[dfh]_0 = 17.7 \times 10^{-6}$ M; λ = 384 nm; pH = 9.84; μ = 0.1 M; t = 25 °C; [Fe(III)]/[OCl^-] = 2.1/1).

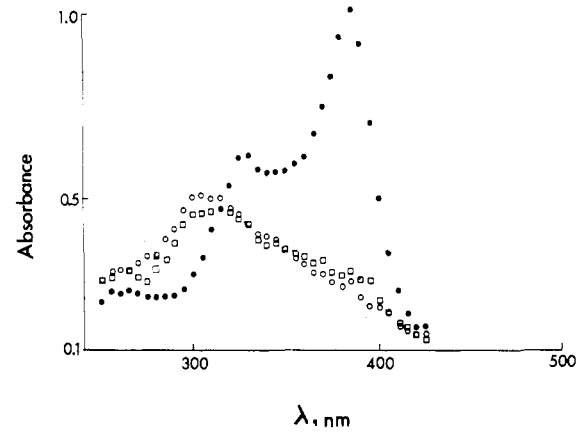

Figure 3. Absorption spectra from stopped-flow experiments (pH = 6.98; t = 25 °C; μ = 0.1 M): (●) 6.45×10^{-6} M dfh + H_2O; (○) 6.45×10^{-6} M dfh + 6.5×10^{-6} M NaOCl; (□) 6.45×10^{-6} M dfh + 6.5×10^{-6} M *m*-chloroperoxybenzoic acid.

dfh with OCl^-. Figure 2 shows a typical "titration curve" at pH 9.8, in which the maximum decrement in absorbance, ΔA, is plotted against the hypochlorite concentration for stopped-flow experiments in which varying amounts of OCl^- are mixed with a fixed amount of dfh. The equivalence of two heme Fe(III) species per OCl^- ion is consistent with results previously obtained on heme oxidation with substituted peroxybenzoic acids,[4] chlorite ion,[10] and iodosobenzene[12] and with the stoichiometric mechanism suggested in Scheme I; however, variations in apparent stoichiometry are found in the presence of a large excess of OCl^-, as described below.

Point spectra for the intermediate state, shown in Figure 3, were obtained by subtracting from the dfh spectrum the maximum absorbance decrement obtained at each wavelength for heme oxidation with a stoichiometric excess of oxidant. The same spectral features have been obtained for reaction with chlorite ion[10] and are observed with selected amine oxides,[25] indicating the same intermediate state to emerge in each system.

Specific kinetic data for the hypochlorite oxidation of deuteroferriheme are given in Table I. In all cases, pseudo-first-order rate constants, denoted k_{obs}, were obtained for the disappearance of dfh in the presence of excess [OCl^-] from plots of ln $(A - A_\infty)$ vs time, where A denotes the absorbance at time t, and A_∞, the minimum absorbance for a given kinetic run, i.e., corresponding to stoichiometric conversion of dfh to the intermediate state. A typical hypochlorite concentration profile showing saturation kinetics is given in Figure 4. Second-order rate constants, designated k', were obtained by dividing k_{obs} by the hypochlorite concentration in regions where k_{obs} is linear in [OCl^-]. Values of k' vary with total heme concentration, however, and, as has

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Table I. Kinetic Data for the Hypochlorite Oxidation of Deuteroferriheme (t = 25 °C; μ = 0.1 M)

buffer pH	10^6[dfh]$_0$,[a] M	α[b]	$10^{-6}k'$,[c] M^{-1} s^{-1}	$10^{-6}k'/\alpha$,[d] M^{-1} s^{-1}	log (k'/α)	$10^{-5}k_D$,[e] M^{-1} s^{-1}	$[CO_3^{2-}]$,[f] M	$10^{-7}k_D/[CO_3^{2-}]$, M^{-2} s^{-1}	k_3,[g] s^{-1}	$10^{-5}(2k_3/K_m)$,[g] M s^{-1}	10^4K_m,[g] M
					Phosphate Buffer						
6.97	3.86	0.467	1.27	2.14	6.33				114	4.3	5.3
	5.16	0.421	1.49	3.54	6.55				117	4.5	5.2
	6.43	0.387	1.27	3.28	6.52				125	4.5	5.5
7.50	5.16	0.259	0.672	2.59	6.41				123	2.6	9.5
	5.16	0.259	0.653	2.52	6.40				96	2.2	8.7
	7.74	0.217	0.578	2.66	6.42				100	3.7	5.4
					Carbonate Buffer						
8.33	3.87	0.125	0.400	3.20	6.51				32	3.5	1.8
8.36	3.86	0.121	0.377	3.16	6.50				28	3.7	1.5
	5.14	0.106	0.382	3.60	6.56				36	3.6	2.0
	6.43	0.0951	0.216	2.27	6.36				26	2.4	2.1
8.50	1.29	0.173	0.488	2.82	6.45						
8.77	3.86	0.0773	0.415	5.37	6.73	3.97	0.0048	8.27	84	5.4	3.1
	5.14	0.0673	0.271	4.03	6.61	1.48		3.08	36	5.5	1.3
	6.43	0.0604	0.312	5.17	6.71	2.79		5.81	27	5.8	0.92
8.78	1.29	0.128	0.572	4.47	6.65						
9.22	3.86	0.0468	0.380	8.12	6.91	5.04	0.0116	4.34	63	8.0	1.56
	5.14	0.0407	0.385	9.46	6.98	5.48		4.72	27	11	0.48
	6.43	0.0364	0.330	9.07	6.96	4.59		3.96	25	13	0.40
9.25	1.29	0.0769	0.400	5.20	6.72						
9.75	2.57	0.0314	0.502	16.0	7.20	8.42	0.0209	4.03	12	25	0.09
	3.86	0.0257	0.333	13.0	7.11	8.58		4.11	11	17	0.13
	5.14	0.0223	0.486	21.1	7.32	5.25		2.51	9	21	0.08
10.26	1.29	0.0247	0.065	2.63	6.42						
	3.87	0.0147	0.513	34.9	7.54	9.52	0.0281	3.39			
					Borate Buffer						
9.12	3.86	0.0523	0.304	5.81	6.76				25	7.7	0.65
	5.14	0.0455	0.0266	5.85	6.77				27	7.6	0.70

[a] [dfh]$_0$ = initial concentration of dfh calculated as monomer. [b] α = [M]/[dfh]$_0$; [M] and [D] denote monomer and dimer concentrations. [c] k' = $-$d[dfh]/([dfh][OCl$^-$]dt) = k_{obs}/[OCl$^-$]. [d] k_m = 3.0 × 10^6 M^{-1} s^{-1} (=k'/α at pH <8.5 (Figure 5)). [e] From eq 7. [f] HCO_3^-, CO_3^{2-} buffer system. [g] From k_{obs} = $2k_3$[OCl$^-$]/(K_m/α + [OCl$^-$]).

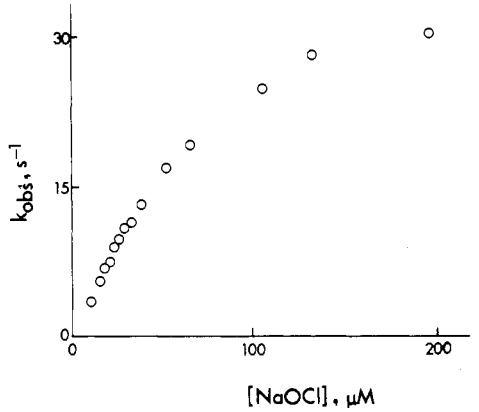

Figure 4. Variation of k_{obs} with hypochlorite concentration ([dfh]$_0$ = 5.14 × 10^{-6} M; pH = 9.25; t = 25 °C; μ = 0.1 M; k_{obs} = $-$d[dfh]/([dfh]dt)).

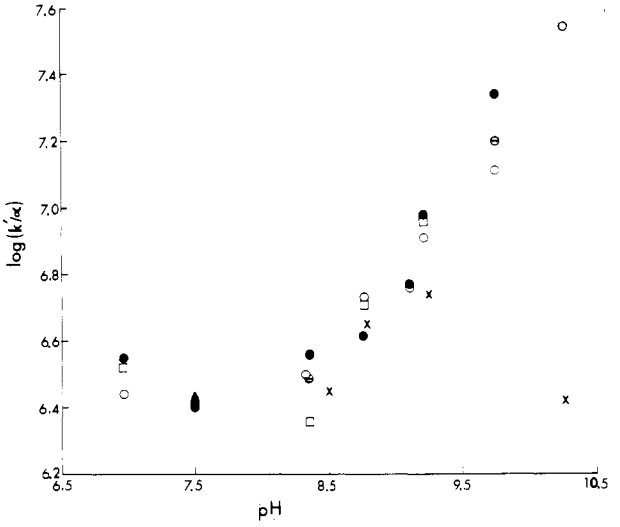

Figure 5. pH dependence of the rate of formation of the "intermediate state" from deuteroferriheme and NaOCl (t = 25 °C; μ = 0.1 M). [dfh]$_0$ × 10^6 (M): ×, 1.29; ⊖, 2.57; ○, 3.86; ●, 5.14–5.16; □, 6.43; ▲, 7.74. Buffer systems: $H_2PO_4^-$, HPO_4^{2-} at pH 6.97, 7.50; HCO_3^-, CO_3^{2-} at pH 8.33, 8.36, 8.77, 9.22, 9.75, 10.26; $B(OH)_3$, $B(OH_4)^-$ at pH 9.12. k' = $-$d[dfh]/([dfh][OCl$^-$]dt); α = [M]/[dfh]$_0$.

been demonstrated in the dfh–H_2O_2 system,[3] the variation is correlated with heme aggregation.

Such aggregation has been studied by Prudhoe and co-workers,[26] who, from spectral measurements, calculated the equilibrium constant K_{eq} = 3.4 × 10^{-2} at 25 °C for dfh dimerization expressed as 2M ⇌ D + H^+, where M and D denote monomeric and dimeric dfh, respectively. If we utilize their conventions, where α is defined as the fraction of dfh existing in monomeric form, the hydrogen ion and heme concentration dependence of α is given by (3), where

$$K_{eq} = [H^+](1 - \alpha)/(2\alpha^2[dfh]_T) \quad (3)$$

[dfh]$_T$ is the total free heme concentration calculated as monomeric Fe(III).[26] From Figure 5, it can be seen that k'/α is essentially constant in the range pH 7–8.5. This is assumed to represent a [dfh]-independent second-order rate constant (k_m) for the hypochlorite oxidation of monomeric dfh.

In our previous studies,[3,9-12] the rate of oxidation of dfh dimer has been regarded as negligible compared to that of heme monomer.[27,28] The observed increase in k'/α shown in Figure 5 from

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(28) Contributions of the dimeric forms of hemes having considerably higher dimerization constants than dfh, such as protoferriheme[29] and mesoferriheme,[23] are suggested to be significant in their oxidation by H_2O_2.[27]

pH to 8.5 to 10.5, however, suggests a possible role of dimeric heme in the overall reactions with hypochlorite. Such a contribution might occur directly via (4), followed by rapid compro-

$$D + OCl^- \xrightarrow{k_4} Fe(III) + FeO + Cl^- \quad (4)$$

portionation (eq 2), where D is assumed to be in the form of an oxo-bridged dinuclear iron(III) species, $Fe^{III}OFe^{III}$. Thus, from Scheme I

$$-d[dfh]_T/dt = 2k_{ox}[M][OCl^-] + 2k_4[D][OCl^-] \quad (5)$$

where $k_4 \ll k_{com} \gg k_{ox}$ with $k_m \equiv 2k_{ox}$ and $k_D \equiv 2k_4$. Therefore

$$k'[dfh]_T = k_m[M] + k_D[D] \quad (6)$$

and since $[dfh]_T = [M] + 2[D]$

$$k_D = 2(k' - k_m\alpha)/(1 - \alpha) \quad (7)$$

If we take $k_m = 3.0 \times 10^6\ M^{-1}\ s^{-1}$, as the value of k'/α in its pH-independent region (Figure 5),[30] a k_D term may be calculated at pH > 8.5 as a function of k' and α, which is determined through solution of the quadratic equation emerging from (3). Results are given in Table I. Although a small pH dependence of k_D is observed, it appears that, in each of the four carbonate buffer systems employed, $k_D/[CO_3^{2-}]$ is essentially constant. This suggests that rate increases above pH 8.5 may reflect carbonate ion enhancement, possibly even a manifestation of general-base catalysis, of hypochlorite oxidation of dfh dimer. Proposal of a mechanism for such catalysis seems unwarranted at present. That dimeric heme is involved in some way, however, is further suggested by the fact that at high pH but at significantly reduced dfh concentrations, where the fraction of the more active monomeric form is increased, k'/α falls to a value near that established at lower pH, which presumably reflects k_m alone. A somewhat lower value of k'/α is observed when the solution is buffered with borate, which may indicate a correspondingly weaker contribution of borate, relative to CO_3^{2-}, in enhancing oxidation of the dimeric species.

The demonstration of saturation kinetics shown in Figure 4 suggests refinement of Scheme I to a modified Michaelis–Menten mechanism, as shown in Scheme II, where Fe^{III} denotes the re-

Scheme II

$$Fe^{III} + OCl^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (Fe^{III}\text{–}OCl^-) \xrightarrow[-Cl^-]{k_3} Fe^{V}O \xrightarrow[Fe^{III}]{k_{com}} Fe^{IV}{}_2O \quad (8)$$

active heme species, (Fe^{III}–OCl^-), a dfh–oxidant (catalyst–substrate) complex, and k_3, the rate constant for the rate-determining step. Attainment of 2/1 heme iron(III)/OCl^- stoichiometry precludes significant catalatic turnover of oxidant such as that observed in the dfh–H_2O_2 system.[2-4,31] Accordingly, $-d([Fe^{III}] + [Fe^{III}\text{–}OCl^-])dt = 2k_3[Fe^{III}\text{–}OCl^-]$. Under conditions where the monomeric form of heme is the only active form

$$k_{obs} = \frac{2k_3[OCl^-]}{K_m/\alpha + [OCl^-]} \quad (9)$$

wherein a plot of $1/k_{obs}$ vs $1/[OCl^-]$ yields a line of slope $K_m/(2\alpha k_3)$ and intercept $1/(2k_3)$. When $[OCl^-] \ll K_m/\alpha$, $k' = 2\alpha k_3/K_m$ and $k_m = 2k_3/K_m$.

The relative pH independence of k'/α between pH 7 and 8.5 implies comparable independence of the ratio $2k_3/K_m$, and indeed, this ratio appears constant within experimental error through this region (Table I). Some variation in the *specific values* of k_3 and K_m becomes apparent, however, primarily with a change from phosphate to carbonate buffer, both k_3 and K_m having larger values

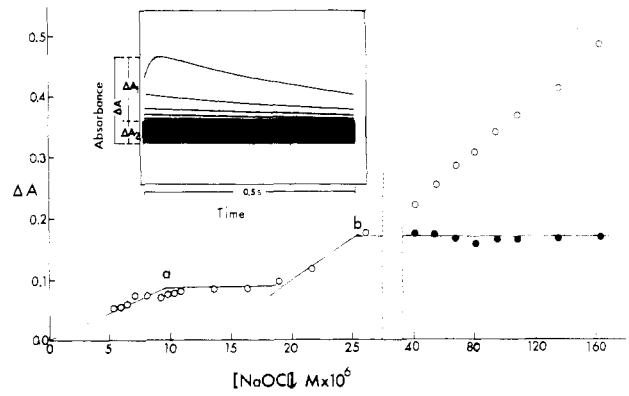

Figure 6. Stopped-flow spectrophotometric measurement of absorbance changes on treatment of deuteroferriheme with excess NaOCl (pH = 10.6; μ = 0.1 M; λ = 384 nm; t = 25 °C; $[dfh]_0 = 19.4 \times 10^{-6}$ M); point a, $[NaOCl]_0 = 9.6 \times 10^{-6}$ M and $[Fe(III)]/[OCl^-] = 2.0$; point b, $[NaOCl]_0 = 2.53 \times 10^{-5}$ M and $[Fe(III)]/[OCl^-] = 0.77$. Insert shows absorbancy change for $[NaOCl]_0 = 4.08 \times 10^{-5}$ M: $\Delta A = 0.22$; $\Delta A_1 = 0.17$; $\Delta A_2 = 0.05$.

in the $H_2PO_4^-$, HPO_4^{2-} system. This effect is reminiscent of specific buffer effects observed in the study of dfh oxidation by H_2O_2, wherein the acceleration of rates of intermediate formation in phosphate buffer were attributed to general-acid catalysis, by $H_2PO_4^-$ ion, of the conversion of catalyst substrate complex to the intermediate state, as reflected in the k_3 term.[9] Alternatively, since, at 25 °C, pK_a = 7.5 for HOCl,[32] the greater value of k_3 in phosphate buffer may be indicative of an effect of molecular hypochlorous acid. This would diminish with increasing pH as OCl^- becomes the predominant form of the oxidizing agent. Enhancement of k_3 also might explain the accompanying enhancement of K_m ($=(k_2 + k_3)/k_1$). The increase in the calculated value of $2k_3/K_m$ in carbonate buffer above pH 8.5 is consistent with the observed rate enhancement tentatively attributed to an effect of heme dimer. It is not surprising that the effect is more pronounced on K_m than on k_3 (Table I), since the kinetic role of dimer might be expected to be manifest more in the k_2/k_1 ratio, which relates to dimer–OCl^- interaction, than in k_3, which describes the collapse of the catalyst–substrate complex to the intermediate state, especially if dissociation of dimer occurs during attack by OCl^- to produce the same catalyst–substrate complex as is obtained via oxidation of monomeric dfh. That relatively small changes in k_3 are observed in carbonate buffer between pH 8.3 and 9.7, where both monomeric and dimeric heme are presumed to be active, seems consistent with this suggestion.

The term "stoichiometry conversion" of dfh to the intermediate state has, until now, referred to the formation of heme oxidation product(s) in which a 2/1 molar ratio of heme iron(III) to hypochlorite is observed. Verification of this ratio via "titration" data (Figure 2) is possible only as long as the formation of the intermediate state is not kinetically coupled to reactions that may consume heme or oxidant such as a catalatic turnover of oxidant, oxidative degradation of the porphyrin ring, or the regeneration of free heme through reduction of oxidized intermediate species. As shown in Figure 2, the maximum ΔA corresponding to formation of the 2/1 species, i.e., the plateau, is maintained in the presence of hypochlorite at concentrations well above that corresponding to stoichiometric equivalence.

A different effect, however, involving a two-stage decrease in optical density, is seen when dfh is oxidized by using much higher concentrations of OCl^-. In such cases, an initial decrease in absorbance is followed by a slower decrement. For 19.4 μM dfh at pH 10.6, the two-stage nature of the reaction is apparent when the initial hypochlorite concentration exceeds 20 μM, and it is shown in the insert in Figure 6 for $[OCl^-]_0$ = 41 μM. Since the two processes are not fully decoupled kinetically, absorbance decrements do not allow independent measurement of each event. Nevertheless, estimates of the magnitudes of the absorbance

(29) Brown, S. B.; Dean, T. C.; Jones, P. *Biochem. J.* **1970**, *117*, 733.
(30) This value of k_m exceeds, by a factor of $\simeq$20, the maximum value of the pH-dependent rate constant for reaction of dfh monomer with ClO_2^-, consistent with the previously proposed mechanism involving formation of OCl^- as a relatively fast-reacting intermediate in the chlorite oxidation of dfh.[10]
(31) Jones, P. In *Oxidases and Related Redox Systems*; King, T. E., Mason, H. W., Morrison, M., Eds.; University Park Press: Baltimore, MD, 1973.
(32) Sillen, L. G. *Spec. Publ.—Chem. Soc.* **1964**, *No. 17*.

changes as a function of initial hypochlorite concentration allow empirical generalizations.

For operational consistency, ΔA_1 is taken as the difference between the initial, maximum absorbance level and that corresponding to the beginning of a continuous band described by successive oscilloscopic traces, the magnitude of which is taken as ΔA_2 (see Figure 6 insert). Figure 6 also shows the total absorbance changes, ΔA, depicted as open circles, as a function of initial hypochlorite concentrations for oxidation of 19.4 μM dfh at pH 10.6 with $[OCl^-]_0$ varying from 5 to 160 μM. Estimated values of ΔA_1, shown as closed circles, appear to be relatively independent of $[OCl^-]_0$. The magnitude of ΔA_2, however, which corresponds to the vertical distance between open and closed points, increases markedly with increasing hypochlorite concentration in this high oxidant concentration region and is undoubtedly due to an increase in the degree of heme destruction originating through oxidant-induced cleavage of the porphyrin ring system. In hemin, this leads to the formation of beliverdin.[33] It is also observed in studies of dfh oxidation by H_2O_2 and peroxybenzoic acids even at much lower $[oxidant]_0/[dfh]_0$ ratios than encountered in the dfh–hypochlorite system.[1,3,4,9,34]

The apparent plateau described by estimated ΔA_1 values suggests a second stoichiometric relationship between dfh and oxidant. A second equivalence point appears to emerge somewhere between 22 and 26 μM $[OCl^-]_0$ (point b, at $[OCl^-]_0 = 2.5 \times 10^{-5}$ M, yields a ratio [heme Fe(III)]/$[OCl^-]$ = 0.77). Since the two processes are not fully decoupled, some early portion of the absorbance change associated with heme destruction undoubtedly contributes to ΔA_1. If this contribution were to remain essentially constant at different hypochlorite concentrations during the time frame of the first (faster) stage of optical density change, then the absorbance decrement corresponding to the stage alone should describe a plateau lying somewhat lower than that depicted by the solid points in Figure 6 and lead to an equivalence point situated to the left of point b and closer to a heme Fe(III)/OCl^- ratio of unity. Speculatively, we suggest formation of an intermediate species having a 1/1 ratio of monomeric heme iron(III) to OCl^-, with titrimetric demonstration of stoichiometry obscured by the accompanying process of heme degradation.

Recent studies by Bretscher and Jones suggest a 1/1 oxidation product to arise through reaction of coproferriheme (cfh) with a variety of substituted peroxybenzoic acids at pH 6, a condition under which cfh, unlike dfh, is reported to exist predominantly as monomer.[15] These workers propose this product to be an analogue of peroxidase–compound I species, which also display 1/1 enzyme/oxidant stoichiometry. Similarly, we propose dfh oxidation at high $[OCl^-]$ to lead to the corresponding compound I analogue, depicted in Scheme I as mononuclear "FeO". The $\Delta A/[OCl^-]$ profile in Figure 6 suggests that such a species may arise through oxidative action of OCl^- on the initially formed comproportion product, depicted as $Fe^{IV}OFe^{IV}$, with loss of Cl^-.

This model for the hypochlorite oxidation of dfh, then, involves formation of a two-electron oxidation product (FeO) that is trapped at relatively low hypochlorite concentrations by free Fe(III) via rapid comproportionation but which is stabilized in mononuclear form under conditions of heretofore unexplored (large excess) concentration of OCl^-. That an alteration from 2/1 to 1/1 stoichiometry had not been previously reported in studies involving other oxidants such as substituted peroxybenzoic acids attests to the relatively high susceptibility of dfh to degradation in their presence. Hypochlorite-induced heme degradation is slow but not insignificant relative to the oxidation process. It presumably leads to a *calculated* [Fe(III)]/$[OCl^-]$ ratio less than 1/1 through contribution of an essentially constant absorbance decrement to the ΔA_1 term. Thus, ΔA_1 is viewed as the sum of two constant absorbance changes, one corresponding to the conversion of dfh to its 1:1 oxidation product (FeO) and a second to partial heme degradation. Such constant absorbance changes, which are independent of $[OCl^-]_0$ at high hypochlorite concentrations, would be expected if heme degradation were to occur via attack of OCl^- on the 2/1 heme/oxidant species rather than on free heme and with a rate law exhibiting the same concentration dependencies as the rate law for hypochlorite oxidation of $Fe^{IV}OFe^{IV}$. Thus, a given absorbance change will occur for conversion of dfh to the initial 2/1 oxidation product, following which, in view of a similarity of rate expressions, the relative rates and, necessarily, relative amounts of oxidation and degradation of $Fe^{IV}OFe^{IV}$ will be constant and equal to the ratio of the respective (similar order) rate constants. Since $[dfh]_0$ remains constant, the absolute magnitudes of the corresponding absorbance changes for these two components of ΔA_1 will also be constant. This model necessarily requires degradation to occur also on the proposed 1/1 oxidation product FeO, with this reaction being the dominant one for heme destruction at very high hypochlorite concentrations. It is interesting to consider this interpretation of dfh behavior in light of that described for coproferriheme (cfh), wherein, on the basis of results of degradation studies and investigations of the rates of heme regeneration, it is presumed that heme destruction occurs predominantly, if not exclusively, through oxidative degradation of the cfh-derived intermediate rather than of free coproferriheme.[15]

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(34) Jones, P.; Prudhoe, K.; Robson, T. *Biochem. J.* **1973**, *135*, 361.