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Mesoferriheme regeneration from its hemoprotein enzyme compound I analog: absorption and kinetic properties of one- and two-electron oxidation products

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Abstract

Spectral and kinetic data obtained in studies of the biphasic in situ regeneration of mesoferriheme (mfh) from oxidized species formed through the NaOCl oxidation of heme are utilized to calculate extinction coefficients of dinuclear iron species which have been proposed as models of peroxidase Compounds I and II (A and B below). For the proposed regeneration mechanism, involving consecutive one-electron redox processes, the calculated values of ϵ_A and ϵ_B are 4×10^4 and 5×10^4 M^{-1} cm^{-1}, respectively.

$$Fe_2^{IV}O \xrightarrow{k_1} Fe^{III}OFe^{IV} \xrightarrow{k_2} Fe_2^{III}O \rightleftharpoons 2Fe^{III}$$

A B mfh(dimer ⇌ monomer)

These values are somewhat greater than those previously calculated for analogous derivatives of deuteroferriheme. Both k_1 and k_2 are independent of pH over the range 6.9–9.1 and of total phosphate buffer concentration from 8 to 50 mM at pH 6.9 suggesting that, under these conditions, there is an absence of significant dissociation of the proposed dinuclear iron species.

Keywords: Kinetics and mechanism; Mesoferriheme regeneration; Hemoprotein enzyme

1. Introduction

Four examples of iron(III) porphyrin IX complexes (ferrihemes) that have been widely studied as models of hemoprotein peroxidase enzymes are shown in Fig. 1. In each system, peroxidatic activity involves oxidation of heme by an oxygen atom donor to produce a reaction intermediate which may be regarded as an analog of peroxidase Compound I [1–4]. Subsequent interaction with a reductant completes the catalytic process and regenerates heme.

A notable difference between the enzyme and the protein-free heme model is the tendency of the latter to undergo aggregation in aqueous solution, specifically, dimerization to an oxo-bridged dinuclear iron complex [5,6]. In studies of the pH and concentration dependence of heme absorption, Brown, Jones, and co-workers have shown that the equilibrium, 2 monomer ⇌ dimer + H_3O^+, is highly dependent on the nature of R, with

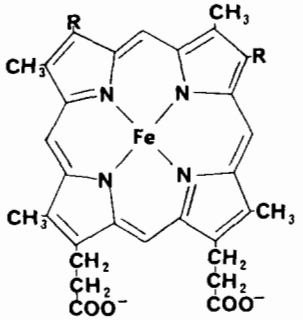

Fig. 1. Ferriheme models of peroxidase enzymes: protoferriheme (pfh), R = −CH=CH$_2$, deuteroferriheme (dfh), R = −H; mesoferriheme (mfh), R = −CH$_2$CH$_3$; coproferriheme (cph), R = −CH$_2$CH$_2$COOH.

dimerization constants equal to 4.5, 0.069 and 0.034 for proto-, meso- and deuteroferriheme, respectively [6–10]. Since the dimeric form is measurably less susceptible than the monomer to attack by O-atom donor species, aggregation leads to reduced catalytic activity

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[11]. For this reason, deuteroferriheme has been widely studied as a model system [11–26]; although recent kinetic investigations of Bretscher and Jones suggest that, of the four model systems shown, coproferriheme is the least prone to aggregation [27].

Previous studies on deutero- and mesoferriheme show a stoichiometric molar ratio $[Fe^{III}]/[R'O] = 2/1$ where Fe^{III} denotes heme calculated as the monomeric species and R'O, a two-electron oxidizing agent [16–28]. This has been interpreted as shown in Eqs. (1) and (2) which depict an initial two-electron oxidation of ferriheme and subsequent comproportionation with heme monomer [16]. Here $Fe^{V}O$ and $Fe_2^{IV}O$ denote, respectively, the initially formed two-electron oxidation product and the comproportionation product without implication as to specific oxidation sites. Indeed, by analogy to catalase and peroxidase oxidation products, the species denoted $Fe^{V}O$ is probably best viewed as an iron(IV)-π-cation radical as opposed to a true Fe^{V} entity [29].

$$Fe^{III} + R'O \longrightarrow Fe^{V}O + R' \quad (1)$$

$$Fe^{V}O + Fe^{III} \longrightarrow Fe_2^{IV}O \quad (2)$$

In peroxidase systems, the enzyme/oxidant ratio is 1:1; thus $Fe_2^{IV}O$ corresponds to a functional, but not structural, analog of peroxidase Compound I. Its formation is compatible with, if not suggested by, the dimerization of protein-free heme itself to oxo-bridged dinuclear iron(III). It is formed through the action of different O-atom donor oxidants on the ferriheme, evidenced by the fact that the same optical spectrum is obtained for the product of reaction of dfh with substituted peroxobenzoic acids [16], chlorite ion [20,24] and hypochlorite ion [30], as well as iodosobenzene and its diacetate [31].

In the absence of added reducing agent, an in situ regeneration of heme is observed. Although the nature of the 'endogenous electron donor' (reductant) is unknown, recent speculation suggests solvent water plays a role [27]. Regeneration is biphasic and characterized kinetically in terms of two first-order processes. The data are compatible with either of two mechanistic models, one involving two forms of intermediate undergoing redox regeneration via parallel processes, and the second involving series reactions emanating from a single heme oxidation product. Temperature dependent studies of the spectrum of the heme oxidation product and of regeneration rates strongly suggest the latter model, here depicted (Eq. (3)) with A denoting the Compound I analog; B a reaction intermediate arising via the one-electron reduction of A and, therefore, a functional analog of enzyme Compound II, and C, regenerated heme in monomer–dimer equilibrium [32].

$$\underset{A}{Fe_2^{IV}O} \xrightarrow{k_1} \underset{B}{Fe^{IV}OFe^{III}} \xrightarrow{k_2} \underset{C}{Fe_2^{III}O} \rightleftharpoons 2Fe^{III} \quad (3)$$

Assuming this mechanism of sequential one-electron redox processes, we have employed optical density measurements and kinetic studies to calculate molar extinction coefficients of the deuteroferriheme derived analogs of the enzyme intermediates [33]. Although mesoferriheme is formally similar to dfh with regard to the stoichiometry of oxidation and biphasic in situ regeneration [28], differences exist in kinetic parameters as well as in susceptibility to dimerization. This, together with the greater sensitivity of mfh to oxidative ring degradation as a competing side reaction [28] prompted us to explore the optical properties of the corresponding mfh derived intermediates, i.e., the proposed two- and one-electron oxidation products denoted $Fe_2^{IV}O$ and $Fe^{III}OFe^{IV}$, respectively, and it is this objective to which the current investigation is directed.

2. Experimental

2.1. Materials and methods

All compounds used for the preparation of buffers and for chemical analysis were of reagent grade. Alkaline solutions of sodium hypochlorite were obtained from Mallindrodt and used to prepare stock solutions which were analyzed iodometrically using standard $Na_2S_2O_3$. Solutions were prepared using freshly distilled water or deionized water that showed a conductance of less than 1 ppm measured as contained NaCl. Hemin (protoferriheme), listed as 3X crystalline was obtained from Nutritional Biochemicals Corporation and used to prepare mesoferriheme via the method of Davies [34] and Brown and Hatzikonstantinou [10] involving hydrogenation over a catalyst consisting of 10% Pd on charcoal in a refluxing solvent system containing 0.05 N KOH in CH_3OH. Purification consisted of crystallization from a solution of $CHCl_3$ and quinine added to glacial acetic acid and sequential washing with EtOH and Et_2O. Typical yields, starting with ~2 g hemin, were of the order of 65% with purity exceeding 99% as indicated by spectral analysis of the pyridine hemochrome.

A typical mfh stock solution was prepared by dissolving 0.058 g of the heme in about 2 ml of 1 N NaOH and diluting to 550 ml with H_2O. Solutions for specific spectral and kinetic studies were prepared shortly before use by dilution of an aliquot part of the stock with buffer.

2.2. Kinetic and spectral studies

Stopped-flow spectrophotometric studies were obtained using a Dionex-D110 spectrophotometer. Buffered heme solutions were mixed with aqueous NaOCl and absorbance changes in the Soret region (389 nm)

were followed using a Tektronix model 5310N or 2221 digital oscilloscope with HC100 plotter. Additional absorbance studies were carried out using a Varian Cary 3 UV–Vis spectrophotometer in which buffered mfh solutions and solutions of their corresponding oxidation products were scanned from 250 to 450 nm.

3. Results and discussion

A typical absorbance profile obtained on a stopped-flow time scale showing the decrease in Soret region absorbance accompanying hypochlorite oxidation of mesoferriheme and subsequent in situ regeneration is shown in Fig. 2. The system is highly sensitive to hypochlorite concentration. An excess of OCl^- expectedly leads to a longer time interval between attainment of the initially formed heme oxidation product and the onset of regeneration, a period corresponding to a steady-state concentration of $Fe_2^{IV}O$. Excess OCl^- also accelerates a side reaction involving degradation of the porphyrin ring leading to incomplete recovery of the original heme absorbance. Previous studies have shown a linear dependence of the percentage degradation on the ratio of $[OCl^-]$ to ($[OCl^-]$ plus [mfh dimer]) [28]. Investigations of Bretscher on coproferriheme indicate degradation to involve oxidant attack on the cph oxidation product [35]. This is consistent with kinetic studies of mfh regeneration in that, within experimental error, the same regeneration rate constants are obtained whether or not the system has incurred ring degradation. Thus, degradation presumably takes place prior to the observed increase in absorbance accompanying regeneration, i.e., during the time that excess OCl^- remains in the system.

The biphasic nature of regeneration is shown in Fig. 3. As previously shown for dfh [32], the accompanying absorbance change can be described by $a_\infty - a = \beta \exp(-k_1 t) + \gamma \exp(-k_2 t)$ where a_∞ and a represent,

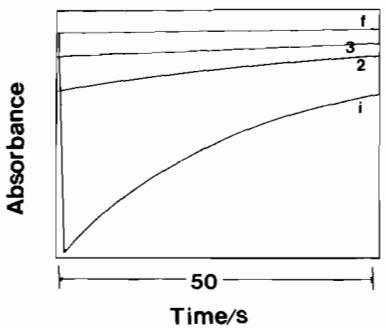

Fig. 2. Stopped-flow kinetic profile of the decrease in absorbance accompanying mfh oxidation by NaOCl and the subsequent in situ regeneration of the heme. $[mfh]_0 = 9.67 \times 10^{-6}$ M; $[NaOCl]_0 = 4.85 \times 10^{-6}$ M; $\lambda = 389$ nm, pH = 6.93, $t = 25$ °C; i denotes initial spectral scan corresponding to the regeneration process; 2, 3 and f denote second, third and final scans.

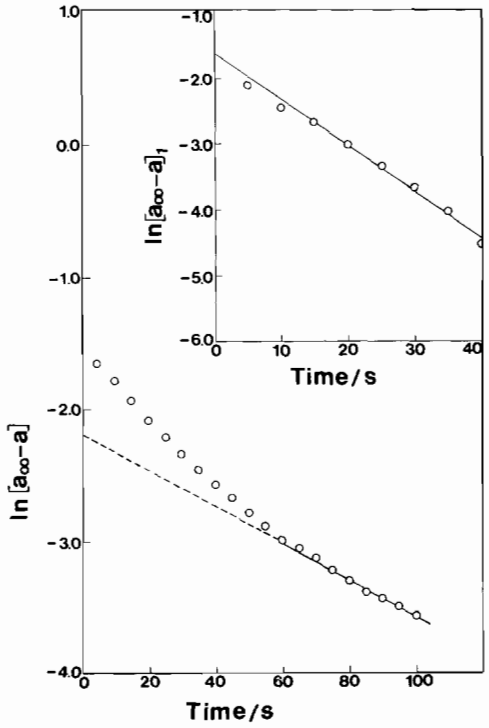

Fig. 3. Biphasic regeneration of mesoferriheme. $[mfh]_0 = 9.67 \times 10^{-6}$ M; $[NaOCl]_0 = 4.85 \times 10^{-6}$ M; $\lambda = 389$ nm, pH = 6.93, $t = 25$ °C. $\ln(a_\infty - a)_2 = -k_2 t + \ln \gamma$; $\ln(a_\infty - a)_1 = -k_1 t + \ln \beta$; $k_1 = 0.066$ s^{-1}, $k_2 = 0.015$ s^{-1}, $\beta = 0.183$, $\gamma = 0.208$. Derived ϵ values are given in Table 1.

respectively, the maximum level of recovered absorbance and the absorbance at time t; with k_1 and k_2 denoting the corresponding first-order rate constants. Coefficients β and γ are interpreted in terms of the series regeneration mechanism as

$$\beta = \frac{lA_0}{k_1 - k_2}[k_1(\epsilon_B - \epsilon_A) - k_2(\epsilon_C - \epsilon_A)] \quad (4)$$

$$\gamma = \frac{lA_0}{k_1 - k_2}[k_1(\epsilon_C - \epsilon_B)] \quad (5)$$

with ϵ terms denoting respective extinction coefficients and l the cell pathlength. Values of k_1, k_2, β and γ are obtained from the data of Fig. 3 where linearity of $(a_\infty - a)$ at long reaction times is interpreted as the contribution of the slower process given by $(a_\infty - a)_2 = \ln \gamma - k_2 t$. The faster process, described by $(a_\infty - a)_1 = \ln \beta - k_1 t$, is then obtained from the region of curvature where $(a_\infty - a) = (a_\infty - a)_1 + (a_\infty - a)_2$. Subsequent determination of A_0 and ϵ_C then allows calculation of ϵ_A and ϵ_B.

In the absence of significant ring degradation, the initial concentration of the original heme oxidation product, A_0, is taken as one-half the original heme concentration calculated as monomeric Fe(III). Since free mfh exists in monomer-dimer equilibrium, and since the degree of dissociation of dimer is concentration

Table 1
Kinetic parameters and extinction coefficients derived from mesoferriheme regeneration; $\lambda = 389$ nm, pH = 6.92, $t = 25$ °C

$10^6[\text{mfh}]_0$[a] (M)	$10^6[\text{NaOCl}]_0$ (M)	α	k_1 (s^{-1})	k_2 (s^{-1})	Δa	β	γ	$10^{-4}\epsilon_C$[b] (M^{-1} cm^{-1})	$10^{-4}\epsilon_B$ (M^{-1} cm^{-1})	$10^{-4}\epsilon_A$[b] (M^{-1} cm^{-1})
6.45[c]	3.25	0.302	0.105	0.031	0.263	0.068	0.214	6.1	5.0	4.0
9.67	4.85	0.258	0.066	0.015	0.354	0.183	0.208	5.7	5.1	4.1
12.9	6.5	0.228	0.110	0.025	0.442	0.109	0.306	5.5	4.7	4.1

[a]Calculated as monomeric Fe(III).
[b]Calculated using $\epsilon_M = 11.8 \times 10^4$ M^{-1} cm^{-1}, $\epsilon_D = 7.2 \times 10^4$ M^{-1} cm^{-1} from Ref. [10].
[c]pH = 6.93.

Table 2
The pH dependence of mesoferriheme regeneration rates; $t = 25$ °C, $\lambda = 389$ nm, $I = 0.1$ M, $[\text{mfh}]_0 = 9.32 \times 10^{-6}$ M, $[\text{NaOCl}]_0 = 9.22 \times 10^{-6}$ M

pH	$10^1 k_1$ (s^{-1})	$10^2 k_2$ (s^{-1})
6.88[a]	1.36	3.20
7.49[a]		2.95
8.13[a]		2.56
8.88[b]	1.34	3.17
9.15[b]	1.41	2.86

[a]Phosphate buffer.
[b]Carbonate buffer.

Table 3
Phosphate concentration dependence of mesoferriheme regeneration rates; $t = 25$ °C, pH = 6.88, $\lambda = 389$ nm, $I = 0.1$ M, $[\text{mfh}]_0 = 9.14 \times 10^{-6}$ M, $[\text{NaOCl}]_0 = 9.08 \times 10^{-6}$ M

$10^3[\text{phosphate}]$[a] (M)	$10^1 k_1$ (s^{-1})	$10^2 k_2$ (s^{-1})
8	1.12	3.30
10	1.20	3.21
20	1.20	3.28
35	1.10	3.47
50	1.19	3.22

[a]Total buffer concentration.

dependent, ϵ_C becomes and 'operational' extinction coefficient also dependent on concentration.

In an earlier definitive study of mesoferriheme absorption and dimerization, Brown and Hatzikonstantinou [10] determined the molar extinction of mfh as a function of concentration from 1.48×10^{-4} to 7.40×10^{-8} M. Our estimation of ϵ_C values in the present study comes from interpolation of these data using the linear dependence of ϵ_C on α, a concentration dependent term defining the fraction of total heme existing in monomeric form. As delineated by Brown et al. [6], $[C] = [M] + 2[D]$ and $\alpha = [M]/[C]$ where C denotes the total mfh concentration calculated as monomer and M and D denote monomer and dimer, respectively. Letting K_d denote the dissociation constant of the dimer, a term K_{obs} is defined as $K_d/[H^+] = [D]/[M]^2$ from which the relation $\alpha^2[C] = (1-\alpha)/(2K_{obs})$ is derived. Thus, values of α as a function of [C] emerge from solution of the quadratic equation with only positive values being significant. Since $\epsilon_C[C] = \epsilon_M[M] + \epsilon_D[D]$, it follows that $\epsilon_C = (\epsilon_M - \epsilon_D/2)\alpha + \epsilon_D/2$. Based upon the referenced data [10] in the Soret region at pH 6.98, $10^{-3}\epsilon_C = 81.8\alpha + 36.3$ ($R = 0.99$) which leads to $\epsilon_M = 11.8 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_D = 7.25 \times 10^4$ M^{-1} cm^{-1}, values comparable to those obtained separately for monomer and dimer extinction in deuteroferriheme [6]. The determination of ϵ_A is based upon these values of ϵ_M and ϵ_D and measurement of the absorbance change accompanying mfh regeneration, i.e. $\Delta a = a_\infty - a = l(\epsilon_C[C] - \epsilon_A A_0)$. Since $A_0 = C_0/2$, $\Delta a/(lC_0) = (\epsilon_M - \epsilon_D/2)\alpha + (\epsilon_D - \epsilon_A)/2$. Values of ϵ_B are then obtained from the ratio β/γ (Eqs. (4) and (5)).

Kinetic parameters along with corresponding extinction coefficients are given in Table 1 for regeneration studies at three separate mfh and OCl^- concentrations at 25 °C and pH 6.92. Although the mfh extinction values of Brown and Hatzikonstantinou [10] were taken at pH 6.98, studies in this laboratory as well as previous reports on dfh and pfh absorption [9,10] indicate a variation of 0.06 pH units to be negligible in terms of derived ϵ values.

Although at the same total heme concentration, temperature, and pH the absorptivity of dfh exceeds that of mfh due to the difference in dimerization equilibria and the fact that monomeric heme has measurable higher extinction than dimer, the corresponding ϵ_A and ϵ_B terms are higher for mfh derived species, i.e., $10^{-4}\epsilon_A \approx 4$ M^{-1} cm^{-1} as opposed to ≈ 2 M^{-1} cm^{-1} for dfh; $10^{-4}\epsilon_B \approx 5$ M^{-1} cm^{-1} versus ≈ 4 M^{-1} cm^{-1} for dfh [33]. The k_1 terms appear to be comparable for reduction of mfh and dfh Compound I analogs, with k_2 for mfh higher by about a factor of 2.

The mfh regeneration rate constants are independent of pH and total buffer concentration over measurable ranges (Tables 2 and 3) indicating that Compound I and II analogs are either not structurally influenced by such changes or that any accompanying structural change does not affect susceptibility to electron transfer in the redox process. This as well as the heme concentration independence of ϵ_A and ϵ_B also suggest

dissociation of $Fe_2^{IV}O$ and $Fe^{III}OFe^{IV}$ to mononuclear iron species to be negligible under these conditions.

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