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Iron-Porphyrin Models of Hemoprotein Enzymes: Mechanism of Decomposition of the Deuteroferriheme Analogues of Peroxidase Enzyme Intermediates

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The iron(III) complex of deuteroporphyrin(IX), deuteroferriheme (dfh), which is a functional analogue of selected peroxidase enzymes, is regenerated spontaneously from an oxidized form of the heme that arises through the reaction of dfh with a variety of O atom donor oxidizing agents such as OCl^-, ClO_2^-, and peroxoacids. This regeneration is followed on a stopped-flow spectrophotometric time scale by observing the recovery of optical density, which follows the absorbance decrement accompanying dfh oxidation in the Soret region (384 nm) of the heme spectrum. The corresponding absorbance change is biphasic, and a graphical treatment of the data leads to the determination of two pseudo-first-order (in dfh) rate constants. Data are compatible with two mechanistic models: one involving two parallel reactions in which two forms of oxidized heme species are simultaneously reduced and the second comprising of two consecutive (series) reactions in which a single form of oxidized heme is reduced to a reaction intermediate that is subsequently reduced to free heme. Kinetic analysis does not allow confirmation or elimination of either mechanism; however, the series model is confirmed through temperature studies. Thus, the spectrum of the dfh oxidation product is independent of temperature, whereas the rate of heme oxidation shows a definite temperature dependence indicating that only one species is produced in the oxidation of dfh. The identity of the reducing agent(s) is unknown; however, the reproducibility of pseudo-fist-order rate constants suggests the participation of solvent water in a rate-determining attack on those species that undergo reduction in the sequential reactions leading to dfh regeneration. A decrease in the extent of regeneration is accompanied by an increase in regeneration rate at high concentrations of heme oxidant. This is most likely due to an oxidant-induced degradation of the porphyrin ring system in which reductant species are produced, thereby providing an additional pathway for dfh regeneration.

Introduction

The catalytic activity of selected hemoprotein enzymes, such as horseradish peroxidase (HRP), has been shown to involve a two-electron oxidation of enzyme by a peroxo substrate to produce an enzyme intermediate (compound I). Compound I then functions as an oxidizing agent of selected species via two consecutive one-electron redox processes, the first proceeding to a second enzyme intermediate (compound II) and the second resulting in enzyme regeneration.[1] The nature and reactivity of such enzyme-derived intermediates has been extensively reviewed.[1-3]

Since the catalysis involves reaction at a heme Fe(III) site, various protein-free iron-porphyrin complexes (Figure 1) have been studied as enzyme models, particularly with regard to the formation and peroxidatic activity of the analogous reaction intermediates.[4-17] However, these enzyme models are subject to dimerization in aqueous solution, the equilibrium between the monomeric heme and its dimer (presumed to exist as an oxo-bridged dinuclear Fe(III) species) having been reported for *proto-deutero-* and *meso*ferrihemes.[18-20] Since dimerization reduces heme reactivity toward oxidizing substrates, deuteroferriheme (dfh), which has a degree of dimerization significantly less than that of the *proto-* and *meso*-derivatives, has seen widespread use as a model system.[21] Its oxidation by O atom donor species, including peroxoacids, iodosobenzene, ClO_2^-, OCl^-, and Me_3N-O, produces a functional (but not stoichiometric) analogue of HRP compound I.[8,12,15,17,22]

Stoichiometrically, HRP compound I is formed through a 1:1 molar interaction of enzyme with peroxide, whereas the analogous oxidized dfh system (to which we refer as the oxidized "intermediate state") displays a 2:1 molar interaction of heme (calculated as monomeric Fe(III)) with a two-electron oxidant. This has been interpreted in terms of a two-electron oxidation of heme Fe(III) followed by comproportionation (Scheme I).[23]

Scheme I

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

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

Despite this stoichiometric variation of catalyst with oxidant, which appears to be a consequence of the tendency of the protein-free heme to form a dinuclear complex, the dfh-derived "intermediate state" mimics the enzyme-derived intermediates in the oxidation of various substrates, including phenols[10] and halide ions,[9,12] as well as in undergoing a slower spontaneous or "in situ" regeneration of catalyst in the absence of added peroxidatic re-

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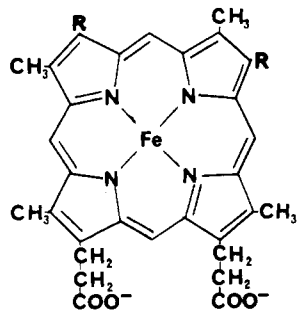

Figure 1. Ferriheme models: protoferriheme, R = $-CH{=}CH_2$; deuteroferriheme, R = H; mesoferriheme, R = $-CH_2CH_3$.

ductant. It is the nature of this regeneration process that comprises the focus of the present study.

One example of spontaneous catalyst regeneration from an enzymatic intermediate has been reported by Hager et al.,[24] who observed a biphasic absorbance change for the decomposition of chloroperoxidase compound I. On the basis of a first-order treatment of data, they proposed a mechanism involving two simultaneous or *parallel* first-order processes assuming the reduction of *two* forms of the oxidized enzyme. More recently, Bretscher and Jones have studied the biphasic regeneration of the iron–porphyrin complex, coproferriheme, from its compound I analogue and concluded that the process involves *series* pseudo-first-order reductions induced by solvent water.[25] It is in the perspective of these conflicting mechanistic schemes that the present study of the spontaneous (biphasic) regeneration of dfh was undertaken.

As we shall show, the kinetic analysis of spectral changes alone does not allow a distinction between parallel and series mechanisms. The present focus, then, is on the question as to whether dfh oxidation leads to an oxidized "intermediate state" consisting of more than one kinetically significant species, in which case regeneration occurs through parallel processes, or leads instead to a single species, in which case regeneration occurs through consecutive (series) reactions. Toward this end, kinetic experiments were done at different temperatures to determine the effect of temperature on the absorption spectrum of the "intermediate state", as well as on its rate of formation.

Since dfh oxidation at high oxidant concentrations has been shown to be accompanied by heme degradation through cleavage of the porphyrin ring system, particularly when H_2O_2 or *m*-chloroperoxobenzoic acid (MCPBA) is employed,[6,21,22] the milder oxidants NaOCl and $NaClO_2$ were used to form the precursor "intermediate state" for the regeneration studies.

Experimental Section

Triply recrystallized hemin was obtained from Nutritional Biochemicals Corp. and subjected to resorcinol melt treatment for the preparation of deuteroferriheme. Extraction and crystallization procedures were as described by Falk,[26] and the dfh was characterized as its pyridine hemochrome derivative, which was found to exceed 99% spectral purity (ϵ = 2.4×10^4 M^{-1} cm^{-1}; λ_{max} = 544 nm). *m*-Chloroperoxobenzoic acid was obtained from Pfalz and Bauer, and solutions 1–2 mM in peroxoacid were prepared and analyzed iodometrically. Sodium hypochlorite was obtained as a 5% solution in NaOH from Mallinckrodt. Sodium chlorite (granular reagent) was obtained from Matheson Coleman and Bell and was shown to be ≥99% pure on the basis of iodometric analysis. Stock solutions having oxidant concentrations between 0.5 and 5.0 mM were prepared and standardized also by iodometric methods. Stock samples were then diluted to appropriate concentrations shortly before their use in stopped-flow experiments. All solutions were prepared by using deionized water, which was passed through a Barnstead mixed-bed ion-exchange column. The conductivity was <0.01 ppm measured as NaCl. All buffer components were of reagent grade. Ionic strengths were adjusted with NaCl. Stopped-flow studies were carried out with a Durrum-Gibson Model D-110 stopped-flow spectrophotometer and oscilloscopic traces obtained with a Tektronix Model 5310N and/or Model 2221 digital storage oscilloscope with HC100 digital plotter. Conventional visible spectra were recorded on a Perkin-Elmer Model 552 spectrophotometer.

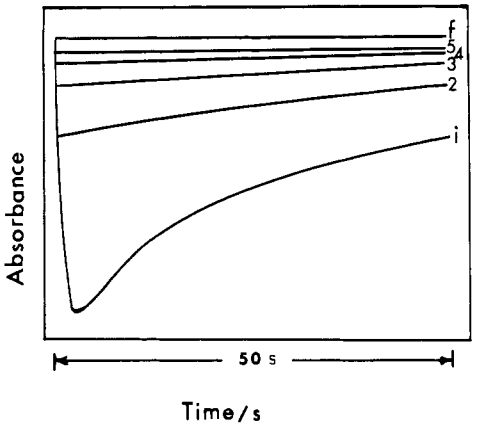

Figure 2. Stopped-flow spectrophotometric trace showing formation of "intermediate state" and regeneration of the heme spectrum. $[dfh]_0$ = 10.3×10^{-6} M; $[OCl^-]_0$ = 6.4×10^{-6} M; pH = 9.25; μ = 0.1 M; t = 25 °C; λ = 384 nm. i, 2, 3, 4, 5, and f denote initial, second, third, fourth, fifth, and final scans each of 50-s duration.

The absorption spectrum of the dfh-derived "intermediate state" was obtained at each temperature as a series of points by subtracting the decrement in absorbance observed upon stopped-flow mixing dfh with oxidant from the absorbance level established with a stopped-flow mixture of dfh with water at different wavelengths. The rate of formation of the "intermediate state" was studied in the presence of excess oxidant and the observed pseudo-first-order (in dfh) rate constants converted to apparent second-order constants according to $k_{app} = k_{obs}/[oxidant]$. Since dfh oxidation occurs predominantly through reaction of heme monomer, the second-order rate constant for disappearance of monomeric heme, k_m, is obtained by dividing k_{app} by a term $n\alpha$, where α represents the fraction of total dfh existing in monomeric form under the specific conditions of temperature, pH, and stoichiometric heme concentration, $[dfh]_0$,[27] and n denotes the stoichiometric equivalence of heme Fe(III) to a single formula unit of oxidant according to Scheme I (n = 2 for OCl^-; n = 4 for ClO_2^-).[6,12,17]

Results

An absorbance–time profile showing recovery of the dfh spectrum following oxidation by OCl^- is shown in Figure 2. That the recovery of optical density represents the actual regeneration of dfh is confirmed by a comparison of kinetic parameters. Thus, a 26.4 μM solution of dfh at pH 9.1 was mixed with an equal volume of 13.2 μM hypochlorite and the solution allowed to stand for 20 min to allow recovery of the optical density lost on formation of the "intermediate state". A sample of the resulting solution (~13.2 μM dfh) was then stopped-flow mixed with 80 μM OCl^-, and the pseudo-first-order decrease in absorbance compared with that obtained on mixing 13.2 μM freshly prepared dfh with 80 μM OCl^- at the same pH. Observed pseudo-first-order rate constants were the same within experimental error: 3.50×10^5 s^{-1} for reaction of OCl^- with freshly prepared dfh; 3.46×10^5 s^{-1} for reaction with the standing solution obtained from a previous mix of dfh with OCl^-, i.e., from "regenerated" dfh.[28]

The biphasic nature of regeneration is empirically evident from a graphical treatment of the data shown in Figure 3 for dfh recovery from the "intermediate state" obtained via oxidation with OCl^- at pH 9.25. Here a denotes the absorbance at time t, and

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(28) Within experimental error, the same kinetic parameters for heme regeneration were also obtained: k_1 = 0.11 s^{-1}, k_2 = 5.7×10^{-3} s^{-1} from "intermediate state" formed by OCl^- oxidation of fresh dfh; k_1 = 0.075 s^{-1}, k_2 = 5.7×10^{-3} s^{-1} from "intermediate state" formed via oxidation of "regenerated" dfh. In both solutions, the regeneration kinetics were measured starting with "intermediate state" formed by adding 6.6 μM OCl^- to 13.2 μM contained dfh.

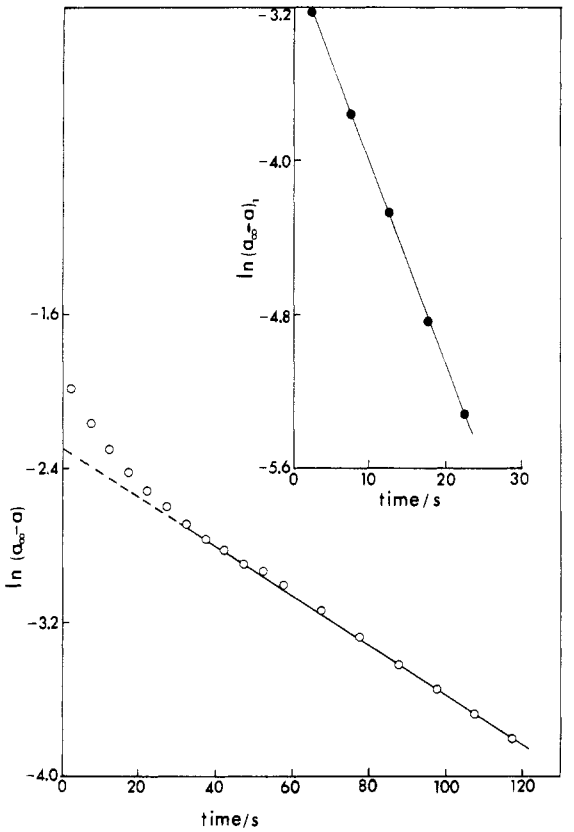

Figure 3. Graphical treatment of deuteroferriheme regeneration. Reaction conditions are as in Figure 2. Open circles show log values for changes in total absorbance; k_2 (=1.28 × 10^{-2} s^{-1}) was determined from the slope of the line. Closed circles show calculated log values for changes in absorbance of faster reacting species (A); the slope yields k_1 (=9.77 × 10^{-2} s^{-1}).

a_∞ denotes the maximum level of recovered absorbance. The plot of ln $(a_\infty - a)$ vs t becomes linear at long times. This line, representing the effect of the slower process, conforms empirically to a first-order reaction and can be written ln $(a_\infty - a)_2$ = ln γ − k_2t, where ln γ is the intercept at t = 0. The absorbance due to the faster process is then defined by difference as $(a_\infty - a)_1$ = $(a_\infty - a) - (a_\infty - a)_2$. As illustrated in Figure 3, ln $(a_\infty - a)_1$ is linear with t and can be written as ln $(a_\infty - a)_1$ = ln $\beta - k_1t$. Hence, the data are entirely consistent with an absorbance of the form

$$(a_\infty - a) = \beta e^{-k_1 t} + \gamma e^{-k_2 t} \quad (3)$$

The absorbance–time profile, then, suggests regeneration to occur via two first-order processes, the faster characterized by rate constant k_1 and the slower by k_2. It is compatible with either of two mechanistic models depicted below.

Here, Scheme II represents parallel reactions leading to regenerated dfh, C, from two different kinetically significant components of the "intermediate state", A and B. In Scheme III, regeneration is viewed as emanating from an "intermediate state" comprised of a single species, A, through reaction intermediate B.

Scheme II

$$A \xrightarrow{k_1} C \quad (4)$$

$$B \xrightarrow{k_2} C \quad (5)$$

Scheme III

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \quad (6)$$

Assuming Beer's law for A, B, and C with $a = a_A + a_B + a_C$,

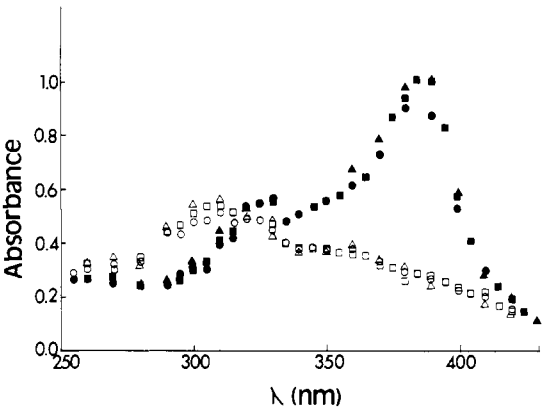

Figure 4. Absorption spectra at different temperatures of dfh and "intermediate state" generated via oxidation by NaOCl (pH= 6.9; μ = 0.1 M). dfh: (▲) 18 °C; (●) 25 °C; (■) 35 °C. "Intermediate state": (△) 18 °C; (○) 25 °C; (□) 35 °C.

each mechanistic model produces an equation of the form shown in (3) with different interpretations of the coefficients β and γ. For the parallel model (Scheme II), $\beta = lA_0(\epsilon_C - \epsilon_A)$ and $\gamma = lB_0(\epsilon_C - \epsilon_B)$: whereas for the series model (Scheme III)

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

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

Here, ϵ_A, ϵ_B, and ϵ_C denote the respective molar extinction coefficients, A_0 and B_0 the initial concentrations of A and B, and l, the cell path length. It is assumed that C_0 = 0 for the parallel model and that $B_0 = C_0 = 0$ in Scheme III. Although β and γ can be evaluated, the designation of a particular reaction scheme is contingent upon a knowledge of the molar extinction coefficient of each species, such data being unavailable at present. It is clear, therefore, that the kinetic treatment alone does not allow distinction between Schemes II and III for heme regeneration. Both parallel and series models are accommodated by the available data, and this limitation in mechanistic interpretation is inherent in any kinetic study of enzyme or heme regeneration based on experimentally determined time dependent absorbance changes.

A distinction between parallel and series models may be made, however, on the basis of studies of the effect of temperature on the rate of formation of "intermediate state" and on its absorption spectrum. Thus, if dfh oxidation produces a single species, it is expected that, barring an effect due to a thermally induced change, the absorption spectrum of the "intermediate state" will be relatively insensitive to temperature. Formation of two forms of oxidized species (A and B in Scheme II) on the other hand, would be expected to lead to a temperature-dependent absorption spectrum due to a temperature-dependent distribution of A and B, although such temperature dependence would be contingent on the existence of a significant activation energy for formation of the "intermediate state" if this distribution were kinetically controlled or a measurable enthalpy of interconversion if thermodynamically controlled.

Figure 4 shows the temperature independence of the point spectrum of the "intermediate state" obtained via treatment of dfh with NaOCl at pH 6.9 and at 18, 25, and 35 °C. Comparable results indicating no significant spectral change with temperature were also obtained at 18, 25, 30, and 35 °C for intermediate generated via heme oxidation by $NaClO_2$. Rate constants do change, however, and data obtained over the range 18–35 °C as well as corresponding activation parameters are given in Table I for the reaction of dfh with both oxidants. It seems unlikely that two forms of reaction intermediate are produced with identical activation energies through the oxidation of dfh by *both* OCl^- and ClO_2^-. We conclude, then, that A denotes a single form of oxidized

Table I. Temperature Dependence of the Rate of Formation of "Intermediate State" via Oxidation of Deuteroferriheme by NaOCl and $NaClO_2$ (pH = 6.9; Ionic Strength (μ) = 0.1 M)

temp, °C	NaOCl $10^{-6}k_m$,[a] M^{-1} s^{-1}	$NaClO_2$ $10^{-4}k_m$,[a] M^{-1} s^{-1}
18	1.09	2.31
25	1.42	3.51
30	2.06	4.95
35	2.71	6.29
E_a,[b] kcal/mol	9.8	10.7
$\Delta H^{\ddagger}$,[c] kcal/mol	9.1	10.2
$\Delta S^{\ddagger}$,[c] cal/(mol K)	+0.36	−1.2

[a] k_m denotes second-order rate constant for attack of oxidant on monomeric heme. For $-d[dfh]/dt = k_{obs}[dfh][oxidant]$, $k_{m(OCl^-)} = k_{obs}/2\alpha$ and $k_{m(ClO_2^-)} = k_{obs}/4\alpha$. α = fraction of heme present as monomeric Fe^{III}. Stoichiometric ratios: $[Fe^{III}]:[OCl^-] = 2:1$; $[Fe^{III}]:[ClO_2^-] = 4:1$. [b] $k_m = Ae^{-E_a/RT}$. [c] $k_m = (kT/h)e^{-\Delta H^{\ddagger}/RT}e^{\Delta S^{\ddagger}/R}$.

Table II. Deuteroferriheme Regeneration, (t = 25 °C; λ = 384 nm; Ionic Strength (μ) = 0.1 M)

pH	$10^6[dfh]_0$,[a] M	$10^6[OCl^-]_0$,[a] M	10^2k_1,[b] s^{-1}	10^2k_2,[c] s^{-1}
7.1	5.18	4.32	7.1	0.65
7.1	10.4	5.03	10.0	1.4
7.1	15.5	10.6	12.1	0.71
7.5	5.16	6.27	7.6	1.32
7.5	10.3	12.1	9.3	1.17
7.5	15.5	18.1	9.2	1.14
8.1	10.4	10.6	11.2	1.18
8.1	15.5	18.6	7.7	1.13
8.7	5.16	7.19	8.4	0.72
8.7	10.3	12.3	12.2	0.86
8.7	15.5	17.6	11.3	0.87
9.3	5.16	5.91	7.8	0.85
9.3	10.3	6.4	9.8	1.28

[a] $[dfh]_0$ and $[OCl^-]_0$ denote initial concentrations employed in forming the "intermediate state". [b] Obtained from plot of ln $(a_\infty - a)_1$ vs t. [c] Obtained from linear portion of plot of ln $(a_\infty - a)$ vs t at large reaction time.

heme and that dfh regeneration proceeds via *series* reactions.

Discussion

If A is presumed to be a two-electron-oxidation product of dfh, regeneration may involve consecutive one-electron reductions, in which case B may be regarded as a functional analogue of enzyme compound II. Such a scheme bears formal similarity to the model of coproferriheme regeneration proposed by Bretscher and Jones in which series reactions were invoked on the basis of biphasic time-dependent absorption data.[25]

The dfh system appears to differ markedly from that of coproferriheme, however, in the apparent aggregate nature of the initial oxidation product A. Thus, if A exists as an oxo-bridged dinuclear species, as suggested by the Fe(III)/oxidant stoichiometry and by analogy to the known dinuclear nature of certain Fe(III)–porphyrins, its one-electron reduction may yield a mixed oxidation state species as depicted in (7), although, again, specific oxidation sites remain conjectural for both A and B.

$$\underset{\text{A}}{Fe^{IV}{}_2O} \xrightarrow{k_1} \underset{\text{B}}{Fe^{III}OFe^{IV}} \xrightarrow{k_2} \underset{\text{(dimer)}}{Fe^{III}{}_2O} \rightleftharpoons \underset{\text{(monomer)}}{2Fe^{III}} \qquad (7)$$
$$\text{C}$$

Although of interest, the nature of the reductant(s) in the regeneration process remains speculative. Previous studies, however, exclude a significant contribution of carboxylic acid in those reactions involving peroxoacid oxidation of heme.[21,29] Also, trace impurities in either dfh, $NaClO_2$, or NaOCl can be ruled out on the basis of the independence of k_1 and k_2 on initial dfh concentration (Table II) and similarities in regeneration rate constants when dfh is oxidized with either NaOCl or $NaClO_2$.

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Table III. Effect of Initial Oxidant Concentration on Deuteroferriheme Regeneration

$10^6[dfh]_0$ = 10.3 M; pH = 9.25

$10^6[NaOCl]_0$, M	% conversion[a]	% regeneration[b]	10^2k_1, s^{-1}	10^2k_2, s^{-1}
2.66	52	>95	7.35	1.22
2.93	57	>95	11.1	1.56
3.20	62	>95	8.71	1.60
5.86	100	>95	7.74	0.89
6.13	100	>95	12.6	1.08
6.39	100	>95	9.77	1.28
9.25	100	>95	9.90	0.86
11.8	100	>95	10.2	0.89
12.0	100	>95	10.2	0.65
14.0	100	>95	12.4	0.59
14.5	100	>95	12.6	0.62

$10^6[dfh]_0$ = 9.3 M; pH = 6.9

$10^6[NaOCl]_0$, M	% conversion[a]	% regeneration[b]	10^2k_1, s^{-1}	10^2k_2, s^{-1}
4.68	100	88	5.20	0.89
9.33	100	78	4.9	0.67

$10^6[dfh]_0$ = 9.80 M; pH = 8.4

$10^6[MCPBA]_0$, M	% conversion[a]	% regeneration[b]	10^2k_1, s^{-1}	10^2k_2, s^{-1}
1.65	34	>95	17.5	1.72
3.30	67	>95	14.1	1.44
4.95	100	95	14.1	1.69
6.60	100	93	13.9	1.88
8.25	100	91	10.8	2.16
11.0	100	80	13.0	2.93
13.8	100	75	17.7	4.18
16.5	100	70	23.8	5.75
19.3	100	68	26.8	7.68
27.5	100	55		18.3[c]
55.0	100	20		55.5[c]

[a] dfh to "intermediate state" assuming a 2:1 ratio of Fe(III) to two-electron oxidant. [b] Estimated degree to which the decrement in absorbance accompanying dfh oxidation is recovered via subsequent regeneration. [c] Absorbance change approaches a single first-order decay.

Table IV. Comparative Regeneration Rates

species	oxidant	10^2k_1, s^{-1}	10^2k_2, s^{-1}
chloroperoxidase[a]	MCPBA	8.8	0.67
deuteroferriheme[b]	MCPBA		0.96–4.4
deuteroferriheme[c]	MCPBA	~15–20	1.5–3.0
deuteroferriheme[c]	OCl^-	5–12	0.6–1.2
deuteroferriheme[c]	ClO_2^-	2–3	~0.6
coproferriheme[d]	MCPBA	62	7.4

[a] Reference 24. [b] Reference 29; one rate constant reported, k_{obs} = rate/[intermediate], where [intermediate] is equated to $[dfh]_0$ over the range 2.7–21 μM at pH = 9.4. [c] This research. [d] Reference 25; tabulated average values of k_1 and k_2 for eight determinations for $[cfh]_0$ = 5–15 μM at pH = 6.0.

Direct involvement of OCl^- in this regeneration can also be eliminated as a result of studies carried out in the presence of morpholine–borane, which rapidly and quantitatively reduces OCl^-[30] without attacking the heme-derived "intermediate state". In particular, introduction of morpholine–borane to a concentration of 7.1 μM in cells containing solutions of "intermediate state" produced from the reaction of 5, 10, and 15 μM dfh with 28 μM NaOCl served only to speed up the observed onset of regeneration, presumably by scavenging excess OCl^- and thereby curtailing reoxidation of regenerated heme. Regeneration rate constants were not affected by the addition of morpholine–borane.

The reproducibility of pseudo-first-order rate constants at moderate initial oxidant concentrations may be suggestive of the involvement of solvent water as a reactant in the rate-limiting steps for regeneration. As seen in Table III, however, high relative

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concentrations of oxidant (MCPBA more than OCl^-) lead to a reduction in the degree to which the heme absorbance is recovered. Heme oxidation in the presence of a large excess of oxidant, particularly H_2O_2 and peroxoacid, has been shown to be accompanied by porphyrin ring degradation and formation of nonabsorbing or low-absorbing products.[25,31] We suggest the marked increase in k_1 and k_2 that occurs with increasing peroxoacid concentration to be a result of porphyrin ring degradation producing reducing ligands, which then attack the oxidized dfh species and provide an additional pathway for decomposition to free heme.[32,33] At the present time, mechanistic details are unknown; however, the fact that higher regeneration rates are observed following heme oxidation by MCPBA than by OCl^- or ClO_2^- (Table IV) probably reflects a peroxoacid induced degradation of the porphyrin ring system.

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(33) Bretscher and Jones suggest the involvement of water in the degradation of coproferriheme–compound I to produce reducing species that promote further decomposition of intermediate.[25]

Notes

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A Comment on the ^1H NMR Spectra of Cobalt(II)-Substituted Superoxide Dismutases with Histidines Deuteriated in the $\epsilon 1$-Position

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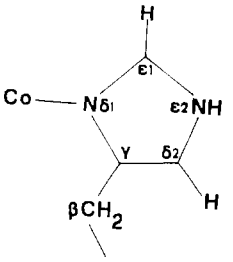
Erythrocyte superoxide dismutase Cu_2Zn_2SOD is a dimeric metalloenzyme containing one copper(II) and one zinc(II) ion bridged by a histidinato residue in each subunit. E_2Co_2SOD and $Cu^I_2Co_2SOD$ are artificial derivatives of the native Cu_2Zn_2SOD[1,2] that permit the investigation through ^1H NMR spectroscopy of the protons of the cobalt domain.[3,4] Cobalt(II) is bound to three histidines and to one aspartate, as shown in the inset of Figure 1; each of the histidines is coordinated through its $N^{\delta 1}$ nitrogen:[5]



The ^1H NMR spectra of the bovine E_2Co_2SOD and $Cu^I_2Co_2SOD$ derivatives are shown in Figure 1. In the $Cu^I_2Co_2SOD$ derivative there are six sharp signals downfield; three of these (shaded in Figure 1) disappear when the spectrum is recorded in D_2O. The latter are assigned as the $N^{\epsilon 2}$ exchangeable protons of the three coordinated histidines. The other three signals are due to $H^{\delta 2}$ of the same histidines that are in a meta-like position with respect to the coordinating nitrogen.[3] Their T_1 and line shape are consistent with signals assigned to meta-like protons of histidines in similar compounds.[6-10] In E_2Co_2SOD one NH signal is missing, probably because it exchanges rapidly on the NMR time scale. One or two broader signals are also present in the spectra, which

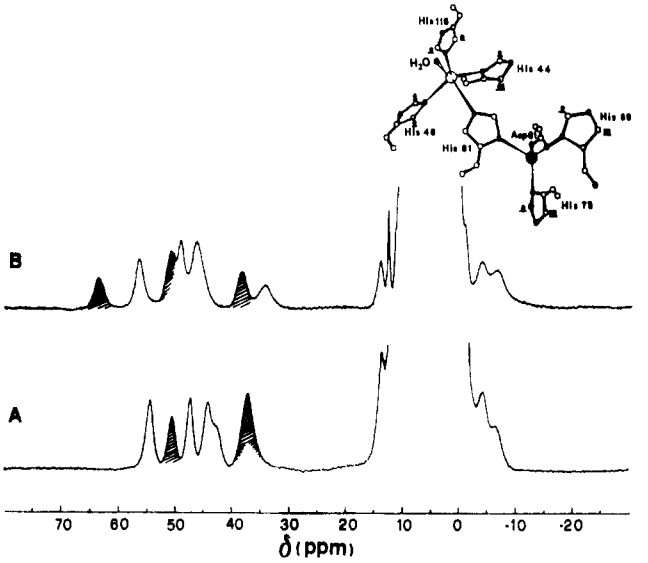


Figure 1. 200-MHz ^1H NMR spectra of bovine E_2Co_2SOD (A) and bovine $Cu^I_2Co_2SOD$ (B). The spectra are recorded in 50 mM acetate buffer, in H_2O, pH 5.5, at 27 °C. The inset shows a scheme of the metal site of SOD.

were tentatively assigned to $H\epsilon 1$ (ortho-like protons) of the above histidines because they are expected to be broader than the meta-like protons.[4,11]

Recently human SOD has been expressed in *Escherichia coli* (HECSOD)[12] and it has been conceivable to prepare a SOD

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