hancement of the spin-spin relaxation times of the three ^{13}C and two ^{31}P resonances of DPG (Table IV). Therefore, the interaction of the Cs^+ ion with DPG most likely involves the carboxylic group on carbon 1 and each of the phosphate groups on carbons 2 and 3 of DPG. The chemical shifts of the ^{13}C and ^{31}P NMR resonances of DPG did not change upon addition of Cs^+, presumably because the interaction with the carboxylate and phosphate groups of DPG occurs via the oxygen atoms and not directly with the reporter nuclei. From our ^{31}P and ^{133}Cs NMR results (Tables I and IV), we conclude that Cs^+ interacts more strongly with DPG than with ADP or ATP. Cs^+ forms a 1:1 complex with DPG with a K_D of 3.8 ± 0.2 mM. This Cs^+ interaction is unique because DPG is reported to bind Mg^{2+}, Zn^{2+}, and Al^{3+} more weakly than does either ADP or ATP, despite the presence of two basic phosphates.[32,43,44] The stronger interaction of Cs^+, relative to Na^+,[45] with DPG may be related to the larger ionic size of Cs^+ and to the capacity of DPG to act as a tridentate ligand. We conclude from our ^{31}P and ^{13}C NMR data (a) that Cs^+ interacts with the phosphate and carboxylate groups of DPG and (b) that the competing ions decrease the observed Cs^+ interaction with DPG.

Comparison of ^{31}P NMR spectra of Cs^+-loaded and Cs^+-free RBC suspensions (Table II) indicated that Cs^+ loading broadened and moved the ^{31}P resonances of DPG (and to a smaller extent the resonances of ATP and P_i) downfield in deoxyRBC but not in CORBC suspensions. Deoxygenation did not cause any significant changes in the peak areas of the ^{31}P resonances of DPG in Cs^+-loaded or Cs^+-free RBC suspensions (data not shown). This lack of change in DPG levels upon deoxygenation is in agreement with previous reports.[30a,46] DPG binds more strongly to deoxyhemoglobin than to oxyhemoglobin (or its analogue, COHb).[30a,33] Moreover, more Mg^{2+} is bound to DPG in CORBC than in deoxyRBC suspensions.[30a] Although less free DPG is available in deoxygenated RBC suspensions to bind Cs^+, DPG is not complexed as fully to Mg^{2+}.[30a] Because the affinity of DPG for Mg^{2+} is higher than that for Cs^+,[32] binding of Cs^+ to free intracellular DPG occurs most strongly in deoxyRBC suspensions. This may explain why the effect of Cs^+ loading on ^{31}P resonances of DPG is more noticeable in deoxyRBC than in CORBC suspensions (Table II). Competition between Cs^+ and hemoglobin for DPG will have the result that smaller amounts of DPG are available to bind to deoxyHb, which, in turn, may result in an increase in the oxygen affinity of hemoglobin. The enhanced oxygen affinity of hemoglobin in the presence of Cs^+ may hinder the release of oxygen to tissues and may provide a mechanism for Cs^+ toxicity.

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Determination of the Molar Extinction Coefficients of the Deuteroferriheme Analogues of Peroxidase Enzyme Compounds I and II

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Absorbance data obtained in the stopped-flow spectrophotometric study of the "in situ" biphasic regeneration of deuteroferriheme (dfh) following its oxidation by NaOCl are utilized to calculate extinction coefficients of species presumed to be involved in series pseudo-first-order redox regeneration processes. Such data are interpreted in terms of a previously proposed mechanism of the type A $\xrightarrow{k_1}$ B $\xrightarrow{k_2}$ C where A denotes the dfh-OCl^- oxidation product; B, a reaction intermediate; and C, the regenerated dfh. Results are consistent with a scheme involving consecutive one-electron reductions of the heme analogues of peroxidase enzyme compounds I and II to regenerate free dfh in a state of monomer-dimer equilibrium. A value $\epsilon \cong 4 \times 10^4$ M^{-1} cm^{-1} at $\lambda = 384$ nm is computed at pH 6.85 and 25 °C for the molar extinction coefficient of the compound II analogue (B), which has been depicted as dinuclear $Fe^{III}OFe^{IV}$. This is intermediate in magnitude between the value $\epsilon \cong 2 \times 10^4$ M^{-1} cm^{-1} calculated for the dfh-derived compound I analogue (A), depicted as $Fe^{IV}{}_2O$, and previously calculated values of extinction coefficients for monomeric and dimeric dfh. Such ϵ values are consistent with observed changes in optical density accompanying heme regeneration and with kinetic studies that show $k_1 > k_2$ by about an order of magnitude.

Introduction

A number of studies of the chemistry of iron(III)-porphyrin (heme) models of peroxidase enzyme systems have been focused on the stoichiometry and rates of formation and the subsequent reactivity of oxidized heme species which are functional analogues of enzyme-derived reaction intermediates.[2-16] Although such investigations are complicated by the tendency of the protein-free hemes to undergo dimerization in aqueous solution with consequent

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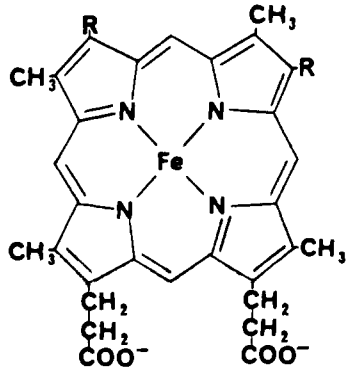

Figure 1. Ferriheme model systems: protoferriheme, R = $-CH{=}CH_2$; deuteroferriheme, R = H; mesoferriheme, R = Et; coproferriheme, R = $-CH_2CH_2COOH$.

decrease in peroxidase-like activity, the degree of aggregation is highly dependent on heme structure and, as a result of its relatively low dimerization constant, deuteroferriheme, dfh, has seen widespread use as an effective model system (Figure 1).[17-21] Largely on the basis of stopped-flow spectrophotometric studies, the stoichiometric mechanism depicted in Scheme I has been proposed for dfh oxidation by two electron oxygen donor substrates.[6,14]

Scheme I

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

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

In this scheme, Fe^{III} denotes the monomeric form of dfh; ROOH, the oxidizing agent; $Fe^{V}O$, an initially formed two-electron-oxidation product without implication as to the specific sites at which oxidation has occurred;[22,23] and $Fe^{IV}{}_2O$, a dinuclear product of comproportionation of $Fe^{V}O$ with free Fe^{III}, which may be regarded as a functional analogue of enzyme compound I. We refer to $Fe^{IV}{}_2O$ as the dfh-derived "intermediate state". Peroxidatic activity of the heme system is dependent on the oxidative action of the intermediate state toward reducing substrates; however, even in the absence of added reducing agent, "in situ" reduction leading to the regeneration of free heme is found to occur.

Recently, this in situ regeneration was reported to display biphasic kinetics with experimental absorbance data fitting eq 3,

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

suggesting two pseudo-first-order processes characterized, respectively, by rate constants k_1 and k_2.[24] Here, a denotes the absorbance at time t and a_∞ the final absorbance level corresponding to regeneration of heme ($a_\infty > a$). It has been noted, however, that the kinetic data alone do not allow a distinction between two mechanistic schemes, one of which involves series, or consecutive, processes depicted as A $\xrightarrow{k_1}$ B $\xrightarrow{k_2}$ C where B denotes a reaction intermediate formed in reduction of the intermediate state, A, to free heme C and the second of which involves parallel reactions depicted as (i) A $\xrightarrow{k_1}$ C and (ii) B $\xrightarrow{k_2}$ C where A and B denote kinetically different forms of the intermediate state.[25-27] Assuming Beer's law to apply with $a = a_A + a_B + a_C$, both series and parallel schemes conform to eq 3, where for the series model

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

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

and for the parallel scheme

$$\beta = lA_0(\epsilon_C - \epsilon_A) \quad (6)$$

$$\gamma = lB_0(\epsilon_C - \epsilon_B) \quad (7)$$

Here A_0 and B_0 denote respective initial concentrations, l denotes cell path length, and ϵ_A, ϵ_B, and ϵ_C denote the respective molar extinction coefficients. It is assumed that $B_0 = 0$ in the series model and that, in both schemes, there is no free dfh present at t_0, the onset of regeneration; i.e., $C_0 = 0$.

Although β and γ are readily determined experimentally, insufficient extinction data exist to allow a distinction between series and parallel mechanisms to be drawn from eqs 4-7 alone. However, on the basis of temperature-dependent studies of the rates of regeneration and the *temperature independence* of the optical spectrum of the intermediate state, obtained by difference in stopped-flow spectrophotometric experiments involving the measured decrease in optical density accompanying heme oxidation, it has been concluded that dfh regeneration proceeds through series, or consecutive, processes rather than via parallel reactions emanating from two forms of the intermediate state.[24] This has led to the proposal of Scheme II, which is consistent with the known tendency of heme to form oxo-bridged dinuclear complexes, as a probable model of regeneration involving consecutive one-electron redox processes. In this scheme, $Fe^{III}OFe^{IV}$ depicts an analogue of (peroxidase) enzyme compound II, a reaction intermediate which is formed via the peroxidatic one-electron reduction of compound I and which subsequently functions as a one-electron oxidant in processes leading to the regeneration of free peroxidase.[22,23,28]

Scheme II

$$\underset{A}{Fe^{IV}{}_2O} \xrightarrow{k_1} \underset{B}{Fe^{III}OFe^{IV}} \xrightarrow{k_2} \underset{C}{Fe^{III}{}_2O} \rightleftharpoons 2Fe^{III} \quad (8)$$

From experimental data and eqs 4 and 5, a procedure is now developed by which extinction coefficients of species A, B, and C are calculated. If the extinction coefficients so calculated appear reasonable, not only will they provide further evidence that the series mechanism is operative, but the calculation will also yield a quantitative property of intermediate species B. Presumably, similar calculations could also be performed with other model systems for heme-containing enzymes.

Experimental Section

Sources of materials, as well as the synthesis, purification, and analysis of deuteroferriheme and the standardization of oxidant solutions, have been previously described.[24] Due to the effectiveness of sodium hypochlorite in oxidizing dfh with minimal side reactions leading to heme degradation, NaOCl was used as the principal oxidizing agent.[15] Rates were measured and difference spectra obtained using a Durrum-Gibson D-110 stopped-flow spectrophotometer in conjunction with a Tektronix

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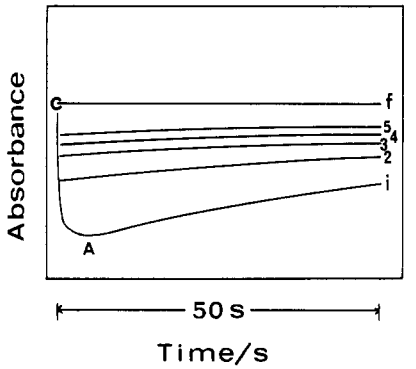

Figure 2. Stopped-flow spectrophotometric trace showing oxidation of dfh (C) to intermediate state (A) and regeneration of heme spectrum. $[dfh]_0 = 5.18 \times 10^{-6}$ M; $[OCl^-]_0 = 5.38 \times 10^{-6}$ M; pH = 7.09; $\mu = 0.1$ M; $t = 25$ °C; $\lambda = 384$ nm; i, 2, 3, 4, 5, and f denote initial, second, third, fourth, fifth, and final scans, each of 50-s duration.

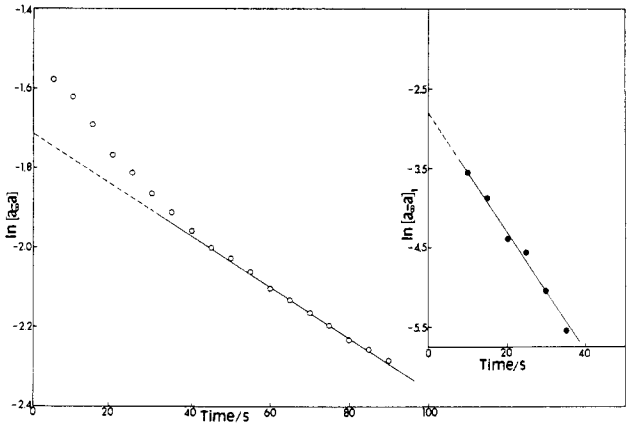

Figure 3. Deuteroferriheme regeneration. $[dfh]_0 = 5.18 \times 10^{-6}$ M; $[OCl^-]_0 = 4.32 \times 10^{-6}$ M; pH = 7.09; $\mu = 0.1$ M; $t = 25$ °C; $\lambda = 384$ nm. Data were treated as in ref 24 by applying eq 3 wherein $a_\infty - a = (a_\infty - a)_1 + (a_\infty - a)_2$. For $\ln (a_\infty - a)$ vs time (open circles), the slope of the line at long reaction times is taken as the rate constant for the slower of two first-order processes, i.e., $\ln (a_\infty - a)_2 = -k_2 t + \ln \gamma$. $k_2 = 6.4 \times 10^{-3}$ s^{-1}. $\gamma = 0.180$. $(a_\infty - a)_1$ values were obtained from $(a_\infty - a) - (a_\infty - a)_2$ in early nonlinear stages of reaction. $\ln (a_\infty - a)_1 = -k_1 t + \ln \beta$. For $\ln (a_\infty - a)_1$ vs time (closed circles), $k_1 = 7.5 \times 10^{-2}$ s^{-1}; $\beta = 0.0608$.

5310N or 2221 digital storage oscilloscope with HC100 digital plotter. Differences in absorbance, Δa, of free heme and the intermediate state (the analogue of compound I) were obtained by measuring the absorbance decrement obtained with a stopped-flow mixture of dfh with NaOCl relative to a dfh–H_2O mixture. In all cases, a slight stoichiometric excess of NaOCl was used to ensure complete conversion of dfh to its oxidized form.

Results and Discussion

Figure 2 depicts a typical stopped-flow absorbance profile showing the decrease in optical density in the Soret region of the heme spectrum due to relatively rapid oxidation of heme by NaOCl, followed by the slower recovery of absorbance accompanying in situ heme regeneration. The values of β, γ, and pseudo-first-order rate constants, k_1 and k_2, are obtained as previously described[24] from a graphical treatment of the absorbance change following formation of the intermediate state, a typical representation of which is shown in Figure 3. Since a small excess of oxidant is used in the initial reaction with dfh, its consumption is responsible for the small time lag frequently observed between completion of formation of the intermediate state and the first sign of an increase in optical density resulting from regeneration. This may lead to an early unreliable value of the absorbance and corresponding $\ln (a_\infty - a)$ term; however, since regeneration occurs through pseudo-first-order processes, this produces no detrimental effect on the determination of kinetic parameters.

Table I. Variation of ϵ_C with Total Stoichiometric Heme Concentration, C_0 (pH = 6.85; $t = 25$ °C; $\mu = 0.1$ M; $l = 1.0$ cm)

$10^6[C]_0$/ M[a]	10^6-[NaOCl]$_0$/ M	α[b]	Δa[c]	$10^{-4}\Delta a/[C]_0$	$10^{-4}\epsilon_C$/ (M^{-1} cm^{-1})[d]
1.85	2.01	0.638	0.184	9.95	10.9
2.78	3.01	0.568	0.248	8.92	10.1
3.70	4.02	0.519	0.320	8.65	9.57
4.63	5.03	0.482	0.384	8.29	9.14
5.55	6.03	0.452	0.448	8.07	8.80
6.48	7.04	0.428	0.488	7.53	8.52
7.40	8.04	0.408	0.544	7.35	8.29
8.33	9.05	0.390	0.560	6.72	8.09

[a] Calculated as monomeric heme Fe(III). [b] Reference 29. [c] $\lambda = 384$ nm. [d] $\epsilon_C = (\epsilon_M - \epsilon_D/2)\alpha + \epsilon_D/2$.

For the calculation of extinction coefficients, the series mechanism is assumed with A depicting the intermediate state and B denoting the product of the pseudo-first-order reduction of A and precursor to regenerated heme, C, which exists in monomer–dimer equilibrium. Thus, ϵ_C represents an *apparent* extinction coefficient which is a composite of extinction coefficients of the monomeric and dimeric forms of dfh. Since the position of dimerization equilibrium is dependent on total stoichiometric heme concentration, ϵ_C will also vary with the concentration of dfh.

In the following discussion, we employ notations similar to those developed by Brown et al.[18] and also utilized in studies of effects of heme aggregation on the peroxidatic action of specific heme model systems.[4] Accordingly, [C] depicts the total stoichiometric concentration of *free* dfh calculated as monomeric Fe^{III}.[29] Thus, setting [M] and [D] as the concentrations of monomeric and dimeric heme respectively, it follows that

$$[C] = [M] + 2[D] \tag{9}$$

If the total absorbance is attributed to dfh, then $a_C = a_M + a_D$. Assuming Beer's law for the absorbance due to [M] and to [D] yields

$$a_C = l(\epsilon_M[M] + \epsilon_D[D]) \tag{10}$$

Since [C] is a stoichiometric concentration, Beer's law does not hold for absorbance due to [C]; i.e., the extinction coefficient obtained would be concentration dependent. However, eq 10 suggests the functional form of Beer's law can be used to define a concentration-dependent "effective" extinction coefficient for C, given as

$$\epsilon_C[C] = \epsilon_M[M] + \epsilon_D[D] \tag{11}$$

Defining α as the fraction of total stoichiometric heme calculated as monomeric Fe^{III} actually present as monomer, i.e.

$$\alpha = [M]/[C] \tag{12}$$

then eqs 9, 11, and 12 yield

$$\epsilon_C = (\epsilon_M - \epsilon_D/2)\alpha + \epsilon_D/2 \tag{13}$$

The concentration dependence of the effective extinction coefficient ϵ_C is thus expressed as a simple linear dependence on α.

The total change in absorbance accompanying the regeneration of heme from intermediate state is represented as $\Delta a = a_\infty - a_0 = l(\epsilon_C[C]_0 - \epsilon_A[A]_0)$ where $[C]_0$ represents the initial concentration of free dfh (before oxidation) or the final dfh concentration (after regeneration) calculated as monomeric Fe^{III} and $[A]_0$, the initial concentration of intermediate state, i.e., that arising from stoichiometric oxidation of heme. Thus, assuming the dinuclear nature of the intermediate state, $[A]_0 = [C]_0/2$ and $\Delta a = l(\epsilon_C - \epsilon_A/2)[C]_0$. Equation 13 then yields

$$\Delta a/(l[C]_0) = (\epsilon_M - \epsilon_D/2)\alpha + (\epsilon_D - \epsilon_A)/2 \tag{14}$$

Values of Δa obtained at different total stoichiometric dfh concentrations $[C]_0$, at pH 6.85 and unit cell length, are listed

(29) Total stoichiometric heme concentration is denoted [T] in refs 18 and 4.

Table II. Values of Extinction Coefficients at Three dfh Concentrations (pH = 7.09; t = 25 °C; μ = 0.1 M; λ = 384 nm)

$10^6[C]_0/M^a$	$10^6[NaOCl]_0/M$	α	k_1/s^{-1}	k_2/s^{-1}	β	γ	$10^{-4}\epsilon_C{}^b$	$10^{-4}\epsilon_A{}^b$	$10^{-4}\epsilon_B{}^b$
5.18	4.32	0.379	0.075	0.0064	0.061	0.180	7.96	2.0	3.89
10.35	5.03	0.287	0.096	0.013	0.030	0.118	6.90	2.0	3.51
15.5	10.6	0.242	0.096	0.0072	0.202	0.238	6.38	2.0	4.18

[a] Calculated as monomeric Fe(III). [b] Calculated utilizing ϵ_D and ϵ_M values at pH 6.85.[19] Units are M^{-1} cm^{-1}.

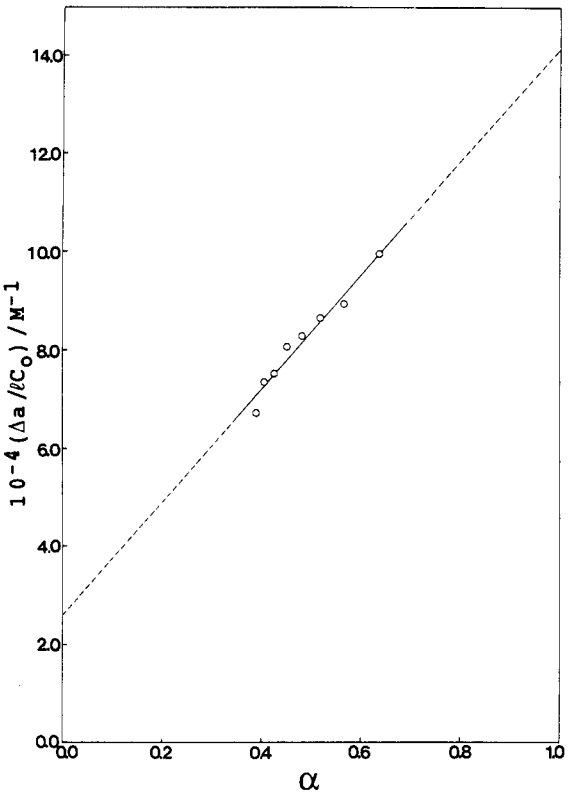

Figure 4. Plot of $\Delta a/(l[C]_0)$ vs α. Experimental conditions are given in Table I. Slope = $\epsilon_M - \epsilon_D/2$ = 11.5 × 10^4 M^{-1} cm^{-1}; intercept (α = 0) = $(\epsilon_D - \epsilon_A)/2$ = 2.6 × 10^4 M^{-1} cm^{-1}; intercept (α = 1) = $\epsilon_M - \epsilon_A/2$ = 14.1 × 10^4 M^{-1} cm^{-1}.

in Table I along with α values calculated from relationships established previously in spectroscopic studies of the pH and concentration dependence of the dfh dimerization equilibrium.[30] A plot of $\Delta a/(l[C]_0)$ vs α is given in Figure 4, wherein $\epsilon_M - \epsilon_D/2$ is obtained from the slope of the line and $(\epsilon_D - \epsilon_A)/2$ from the intercept at α = 0. The term $\epsilon_M - \epsilon_A/2$ also emerges directly from an extrapolation to α = 1, where, conceptually, $[C]_0$ = [M].

The calculation of the molar extinction coefficients also involves a consideration of the results of Brown et al. on the pH dependence of the dimerization equilibrium in deuteroferriheme.[18] By interpolation of the results of ref 18, the value ϵ_D = 7.2 × 10^4 M^{-1} cm^{-1} is obtained for the dfh dimer at pH 6.85 and 25 °C. From the intercept at α = 0 in Figure 4 (2.6 × 10^4 M^{-1} cm^{-1}), one then calculates ϵ_A = 2.0 × 10^4 M^{-1} cm^{-1} under the same conditions of temperature and pH. The slope of the line in Figure 4 (11.5 × 10^4 M^{-1} cm^{-1}) is then used to calculate the value ϵ_M = 15 × 10^4 M^{-1} cm^{-1}. This value compares favorably with the interpolated value $\epsilon_M \sim$ 12.5 × 10^4 M^{-1} cm^{-1} obtained from the results of Brown et al.[18]

As indicated above, ϵ_C is an effective extinction coefficient dependent upon the degree of dimerization, which, in turn, is dependent upon pH and total heme concentration expressed as $[C]_0$. Values of ϵ_C, listed in Table I, were calculated at various α values from eq 13 using the above value for ϵ_D and the measured slope (11.5 × 10^4 M^{-1} cm^{-1}) of the line shown in Figure 4.

The ratio of eqs 4 and 5 is then utilized to calculate $\epsilon_B = (\beta k_1\epsilon_C + \gamma k_2\epsilon_C + \gamma\epsilon_A(k_1 - k_2))/(k_1(\beta + \gamma))$ from a knowledge of ϵ_A and a determination of ϵ_C, β, γ, k_1, and k_2 under specific experimental conditions. The value of ϵ_B may be expected to be pH dependent but not heme concentration dependent if B is a single kinetically significant species. Data of Table II, compiled at pH 7.09 in regeneration studies conducted at $[C]_0$ = 5.2, 10.3, and 15.5 μM, indeed suggest ϵ_B to be independent of total dfh concentration.[31]

The assignment of B as a dinuclear compound II analogue, tentatively depicted as $Fe^{III}OFe^{IV}$ in Scheme II and, therefore, in an oxidation state intermediate between free heme and its compound I analogue, is consistent both with the calculated value of ϵ_B ($\sim$4 × 10^4 M^{-1} cm^{-1}), which is intermediate between ϵ_A and the range of ϵ_C values obtained, and with the absorbance trend observed for dfh regeneration, i.e., monatonic, with k_1 greater than k_2 by about 1 order of magnitude. Thus, extinction data appear altogether consistent with the proposed series mechanism suggesting consecutive one-electron reductions of the functional analogues of enzyme compounds I and II to the monomer–dimer equilibrium state of deuteroferriheme iron(III).

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(30) dfh dimerization is $[H^+]$ dependent according to $2M \rightleftharpoons D + H^+$ where $K_{dim} = [D][H^+]/[M]^2$. Defining $K_{obs} = K_{dim}/[H^+]$, it follows from the definition of α that $K_{obs} = (1 - \alpha)/(2\alpha^2[C])$. Thus, α is calculated from the resulting quadratic equation with only positive values being significant.[2,4]

(31) ϵ_M and ϵ_D values used to calculate ϵ_C and ϵ_A are those computed at pH 6.85. Brown et al.[18] have shown variations in ϵ_M and ϵ_D to be small in this range (pH 6.8–7.1).