115305-72-1; **5**, 78274-94-9; **6-H**·$2CH_2Cl_2$, 115290-48-7; **7**, 115290-49-8; **8-D**, 115290-52-3; **8-H**, 115290-50-1; **8-H** (Cl salt), 115290-61-4; **10**, 115290-54-5; **11**, 115290-56-7; **12**, 115290-58-9; **13**, 115290-60-3; Me_3Al, 75-24-1; CH_4, 74-82-8; CH_3CH_3, 74-84-0; CH_3CO, 75-07-0; perdeuteriotrimethylaluminum, 5630-35-3.

Supplementary Material Available: Table S-I, anisotropic thermal parameters for **3**; Table S-II, calculated hydrogen atom positions for **3**; Table S-III, anisotropic thermal parameters for **6**; Table S-IV, calculated hydrogen atom positions for **6** (10 pages); Tables S-V and S-VI, calculated and observed structure factors for **3** and **6**, respectively (92 pages). Ordering information is given on any current masthead page.

Intramolecular Long-Range Electron Transfer in the α-Hemoglobin Subunit

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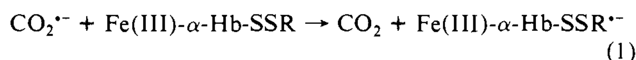
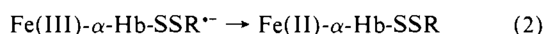
Contribution from the Department of Chemistry, Nuclear Research Centre-Negev, Beer-Sheva 84965, Israel, and Department of Chemistry, Division of Biological Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received December 21, 1987*

Abstract: The single sulfhydryl group (Cys-104) of the isolated human α-hemoglobin chain was converted to a mixed disulfide with 5,5′-dithiobis(2-nitrobenzoic acid). The mixed disulfide on the protein can be reduced to its radical anion with the pulse radiolytically generated formate radical, $CO_2^{\bullet-}$. The electron of the disulfide radical anion then transfers to the protein heme group at a nominal distance of approximately 12 Å with an intramolecular rate constant of 188 ± 23 s^{-1} at room temperature and pH 7. The disulfide radical anions formed from 5,5′-dithiobis(2-nitrobenzoic acid) and from the mixed disulfide on the protein have similar E_{m7}, −42 ± 1 and −41 ± 1 mV, respectively.

It is now apparent that intramolecular electron transfer can occur over relatively long distances in proteins (Table II and ref 2–7). While a number of factors may affect such long-range electron transfers (LRET)[1]—among them the distance, geometric disposition and redox potential between donor, and acceptor and the solvent/structural reorganization accompanying the transfer[8]—there is insufficient data to assess the relative importance of any one factor for LRET in proteins. Since LRET may play an important role in (i) physiologically significant electron-transfer reactions, (ii) damage of proteins and nucleic acids by ionizing radiation, and (iii) the potentially destructive reactions of metabolically generated free radicals, it is important to explore the mechanism(s) of LRET in biological macromolecules. Most protein LRET studies have involved electron transfer between metal centers held apart, at presumably fixed distances. There are fewer examples with an organic free radical as the electron donor and/or acceptor. Prütz and co-workers[9] reported that the tryptophan radical oxidizes tyrosine within the same peptide or protein, and we[10] have shown that the disulfide bond(s) of RNase A can be reduced to the disulfide radical anion ($RSSR^{\bullet-}$) in an intramolecular process. In this paper we report that the organic radical $RSSR^{\bullet-}$, formed by formate radical ($CO_2^{\bullet-}$) reduction of a mixed disulfide (eq 1) attached to cysteine-104 of the isolated α-hemoglobin subunit, can be an electron donor to the heme group in an intramolecular electron transfer (eq 2) over approximately 12 Å.[11]



$$CO_2^{\bullet-} + \text{Fe(III)-}\alpha\text{-Hb-SSR} \rightarrow CO_2 + \text{Fe(III)-}\alpha\text{-Hb-SSR}^{\bullet-} \qquad (1)$$



$$\text{Fe(III)-}\alpha\text{-Hb-SSR}^{\bullet-} \rightarrow \text{Fe(II)-}\alpha\text{-Hb-SSR} \qquad (2)$$

Experimental Methods and Materials

We treated (carbon monoxy)hemoglobin A, isolated from freshly drawn human blood by the procedure of Uchida and co-workers,[12] with *p*-mercuribenzoate[13] and separated the α-hemoglobin subunit with the following procedure suggested by Alpert. The treated protein was absorbed to a carboxymethyl-cellulose (Whatman C-52) column (1.5 × 30 cm), washed with 10 mM phosphate, pH 7.0, to remove the β-chain, and washed again with 1 mM mercaptoethanol and 1 mM phosphate buffer, pH 7.0, to remove the mercurial. We then eluted the still-adsorbed α-Hb-CO from the resin with a 40 mM Tris, pH 8.0, buffer, dialyzed the eluate against 5 mM phosphate, pH 7.0, and stored it at 4 °C. On the day just before its use, we oxidized the protein for 20 min at 4 °C with a 2-fold molar excess of ferricyanide and then removed ferri- and ferrocyanide with a Dowex 1X-8 column (1 × 5 cm).[12] We next incubated the Fe(III)-α-Hb with a 2-fold excess of DTNB at 4 °C for 1 h, removed the small ligands by dialysis against 5 mM phosphate, 1 M

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(1) Abbreviations used are the following: $CO_2^{\bullet-}$, the formate radical; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); e_{aq}^-, the hydrated electron; $H^\bullet$, the hydrogen atom; LRET, long-range electron transfer; $OH^\bullet$, the hydroxyl radical; RNase, pancreatic ribonuclease A; $RSSR^{\bullet-}$, the disulfide radical anion; α-Hb, the isolated α-subunit of human hemoglobin A; Fe(III)-α-Hb-SSR, the mixed-disulfide derivative of oxidized α-Hb formed by the reaction of the subunit with DTNB; Fe(III)-α-Hb-$SSR^{\bullet-}$, the disulfide radical anion form of oxidized Fe(III)-α-Hb-SSR; Fe(II)-α-Hb-SSR, reduced deoxy-α-Hb-SSR.

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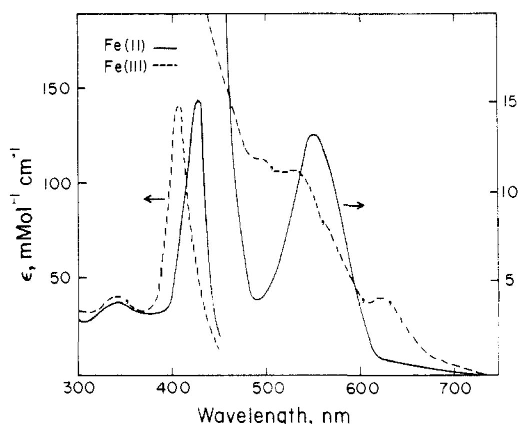
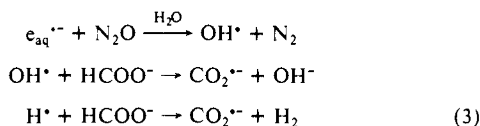


Figure 1. Spectra of Fe(III) (---) and Fe(II) (—) forms of α-Hb-SSR, the mixed-disulfide derivative of the human α-hemoglobin chain, pH 7.0.

glycine buffer, pH 7.0, and estimated the α-Hb concentration from the absorbance of the cyanide complex and the extinction coefficient of intact cyanomethemoglobin.[14] The next day we centrifuged this stock solution to remove the approximately 10% of the protein denatured overnight. After dilution into the appropriate solution, the sample was bubbled gently with N_2O for 5 min, and then N_2O was directed onto the stirred solution surface for another 1 h. From the absorbance of the sample it was clear that deoxygenation had not resulted in significant denaturation. The sample solution was then used within 0.5 h, after which the spectrum was taken again to ensure that no denaturation had occurred during the experiment.

Water with a resistivity of 18 MΩ was obtained from a Millipore Q apparatus. All other chemicals were purchased and used with no further purification.

The electron-transfer reaction was initiated by pulse radiolytically generated radicals. When a short pulse (100–500 ns) of high-energy electrons (ca. 4 MeV from the two Varian linear accelerators at The Ohio State University and the Hebrew University, Jerusalem) interacts with water, the overwhelmingly predominant species of an aqueous protein solution, the primary radicals produced are the hydrated electron ($e_{aq}^{\bullet-}$), the hydroxyl radical ($OH^\bullet$), and the hydrogen atom ($H^\bullet$). With N_2O and formate also present in solution, all the primary radicals are converted to the formate radical ($CO_2^{\bullet-}$) by the series of reactions shown in eq 3.



$$e_{aq}^{\bullet-} + N_2O \xrightarrow{H_2O} OH^\bullet + N_2$$
$$OH^\bullet + HCOO^- \rightarrow CO_2^{\bullet-} + OH^-$$
$$H^\bullet + HCOO^- \rightarrow CO_2^{\bullet-} + H_2 \qquad (3)$$

Solutions of Fe(III)-α-Hb-SSR (ca. (1–10) × 10^{-5} M) with 0.1–0.2 M formate and 0.1–0.2 M glycine were prepared shortly before use, saturated with N_2O, and transferred under a slight argon pressure to a 1-cm cell. (There was no change in the visible spectrum of the hemoglobin preparation upon addition of formate, and we conclude that there is little likelihood of complex formation between formate and heme iron.) The $CO_2^{\bullet-}$ concentration was between 0.5 and 2.5 μM, dependent on the chosen pulse length of the electron beam, but was never greater than 5% of the Fe(III)-α-Hb-SSR concentration. The α-Hb-SSR transients were monitored at various wavelengths: 420 nm, near the isosbestic point between Fe(III)-α-Hb and Fe(II)-α-Hb and where the disulfide anion radical absorbs strongly;[15] 450 and 570 nm, where the disulfide radical anion does not absorb appreciably and the absorption increases on reduction of the heme iron; 500 and 630 nm, where the absorption decreases on reduction of the Fe(III) heme.

We performed cyclic voltammetry and differential-pulse polarography with a Model 173D potentiostat/galvanostat, a Model 179 digital coulometer, a Model 175 universal programmer, and a Model 174A polarographic analyzer, all from Princeton Applied Research Corp. (Princeton, NJ). The experiments were conducted in a three-electrode glass cell (Metrohm) with a glassy-carbon working electrode of 0.2-cm^2 exposed area. Before every run, the working electrodes were washed with 50% nitric acid, rinsed with water, polished, and rinsed with water once again. All solutions contained 0.1 M KCl and 5 mM phosphate buffer,

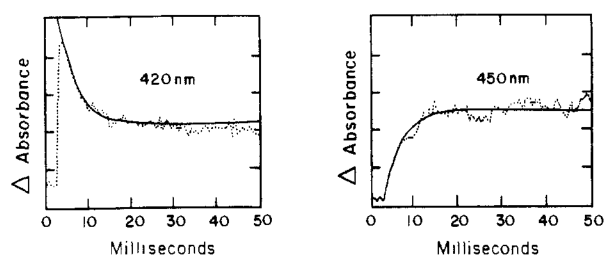


Figure 2. Reaction of the formate radical, $CO_2^{\bullet-}$, with Fe(III)-α-Hb-SSR, the mixed-disulfide derivative of the human α-hemoglobin chain. Experiments were performed in N_2O-saturated 50 mM $HCOO^-$, 10 mM phosphate buffer, pH 7.0. The heme concentration was 5 × 10^{-5} M and the initial radical concentration 1.9 μM: left, 420 nm; right, 450 nm. The solid lines are the calculated best fit first-order time profiles.

pH 7.0, and were degassed for 1 h. No mediator was used in any of these experiments.

Results and Discussion

The α-chain of human hemoglobin A has one sulfhydryl, that of cysteine-104. The mixed-disulfide derivative formed in the reaction of that single sulfhydryl with DTNB has a spectrum close to that of the native subunit,[16,17] except for the additional disulfide absorption band centered near 325 nm (Figure 1). The spectral similarity between α-Hb and α-Hb-SSR in the visible region suggests no major structural change around the heme group due to the DTNB modification. We monitored reduction of the heme iron at 450, 500, 570, and 630 nm on the basis of the spectral differences between Fe(III) and Fe(II) forms of the protein. The disulfide radical anion $RSSR^{\bullet-}$ absorbs strongly between 390 and 420 nm;[15] and since oxidized and reduced forms of α-Hb have an isosbestic point near 420 nm, we chose this wavelength to monitor reaction 1, $CO_2^{\bullet-}$ reduction of the disulfide bond in Fe(III)-α-Hb-SSR. There is no evidence in the literature that $CO_2^{\bullet-}$ could reduce any group other than the disulfide and the heme in Fe(III)-α-Hb-SSR with a concomitant large absorbance change at 420 nm.[15]

As seen in Figure 2, pulse radiolytic introduction of $CO_2^{\bullet-}$ into a solution of Fe(III)-α-Hb-SSR results in a rapid absorption increase at 420 nm followed by a slower first-order absorption decay with a rate constant of approximately 200 s^{-1}. Since 420 nm is close to an isosbestic point between oxidized and reduced α-hemoglobins and since only reduction at the heme or at the disulfide could cause so large an absorbance increase, the faster process should be due to 1-electron heme reduction, disulfide reduction, or simultaneous heme and disulfide reduction. At 450 nm, where $RSSR^{\bullet-}$ absorption is negligible, one major process is seen (Figure 2), a slower absorption increase with an apparent first-order rate constant of approximately 200 s^{-1}. Since this must be due to heme reduction, which is accompanied by an absorbance increase at 450 nm, we conclude that the faster process seen at 420 nm but not at 450 nm is due to disulfide bond reduction. On the other hand, the slower process at 420 nm also proceeds with a rate constant near 200 s^{-1} and, therefore, must be associated with heme reduction. Hence, we propose that the faster process at 420 nm is primarily reaction 1 and that the slower process observed at both 420 and 450 nm is reaction 2. The absorbance decline at 420 nm reflects the simultaneous oxidation of the disulfide radical anion and reduction of the heme group.

If the faster reaction is the reduction of eq 1, then it should be second order with an apparent rate constant dependent on protein concentration. If the slower reaction is the intramolecular process of eq 2, then it should be truly first order and independent of the protein concentration. The apparent rate constant of the rapid phase seen at 420 nm is in fact linearly dependent on the Fe(III)-α-Hb-SSR concentration (data not shown), from which

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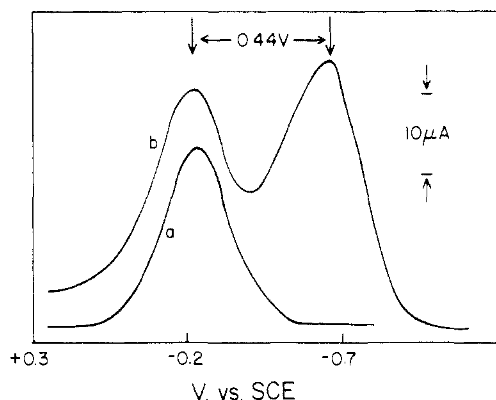

Figure 3. Differential-pulse polarography of the human hemoglobin α-chain. Experimental conditions are those described in Experimental Methods and Materials: bottom curve (a), Fe(III)-α-Hb, no mixed disulfide; top curve (b), Fe(III)-α-Hb-SSR, with mixed disulfide.

dependence we have obtained the second-order rate constant of 2.2×10^8 M^{-1} s^{-1} for reduction by $CO_2^{\bullet-}$. The second, slower process has an observed rate constant of 188 ± 23 s^{-1} and is independent of the wavelength chosen for observation—420, 450, 570, and 630 nm—and of the protein concentration over the range of (1–10) × 10^{-5} M. Therefore, we conclude that we have indeed observed the two reactions 1 and 2.

Because $CO_2^{\bullet-}$ might also reduce some other non-disulfide group[15] that in turn could be an electron donor in the slow process we had ascribed to disulfide radical reduction of the heme, we looked into $CO_2^{\bullet-}$ reduction of the unmodified subunit, Fe(III)-α-Hb. Under the same conditions of the above experiments, there is one pseudo-first-order transient with an apparent rate constant linearly dependent on the protein concentration to yield the second-order rate constant of 2.1×10^9 M^{-1} s^{-1}. Importantly, there is no slower kinetic process, one that might have arisen from the reduction of an additional (nondisulfide) protein group that in turn could donate the electron to the heme. This supports our assumption that the disulfide radical anion of Fe(III)-α-Hb-$SSR^{\bullet-}$ is the only electron donor in the slow intramolecular reduction of the heme group, reaction 2. This control experiment does, however, raise the question of why we did not observe more of the rapid heme reduction in the $CO_2^{\bullet-}$ reaction with Fe(III)-α-Hb-SSR, the disulfide modified protein. The spectrum of the disulfide modified protein suggests no large structural difference between Fe(III)-α-Hb and Fe(III)-α-Hb-SSR but does not rule out a more subtle change that might affect the reactivity of the heme. Any answer to this question must, however, also take into account the possibility that the fast $CO_2^{\bullet-}$ reductions of the heme in Fe(III)-α-Hb and the disulfide in Fe(III)-α-Hb-SSR could themselves occur indirectly through a transient too unstable to be observed.[15]

To determine the redox potential difference that drives the observed intramolecular electron transfer, we performed cyclic voltammetry on solutions of α-Hb and α-Hb-SSR at protein concentrations near 10^{-4} M. The waves obtained with sweep rates varying from 2 to 100 mV/s were well-defined, although, due perhaps to slow redox reactions and/or protein absorption onto the electrode, too broad for us to assign redox potentials. We obtained better results with differential-pulse polarography. At a scan rate of 2 mV/s and a modulation amplitude of 25 μV we saw two well-defined peaks with Fe(III)-α-Hb-SSR (Figure 3) at +0.030 and −0.41 V (vs NHE). On the basis of the midpoint potentials obtained with α-Hb and DTNB alone (Table I), we conclude that the more positive potential is that of the heme group and the more negative that of the disulfide. The apparent potential difference between the disulfide radical anion and the Fe(III) heme group, the driving force for the electron transfer, is approximately 0.44 V. We also note that there is little change in the two midpoint potentials when the disulfide is attached to the protein, further evidence against a large structural change upon cysteine modification.

Table I. Pulse Polarographic Potentials of Native and DTNB-Modified α-Hemoglobin Subunit

	E_{m7}, V vs NHE	
	Fe^{3+}/Fe^{2+}	$S\text{-}S/S\text{-}S^{\bullet-}$
HS(Cys-104)-α-Hb(Fe^{3+})	0.05 ± 0.01	
NBS-S(Cys-104)-α-Hb(Fe^{3+})	0.03 ± 0.01	−0.41 ± 0.01
DTNB		−0.42 ± 0.01

Table II. Selected Intramolecular Rate Constants

donor/acceptor	syst	δE_{m7},[a] V	d,[b] Å	k,[c] s^{-1}
Zn(II)*/Fe(III)[d]	Zn–Hb/cyt b_5 complex	1.0	7	8000
Fe(II)/Fe(III)[e]	cyt b_5/cyt c complex	0.2	8	1700
porph*/Fe(III)[e]	porph–cyt c/cyt b_5 complex	0.4	8	50000
Zn(II)*/Fe(III)[e]	Zn–cyt c/cyt b_5 complex	0.8	8	500000
$porph^-$/Fe(III)[e]	porph–cyt c/cyt b_5 complex	1.1	8	8000
Ru(II)/Fe(III)[f]	Ru–cytochrome c	0.15	12	40
Fe(II)/Ru(III)[g]	Ru–cytochrome c	0.18	12	<0.001
Ru(II)/Cu(II)[h]	Ru–azurin	0.24	12	2
$S\text{-}S^{\bullet-}$/Fe(III)[i]	Fe(III)-α-Hb-SSR	0.44	12	200
Ru(II)/Fe(III)[j]	Ru–hemoglobin	−0.02	13	0.02
α-Zn(II)*/ β-Fe(III)[k]	Zn/Fe–hemoglobin	0.8	20	100

[a] Difference in the redox potentials of donor and acceptor in volts. [b] Estimated edge-to-edge distance between redox centers in angstroms. [c] Apparent first-order rate constant in reciprocal seconds. [d] Reference 21. [e] Reference 22. [f] Reference 23. [g] Reference 24. [h] Reference 25. [i] This report. [j] Reference 26. [k] Reference 27.

Linear disulfide radical anions are normally quite unstable; e.g., the cystamine $RSSR^{\bullet-}$ decays with a reported first-order rate constant of 0.35×10^6 s^{-1}.[18] For this reason there has, to date, been no direct measurement of an RSSR/$RSSR^{\bullet-}$ midpoint potential, although there is a recent calculation of the dithiothreitol and lipoamide cyclic radical anion E_{m7}, both −1.6 V.[19] Our ability to measure a midpoint potential for the disulfide radical anion of DTNB is due to its remarkably slow rate of decay.[20] The much more positive potential of the DTNB $RSSR^{\bullet-}$ presumably accounts for its greater stability when compared with other disulfide radical anions and may be due to stabilization of the anionic radical by the nitrophenyl ring.

To place our results into perspective, we have compiled rate constants from the literature for LRET in a variety of proteins (Table II). Our results fall within the range of rate constants found by others. We should note that although the heme and the cysteine-104 sulfur are separated by approximately 12 Å in the crystalline hemoglobin tetramer,[11] the value that we have assigned in Table II, our results were obtained with Fe(III)-α-Hb-SSR, the isolated and then modified α subunit, in solution. We do not know what structural differences we should, therefore, expect, although the evidence available suggests little alteration around the heme group due to the covalent modification. From the compiled results of Table II we note that there is no apparent relationship between the rate of the electron transfer and the redox potential difference nor between the rate and the distance between redox centers, although there does appear to be an overall trend

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of declining rate constants with increasing distance. While we might expect such a trend, the apparent lack of any direct correlation most presumably arises from the pecularities of each protein system.

Since most literature examples of LRET in proteins involve two metal redox sites, it may not be recognized generally that there are a number of non-metal sites on proteins that, given the proper reagent, can be reduced or oxidized.[15] Moreover, the radicals so formed can participate in intramolecular electron-transfer reactions.[9,10] Hence, it is imperative to verify both the electron donor and acceptor in a protein intramolecular redox reaction. In the experiments we report here, we have measured spectral changes associated with both the disulfide radical anion and the α subunit heme group. Moreover, since we have found that $CO_2^{\bullet-}$ reduces the heme group in Fe(III)-α-Hb with no attached disulfide and predominantly the disulfide of Fe(III)-α-Hb-SSR, unwanted side reactions due to "contaminating" radical reduction at other protein sites most presumably do not occur in this system.

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Registry No. $CO_2^{\bullet}$, 2564-86-5; heme, 14875-96-8; cysteine, 52-90-4.

Oxoferryl Complexes of the Halogenated (Porphinato)iron Catalyst: (Tetrakis(2,6-dichlorophenyl)porphinato)iron

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Abstract: Oxidation of (hydroxo)(tetrakis(2,6-dichlorophenyl)porphinato)iron(III) [TPP(2,6-Cl)Fe^{III}OH] by *m*-chloroperoxybenzoic acid (mCPBA) in THF or DMF yields the THF and DMF adducts of the oxoferryl complex TPP(2,6-Cl)Fe^{IV}=O. Addition of 10-fold molar excess of 1-methylimidazole (1-MeIm) to the THF adduct gives the corresponding 1-MeIm complex. ^{1}H NMR of the DMF-d_7 adduct (200 MHz, DMF-d_7, -50 °C) shows proton resonances consistent with an oxoferryl S = 1 structure: 14 ppm (pyrrole H), 4.01 ppm (phenyl *m*- and *p*-H) in a ratio of 2:3. The UV-vis spectra (-40 °C) of the red solutions show the band at ~550 nm which is typical of the ferryl oxidation state: (THF) TPP(2,6-Cl)Fe^{IV}=O, λ_{max}($\epsilon \times 10^{-3}$) 417 (169.5), 551 (10.6), 622 (3.4), 653 (1.4) nm; (DMF) TPP(2,6-Cl)Fe^{IV}=O, λ_{max} ($\epsilon \times 10^{-3}$) 419 (157.6), 554 (11.1), 632 (3.5) nm; (1-MeIm) TPP(2,6-Cl)Fe^{IV}=O, λ_{max} ($\epsilon \times 10^{-3}$) 421 (157.5), 561 (13.2), 624 (3.3), 661 (1.8) nm. Resonance Raman bands in the region of the Fe=O stretch provide support for the oxoferryl structure with frequencies that correlate inversely with the strength of the sixth axial ligand: THF (841 cm^{-1}) < DMF (829 cm^{-1}) < 1-MeIm (818 cm^{-1}). The Mössbauer spectra, similar to those of other ferryl S = 1 complexes, establish the ferryl oxidation state of iron. While quadrupole splittings vary inversely with axial ligand strength (THF (2.08 mm s^{-1}) < DMF (1.81 mm s^{-1}) < 1-MeIm (1.35 mm s^{-1})), isomer shifts are essentially constant. Resonance Raman and Mössbauer data are rationalized by a rearrangement of bonding electron density from Fe $3d_{x^2-y^2}$ to $3d_{z^2}$ with increasing axial ligand bonding. The Fe=O bond weakens, decreasing $\nu_{Fe=O}$, and the axial electric field gradient decreases, giving smaller quadrupole splitting. However, little net change in electron density at Fe results in constant δ.

Chloro(tetrakis(2,6-dichlorophenyl)porphinato)iron(III) [TPP(2,6-Cl)FeCl] is frequently employed as a monooxygen transfer catalyst because of the resistance of the porphyrin ring to oxidative degradation and the high reactivity of the intermediate compound I analogue. Hydroxylations of alkanes[1] and stereospecific epoxidations of olefins[2-4] have been described and the high number of turnovers achievable with this catalyst has also enabled the "suicide" formation of N-alkylated porphyrin complexes to be extensively studied.[5-8] Despite interest in the catalytic properties of TPP(2,6-Cl)Fe^{III} and a number of reports in which reactions were carefully monitored by UV-vis spectroscopy,[1,5,7] no complex with iron in the ferryl oxidation state has been directly observed or characterized. Nevertheless, physicochemical data on high-valent complexes should prove important in understanding the chemistry of the one- and two-electron oxidized species of this useful catalyst as well as other bifacially hindered (porphinato)iron complexes currently used to probe mechanisms of biological oxidations. Hence we have attempted to develop conditions under which observable high-valent complexes of iron 2,6-dichlorophenyl porphyrin can be generated and studied. We report here the characterization of the oxoferryl compound II analogue, TPP(2,6-Cl)Fe^{IV}=O, generated by the oxidation of the hydroxo complex with mCPBA and stabilized by axially ligated dimethylformamide (DMF), tetrahydrofuran (THF), or 1-methylimidazole (1-MeIm). Observation of transients in UV-vis and ^{1}H NMR spectra during the oxidation reactions has provided insights into the pathway leading to the oxoferryl complex.

Results

Oxidation of TPP(2,6-Cl)FeOH in DMF and THF. Oxidation of TPP(2,6-Cl)FeOH with mCPBA in DMF-d_7 at -50 °C yielded a deep red solution with an ^{1}H NMR spectrum consisting of

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