Photoinduced Oxidation of Microperoxidase-8: Generation of Ferryl and Cation-Radical Porphyrins

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Abstract: The electron-transfer reactivity of microperoxidase-8 (MP8), the heme octapeptide derived from enzymatic cleavage of cytochrome *c*, has been studied by nanosecond flash photolysis methods. Ferric MP8 is rapidly oxidized by photogenerated $Ru(bpy)_3^{3+}$ in acidic solutions to a ferric cation-radical porphyrin ($k \sim 5.6 \times 10^9$ M^{-1} s^{-1}); the oxidation product in alkaline solutions is ferryl MP8 ($k \sim 2.2 \times 10^9$ M^{-1} s^{-1}). Numerical simulations of the kinetics for the direct oxidation of ferric to ferryl MP8 predict a marked pH effect on the rate of reaction in alkaline solutions; however, only a very weak pH dependence is observed in the range 7−8.5, indicating that the ferryl species is generated by intramolecular electron transfer within a ferric cation-radical porphyrin. Transient spectra taken between pH 6 and 8.5 show increasing ferryl absorption as the pH is increased, demonstrating a pH-dependent equilibrium between the two oxidized forms of MP8.

Introduction

High-valent iron porphyrins have been implicated in the catalytic cycles of many metalloenzymes, including the peroxidases, catalases, and cytochromes P-450.[1−3] The ability of these enzymes to oxidize a wide variety of substrates with high specificity has generated considerable interest in the chemistry of their high-valent forms[4,5] as well as in the structures and properties of synthetic analogs.[4−10] Peroxidase compounds **I** and **II** participate in enzymatic catalysis; compound **I** contains both a ferryl (Fe^{IV}═O) ion and an organic radical, derived either from an amino acid residue (cytochrome *c* peroxidase, CcP)[11] or from the porphyrin (horseradish peroxidase, HRP).[12] The properties of CcP compound **II**, formed by reaction of compound **I** with single-electron reductants, indicate that it is an equilibrium mixture of Fe^{IV}═O and Fe^{III} tryptophan-radical species.[11,13] If no readily oxidizable amino acid residues are available, it is possible that a porphyrin radical plays a functional role in compound **II**; however, in HRP and other heme proteins, the ferric porphyrin radical form of compound **II** has thus far eluded detection.[14] While compounds **I** and **II** have similar one-electron reduction potentials, compound **I** is much more reactive toward single-electron reductants; electron transfer to the oxidized porphyrin or amino acid of compound **I** is *ca*. 1000 times more rapid than reduction of the ferryl ion in compound **II**.[13,14] A similar trend also has been observed in the reverse reaction, in which oxidation of compound **II** to generate compound **I** is observed to be more rapid than the oxidation of met-aquo forms to compound **II**.[15]

We have been exploring the use of photoinduced electron-transfer reactions to generate analogs of high-valent metalloenzyme intermediates in heme enzyme models. Stepwise oxidation of $(P)Fe^{III}-OH$ or $(P)Fe^{III}-OH_2$ (P = porphyrin) to $(P)Fe^{IV}$═O and cationic $(P^{\bullet+})Fe^{IV}$═O generates species whose active oxygen atoms are derived from water instead of peroxides or other active forms of dioxygen.[16] Our work has centered on microperoxidase-8 (MP8), the heme-containing octapeptide obtained from proteolytic cleavage of horse heart cytochrome *c*, which has been shown to be a functional peroxidase enzyme model.[17−21] MP8 contains the heme prosthetic group as well as amino acid residues 14−21 of horse heart cytochrome *c*. The imidazole of His18 occupies one of the axial binding sites, leaving the other axial site for exogenous ligands such as water or hydroxide.[17] We have found that the MP8 ferric porphyrin radical is readily prepared by a photoinduced single-electron oxidation in acidic aqueous media. Interestingly, our kinetics data suggest that this ferric porphyrin radical undergoes intramolecular electron transfer to form ferryl MP8 in alkaline solution.

Experimental Section

Materials. Microperoxidase-8 was prepared from horse heart cytochrome *c* (Sigma) by a modification of literature procedures.[18,19] Cytochrome *c* (500 mg) and 13 mg of pepsin (Sigma) were dissolved in 20 mL of water. The pH was adjusted to 2.1 by addition of 1 M HCl. After 30 min, another portion of pepsin (13 mg) was added. After standing overnight, the solution was brought to pH 9.1 by addition of

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ammonium hydroxide (Fischer), and then trypsin (18 mg) (Sigma) was added. The resulting red solution was allowed to stand at 37 °C overnight. Crude MP8 was precipitated by addition of solid ammonium sulfate (29 g) and collected by centrifugation. The supernatant was discarded and the remaining red solid was dissolved in water. Residual ammonium sulfate was removed by washing with water in an Amicon ultrafiltration cell (YM1 membrane). The crude MP8 was purified by FPLC chromatography using a Pharmacia Pep-RPC 16/10 column running a 12−36% acetonitrile/water gradient. FPLC solvents contained 0.1% trifluoroacetic acid. Purified MP8 was lyophilized and stored in a refrigerator. *N*-Acetyl MP8 was prepared by slow addition of a 500-fold excess of acetic anhydride (Aldrich) to a solution of purified MP8 in 0.1 M potassium bicarbonate solution. Excess bicarbonate was removed by repeated washing with water in an Amicon stirred cell and the acetylated peptide was purified by reverse-phase FPLC. The concentrations of stock solutions of MP8 were determined spectrophotometrically ($\epsilon_{396} = 1.57$ mM^{-1} cm^{-1} at pH 8).[18] Ferryl MP8 was prepared chemically by addition of 3% hydrogen peroxide (VWR) to a solution of ferric MP8 in pH 9.5 buffer. Purified MP8 and *N*-acetyl MP8 were assayed for purity and fully characterized by mass spectrometry and NMR methods.[20] Distilled water was further purified by a Millipore Nanopure system before use. Tris(2,2′-bipyridine)-ruthenium(II) chloride (Strem) was used as received. Hexaammineruthenium(III) chloride (Strem) was recrystallized from warm hydrochloric acid solutions.

Methods. Absorption spectra were recorded on an HP-8452 spectrophotometer. Flash photolysis measurements were performed on stirred samples contained in sealed quartz cuvettes deoxygenated by repeated evacuation followed by backfilling with purified argon on a vacuum line. The excitation source for flash photolysis measurements at 480 nm was a Lambda-Physik FL 3002 dye laser using coumarin 480 dye (Exciton) pumped by a Lambda-Physik LPX 210i XeCl excimer laser. Dye laser output was attenuated by passage through crossed polarizing filters. Typically, 1 mJ/pulse laser energies were used. The pulse energy was monitored for consistency by a photodiode and a discriminator. Probe light for transient absorption spectra was provided by a 75-W continuous-wave arc lamp (PTI Model A 1010). Probe wavelengths were selected for detection and monitored by an ISA monochromator fitted with a Hamamatsu 5-stage photomultiplier tube. PMT output was amplified and then processed by a Sony/Tektronix RTD 710 digitizer interfaced to an IBM personal computer.

Results and Discussion

The electronic absorption spectrum of resting MP8 is typical of a high-spin ferric heme (Soret at 396 nm, Q-bands at 510 and 623 nm). Addition of 1 molar equiv of hydrogen peroxide to an alkaline solution (pH > 9) of MP8 results in the formation of (P)Fe^{IV}═O,[21] whose electronic spectrum features a red-shifted Soret band (406 nm) and absorptions at 519 and 548 nm analogous to those found in the spectra of ferryl myoglobin[16,22,23] and compound **II** of horseradish peroxidase.[15] The (P)Fe^{IV}═O/(P)Fe^{III}−OH$_2$ MP8 difference spectrum exhibits a large bleach centered at 390 nm and a positive absorption change with a maximum at 413 nm (Figure 1).

We have generated (P)Fe^{IV}═O by photooxidation of ferric MP8 (eq 1a−c). Irradiation of Ru(bpy)$_3^{2+}$ in the presence of

$$\mathrm{Ru(bpy)_3^{2+}} \xrightarrow{h\nu} \mathrm{Ru(bpy)_3^{2+*}} \quad (1a)$$

$$\mathrm{Ru(bpy)_3^{2+*} + Ru(NH_3)_6^{3+} \rightarrow Ru(bpy)_3^{3+} + Ru(NH_3)_6^{2+}} \quad (1b)$$

$$\mathrm{Ru(bpy)_3^{3+} + (P)Fe^{III}{-}OH_2 \rightarrow (P^{\bullet+})Fe^{III}{-}OH_2/(P)Fe^{IV}{=}O + 2H^+ + Ru(bpy)_3^{2+}} \quad (1c)$$

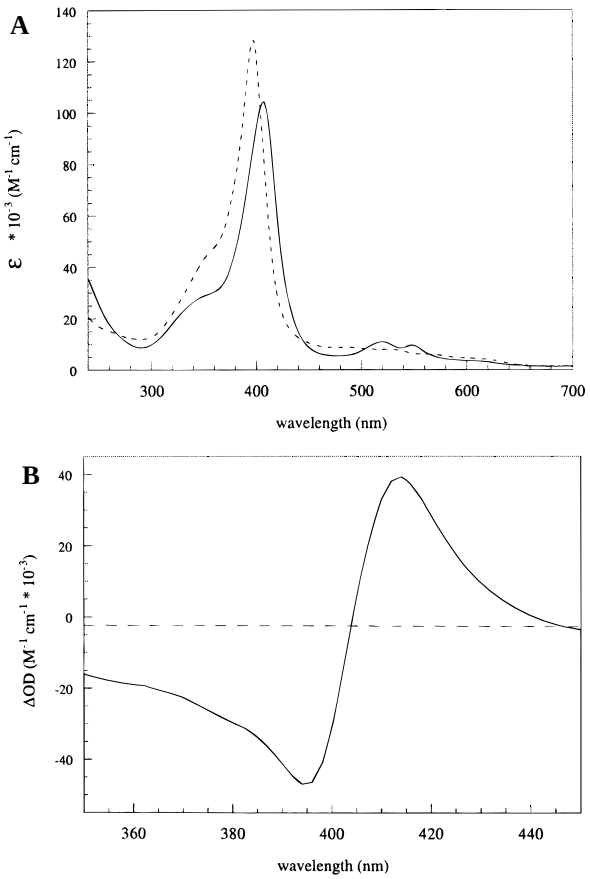

Figure 1. (A) Absorption spectra of resting Ac-MP8 in pH 10, 0.1 M borate buffer (broken line) and ferryl Ac-MP8 prepared by addition of excess hydrogen peroxide at 0 °C (solid line). (B) Ferryl−ferric difference spectrum in the Soret region.

excess Ru(NH$_3$)$_6^{3+}$ produces Ru(bpy)$_3^{3+}$ ($E°$(Ru$^{3+/2+}$) = 1.25 V vs NHE) in less than 1 μs.[24] In the absence of ferric MP8, the photogenerated Ru(bpy)$_3^{3+}$ reacts with Ru(NH$_3$)$_6^{2+}$ to regenerate the starting complexes on a millisecond time scale (eq 1a,b). The presence of micromolar ferric MP8 accelerates the decay of Ru(bpy)$_3^{3+}$ and gives rise to absorption changes at 410 and 390 nm that are attributable to the formation of (P)-Fe^{IV}═O at pH > 6 (Figure 2). The ferryl/ferric difference spectrum strongly resembles the transient absorption spectrum obtained 300 μs after laser irradiation (Figure 3c). Ferryl MP8 features a well-defined Q-band doublet with maxima at 521 and 548 nm. The transient absorption spectrum at pH 8 exhibits similar features at 524 and 552 nm, consistent with transient generation of (P)Fe^{IV}═O (Figure 4). Under these conditions, the ferryl species persists for several milliseconds.

The oxidation of ferric MP8 by Ru(bpy)$_3^{3+}$ exhibits pseudo-first-order kinetic behavior. A plot of the observed rate constant *vs* the concentration of ferric MP8 is linear over a wide concentration range (5−100 μM), and the slope yields a second-order rate constant of 2.2 × 10^9 M^{-1} s^{-1} at pH 8 (Figure 5). The rate of appearance of (P)Fe^{IV}═O after laser photolysis is essentially pH independent over the pH 7−9 range (pH 7, 2.7 × 10^9 M^{-1} s^{-1}; pH 7.5, 2.2 × 10^9 M^{-1} s^{-1}; pH 8.5, 2.0 × 10^9 M^{-1} s^{-1}). The rate of ferryl generation exhibits a small solvent isotope effect, $k_H/k_D = 1.7$, consistent with the presence of a hydroxide or aquo ligand on ferric MP8 that is deprotonated to the oxo ligand of ferryl MP8.

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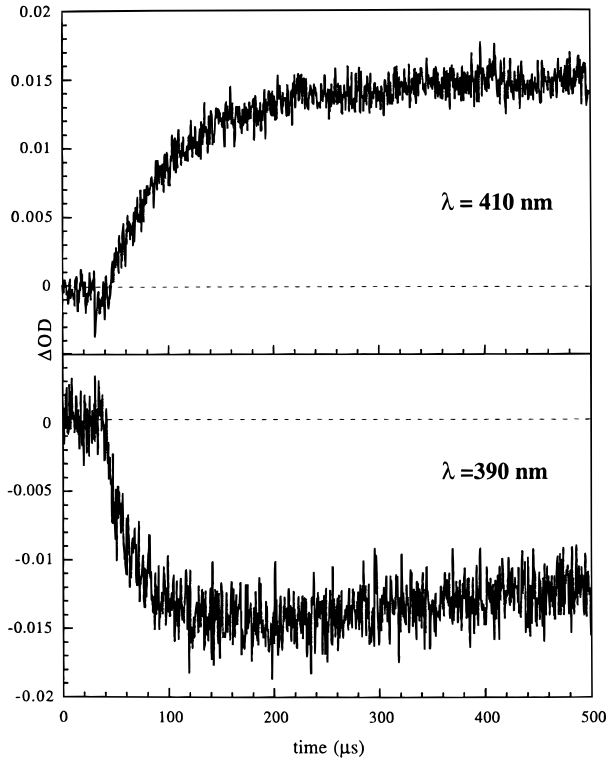

Figure 2. Single-wavelength transient absorption spectra in the (P)-Fe^{III} (390 nm) and (P)Fe^{IV} (410 nm) Soret regions obtained after flash photolysis of a 7 μM solution of resting MP8 with 5 mM $Ru(bpy)_3^{2+}$ and 10 mM $Ru(NH_3)_6^{3+}$ in 0.1 M phosphate buffer (pH 8).

Oxidation of (P)$Fe^{III}-OH_2$ by $Ru(bpy)_3^{3+}$ in solutions below pH 6 does not result in transient generation of ferryl MP8, and flash photolysis measurements are consistent with the production of the ferric cation-radical porphyrin. The transient absorption spectrum taken 300 μs after laser photolysis of a pH 6 solution (Figure 3a) shows a Soret-region bleach whose minimum coincides with the ferric Soret-band maximum (396 nm). No OD increase is observed in the Soret region, indicating the production of a porphyrin species with a weaker Soret band. Electrochemical oxidation of Fe^{III}(TPP)−Cl gives $[Fe^{III}(TPP)-Cl]^{\bullet+}$, whose absorption spectrum features a broadened Soret band with a lower extinction coefficient than that of the neutral Fe^{III} species.[25] An examination of the transient absorption spectrum in the visible region reveals broad absorptions at 587 and 650 nm (Figure 4). The absorption spectra of cation-radical porphyrins typically exhibit broadened, red-shifted Q-bands.[25] No solvent isotope effect was observed on the rate of oxidation and an apparent second-order rate constant of 5.6×10^9 M^{-1} s^{-1} was obtained from a concentration dependence study (Figure 5). Ferric deuteroporphyrin dimethyl ester (Fe^{III}(DPDE)) reacts rapidly with radiolytically generated peroxyl radicals in mixed organic-aqueous solvents to give $Fe^{III}(DPDE)^{\bullet+}$ ($k \sim 2.6 \times 10^8$ M^{-1} s^{-1}).[26−28] Peroxyl radicals have reduction potentials in the 1.0−1.1 V range, similar to that of $Ru(bpy)_3^{3+}$.[29]

It is apparent that oxidation of ferric MP8 at low pH results in exclusive formation of the porphyrin cation-radical product (eq 2a). At low pH, equilibrium 2b lies to the left; deprotonation

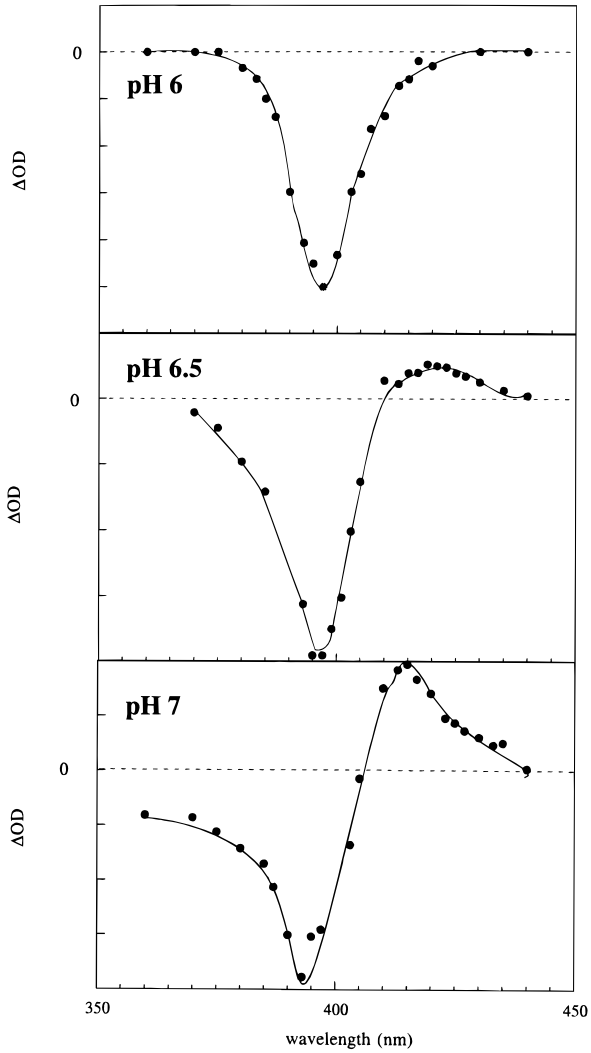

Figure 3. Soret-region transient absorption spectra taken 300 μs after laser photolysis at pH 6, 6.5, and 7. Each sample contains 7 μM MP8, 10 μM $Ru(bpy)_3^{2+}$, and 10 mM $Ru(NH_3)_6^{3+}$ in 0.1 M phosphate buffer.

of the radical aquo ligand is not favored, and the radical species is effectively trapped. At alkaline pH, initial oxidation of the

$$Ru(bpy)_3^{3+} + (P)Fe^{III}-OH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ru(bpy)_3^{2+} + (P^{\bullet+})Fe^{III}-OH_2 \quad (2a)$$

$$(P^{\bullet+})Fe^{III}-OH_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (P)Fe^{IV}{=}O + 2H^+ \quad (2b)$$

$$Ru(bpy)_3^{3+} + (P)Fe^{III}-OH_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Ru(bpy)_3^{2+} + (P)Fe^{IV}{=}O + 2H^+ \quad (2c)$$

porphyrin is followed by rapid conversion to the ferryl product; the buildup of radical species is no longer observed. Oxidation of the porphyrin is not a proton-coupled redox process, and therefore its associated rate constants (k_1 and k_{-1}) are predicted to be insensitive to solution pH and isotopic composition, in line with the experimental findings. The single-electron oxidation of chromium(III) porphyrins to the corresponding chromium(IV)−oxo porphyrins proceeds through an analogous chromium(III) cation-radical porphyrin intermediate whose optical spectrum has been observed by pulse radiolytic meth-

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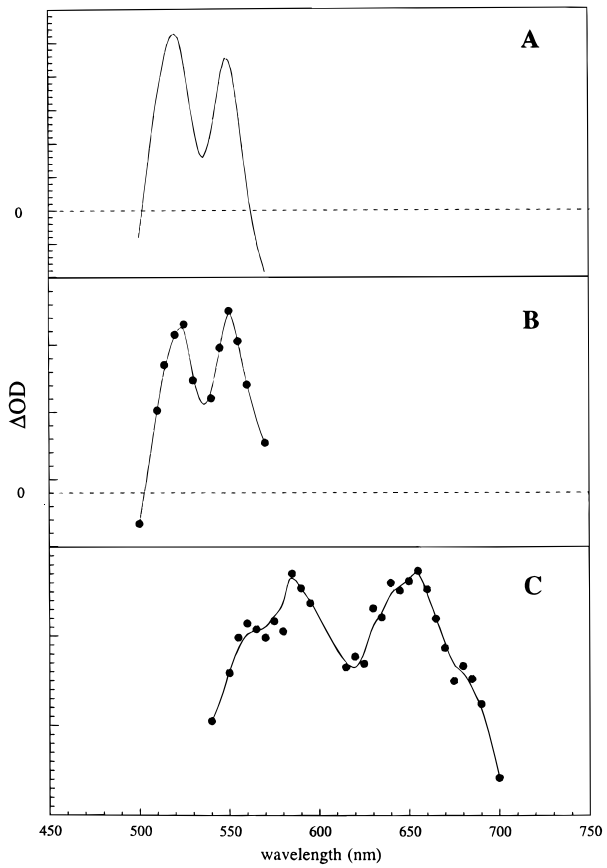

Figure 4. (A) Ferryl−ferric difference spectrum in the Q-band region. Ferryl MP8 was generated by addition of hydrogen peroxide to a solution of ferric Ac-MP8 in pH 9.5 borate buffer at 0 °C. (B [C]) Q-band region transient absorption spectrum obtained 300 μs after laser irradiation of solutions containing 30 mM MP8, 20 mM $Ru(bpy)_3^{2+}$, 10 mM $Ru(NH_3)_6^{3+}$ in 0.1 M phosphate buffer, and pH 8 and [pH 6].

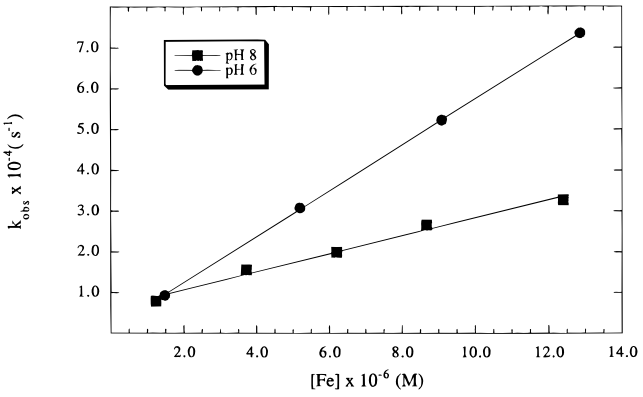

Figure 5. Rates of oxidation of ferric MP8 to $(P^{\bullet+})Fe^{III}-OH_2$ (pH 6) and $(P)Fe^{IV}=O$ (pH 8) as a function of $[Fe^{III}]$: $[Ru(bpy)_3^{2+}]$, 10 μM; $[Ru(NH_3)_6^{3+}]$, 10 mM.

ods.[30] Transient absorption spectra taken 300 μs after excitation exhibit a gradual growth of the 420 nm $Fe^{IV}=O$ absorption relative to the 396 nm bleach as the pH increases from 6 to 8.5 (Figure 4a−c). We interpret this as a pH-dependent shift of the equilibrium between the two redox forms of oxidized MP8 (eq 2b). It has been shown that electrochemically generated $[Fe^{III}(TMP)(ClO_4)]^{\bullet+}$ undergoes a similar transformation to a ferryl species upon metathesis of the perchlorate ligand to hydroxide.[31]

The rate law for the mechanism described by eq 2 (with pseudo-first-order conditions for $(P)Fe^{III}-OH_2$ and $Ru(bpy)_3^{2+}$) predicts biexponential kinetics of ferryl MP8 formation. The conversion of a ferric aquo or hydroxo complex to a ferryl species involves coupled proton and electron transfers, and whether the processes are concerted or sequential, the observed rate should be strongly dependent on pH and solvent isotope (H/D) composition. The observed ferryl MP8 formation rates are only weakly dependent on these two factors indicating that neither eq 2b nor eq 2c is important in the rate-determining step of the reaction. If we assume that eq 2c does not contribute to the observed reaction (i.e., $k_3[(P)Fe^{III}-OH_2] + k_{-3}[Ru(bpy)_3^{2+}] \ll k_1[(P)Fe^{III}-OH_2] + k_{-1}[Ru(bpy)_3^{2+}]$) then the two rate constants in the biexponential formation of ferryl MP8 are given by eq 3.

$$\nu_1 = k_1[Fe^{III}MP8] + \frac{k_{-1}[Ru(bpy)_3^{2+}]k_{-2}}{k_2 + k_{-2}} \qquad (3a)$$

$$\nu_2 = k_2 + k_{-2} \qquad (3b)$$

The intramolecular equilibration between ferryl MP8 and the ferric porphyrin radical (eq 2c), although intrinsically slow, is still likely to be faster that the bimolecular oxidation of ferric MP8 by $Ru(bpy)_3^{3+}$ (i.e., $k_2 + k_{-2} \gg k_1[(P)Fe^{III}-OH_2] + k_{-1}[Ru(bpy)_3^{2+}]$. Hence, the amplitude of the exponential corresponding to ν_2 will be negligible and the overall reaction will appear to be a single-exponential process. The expression for ν_1 correctly predicts the linear dependence on the total MP8 concentration, the observed nonzero intercept, as well as the weak dependence on pH and H/D composition of the solvent.

Our findings strongly indicate that ligand-centered oxidation of both hydroxo and aquo MP8 is the rate-limiting step in the formation of $(P)Fe^{IV}=O$. Although ferric porphyrins generally have ligand-centered reduction potentials somewhat higher than their metal-centered potentials,[32,33] the porphyrin is oxidized more rapidly by $Ru(bpy)_3^{3+}$ than the ferric center. The difference in electron-transfer rates is attributable to the proton-coupled oxidation of the ferric center to a ferryl species. It is likely that the large reorganization energy for concerted $Fe^{III/IV}=O$ interconversions makes stepwise mechanisms more favorable and may account for the sluggish reactivity of compound **II** ferryl species.[34] Our data indicate that $(P)Fe^{IV}=O$ is not a kinetically competent oxidant and that its reactions are governed by a slow interconversion between the metal−oxo form and the highly reactive ferric radical species.

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