Manganese Microperoxidase-8

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Received September 13, 1997

Demetalation of Fe(III) microperoxidase-8 (MP8) by anhydrous HF gives metal-free MP8, a convenient starting material for a wide variety of metal-substituted MP8 derivatives, including Mn(III)MP8. Mn(III)MP8 was produced by treatment of metal-free MP8 with manganous acetate in aerated aqueous solution; it was characterized by mass spectrometry and UV−visible absorption spectroscopy. Resonance Raman spectra suggest that Mn(III)-MP8 contains histidine and water axial ligands at neutral pH. The Mn(IV)═O derivative is readily prepared by oxidation of Mn(III)MP8 with hydrogen peroxide or $Ru(bpy)_3^{3+}$ (bpy = 2,2′-bipyridine).

Introduction

Manganese has been substituted for iron in several heme proteins.[1−3] Investigations of manganese and iron peroxidases have revealed a striking difference in the reactivities of the two families of enzymes, with the former being much less efficient for reduction of hydrogen peroxide.[4,5] In an effort to shed light on these reactivity differences, we elected to examine the spectroscopy and redox chemistry of manganese derivatives of microperoxidases such as MP8.[6−10] Much work has been done on Fe(III)MP8, but there have been no previous reports of MPs containing other transition metals.

One method of substitution of metals in heme proteins involves the removal of the native heme followed by reconstitution of the apoprotein with the appropriate metal-substituted porphyrin.[11,12] The covalent nature of the protein−heme linkage in *c*-type cytochromes and their heme peptides, however, disfavors this approach. An alternative method involves treatment of *c*-type hemes with anhydrous HF to produce free-base porphyrin heme *c* species, which can then be reconstituted by treatment with the appropriate metal salt.[3] This procedure provides a convenient synthetic route to manganese(III) microperoxidase-8. Reaction of Mn(III)MP8 with H_2O_2 or photogenerated $Ru(bpy)_3^{3+}$ (bpy = 2,2′-bipyridine) affords the Mn(IV)═O form of MP8 for comparisons with the ferryl states of MP8[13] and HRP.[14]

Results and Discussion

Preparation and Characterization of Metal-Free MP8 and Mn(III)MP8. Treatment of lyophilized solid MP8 with anhydrous HF at −78 °C results in the formation of a deep purple solution. After the removal of iron fluoride salts from the crude preparation by gel filtration chromatography in the dark, a purple solution of metal-free MP8 is obtained. The absorption spectrum of metal-free MP8 has a very broad Soret band with a maximum at 383 nm as well as other features (508, 542, 574, and 623 nm) characteristic of free-base porphyrins.[3,15,16] As expected, bright red emission is visible from solutions of metal-free MP8 under ultraviolet irradiation.[16] The identity of metal-free MP8 was confirmed by ^{232}Cf plasma-desorption mass spectrometry, which showed a parent ion peak at 1452 amu, along with features corresponding to the sodium adduct (M^+ + 23) and a 1409-amu fragment possibly attributable to a decarboxylation fragment. No residual unreacted FeMP8 was detected by mass spectrometry.

The crude reaction mixture that results from treating metal-free MP8 with manganous acetate is a distinctive orange color. This species constitutes the major band that elutes from reverse-phase chromatographic materials. The purity of MnMP8 was assayed by analytical FPLC. The material comprising the major FPLC band was identified as MnMP8 by ^{232}Cf plasma-desorption mass spectrometry. Since Mn(III) porphyrins are commonly six-coordinate, a water molecule could be axially coordinated in the complex (mass 1506 amu). Indeed, a mass of 1507 amu was observed, corresponding to the protonated aquo parent ion. The absorption spectrum of purified Mn(III)-MP8 has a distinctive split Soret band, with one component appearing at 368 nm and a weaker one at 463 nm (Figure 1). The extinction coefficient at 368 nm is 110 mM^{-1} cm^{-1} at pH 2. As the pH is increased, the intensity of the Soret component

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S0020-1669(97)01166-X CCC: $15.00
Published on Web 04/01/1998

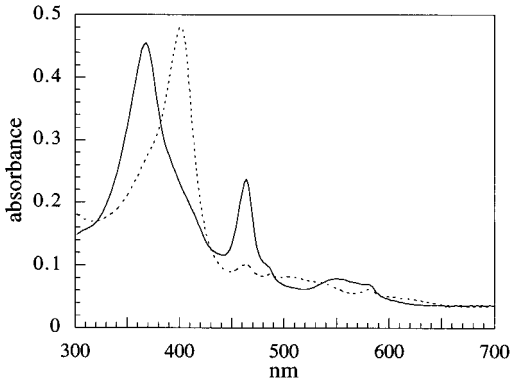

Figure 1. Absorption spectra of Mn(III)MP8 (solid line) and Mn(IV)═O MP8 (dotted line) generated by H_2O_2 oxidation.

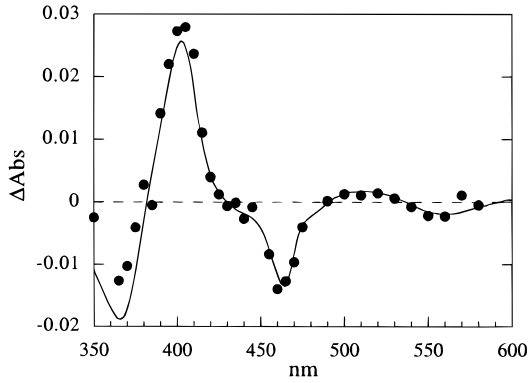

Figure 2. Comparison of transient and static Mn(IV)═O − Mn(III) MP8 spectra; ΔOD values obtained from single-exponential fits of kinetics traces (dots) superimposed on the static difference spectrum (solid line).

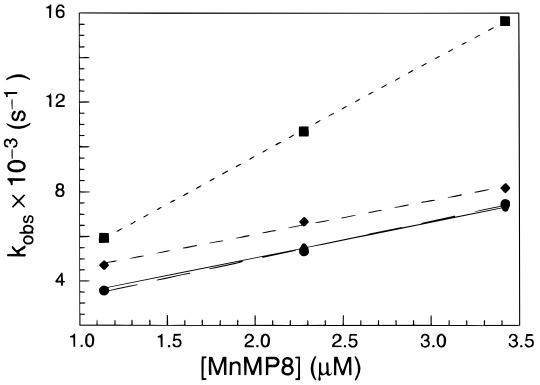

Figure 3. Plot of observed pseudo-first-order rate constants for oxidation of MnMP8 by $Ru(bpy)_3^{3+}$ as a function of MnMP8 concentration at pH 6, 7, 8, and 8.5. Lines are best fits from least-squares fitting. Apparent second-order rate constants ($k \times 10^{-9}$ M^{-1} s^{-1}) are pH 6 (•), 1.7; pH 7 (●), 1.8; pH 8 (◆), 1.6; pH 8.5 (■), 4.2.

at 368 nm increases, while the 460-nm absorbance decreases (p*K* 4.5). We attribute this change in the spectrum to deprotonation and axial binding of the His18 ligand (the analogous change in Fe(III)MP8 occurs at pH 2−3).[6,7,17] Split Soret bands such those observed in Mn(III)MP8 and other manganese(III) porphyrins are attributable to inequivalent interactions between the porphyrin π_x and π_y LUMOs and dπ electrons of the manganese ion.[18,19] The resonance Raman spectrum (457.9-nm excitation) of Mn(III)MP8 in neutral aqueous solution features high-frequency porphyrin skeletal modes at 1378 (ν_4), 1496 (ν_3), 1570 (ν_2), and 1620 cm^{-1} (ν_{10}). In contrast to resonance Raman spectra of ferric MP8 in neutral solutions, which exhibit broadened spin-state marker bands due to the presence of a mixture of high- and low-spin states,[17,20] Raman spectra of Mn(III)MP8 feature sharp peaks that suggest a highly homogeneous sample. The positions of ν_3 and ν_2 are sensitive to the coordination geometry about the Mn(III) ion and are most similar to those of 6-coordinate Mn(III) heme species,[21] supporting mass spectrometric evidence for water binding.

Studies of the intermediate formed by reaction of Mn(III) porphyrins with peroxides indicate that it is a Mn(IV) oxo (Mn(IV)═O) species rather than a Mn(IV) hydroxide complex (Mn(IV)−OH).[22] Treatment of Mn(III)MP8 with an excess of hydrogen peroxide generates a fleeting intermediate whose absorption spectrum reverts within seconds to that of the starting Mn(III)MP8 with little observed decomposition. The intermediate is likely to be Mn(IV)═O MP8, because the single Soret band at 401 nm in its spectrum (Figure 1) resembles that of the oxidized form of MnHRP, which has a single Soret peak at 412 nm.[1,22] The spectrum of the intermediate also is similar to that of Mn(IV) hematoporphyrin IX.[23] Addition of sodium dithionite to an anaerobic solution of Mn(III)MP8 yields another species with a Soret band in a "normal" position (424 nm); this species, presumably, is the one-electron-reduced complex, Mn(II)MP8.

Photoinduced Oxidation of Mn(III)MP8. Laser flash photolysis of Mn(III)MP8 samples containing 10 μM $Ru(bpy)_3^{2+}$ and 10 mM $Ru(NH_3)_6^{3+}$ in neutral buffered solution results in the appearance of a transient absorption spectrum that is in excellent agreement with the difference spectrum calculated from peroxide oxidation of Mn(III)MP8 (Figure 2). The appearance of the Mn(IV)═O signal obeys first-order kinetics; the slope of the observed pseudo-first-order rate constant versus the Mn(III)MP8 concentration yields an apparent second-order rate constant (ca. 1.5×10^9 M^{-1} s^{-1}) that is roughly independent of pH (Figure 3), although the oxidation reaction is slightly faster in solution buffered at pH 8.5 (4×10^9 M^{-1} s^{-1}). Since Mn−porphyrin aquo complexes typically have p*K*s that are ∼1 unit lower[24] than those of the analogous Fe porphyrins (the p*K* of Fe(III)(H_2O)MP8 is 9.6),[8,17] Mn(III)MP8 may be mainly a manganese−hydroxo porphyrin at pH 8.5. Unfortunately, the instability of $Ru(NH_3)_6^{3+}$ prevented further exploration of the pH dependence of Mn(III)MP8 oxidation in the alkaline region. The lack of pH dependence between pH 6 and 8 and the observation that the rate constants are similar to those for oxidation of Fe(III)MP8 suggest that electron transfer from the porphyrin ring precedes metal−oxo formation.[13] We do not, however, have any direct spectroscopic evidence for a Mn(III)−porphyrin radical.

Our finding that Mn(IV)═O MP8 is readily formed by one-electron oxidation of Mn(III)MP8 suggests that the low reactivity of Mn(III) heme proteins with peroxides is attributable to enhanced kinetic stability of the Mn(III)−peroxo unit.[19] We suspect that Mn porphyrins are relatively inefficient at cleaving peroxide because of reduced "push" from the other axial

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ligand.[25] Indeed, the metal−imidazole stretch of Mn(II)Mb is at significantly lower frequency than that of Fe(II)Mb,[21] indicating that axial bonding is weaker in the Mn(II) species; additionally, although Raman measurements are not available for the corresponding Mn(III) complex, it is likely that axial elongation in the d^4 high-spin system would further weaken the axial Mn−N (imidazole) bond. These diminished axial ligand interactions reduce the effectiveness of the proximal histidine as an electron donor, disfavoring O−O cleavage. Finally, it is interesting to note that there are manganese analogues of Fe-containing superoxide dismutases (SOD) and catalases, but there are no natural Mn-containing peroxidases.[26] Moreover, only MnSOD has a mononuclear active site; Mn catalases all contain a binuclear bridged manganese core that is responsible for peroxide reactivity.[27]

Experimental Section

Materials. Deionized water was purified with a Barnstead Nanopure system before use. Fe(III)MP8 was prepared from cytochrome *c* by enzymatic digestion and purified by FPLC as described previously.[13]

Methods. Preparation of Mn(III)MP8. Lyophilized Fe(III)MP8 in a Teflon reaction container was attached to an HF line and cooled with a dry ice acetone bath. Sufficient HF was condensed into the reaction vessel to cover the solid MP8. A purple solution results immediately. The HF solution was allowed to stand for 5 min at dry ice temperature and then allowed to warm to room temperature, after which time the HF was removed in vacuo. A brownish-purple residue remained in the vessel. The residue was treated with 0.1 M ammonium acetate buffer, pH 5, resulting in a purple solution, which was passed down either a Sephadex G-10 or G-25 (Pharmacia) column, which removed the iron salts from the free-base solution. Care was taken to minimize the exposure of the free base to light, as many free-base porphyrins are light-sensitive. Gel-filtered solutions of metal-free MP8 were used immediately for metalation reactions by addition of manganous acetate (Aldrich) to give a roughly 10 mM solution. The metalation reaction mixture was warmed to 40 °C in a water bath and allowed to stand for 2 h, then removed and allowed to stand in the dark for 2 days. The progress of Mn insertion was monitored by UV−vis spectroscopy. After 2 days, the crude reaction mixture was washed with 0.1% TFA and concentrated in an Amicon stirred cell. The concentrated crude mixture was fractionated on a Pharmacia PepRPC column using a standard FPLC protocol (2.5 mL/min flow rate).[13] The major orange fraction was collected and analyzed by mass spectrometry and UV−visible spectroscopy.

Measurements. UV−visible spectra were taken on a Hewlett-Packard 8452A diode array spectrophotometer. Raman spectra were collected with a SPEX 750 M Raman monochromator equipped with a cooled CCD array. Excitation light (457.9 nm) was provided by a Spectra Physics Innova continuous-wave argon ion laser running at 200 mW. Plasma lines were removed from the excitation beam by passage through a prism followed by tight focusing. A 20-μm aperture was used to reject off-axis light. The excitation beam was directed to the sample contained in a 1-cm-pathlength quartz fluorescence cuvette. Scattered light was collected by a lens and focused on the monochromator aperture using 1:1 imaging geometry. Typical acquisition times were approximately 1 min. Artifacts due to cosmic rays were manually removed from spectra. Pulsed laser excitation at 480 nm for transient absorption experiments was provided by a Lambda-Physik FL3002 using coumarin 480 dye (Exciton) pumped by a Lambda-Physik LPX 210i XeCl excimer laser (308 nm). Pulse energies of 1 mJ were typically used. Probe light for transient absorption measurements was provided by a 75-W continuous-wave arc lamp (PTI model A 1010) focused on the aperture of an ISA monochromator fitted with a Hamamatsu 5-stage photomultiplier tube. Time-resolved PMT output was amplified by a fast amplifier and processed by a Sony/Tektronix RTD 710 digitizer interfaced to an IBM personal computer. The arc lamp and laser beams were manually made coincident in the sample cuvette by aligning the parallel counter-propagating beams. Kinetics data were evaluated using a nonlinear least-squares fitting program.

Acknowledgment. We thank Jane Saunders, Gary Hathaway, and Peter Green for assistance with mass spectrometry. S.A. was a participant in the Caltech SURF program. D.W.L. acknowledges a fellowship from the Parsons Foundation. This work was supported by the NSF.

Supporting Information Available: The metal-free MP8 absorption spectrum; a chromatographic trace of Mn(III)MP8; mass spectra of metal-free MP8 and Mn(III)MP8; and the resonance Raman spectrum of Mn(III)MP8 (4 pages). Ordering information is given on any current masthead page.

IC971166C

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