Electrochemical Generation and Reactions of Ferrylmyoglobins in Water and Microemulsions

Anthony C. Onuoha, Xiaolin Zu, and James F. Rusling*

Contribution from the Department of Chemistry, University of Connecticut, U-60, 215 Glenbrook Rd., Storrs Connecticut 06269-4060

Received November 18, 1996[⊗]

Abstract: Ferrylmyoglobin species, which are active oxidant forms of the protein myoglobin, were obtained by electrochemical reduction of metmyoglobin [$MbFe^{III}$] in the presence of oxygen in aqueous neutral buffer and in microemulsions of oil, water, and cationic surfactant. Reduction of myoglobin at −0.4 V vs SCE catalyzed the reduction of oxygen to hydrogen peroxide at the electrode. Hydrogen peroxide oxidizes metmyoglobin in solution to give the radical ferrylmyoglobin $^{\bullet}X-MbFe^{IV}=O$, which is known to decay rapidly to the non-radical $MbFe^{IV}=O$. This complex reduction−oxidation process converted nearly all of the 30 μM myoglobin in a spectroelectrochemical cell to ferrylmyoglobins in 15 min in pH 7.3 buffer and in 18 min in microemulsions. Characteristic ferryl heme absorbance bands near 421, 548, and 584 nm were used to identify products. Confirmation of ferrylmyoglobins was provided by reductions to metmyoglobin with ascorbate and by myoglobin-mediated electrochemical epoxidation of styrene. Fiftyfold higher yields of styrene oxide and benzaldehyde were achieved in a microemulsion compared to electrochemical or chemical methods in pH 7.4 buffer. The electrochemical approach described may also prove useful for investigating active catalytic species of heme enzymes such as cytochrome P450.

Introduction

While the iron heme protein myoglobin (Mb) plays an essential role in oxygen storage and transport in mammalian muscle, it can also catalyze oxidations of organic molecules.[1−5] The active oxidants in catalytic cycles are oxidized forms of Mb, which can be formed by reacting metmyoglobin [$MbFe^{III}$] with hydrogen peroxide:[6−13]

$$X-MbFe^{III} + H_2O_2 \rightarrow {}^{\bullet}X-MbFe^{IV}=O + H_2O \quad (1)$$

$$2\,{}^{\bullet}X-MbFe^{IV}=O + H_2O_2 \rightarrow 2HX-MbFe^{IV}=O + O_2 \quad (2)$$

In eq 1, $X-MbFe^{III}$ represents metmyoglobin and $^{\bullet}X-MbFe^{IV}=O$ is the ferrylmyoglobin radical. One oxidizing equivalent from H_2O_2 is used at the iron center, and the other creates a free radical located on an amino acid residue (X), possibly tyrosine-103, on the surface of the protein.[14,15]

The ferrylmyoglobin radical $^{\bullet}X-MbFe^{IV}=O$ is a transient species with a half-life of about 30 s at medium pH and room temperature.[3c,8,12] It decays via eq 2 and other possible pathways[7,8] to a relatively stable species $HX-MbFe^{IV}=O$, which we shall call ferrylmyoglobin and for simplicity denote below as $MbFe^{IV}=O$. The presence of low-spin $Fe^{IV}=O$ heme in ferrylmyoglobin was confirmed[16−18] by Raman, X-ray absorption, Mossbauer, and NMR spectroscopy. $MbFe^{IV}=O$ is similar to peroxidase compound II[6,12] and to a proposed active intermediate in cytochrome P450 (cyt P450)-catalyzed oxidations.[19−23]

Ferrylmyoglobin radical formed by treatment of metmyoglobin with hydrogen peroxide has been used to oxidize organic substrates via the ferryl oxygen and the radical on the protein surface.[1,3,5] It also reacts with lipids and lipoproteins, and may be involved in disease-causing chemical events.[2,3b,4,11,15,24]

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1997.

(1) Ortiz de Montellano, P. R.; Catalano, C. E. *J. Biol. Chem.* **1985**, *260*, 9265−9271.
(2) Arduini, A.; Eddy, L.; Hochstein, P. *Arch. Biochem. Biophys.* **1990**, *281*, 41−43.
(3) (a) Rao, S. I.; Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 803−809. (b) Rao, S. I.; Wilks, A.; Hamberg, M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1994**, *269*, 7210−7216. (c) Choe, Y. S.; Rao, S. I.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **1994**, *314*, 126−131.
(4) Rice-Evans, C. In *Trace Elements and Free Radicals in Oxidative Diseases;* Favier, A. E., Neve, J., Faure, P., Eds.; AOCS Press: Champaign, IL, 1994; pp 92−99.
(5) Kelman, D. J.; DeGray, J. A.; Mason, R. P. *J. Biol. Chem.* **1994**, *269*, 7458−7463.
(6) George, P.; Irvine, D. H. *Biochem. J.* **1952**, *52*, 511−517.
(7) (a) King N. K.; Winfield, M. E. *J. Biol. Chem.* **1963**, *238*, 1520−1528. (b) King N. K.; Winfield, M. E. *Aust. J. Biol. Sci.* **1966**, *19*, 211−217.
(8) Yonetoni, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974−1979.
(9) (a) Galaris, D.; Cadenas, E.; Hochstein P. *Free Radical Biol. Med.* **1989**, *6*, 473−478. (b) Galaris, D.; Cadenas, E.; Hochstein P. *Arch. Biochem. Biophys.* **1989**, *273*, 497−504. (c) Galaris, D.; Eddy, L.; Arduini, A.; Cadenas, E.; Hochstein, P. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 1162−1168.
(10) Giuvlivi, C.; Cadenas, E. *FEBS Lett.* **1993**, *332*, 287−290.
(11) Turner, J. J. O.; Rice-Evans, C. A.; Davies, M. J.; Newman, E. S. R. *Biochem. J.* **1991**, *277*, 833−837.
(12) Davies, M. J. *Biochem. Biophys. Acta* **1991**, *1077*, 86−90.
(13) Dee, G.; Rice-Evans, C. A.; Obeyesekera, S.; Meraji, S.; Jacobs, M.; Bruckdorfer, K. R. *FEBS Lett.* **1991**, *294*, 38−42.
(14) Davies, M. J. *Free Radical Res. Commun.* **1990**, *10*, 361−370.
(15) Turner, J. J. O.; Rice-Evans, C. A.; Davies, M. J.; Newman, E. S. R. *Biochem. Soc. Trans.* **1990**, *18*, 1056−1059.
(16) (a) Sitter, A. J.; Reczek, C. M.; Terner, J. *Biochem. Biophys. Acta* **1985**, *828*, 229−235. (b) Chance, M.; Powers, L.; Kumar, C.; Chance, B. *Biochemistry* **1986**, *25*, 1259−1265.
(17) (a) Schulz, C. E.; Chiang, R.; Debrunner, P. G. *J. Phys. Colloq.* **1979**, *40*, 534−536. (b) Foote, N.; Gadsby, P. M. A.; Greenwood, C.; Thomson, A. J. *Biochem. J.* **1989**, *261*, 515−522.
(18) La Mar, G. N.; De Ropp, J. S.; Latos-Grazynski, L.; Balch, A. L.; Johnson, R. B.; Smith, K. M.; Parish, D. W.; Cheng, R.-J. *J. Am. Chem. Soc.* **1985**, *107*, 782−787.
(19) Wislocki, P. G.; Miwa, G. T.; Lu, A. Y. H. In *Enzymatic Basis of Detoxication*; Jakoby, W. B., Ed.; Academic Press: New York, 1980; Vol. 1, pp 135−182.
(20) Guengerich, F. P.; MacDonald, T. L. *Acc. Chem. Res.* **1984**, *17*, 9−16.
(21) Ortiz de Montellano, P. R., Ed. *Cytochrome P450*, 2nd ed.; Plenum: New York, 1995.
(22) Schenkman, J. B.; Greim, H., Eds. *Cytochrome P450*; Springer-Verlag, Berlin, 1993.
(23) Omura, T.; Ishimura, Y.; Fujii-Kuriyama, Y., Eds. *Cytochrome P450*, 2nd ed.; Kodansha/VCH: Tokyo, 1993.
(24) Kanner, J.; Harel, S. *Arch. Biochem. Biophys.* **1985**, *237*, 314−321.

S0002-7863(96)04007-3 CCC: $14.00

Ferrylmyoglobins have been implicated in oxidative cellular damage in coronary heart disease.[4,9,10,12,15]

The catalytic reactivity of Mb is similar to liver cyt P450s, which may catalyze oxidative carcinogenic activation of pollutants.[19−23] Like cyt P450, Mb employs high-valent heme iron to catalyze oxidations. Although Mb is less completely stereo- and regiospecific in its reactivity, catalytic processes are likely to be similar. Thus, the more readily available myoglobin provides a useful comparative model for cyt P450 in investigations of the chemistry of pollutant-caused disease mechanisms.

Ferrylmyoglobin and ferrylmyoglobin radical can be detected by visible absorption spectroscopy.[9a,b,11] In neutral aqueous solutions, the Soret visible absorption band of the ferryl heme occurs at a characteristic wavelength of 421 nm.[9c,11,15] The maximum is shifted red from that of metmyoglobin. Distinct absorption bands of ferrylmyoglobin also occur at about 548 and 584 nm.[9a,b]

In this paper, we report electrochemically driven generation of ferrylmyoglobins detected by visible absorbance spectroelectrochemistry. The process involves electrochemical reduction of oxygen meditated by myoglobin, producing hydrogen peroxide, which in turn oxidizes metmyoglobin to ferrylmyoglobins. This approach was also used for myoglobin-mediated oxidation of styrene.

Experimental Section

Chemicals. Lyophilized horse heart myoglobin was from Sigma. Didodecyldimethylammonium bromide (DDAB, 99%), cetyltrimethylammonium bromide (CTAB, >99%), and dodecane (99%) were from Eastman Kodak. Styrene, styrene oxide, and benzaldehyde were from Aldrich. Hydrogen peroxide (30%) was from J. T. Baker. Buffers were 0.05 M phosphate (pH 7.3 or 7.4) or 0.05 M acetate buffer (pH 4.8), and contained 0.05 M NaBr. All other chemicals were reagent grade.

Microemulsions were prepared as described previously,[25,26] and have been characterized as continuous in both oil and water.[25,27] Percent compositions (wt %) were DDAB/water/dodecane (13/28/59) and CTAB/water/1-pentanol/tetradecane (17.5/35/35/12.5). Specific conductivities of microemulsions were as follows: DDAB, 0.12×10^{-3} Ω^{-1} cm^{-1}; CTAB, 2.0×10^{-3} Ω^{-1} cm^{-1}.

Apparatus and Procedures. For spectroelectrochemistry, a Princeton Applied Research Corp. (PARC) Model 273 electrochemical analyzer was coupled to a Perkin Elmer Lambda 6 UV−visible spectrophotometer. The three-electrode cell was a quartz UV−vis cuvette (Uvonics, Inc.). It employed a Vycor tipped saturated calomel reference electrode (SCE), a Pt wire counter electrode, and a small block ($2.5 \times 0.6 \times 0.3$ cm) of pyrolytic graphite (HPG-99, Union Carbide) as the working electrode, with electroactive basal and edge planes. The top of the working electrode was attached to a conducting cable with silver epoxy, which was then coated with non-conducting epoxy. Cyclic voltammetry was done as described previously[25] with use of a basal plane disk PG working electrode ($A = 0.28$ cm^2). In aerobic experiments, measured volumes of oxygen gas were added via a syringe to solutions in a sealed cell that had been previously degassed with purified nitrogen. Potentials are reported vs SCE.

Prior to use, pyrolytic graphite (PG) electrodes were polished for 2 min with a 0.3-μm α-alumina dispersion on a billiard cloth on a metallographic polishing wheel, then sonicated for 2 min in distilled water. The resulting mirror-like surface was wiped dry with a Kimwipe before use.

The spectroelectrochemical cell had a Teflon cap with holes for reference, counter, and working electrodes. A fourth hole held an inlet for oxygen or nitrogen. The SCE reference electrode terminating in a 0.3-cm-diameter Vycor frit was immersed to a depth of 2 cm in the cell. The working electrode was positioned flat against one side of the cuvette, out of the light path.

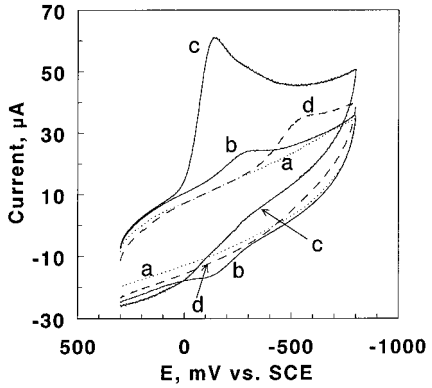

Figure 1. Cyclic voltammograms on a bare PG electrode at 100 mV s^{-1} in pH 4.8 acetate buffer containing 50 mM NaBr: (a) background with no myoglobin or oxygen present; (b) 30 μM Mb in anaerobic buffer; (c) 30 μM Mb after 15 mL of O_2 was added; and (d) after 15 mL of O_2 was added, no Mb present.

In typical experiments, 3.0 mL of 30 μM Mb was pipetted into the cell with electrodes in place. A gentle flow of oxygen was continuously passed through the solution. An initial spectrum of MbFe^{III} was recorded, and electrolysis was begun at a potential of −0.4 V vs SCE. Spectra of products were recorded at various times. Coulometric charge vs time curves were obtained with the PARC Model 273 Electrochemistry System during spectroelectrochemistry and were corrected for background by subtracting the charge in equivalent experiments without myoglobin.

Hydrogen peroxide was monitored by using Quantofix Peroxide 100 test sticks (Macherey-Nagel GmbH & Co., Germany). The detection limit is 0.03 mM.

Oxidations of styrene and reductions of MbFe^{II}−O_2 employed a PARC Model 273 potentiostat and a water-jacketed, divided cell with a carbon felt (WDF, Union Carbide, $2 \times 1.5 \times 0.6$ cm) working electrode, an 8 cm long, 0.5 cm diameter spectroscopic carbon rod as counter electrode, and a saturated calomel reference electrode (SCE). The cell was open to air as a source of oxygen and temperature was controlled with a circulating water bath.

Gas chromatography (GC) and gas chromatography−mass spectrometry (GC/MS) were used to determine organic electrolysis products, using methods and equipment similar to those described previously.[26] Styrene oxide and benzaldehyde were used as external standards for quantitation by GC. Identities of styrene oxidation products in reaction mixtures were confirmed by GC/MS.

Results

Voltammetry. Cyclic voltammograms of 30 μM metmyoglobin in anaerobic pH 4.8 buffer at 100 mV s^{-1} at polished basal plane PG electrodes (Figure 1b) gave a reduction peak at −300 mV vs SCE and an oxidation peak that was 160 mV more positive. The 160 mV peak separation suggests slow electron transfer between myoglobin and the electrode, similar to that observed on an indium tin oxide electrode in neutral solutions.[29] The peak potentials are consistent with redox reactions involving the heme Fe^{III}/Fe^{II} couple of the protein.

After passing 15 mL of oxygen through a previously degassed metmyoglobin solution in a sealed cell, a substantial increase in the reduction current for MbFe^{III} was observed (Figure 1c). This was accompanied by a positive shift of the peak to −140 mV and the disappearance of the oxidation peak for MbFe^{II}. The ratio of the reduction peak in the presence and absence of oxygen decreased as the scan rate was increased. In the absence

(25) Onuoha, A. C.; Rusling, J. F. *Langmuir* **1995**, *11*, 3296−3301.
(26) Gao, J.; Rusling, J. F.; Zhou, D.-L. *J. Org. Chem.* **1996**, *61*, 5972−5977.
(27) Ceglie, A.; Das, K. P.; Lindman, B. *Colloids Surfaces* **1987**, *28*, 29−40.
(28) Bard, A. J.; Faulkner, A. J. *Electrochemical Methods*; Wiley: New York, 1980.
(29) King, B. C.; Hawkridge, F. M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1992**, *114*, 10603−10608.

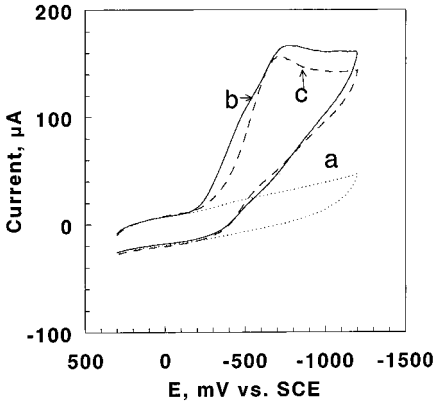

Figure 2. Cyclic voltammograms on a bare PG electrode at 100 mV s^{-1} in pH 7.3 phosphate buffer containing 50 mM NaBr: (a) 30 μM Mb in anaerobic buffer; (b) 30 μM Mb, buffer saturated with O_2; and (c) buffer saturated with O_2, no Mb present.

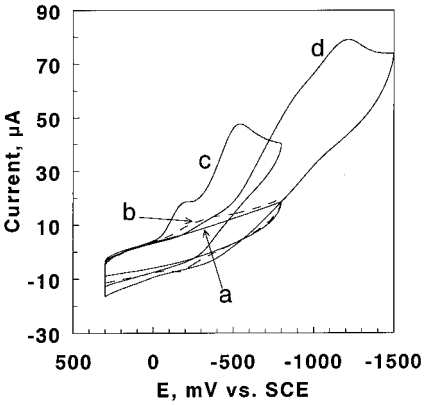

Figure 3. Cyclic voltammograms on a bare PG electrode at 100 mV s^{-1} in DDAB microemulsion [DDAB/water/dodecane (13/28/59)]: (a) background with no myoglobin or oxygen present; (b) 30 μM Mb, anaerobic; (c) 30 μM Mb after 5 mL of O_2 was added; and (d) after 5 mL of O_2 was added, no Mb present.

of metmyoglobin, a peak for direct reduction of oxygen was observed at about −600 mV (Figure 1d).

Metmyoglobin in anaerobic pH 7.3 buffer did not give CV reduction or oxidation peaks on polished basal plane PG electrodes (Figure 2a). This is similar to behavior on rough PG, Au, or Pt electrodes between pH 5.5 and 8.[30] With oxygen and Mb present in the pH 7.3 buffer, a large reduction peak was found at a potential of about −750 mV (Figure 2b). The reduction current begins to increase at about −190 mV vs SCE, and the shoulder on the rising portion of curve 2b suggests catalysis by Mb. This catalytic reaction occurs at potentials slightly positive of the direct reduction of oxygen (Figure 2c).

Cyclic voltammograms of metmyoglobin in an anaerobic DDAB microemulsion showed CV reduction−oxidation peaks separated by >100 mV (Figure 3b). When oxygen was injected into the protein-containing medium, the reduction curve (Figure 3c) showed two peaks at −180 and −530 mV, and no oxidation peak. The ratio of the first peak in the presence and absence of oxygen decreased with increasing scan rate. CVs in the CTAB microemulsion gave similar evidence for myoglobin-mediated reduction of oxygen. The second peak at −530 mV is attributed to reduction of oxygen. This reduction may be facilitated by accumulation of oxygen in a film of adsorbed

(30) (a) Rusling, J. F.; Nassar, A-E. F. *J. Am. Chem. Soc.* **1993**, *115*, 11891−11897. (b) Nassar, A-E. F.; Willis, W. S.; Rusling, J. F. *Anal. Chem.* **1995**, *67*, 2386−2392. (c) Nassar, A-E. F., Narikiyo, Y.; Sagara, T.; Nakashima, N.; Rusling, J. F. *J. Chem. Soc., Faraday Trans.* **1995**, *91*, 1775−1782.

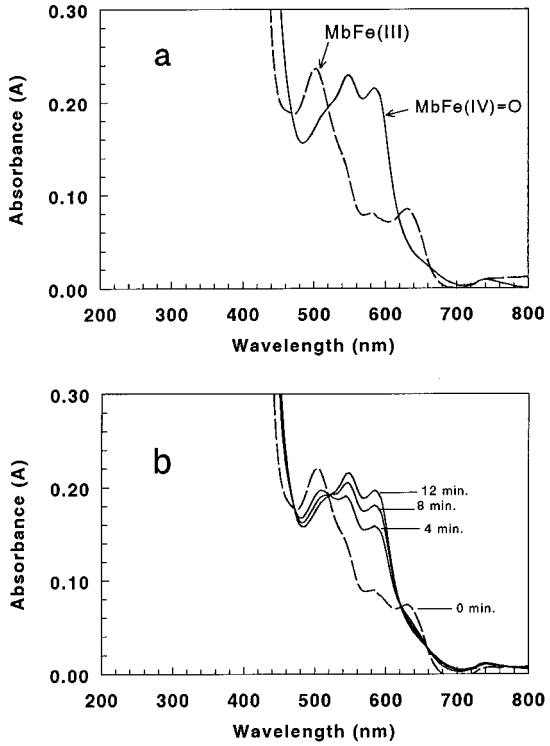

Figure 4. Visible absorption spectra in pH 7.3 buffer of (a) 30 μM $MbFe^{III}$ and $MbFe^{IV}$=O formed by addition of 0.6 mM H_2O_2 to the $MbFe^{III}$ solution and (b) 30 μM $MbFe^{III}$ before (0 min) and after 4−12 min of controlled potential electrolysis at −0.4 V vs SCE in buffer saturated with O_2.

DDAB and Mb on the electrode, since it occurs at a similar potential to that in Mb−DDAB films deposited onto PG electrodes and used in buffer solutions.[25] Without myoglobin, the reduction peak for oxygen in microemulsions was at −1.2 V (Figure 3d).

Spectroelectrochemistry at pH 7.3. Visible absorption spectra of ferrylmyoglobin are well characterized in the medium pH range,[9,11,15] so we first discuss results at pH 7.3. The reaction of metmyoglobin with hydrogen peroxide was used to obtain standard spectra. The $MbFe^{IV}$=O and $^{\bullet}X-MbFe^{IV}$=O formed were detected simultaneously by the characteristic bands at 548 and 584 nm after addition of hydrogen peroxide, accompanied by the decrease of the 503- and 631-nm peaks of $MbFe^{III}$ (Figure 4a). Simultaneously, the Soret band shifted from 408 nm for metmyoglobin to 421 nm, again characteristic of the ferrylmyoglobin species. $MbFe^{IV}$=O and $^{\bullet}X-MbFe^{IV}$=O cannot be distinguished in these experiments, but $^{\bullet}X-MbFe^{IV}$=O is known to decay (eq 2) to $MbFe^{IV}$=O rapidly, and spectra after several minutes reflect mainly the latter species.

Electrochemical reduction of metmyoglobin in aerobic pH 7.3 phosphate buffer was done in the spectroelectrochemical cell at −0.4 V, a potential on the rising portion of the reduction peak in the voltammograms (cf. Figure 2). After several minutes of electrolysis under oxygen flow, the 503- and 631-nm bands of $MbFe^{III}$ decreased and bands at 548 and 584 nm characteristic[9,11,15] of the ferrylmyoglobins appeared (Figure 4b). These changes were accompanied by isosbestic points at 521 and 612 nm, characteristic of transformation of $MbFe^{III}$ to $MbFe^{IV}$=O with no stable intermediates.

After about 17 min of electrolysis, a gradual decrease in absorbance of the ferrylmyoglobin bands was observed with time (Figure 5a). The Soret band of $MbFe^{III}$ at 408 nm, converted to the 421-nm ferryl heme band during the electrolysis (Figure 5b), also decreased with time after 17 min. If

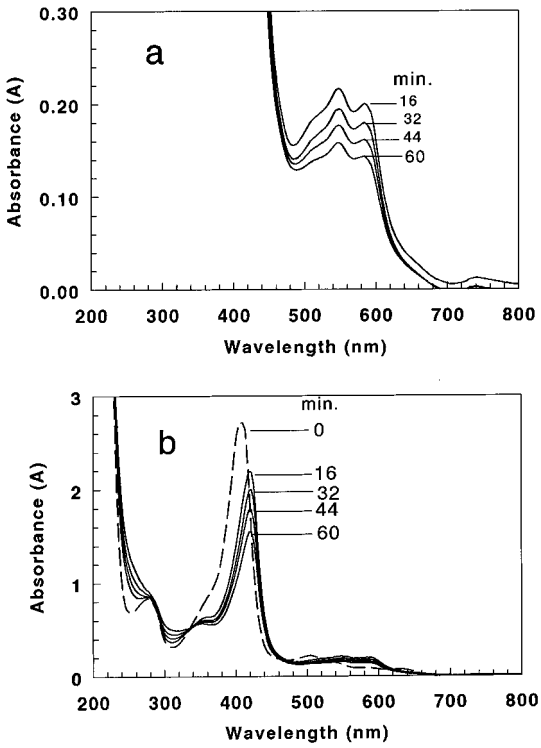

Figure 5. Visible absorption spectra in pH 7.3 buffer of (a) the 500−700-nm region after 16−60 min of controlled potential electrolysis of 30 μM $MbFe^{III}$ at −0.4 V vs SCE in buffer saturated with O_2 and (b) the Soret band region showing 30 μM $MbFe^{III}$ prior to bulk electrolysis (0 min.) and after 16−60 min of electrolysis as in part a.

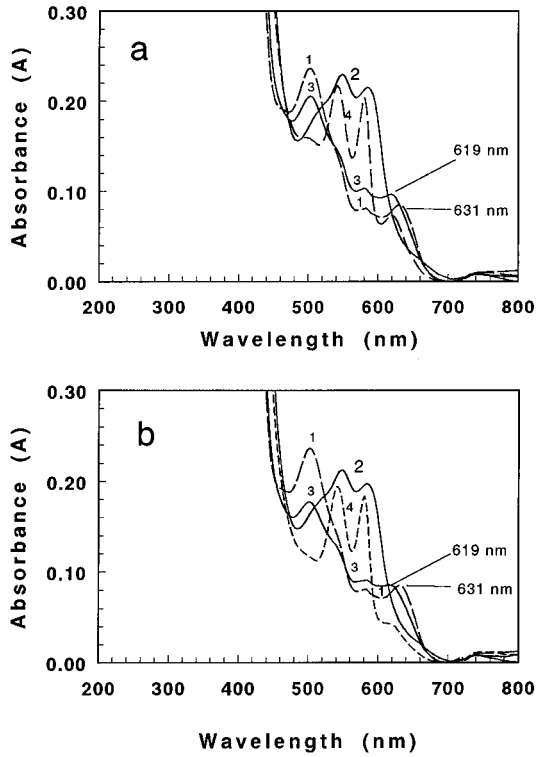

Figure 6. Spectra in pH 7.3 buffer: (a) (a.1) 30 μM $MbFe^{III}$, (a.2) $MbFe^{IV}$=O formed by the addition of 0.6 mM H_2O_2 to 30 μM $MbFe^{III}$, (a.3) $MbFe^{III}$ obtained after $MbFe^{IV}$=O was reduced with 1.1 mM ascorbic acid, and (a.4) $MbFe^{II}-O_2$ obtained after addition of 3.4 mM ascorbic acid; (b) (b.1) 30 μM $MbFe^{III}$, (b.2) after 16 min of controlled potential electrolysis of 30 μM $MbFe^{III}$ at −0.4 V vs SCE in buffer saturated with O_2, (b.3) after addition to b.2 of 1.71 mM ascorbic acid, and (b.4) after addition to b.3 of 7.66 mM ascorbic acid.

electrolysis was terminated after 15−17 min, the 421-, 548-, and 584-nm bands were stable for about 1 h.

Charge passed through the electrochemical cell increased linearly with time during these spectroelectrochemical experiments. After 15 min of electrolysis, the background corrected charge was 232 mC. Using Faraday's law, this charge corresponds to 25 electrons/mol of metmyoglobin initially present. These coulometric findings are also consistent[31a] with the catalytic electrochemical reduction of oxygen.

No ferrylmyoglobin was formed when oxygen was absent from the pH 7.3 solution. The absence of changes in the Soret band of metmyoglobin indicated that very little redox conversion occurred under such conditions. Thus, reduction of metmyoglobin appears to be facilitated via the kinetic influence of the follow up reaction of oxygen with $MbFe^{II}$, which uses up this reduction product as soon as it is formed.

Further evidence supporting the generation of ferrylmyoglobins was obtained via reduction of the oxidized product with ascorbic acid.[9b,10] Addition of ascorbic acid to a solution of chemically produced ferrylmyoglobins (Figure 6.a.2) resulted in a spectrum similar to that of metmyoglobin (Figure 6a.3, cf. Figure 6a.1). The shift in the original metmyoglobin 631-nm band to 619 nm after reduction of ferrylmyoglobin by ascorbate has been attributed to a small amount of oxidative damage to the protein during the chemical oxidation−reduction cycle.[9b] This damage may also cause the metmyoglobin absorbance to be smaller after the oxidation−reduction (Figure 6a.3, cf. Figure 6a.1). Addition of more ascorbic acid to this sample reduced metmyoglobin, giving the spectrum of $MbFe^{II}-O_2$ (Figure 6a.4).

Nearly identical results were found when ascorbic acid was added to electrochemically generated ferrylmyoglobins (Figure 6b.2). Ascorbic acid was added after allowing time for $^{\bullet}X-MbFe^{IV}$=O to decay fully to $MbFe^{IV}$=O. The ferryl species formed electrochemically was reduced to $MbFe^{III}$ by ascorbate (Figure 6b.3). Subsequently, this sample was reduced with additional ascorbate to $MbFe^{II}-O_2$ (Figure 6b.4).

When the pH 7.3 solution in which ferrylmyoglobins were formed was converted to pH 4.8 by adding acid, a major band at 585 nm was found. The spectrum was identical to that observed after reacting hydrogen peroxide with metmyoglobin in pH 4.8 buffer. Conversion of either of these pH 4.8 solutions to pH 7.3 did not regenerate the ferryl spectrum observed originally at pH 7.3.

Spectroelectrochemistry at pH 4.8. Ferrylmyoglobin in acidic solutions is much less stable than at neutral pH. Initial rapid formation of a peak at 525 nm in low-temperature reactions of hydrogen peroxide with metmyoglobin was attributed to a protonated ferrylmyoglobin, $(H^+)MbFe^{IV}$=O.[7b,17b,31b] This was rapidly replaced by a band at 585 nm, indicative of irreversible decomposition of $(H^+)MbFe^{IV}$=O. It was not possible to reduce this decomposition product to metmyoglobin.

Spectra obtained after adding H_2O_2 to metmyoglobin in pH 4.8 buffer (Figure 7a.2) had a major peak at 585 nm, with a small shoulder at about 560 nm. This is the same as the spectrum of the decomposition product of $(H^+)MbFe^{IV}$=O.[7] Addition of reducing agents to solutions of the 585 nm absorbing species did not regenerate the $MbFe^{III}$ spectrum. Reduction with ascorbate leads to disappearance of the 585-nm peak (Figure 7a.3) without clear indication of the reduced myoglobin product.

Spectra after electrolysis of metmyoglobin at −0.4 V in pH 4.8 buffer (Figure 7b.2) were similar to that of the chemically oxidized species (Figure 7a.2). The spectrum had a major peak

(31) (a) Bard, A. J.; Santhananam, K. S. V. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1970; Vol. 4, pp 215−315. (b) Wittenberg, J. B. *J. Biol. Chem.* **1978**, *253*, 5694−5695.

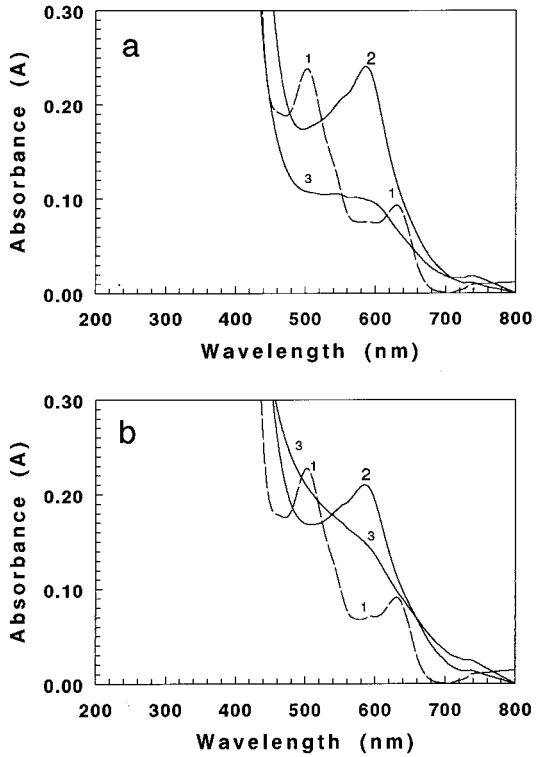

Figure 7. Spectra in pH 4.8 buffer: (a) (a.1) 30 μM $MbFe^{III}$, (a.2) after addition of 0.3 mM H_2O_2 to 30 μM $MbFe^{III}$, and (a.3) after addition to a.2 of 30 mM ascorbic acid; (b) (b.1) 30 μM $MbFe^{III}$, (b.2) after 20 min of controlled potential electrolysis of 30 μM $MbFe^{III}$ at −0.4 V vs SCE in buffer saturated with O_2, and (b.3) after addition to b.2 of 8.3 mM ascorbic acid.

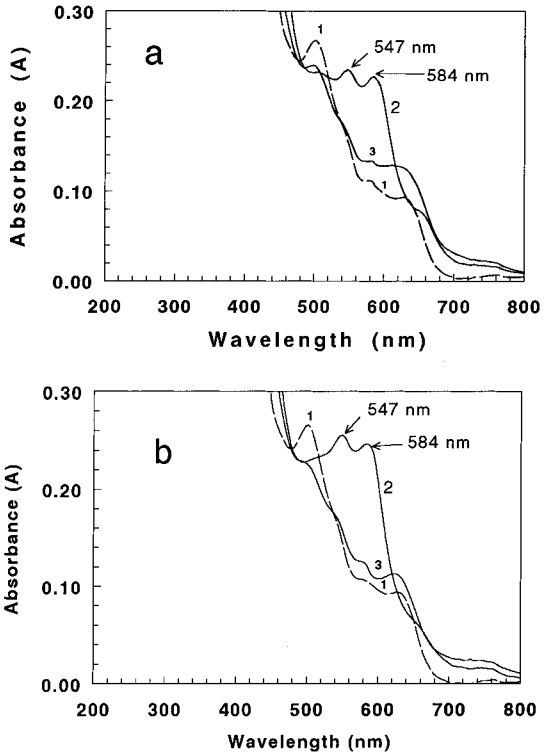

Figure 8. Spectra in DDAB microemulsion [DDAB/water/dodecane (13/28/59)]: (a) (a.1) 30 μM $MbFe^{III}$, (a.2) $MbFe^{IV}$=O formed by the addition of 0.03 mM H_2O_2 to 30 μM $MbFe^{III}$, and (a.3) after $MbFe^{IV}$=O was reduced with 0.17 mM ascorbic acid; (b) (b.1) 30 μM $MbFe^{III}$, (b.2) after 18 min of controlled potential electrolysis of 30 μM $MbFe^{III}$ at −0.4 V vs SCE in buffer saturated with O_2, and (b.3) after addition to b.2 of 1 mM ascorbic acid.

at 585 nm, and a shoulder at 560 nm, again suggesting decomposition of $(H^+)MbFe^{IV}$=O. The second derivative of this spectrum showed only a small band at 525 nm, indicative of a very small amount of $(H^+)MbFe^{IV}$=O. Reduction with ascorbate did not give the $MbFe^{III}$ spectrum (Figure 7b.3).

Coulometric results were consistent with a catalytic electrochemical process,[31a] as at pH 7.4. Charge passed through the electrochemical cell increased linearly with time, and 174 mC passed after 15 min. This corresponds to 20 electrons/mol of metmyoglobin initially present.

Spectroelectrochemistry in Microemulsions. Microemulsions are clear microheterogeneous mixtures of oil, water, and surfactant with intriguing possibilities as low-toxicity media for organic chemical reactions, including kinetic control and rate enhancement.[32,33] If ferrylmyoglobins could be generated in this medium, oxidations of water-insoluble substrates would be possible. Microemulsions made with cationic surfactants (see Experimental Section) were examined because we had previously shown that metmyoglobin could be reduced electrochemically in such media.[25] Soret bands of $MbFe^{III}$ were at 406 nm in the DDAB and 401 nm in the CTAB microemulsion, consistent with the possibility of small amounts of unfolding of the protein, but not with full denaturation, which gives a band at <390 nm. Native Mb in pH 7 buffer has a Soret band at about 408 nm.

Addition of hydrogen peroxide to metmyoglobin in a microemulsion gave a species with bands at 421, 547, and 584 nm (Figure 8a.2). This spectrum was very similar to that of the ferrylmyoglobins formed at pH 7.3 (cf. Figures 4 and 6). Reduction of this species with ascorbate in the microemulsion gave a spectrum similar to that of metmyoglobin (Figure 8a.3).

Electrolysis of metmyoglobin in a DDAB microemulsion at −0.4 V vs SCE produced a spectrum similar to that found by reaction of metmyoglobin with H_2O_2 (Figure 8b.2). A similar spectrum was observed in the CTAB microemulsion. Reduction of this species with ascorbate gave a spectrum resembling that of metmyoglobin (Figure 8b.3). Again, coulometry suggested a catalytic electrochemical process.[31a] Charge increased linearly with time, and 162 mC passed through the cell in 18 min. This corresponds to 19 electrons/mol of metmyoglobin initially present.

Detection of Hydrogen Peroxide during Electrolyses. Quantofix Peroxide 100 sticks inserted into buffers and microemulsions gave positive tests for peroxide after electrolyses. No hydrogen peroxide was detected before electrolysis. With 30 μM metmyoglobin in buffers or microemulsions, about 0.3 mM H_2O_2 was found after 30 min of electrolyses (Table 1).

As controls, electrolyses were done for 30 min at −0.4 V vs SCE under oxygen flow in the absence of metmyoglobin. Much smaller amounts of hydrogen peroxide were formed compared to electrolyses in the presence of metmyoglobin (Table 1).

Oxidation of Styrene. Myoglobin mediates the oxidation of styrene to styrene oxide and benzaldehyde in a process utilizing hydrogen peroxide (eq 1) to produce $^{\bullet}X-MbFe^{IV}$=O.[1,3a,c] We examined the possibility of driving this reaction by electrolyzing myoglobin at a carbon felt cathode in the presence of oxygen and styrene. Product analyses after 5 h of electrolysis

(32) (a) Fendler, J. H. *J. Phys. Chem.* **1980**, *84*, 1485−1491. (b) Fendler, J. H. *Membrane Mimetic Chemistry*, Wiley: New York, 1982. (c) Bourrel, M.; Schechter, R. S. *Microemulsions and Related Systems*; Marcel Dekker: New York, 1988. (d) O'Connor, C. J.; Lomax, T. D.; Ramage, R. E. *Adv. Colloid Interface Sci.* **1984**, *20*, 21−97.

(33) (a) Rusling, J. F. In *Modern Aspects of Electrochemistry*; Conway, B. E., Bockris, J. O'M., Eds.; Plenum Press: New York, 1994; No. 26, pp 49−104. (b) Rusling, J. F. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1994; Vol. 18, pp 1−88.

Table 1. Amounts of Hydrogen Peroxide after 30 min of Electrolyses at −0.4 V vs SCE[a]

medium	[Mb], μM	H_2O_2 found, mM
pH 7.3 buffer	0	0.03
pH 7.3 buffer	30	0.30
pH 4.8 buffer	0	0.09
pH 4.8 buffer	30	0.30
DDAB microemulsion	0	0.09
DDAB microemulsion	30	0.30

[a] In a 3-mL solution in a spectroelectrochemical cell; detection limit for H_2O_2 is 0.03 mM.

Table 2. Product Yields in Myoglobin-Mediated Oxidation of Styrene[a]

		yield, μmol/25 mL		
method	T, °C	styrene oxide	benzaldehyde	total
H_2O_2,[b] pH 7.4	0	2.1	0.6	2.7
H_2O_2, pH 7.4	15	0.5	0.2	0.7
$E = -0.65$ V, pH 7.4	2	6.8	2.0	8.8
$E = -0.65$ V, pH 7.4	15	0.8	0.5	1.3
$E = -0.65$ V, CTAB microemulsion[c]	15	23.4	25.4	48.8

[a] Oxidations of 10 mM styrene per 25 mL of aerobic solution containing 80 μM myoglobin, analyzed by gas chromatography after 5 h; chemical oxidations employed 0.6 mM H_2O_2. [b] Data from ref 1. [c] Composition by weight was CTAB/water/1-pentanol/tetradecane (17.5/35/35/12.5).

at −0.65 V vs SCE showed that larger amounts of styrene oxide and benzaldehyde (Table 2) were afforded in neutral buffer solutions by electrolysis than by chemical reaction with hydrogen peroxide. The 548- and 584-nm absorption bands of ferrylmyoglobins were observed for samples withdrawn from the electrolysis chamber. Furthermore, electrolysis in a microemulsion gave 50-fold better yields than chemical oxidation or electrolysis in pH 7.4 buffer (Table 2). No styrene oxide or benzaldehyde were formed when myoglobin was absent from the electrolysis solutions, or when H_2O_2 was used alone.

Electrochemical Reduction of $MbFe^{II}-O_2$. $MbFe^{II}$ reacts rapidly with oxygen to give $MbFe^{II}-O_2$.[34] The following experiment was done to test the possibility of electrochemical reduction of this complex to yield hydrogen peroxide.

Anaerobic 40 μm Mb in pH 7.4 buffer (10 mL) in an electrolysis cell was reduced chemically to $MbFe^{II}$ by adding excess sodium dithionite.[35a] Production of $MbFe^{II}$ was confirmed by observation of its Soret band at 431 nm and a band at 560 nm.[34] Oxygen was then bubbled through the solution to convert $MbFe^{II}$ to $MbFe^{II}-O_2$ and to help destroy the remaining dithionite. $MbFe^{II}-O_2$ was detected by its Soret band at 414 nm and two sharp bands at 543 and 581 nm. Spectra of a control sample of $MbFe^{II}-O_2$ showed little change over 1 h, but after several hours some $MbFe^{III}$ was detected, presumably from autoxidation of $MbFe^{II}-O_2$.[34]

Electrolysis of the $MbFe^{II}-O_2$ solution at a carbon felt cathode[35b] at −0.4 V vs SCE was now begun. During electrolysis, the Soret band shifted from 414 nm to the 421 nm characteristic of ferrylmyoglobins (Figure 9a). The sharp bands at 543 and 581 nm decreased and broadened with time, and shifted to 547 and 584 nm after 120 min of electrolysis (Figure 9b). Hydrogen peroxide was detected at 1 mM after 60 min of electrolysis and >1 mM after 120 min. These results suggest that $MbFe^{II}-O_2$ is reduced to yield hydrogen peroxide, which

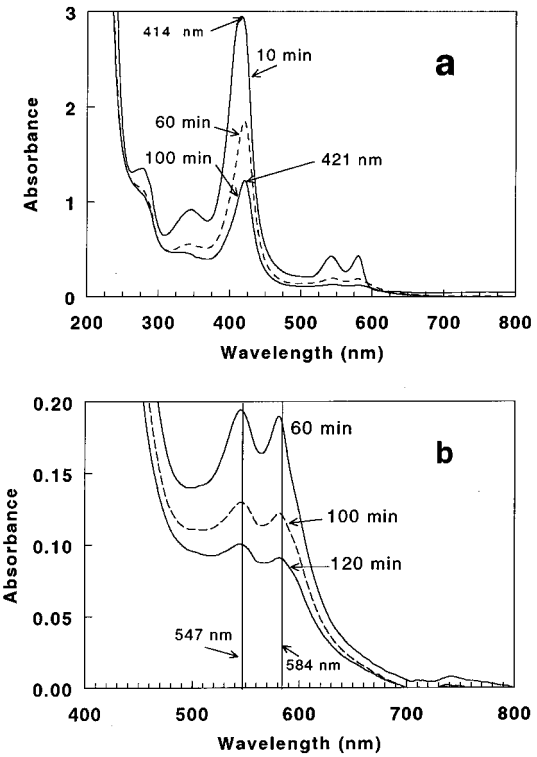

Figure 9. Spectra during electrolysis at −0.4 V vs SCE of $MbFe^{II}-O_2$ in pH 7.4 buffer formed by reduction of 40 μM $MbFe^{III}$ with excess sodium dithionite followed by addition of oxygen: (a) UV−vis spectrum showing change in the Soret band from 414 nm for $MbFe^{II}-O_2$ to 421 nm for $MbFe^{IV}$=O; and (b) visible region showing development of bands at 547 and 584 nm during electrolysis.

then reacts with reduced Mb species in the solution to yield ferrylmyoglobins.

The above conclusion was supported by observing the spectrum after adding more metmyoglobin to the solution after 120 min of electrolysis. This resulted in growth of the bands at 421, 547, and 584 nm and a final spectrum identical with that of $MbFe^{IV}$=O, presumably formed in greater amounts by oxidation of the newly added metmyoglobin by hydrogen peroxide in the solution.

Discussion

Electrolysis at pH 7.3. Cyclic voltammograms showed (Figure 2a) that in the absence of oxygen, electron transfer to $MbFe^{III}$ from basal plane PG electrodes is very slow in the medium pH range, as reported previously.[30] In the presence of oxygen, a large chemically irreversible peak appears (Figure 2b). This wave has a large shoulder about 50 mV positive of the direct reduction wave of oxygen (Figure 2c), suggesting that metmyoglobin mediates the reduction of oxygen. The 25 electrons/mol of metmyoglobin found from coulometry after 15 min. and the linear charge vs time curves are consistent with such a catalytic electrochemical process.[31a]

Spectroelectrochemical experiments clearly demonstrate the formation of ferrylmyoglobins by appearance of new bands at 421, 548, and 584 nm during electrolysis of metmyoglobin in the presence of oxygen (Figures 4−6). Since $^{\bullet}X-MbFe^{IV}$=O decays rather rapidly ($t_{1/2} \sim 30$ s)[6−8,15] with respect to its rate of formation in this experiment, it is likely that the spectra represent mainly $MbFe^{IV}$=O. This is the same species formed by reaction of metmyoglobin with hydrogen peroxide, and confirmed as $MbFe^{IV}$=O by ESR, Raman, NMR, and Mossbauer spectroscopy.[11,12,15−18] If electrolysis was stopped after about 15 min, $MbFe^{IV}$=O was stable for an hour.

(34) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands;* North Holland: Amsterdam, 1971.

(35) (a) Nassar, A-E. F.; Bobbitt, J. M.; Stuart, J. D.; Rusling, J. F. *J. Am. Chem. Soc.* **1995**, *117*, 10986−10993. (b) A large surface area carbon felt cathode was used to electrolyze $Fe^{II}-O_2$ more rapidly than the decomposition of protein species.

The electrochemically generated species with bands at 421, 548, and 584 nm could be reduced with ascorbate to metmyoglobin and $MbFe^{II}-O_2$ (Figure 6). This provides additional evidence for an oxidized form of metmyoglobin, i.e. $MbFe^{IV}=O$. Reactions of $MbFe^{IV}=O$ and $MbFe^{III}$ with ascorbate involve:[4,9b]

$$MbFe^{IV}=O + AH^- + H^+ \rightarrow MbFe^{III} + A^{\bullet -} + H_2O \quad (3)$$

$$MbFe^{III} + 2O_2 + 4AH^- \rightarrow MbFe^{II}-O_2 + 4A^{\bullet -} + 2H_2O \quad (4)$$

where AH^- = ascorbate, and $A^{\bullet -}$ = ascorbyl radical, respectively.

Electrolysis in Microemulsions. Voltammetry showed that electron transfer to metmyoglobin is faster in the microemulsions than in pH 7.3 buffer. However, electron transfer is somewhat slower than the nearly reversible reaction obtained with Mb imbedded in insoluble surfactant films.[25,30] Nevertheless, similar factors involving protein−surfactant and electrode−surfactant interactions may be operative in the surfactant films and the DDAB microemulsion. In particular, adsorption of proteinaceous macromolecules onto the electrode from the Mb solution may be inhibited by adsorption of surfactant, thus facilitating electron transfer that would otherwise be blocked by the macromolecular adsorbates.[30b]

In the presence of oxygen, voltammetric (Figure 3) and coulometric results (linearity with time and the 19 electrons/mol of metmyoglobin in 18 min) were again consistent with reduction of oxygen by electrochemical catalysis.

Reaction of myoglobin with hydrogen peroxide in microemulsions gave absorbance bands at 421, 547, and 584 nm (Figure 8a). These bands are very similar to those of ferrylmyoglobins at pH 7.3. This finding, along with the observation of a spectrum similar to that of metmyoglobin after reducing this species with ascorbate, strongly suggests that $MbFe^{IV}=O$ was formed in the microemulsion.

Observation of bands at 421, 547, and 584 nm (Figure 8b) after electrolysis of metmyoglobin in oxygen-saturated microemulsions suggests electrochemical formation of ferrylmyoglobins. The species formed could also be reduced to metmyoglobin with ascorbate. A small amount of decomposition of the protein during oxidation−reduction is the probable cause of lower absorbances in the metmyoglobin spectra in Figures 8a.3 and 8b.3.

Electrolysis at pH 4.8. At pH 4.8 and the ionic strength used, a fraction of the $MbFe^{III}$ exists in a partly unfolded form resembling a "molten globule".[36] This form of the protein has 5 or 6 protonated histidines, which reside unprotonated in hydrophobic pockets in the native protein. It is likely that this "molten globule" form of the protein accepts electrons from the electrode more readily than the native form. This results in the observed voltammetric signal at pH 4.8 on PG electrodes, while no CV is observed at pH 7.3. This is consistent with a recent study of electron transfer to metmyoglobin in DDAB and phosphatidylcholine films,[37] which demonstrated partial unfolding of the protein at pH <5 correlated with an increase in protein electroactivity in the films.

Despite better electron transfer properties of the partly unfolded myoglobin, ferrylmyoglobin is unstable at this pH. Very little ferrylmyoglobin was observed at pH 4.8 after electrolysis in the presence of oxygen. The absorbance band at 585 nm (Figure 7) is consistent with the known irreversibly formed decomposition product of $(H^+)MbFe^{IV}=O$.[7] Voltammetry (Figure 1) and coulometry were again consistent with reduction of oxygen by electrochemical catalysis.

Pathway of Ferrylmyoglobin Formation. Previous ESR spectroscopy studies of myoglobin in sodium bromide solutions and DDAB films confirmed that the axial ligand of $MbFe^{III}$ is water.[37b] Thus, our experiments begin with metmyoglobin in solution. Electrochemical and spectroelectrochemical results suggest the formation of ferrylmyoglobin in neutral buffers and microemulsions by a multistep reduction−oxidation pathway. This involves electrochemical reduction of metmyoglobin, myoglobin-mediated formation of hydrogen peroxide, and chemical oxidation of metmyoglobin by hydrogen peroxide. Much more hydrogen peroxide was formed during electrolyses with myoglobin present than without myoglobin (Table 1), supporting a mediating role for the protein.

Scheme 1 is consistent with our results. In Scheme 1, an electron from the electrode first reduces metmyoglobin (eq 5) to $X-MbFe^{II}$, which reacts rapidly with oxygen to give the physiologically important $X-MbFe^{II}-O_2$ (eq 6). In neutral solutions, this reaction has a rate constant[38] of 2×10^7 M^{-1} s^{-1}. The autoxidation of $MbFe^{II}-O_2$ occurs with a rate constant[38] of 2×10^{-6} s^{-1}, and is too slow to be involved in the catalytic production of hydrogen peroxide.

Scheme 1

$$X\text{–}MbFe^{III} + e \longrightarrow X\text{–}MbFe^{II} \quad \text{(at electrode)} \quad (5)$$

$$X\text{–}MbFe^{II} + O_2 \longrightarrow X\text{–}MbFe^{II}\text{–}O_2 \quad (6)$$

$$X\text{–}MbFe^{II}\text{–}O_2 + 2e + 2H^+ \longrightarrow X\text{–}MbFe^{II} + H_2O_2 \quad \text{(at electrode)} \quad (7)$$

$$H_2O_2 + X\text{–}MbFe^{III} \longrightarrow {}^{\bullet}X\text{–}MbFe^{IV}{=}O + H_2O \quad (1)$$

$$2\,{}^{\bullet}X\text{–}MbFe^{IV}{=}O + H_2O_2 \longrightarrow 2HX\text{–}MbFe^{IV}{=}O + O_2 \quad (2)$$

Hydrogen peroxide is apparently produced by electrochemical reduction of $MbFe^{II}-O_2$ (eq 7).[39] A catalytic cycle involving eqs 6 and 7 is suggested by large cathodic currents in the presence of oxygen at the potentials of $MbFe^{III}$ reduction, by linear charge vs time curves and the large number of electrons/mole of metmyoglobin found during electrolysis, by detection of significant hydrogen peroxide in Mb solutions after electrolysis (Table 1), and by detection of hydrogen peroxide and ferrylmyoglobins from electrolytic reduction of $MbFe^{II}-O_2$.

Hydrogen peroxide, a major product of the electrochemical reduction, diffuses away from the electrode and reacts with $MbFe^{III}$ in solution to give $^{\bullet}X-MbFe^{IV}=O$. The rate constant for this well-known reaction at pH 7 and 25 °C was estimated[38] at 440 M^{-1} s^{-1}. Rapid decay of the radical species[3c,7a,8] with $t_{1/2} \sim 30$ s at room temperature, coupled with its relatively slow electrochemical formation, suggests that the observed spectral characteristics are due mainly to $MbFe^{IV}=O$. Hydrogen peroxide also reacts with $MbFe^{II}$ to yield ferrylmyoglobin,[3c,40] but this reaction with rate constant[38] 4×10^3 M^{-1} s^{-1} is too slow to complete with the very rapid oxygenation of $MbFe^{II}$ (eq 6).

(36) (a) Brunori, M.; Giacometti, G. M.; Antonini, E.; Wyman, J. *J. Mol. Biol.* **1972**, *63*, 139−152. (b) Goto, Y.; Fink, A. L. *J. Mol. Biol.* **1990**, *214*, 803−805. (c) Yang, A.-S.; Honig, B. *J. Mol. Biol.* **1994**, *237*, 602−614 and references therein.

(37) (a) Nassar, A-E. F.; Zhang, Z.; Hu, N.; Rusling, J. F.; Kumosinski, T. F. *J. Phys. Chem.* Submitted for publication. (b) Nassar, A-E. F.; Zhang, Z.; Chynwat, V.; Frank, H. A.; Rusling, J. F.; Suga, K. *J. Phys. Chem.* **1995**, *99*, 11013−11017.

(38) Wazawa, T.; Matsuoka, A.; Tajima, G.; Sugawara, Y.; Nakamura, K.; Shikama, K. *Biophys. J.* **1992**, *63*, 544−550 and references therein.

(39) (a) Reduction of oxygen may also be facilitated partly by mass transport with $MbFe^{III}$, as suggested previously.[39b] (b) King, B. C.; Hawkridge, F. M. *Talanta* **1989**, *36*, 331−334.

(40) Whitburn, K. D. *Arch. Biochem. Biophys.* **1987**, *253*, 419−430.

$MbFe^{IV}$═O was stable in neutral buffer and microemulsions for about an hour, consistent with its decomposition rate constant[38] of 0.001 s^{-1} at pH 7. $MbFe^{IV}$═O oxidized ascorbate, as demonstrated by detection of $MbFe^{III}$ (Figures 6 and 8). Ferrylmyoglobins formed *in situ* in an electrochemical cell oxidized styrene, as discussed in more detail below.

Scheme 1 explains how the strong *oxidant* ferrylmyoglobin radical can be formed efficiently during electrochemical *reduction* of metmyoglobin in the presence of oxygen. Non-protein oxo-ferrylporphyrins have been produced previously by electrochemical oxidation of Fe^{III}−OH porphyrin derivatives at high pH.[41] In the method described herein, ferrylmyoglobins are formed by a catalytic reduction−oxidation pathway under conditions where the protein retains a native or near-native structure.

The electrochemical method described gives high yields of $MbFe^{IV}$═O because of the heterogeneous catalytic reduction of oxygen (eqs 5−7). Rapid catalytic production of hydrogen peroxide at the electrode eventually results in oxidation of nearly all the myoglobin in the solution to ferrylmyoglobins. While metmyoglobin and perhaps ferrylmyoglobins are also reducible at the electrode, these reductions produce $MbFe^{II}$, which participates in the catalytic reduction of oxygen until nearly all the metmyoglobin has been oxidized to ferrylmyoglobin. After this time, the ferrylmyoglobin concentration decreases, most likely because it is reduced to another product and/or destroyed by excess hydrogen peroxide.[3]

Oxidation of Styrene. Detailed mechanistic studies of styrene oxidation by myoglobin−hydrogen peroxide showed that the reaction features two epoxidation mechanisms. One pathway involves the protein radical, and yields an epoxide with oxygen incorporated from molecular oxygen.[1,3a] The second involves oxidation by the oxo-ferryl group. The product incorporates an oxygen atom from hydrogen peroxide, but *this reaction also requires the protein radical*.[3c] Non-radical $MbFe^{IV}$═O does not oxidize styrene. Also, these reactions do not occur with hydrogen peroxide alone. Thus, the ability to oxidize styrene by electrochemical reduction of metmyoglobin in the presence of oxygen (Table 2) supports the formation of the radical $^{\bullet}X-MbFe^{IV}$═O in Scheme 1 (eq 1).

Electrochemically-driven myoglobin-mediated oxidation of styrene gave better yields of products than the analogous chemical oxidation in neutral buffer solutions (Table 2). Electrochemically-driven oxidation in the microemulsion gave dramatically better yields than in the buffer. This is probably because of the much better solubility of reactants styrene and oxygen in the microemulsion than in water.

Conclusions

Our results demonstrate that ferrylmyoglobin species can be produced in a complex reduction−oxidation pathway driven by the electrochemical reduction of metmyoglobin in the presence of oxygen in aqueous neutral buffer or microemulsions. Myoglobin mediates the reduction of oxygen to hydrogen peroxide (Scheme 1), which eventually oxidizes nearly all of the myoglobin in solution to ferrylmyoglobin. Good yields of ferrylmyoglobin are obtained by controlled continuous production of hydrogen peroxide at the cathode, which may provide advantages in protein stability over the addition of hydrogen peroxide in a single batch.

Oxidations with ferrylmyoglobins were demonstrated. Microemulsions were shown to be useful for reactions of a water-soluble protein with an oil-soluble suspected carcinogen, styrene. Product yields in the microemulsion were 50-fold greater than in neutral buffer with use of either the electrochemical method or chemical oxidation. The method described herein may also prove useful for the study of catalytically active higher oxidation states of other heme enzymes, such as cytochrome P450.

Acknowledgment. This work was supported by Grant No. ES03154 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Contents are solely the responsibility of the authors and do not necessarily represent official views of NIEHS, NIH. The authors thank the University of Connecticut staff Al Brown, Joe Csiki, and Walter Dann of the Technical Services Department for construction of cells and electrodes and Dennis Shannon of the Department of Environmental Health and Safety for the gift of Quantofix Peroxide test sticks.

JA964007L

(41) (a) Calderwood, T. S.; Lee, W. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 8272−8273. (b) Calderwood, T. S.; Bruice, T. C. *Inorg. Chem.* **1986**, *25*, 3722−3724.