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Kinetics of Reduction of Metmyoglobins by Ascorbate. Effect of the Modification of the Heme Distal Side, Heme Propionates, and 2,4-Substituents of Deuterohemin

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Rate constants are reported for the reduction by ascorbate of BrCN-modified metmyoglobin and metmyoglobin reconstituted with 2,4-disubstituted deuterohemin and with protohemin dimethyl ester. Modification of the heme distal histidylimidazole by cyanogen bromide and of the heme propionates to their methyl esters accelerated the reduction rate compared with that for its native form, suggesting that the structural change in the iron site from hexa- to pentacoordination is an important factor. The logarithm of the second-order rate constants for the reduction of the metmyoglobin reconstituted with a 2,4-disubstituted deuterohemin (-CHO, $-COC_2H_5$, $-COCH_3$, $-CH{=}CH_2$, and -H) is linearly correlated with the pK_3 of the acid-dissociation constants of the porphyrin monocation. This suggests that the electronic factor is predominant for these substituents. Mesohemin reconstituted myoglobin is an exception, perhaps for steric reasons.

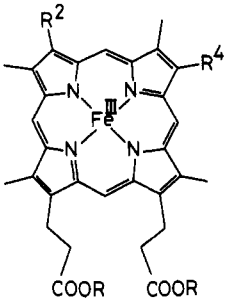
Introduction

Reduction kinetics of metmyoglobins (metMb) have been studied extensively by using inorganic reagents[1] and a few organic reductants.[2-4] The native metMb has Fe(III) protoporphyrin IX, $Fe^{III}PP$, as a prosthetic group. Reduction kinetics of cobalt(III) and manganese(III) myoglobins by dithionite ions have also been investigated.[5]

The X-ray crystallographic study of sperm whale metMb has shown that the distal histidylimidazole makes a hydrogen bond to the coordinated water and that the heme propionates make hydrogen bonds to Arg-45 and His-97, thereby stabilizing the structure of the heme pocket.[6] We have previously compared the reactivity of the reduction by ascorbate of sperm whale skeletal muscle and horse heart metMb,[4] the latter being more reactive. We suggested that the hydrogen bonds of Arg-45 to the heme propionate and to Asp-60 are important to their reactivity differences. The Arg residue has mutated to Lys in horse heart metMb.

The reduction of metMb(H_2O) to deoxymyoglobin (deoxyMb) must be accompanied by a structural change in the iron site. The hexacoordinated metMb(H_2O) becomes a pentacoordinated deoxyMb.[7] Recent investigations into the redox reactions of hemoproteins are concerned with the spin change,[1d,8] the geometry change[9] in the heme iron site, and the extent of exposure of the heme.[10]

Chart I



	R^2, R^4	R
PP	$CH{=}CH_2$	H
PPDME	$CH{=}CH_2$	CH_3
DP	H	H
MP	C_2H_5	H
DADP	$COCH_3$	H
DPDP	COC_2H_5	H
DFDP	CHO	H

Reconstitution of a 2,4-disubstituted deuterohemin with apomyoglobin has been widely used to investigate the oxygen affinity of deoxymyoglobin.[11] Therefore, we decided to study the kinetics of the reduction by ascorbate (HA^- and A^{2-}) of the following three kinds of sperm whale metmyoglobins. The first is a derivative in which the distal histidylimidazole has been modified by cyanogen bromide (BrCN). In this form the distal histidyl residue cannot make a hydrogen bond to the coordinated water, and the sixth coordination site of iron is vacant.[12] The second is a derivative in which the heme propionates are replaced by their methyl esters. Hydrogen bonds to Arg-45 and His-97 are not possible. The third is myoglobin reconstituted with hemin in which the vinyl groups at the 2- and 4-positions are replaced by formyl, propionyl, acetyl, ethyl, or hydrogen. Electronic and steric effects are ex-

(1) (a) Lambeth, D. O.; Palmer, G. *J. Biol. Chem.* **1973**, *248*, 6095. (b) Cassat, J. C.; Marini, C. P.; Bender, J. W. *Biochemistry* **1975**, *14*, 5470. (c) Huth, S. W.; Kimberly, K. E.; Piszkiewicz, D.; Fleischer, E. B. *J. Am. Chem. Soc.* **1976**, *98*, 8467. (d) Cox, R. P.; Hollaway, M. R. *Eur. J. Biochem.* **1977**, *74*, 575. (e) Olivas, E.; deWaal, D. J. A.; Wilkins, R. G. *J. Biol. Chem.* **1977**, *252*, 4038. (f) Eaton, D. R.; Wilkins, R. G. *J. Biol. Chem.* **1978**, *253*, 908. (g) Itzkowitz, M.; Haim, A. *Bioinorg. Chem.* **1978**, *9*, 323. (h) Augustin, M. A.; Yandell, J. K. *Inorg. Chim. Acta* **1979**, *37*, 11. (i) Balahura, R. J.; Wilkins, R. G. *Biochim. Biophys. Acta* **1983**, *724*, 465.

(2) (a) Cox, R. P. *Biochem. J.* **1977**, *167*, 493. (b) Simic, M. G.; Taub, I. A. *Biophys. J.* **1978**, *24*, 285. (c) Van Leeuwen, J. W.; Van Dijk, C.; Grande, H. J.; Veeger, C. *Eur. J. Biochem.* **1982**, *127*, 631.

(3) Bradic, Z.; Tsukahara, K.; Wilkins, P. C.; Wilkins, R. G. In *Frontiers in Bioinorganic Chemistry*; Xavier, A. V., Ed.; VCH Publishers: Weinheim, FRG, 1986; p 336.

(4) (a) Tsukahara, K.; Yamamoto, Y. *J. Biochem.* **1983**, *93*, 15. (b) Tsukahara, K. *Inorg. Chim. Acta* **1986**, *124*, 199.

(5) (a) Hambright, P.; LeMelle, S.; Alston, K.; Neta, P.; Newball, H.; DiStefano, S. *Inorg. Chim. Acta* **1984**, *92*, 167. (b) Langley, R.; Hambright, P.; Alston, K.; Neta, P. *Inorg. Chem.* **1986**, *25*, 114.

(6) (a) Kendrew, J. C.; Dickerson, R. E.; Strandberg, B. E.; Hart, R. G.; Davies, D. R.; Phillips, D. C.; Shore, V. C. *Nature (London)* **1960**, *185*, 422. (b) Takano, T. *J. Mol. Biol.* **1977**, *110*, 537.

(7) (a) Nobbs, C. L.; Watson, H. C.; Kendrew, J. C. *Nature (London)* **1966**, *209*, 339. (b) Takano, T. *J. Mol. Biol.* **1977**, *110*, 569.

(8) (a) Kadish, K. M.; Su, C. H. *J. Am. Chem. Soc.* **1983**, *105*, 177 and references therein. (b) Dose, E. V.; Tweedle, M. F.; Wilson, L. J.; Sutin, N. *J. Am. Chem. Soc.* **1977**, *99*, 3886. (c) Fischer, M. T.; Sligar, S. G. *J. Am. Chem. Soc.* **1985**, *107*, 5018.

(9) (a) Mauk, A. G.; Gray, H. B. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 206. (b) Crutchley, R. J.; Ellis, W. R., Jr.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002. (c) Dixon, D. W.; Barbush, M.; Shirazi, A. *J. Am. Chem. Soc.* **1984**, *106*, 4638. (d) Dixon, D. W.; Barbush, M.; Shirazi, A. *Inorg. Chem.* **1985**, *24*, 1081. (e) Shirazi, A.; Barbush, M.; Ghosh, S.; Dixon, D. W. *Inorg. Chem.* **1985**, *24*, 2495.

(10) Meyer, T. E.; Kamen, M. D. *Adv. Protein Chem.* **1982**, *35*, 105.

(11) (a) Kawabe, K.; Imaizumi, K.; Imai, K.; Tyuma, I.; Ogoshi, H.; Iwahara, T.; Yoshida, Z. *J. Biochem.* **1982**, *92*, 1703. (b) Chang, C. K.; Ward, B.; Ebina, S. *Arch. Biochem. Biophys.* **1984**, *231*, 366 and references therein.

(12) (a) Shiro, Y.; Morishima, I. *Biochemistry* **1984**, *23*, 4879. (b) Morishima, I.; Shiro, Y.; Wakino, T. *J. Am. Chem. Soc.* **1985**, *107*, 1063 and references therein.

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pected. We have also studied the reduction kinetics by ascorbate of (protoporphyrin IX)-, (deuteroporphyrin IX)-, and (mesoporphyrin IX)bis(pyridine)iron(III) complexes in aqueous Triton X-100, where no steric effect of the 2,4-substituents is expected. A preliminary study of one such reaction has been published.[13] Abbreviations of the porphyrins are shown in Chart I.

Experimental Section

Reagents. Meso- and deuteroporphyrin IX were prepared from protohemin (Wako Pure Chemical Industries, Ltd.) as previously described[14,15] and purified by chromatography on an alumina column (grade IV) after they were converted to the dimethyl esters.[14] Meso- and deuterohemin were prepared by incorporation of iron into the porphyrins after the hydrolysis of the esters by 6 M HCl. Diacetyl-,[16] dipropionyl-,[16] and diformyldeuterohemin[17] were prepared by a previously reported method. These hemins were purified by partition chromatography on a Celite column.[18] The purity of the hemins and porphyrins thus obtained was checked spectrophotometrically.

Sperm whale skeletal muscle myoglobin (type II, Sigma) was purified as previously described.[4] Apomyoglobin (apoMb) was prepared from metMb by the acid–butanone method of Teale[19] and purified as described by Caughey, et al.[18] Recombination of hemin[20] and iron(III) protoporphyrin IX dimethyl esters, Fe^{III}PPDME,[21] with apoMb was carried out by using a slightly modified method that has already been published. The mixture of hemin or Fe^{III}PPDME with apoMb was kept in a refrigerator at 4 °C for at least 24 h and was subjected to a dialysis and to CM-52 cellulose column chromatography. The concentrations of the reconstituted metMb were determined spectrophotometrically.[21,22] The yield of metMb reconstituted with Fe^{III}PPDME was about 15% and that of other reconstituted metMb was 40–50%. BrCN-modified metMb was prepared in situ by the method previously described.[12]

Sodium L-ascorbate was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. All the solutions used for measurements were prepared from redistilled water. A solution of sodium ascorbate was freshly prepared in an argon atmosphere. Stock solutions of hemin in Triton X-100 were prepared by dissolving hemin in 8% w/w of Triton X-100 containing a small amount of 0.1 M NaOH and by neutralizing with 0.1 M HCl. The solution was filtered through a 0.45 μm membrane. Fe(edta)$^{2-}$ was prepared in situ as follows: $Fe(ClO_4)_2 \cdot 6H_2O$, which was standardized spectrophotometrically as the 1,10-phenanthroline complex (ϵ_{511} = 11 100 M^{-1} cm^{-1}), Na_2H_2edta, and buffer were purged with argon gas. Na_2H_2edta (in 20% excess) was added to the $Fe(ClO_4)_2 \cdot 6H_2O$. Buffer was then added to the solution of Fe(edta)$^{2-}$. All other chemicals used were of guaranteed grade.

Equilibrium Measurements. Acid dissociation constants of the heme-linked water molecule of metMb ((1.5–2.0) × 10^{-5} M) were determined spectrophotometrically at 25 °C, at an ionic strength (I) of 0.30 M (NaCl), and at pH 6.0–10.1 (a 0.10 M sodium phosphate buffer for below pH 7 and a 0.20 M Tris–HCl buffer for others; Tris = tris(hydroxymethyl)aminomethane). Wavelengths used for measurements were 390–400, 410–430, 440–460, 550–560, and 600 nm.

The formation constants of the bis(pyridine) adducts of hemin were determined spectrophotometrically (370–420 nm) at 25 °C, I = 0.50 M (NaCl), pH 7.34 and 7.85 (a 0.10 M Tris–HCl buffer), [hemin] = 5.0 × 10^{-6} M, and [pyridine] = (1.0–8.0) × 10^{-2} M in aqueous 2% Triton X-100.

Kinetic Measurements. All the reactions were carried out in an argon atmosphere. The reactions were initiated by injecting the solution of sodium ascorbate into the solution containing metMb, NaCl, and a Tris–HCl buffer over a pH range from 7.19 to 8.62. The change in absorbance with time was followed with either a Hitachi 200-20 or a Shimadzu UV 140-02 spectrophotometer. The wavelengths used for the measurements were 556 (reconstituted PPmetMb), 542 (DPmetMb), 541 (MPmetMb), 454 (DFDPmetMb), 447 (DADPmetMb), 432 (DPDPmetMb), and 423 nm (PPDMEmetMb). In order to ensure pseudo-first-order conditions, sodium ascorbate was used in excess over metMb. The initial concentrations of sodium ascorbate and metMb were (0.010–1.00) × 10^{-1} M and (0.12–1.00) × 10^{-4} M, respectively.

The temperature was controlled at 25.0 ± 0.1 °C and the ionic strength was adjusted to 0.30 M with NaCl.

The pH of the solution was measured on a Hitachi-Horiba F-7 pH meter. The concentration of hydrogen ion was calculated from the pH by using the activity coefficient of H^+, which was evaluated by means of the Davies equation.[23]

The kinetics for the reductions by ascorbate of hemin–bis(pyridine) adducts in 2% Triton X-100 were carried out at 25 °C, I = 0.50 M (NaCl), pH 7.34 and 7.85 (a 0.10 M Tris–HCl buffer), $[hemin]_0$ = 5.0 × 10^{-6} M, $[py]_0$ = (1.0–8.0) × 10^{-2} M, and $[HA^-]_0$ = (0.70–2.98) × 10^{-3} M in an argon atmosphere. The reactions were followed at 422, 410, and 407 nm for the (PP)-, (MP)-, and (DP)Fe^{III} systems, respectively.

The reduction by Fe(edta)$^{2-}$ was followed at 556 nm with a Union Giken RA-400 stopped-flow spectrophotometer. Fe(edta)$^{2-}$ ((0.5–1.0) × 10^{-2} M) was used in excess over metMb (5.0 × 10^{-5} M) at pH 7 (a 0.1 M phosphate buffer) and I = 0.2 M.

Table I. pK Values for the Reconstituted Sperm Whale metMb(H_2O) and pK_3 Values for the 2,4-Substituted Deuteroporphyrin IX Dimethyl Esters at 25 °C

porphyrin	pK	pK_3[a]
MP	9.40 ± 0.10,[b] 9.3–9.4[c]	5.85
DP	9.25 ± 0.10,[b] 9.10 ± 0.05[d]	5.5
PP(native)	9.16 ± 0.03,[e] 9.04,[f] 8.95 ± 0.05[c]	4.80
PPDME	7.90 ± 0.15,[b] 7.5[g]	4.80
DPDP	7.87 ± 0.05[b]	3.2
DADP	7.80 ± 0.10,[b] 7.60 ± 0.10[d]	3.35
DFDP	7.31 ± 0.05[b]	3.0

[a] For the equilibrium $H_3P^+ \rightleftharpoons H_2P + H^+$ in 2.5% sodium dodecyl sulfate.[24] [b] At I = 0.30 M. This work. [c] At 20 °C and I = 0.2 M: Brunori, M.; Amiconi, G.; Antonini, E.; Wyman, J.; Zito, R.; Rossi-Fanelli, A. *Biochim. Biophys. Acta* **1968**, *154*, 315. [d] McGrath, T. M.; La Mar, G. N. *Biochim. Biophys. Acta* **1978**, *534*, 99. [e] At I = 0.30 M.[4] [f] At I = 0.20 M: George, P.; Hanania, G. I. H.; Irvine, D. H.; Abu-Issa, I. *J. Chem. Soc.* **1964**, 5689. [g] For horse heart metMb at 23 °C and I = 0.01 M: Tamura, M.; Woodrow, G. V., III; Yonetani, T. *Biochim. Biophys. Acta* **1973**, *317*, 34.

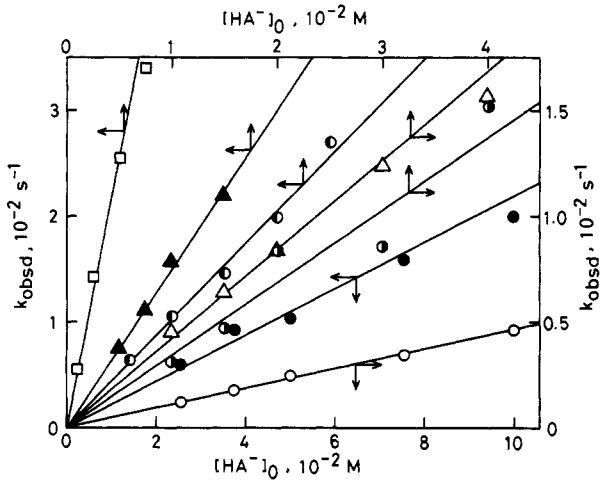

Figure 1. Plot of k_{obsd} vs. $[HA^-]_0$ for the reduction by ascorbate of the reconstituted metMb and BrCN-modified metMb at 25 °C and I = 0.30 M (NaCl): (○) DPmetMb at pH 8.36; (●) MPmetMb at pH 8.62; (◑) DADPmetMb at pH 7.19; (△) DPDPmetMb at pH 7.19; (◐) DFDPmetMb at pH 7.64; (▲) PPDMEmetMb at pH 7.19; (□) BrCN-modified metMb at pH 7.19.

Results

The pK values for the acid dissociation equilibrium

$$metMb(H_2O) \overset{K}{\rightleftharpoons} metMb(OH) + H^+ \qquad (1)$$

(13) Tsukahara, K.; Okazawa, T.; Yamamoto, Y. *Chem. Lett.* **1986**, 1247.
(14) Yonetani, T.; Asakura, T. *J. Biol. Chem.* **1968**, *243*, 4715.
(15) Chu, T. C.; Chu, E. J.-H. *J. Am. Chem. Soc.* **1952**, *74*, 6776.
(16) Caughey, W. S.; Alben, J. O.; Fujimoto, W. Y.; York, J. L. *J. Org. Chem.* **1966**, *31*, 2631.
(17) Sono, M.; Asakura, T. *Biochemistry* **1974**, *13*, 4386.
(18) Caughey, W. S.; Fujimoto, W. Y.; Bearden, A. J.; Moss, T. H. *Biochemistry* **1966**, *5*, 1255.
(19) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543.
(20) Ogoshi, H.; Kawabe, K.; Mitachi, S.; Yoshida, Z.; Imai, K.; Tyuma, I. *Biochim. Biophys. Acta* **1979**, *581*, 266.
(21) Tamura, M.; Asakura, T.; Yonetani, T. *Biochim. Biophys. Acta* **1973**, *295*, 467.
(22) Makino, R.; Yamazaki, I. *Arch. Biochem. Biophys.* **1974**, *165*, 485.
(23) Davies, C. W. *J. Chem. Soc.* **1938**, 2093.
(24) Hambright, P. In *Porphyrins and Metalloporphyrins*; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; p 233.

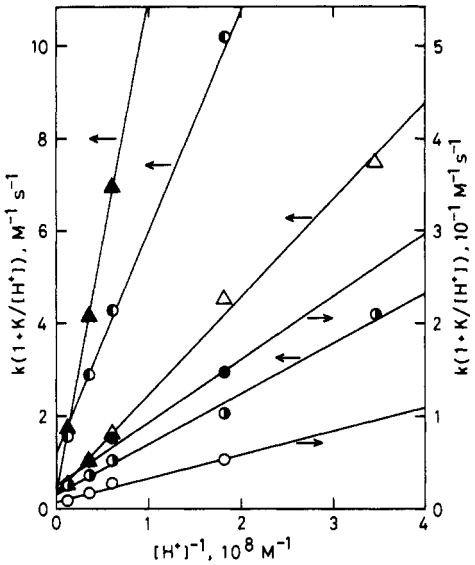

Figure 2. Plot of $k(1 + K/[H^+])$ vs. $[H^+]^{-1}$ for the reduction of the reconstituted metMb by ascorbate at 25 °C and $I = 0.30$ M (NaCl): (○) DPmetMb; (●) MPmetMb; (◑) DADPmetMb; (△) DPDPmetMb; (◐) DFDPmetMb; (▲) PPDMEmetMb.

are listed in Table I along with those reported in the literature.

Reduction of Reconstituted Metmyoglobin by Ascorbate. The absorption maxima of the reaction products were 544, 542, 560, 563, 575, and 556 nm for MP-, DP-, DPDP-, DADP-, DFDP-, and PPDME derivatives, respectively, and are characteristic of deoxyMb:

$$2\text{metMb} + \text{ascorbate} \rightarrow 2\text{deoxyMb} + \text{dehydroascorbic acid} \quad (2)$$

Five isosbestic points were observed in the 350–700-nm region for each system. The plots of $-\ln (A_\infty - A_t)$ vs. time were linear for at least 80% completion, where A_∞ and A_t represent the absorbance at infinity and time t, respectively. The observed first-order rate constants (k_{obsd}) obtained from the slopes of the straight lines showed a linear dependence on the initial concentrations of ascorbate ($[HA^-]_0$) as shown in Figure 1. Thus, the rate law is described as

$$-d[\text{metMb}]/dt = k[\text{metMb}][HA^-]_0 \quad (3)$$

The second-order rate constant, k, increased as the pH increased (pH 7.19–8.62). The pH dependence of k can be described by the following equations in addition to eq 1:

$$HA^- \rightleftharpoons A^{2-} + H^+ \quad (4)$$

$$\text{metMb}(H_2O) + HA^- \xrightarrow{k_1} \text{deoxyMb} + \text{radical} \quad (5)$$

$$\text{metMb}(H_2O) + A^{2-} \xrightarrow{k_2} \text{deoxyMb} + \text{radical} \quad (6)$$

$$2\,\text{radical} \xrightarrow{\text{fast}} H_nA^{n-2} + \text{dehydroascorbic acid} \quad (7)$$

metMb(OH) is much less reactive than metMb(H_2O).[3,4] The above mechanism produces eq 8 for the second-order rate constant.

$$k = \frac{k_1 + k_2K_a/[H^+]}{1 + K/[H^+]} \quad (8)$$

Plots of $k(1 + K/[H^+])$ vs. $[H^+]^{-1}$ gave straight lines (Figure 2). The second-order rate constants k_1 and k_2 were obtained from the intercept and the slope of these straight lines, respectively. The pK_a value of 11.11 ± 0.03 was used for later calculations.[4] All the results are tabulated in Table II.

Reduction of BrCN-Modified Metmyoglobin by Ascorbate. When the native metMb (1.0×10^{-4} M) was treated with a one- to twofold excess of BrCN at 25 °C, $I = 0.30$ M (NaCl), and pH 7.19–7.87 (a 0.20 M Tris–HCl buffer), the absorbance at 504 nm increased, reached a maximum in about 15 min (with isosbestic points at 573, 652, and 695 nm), and then gradually decreased.[13] Therefore, for kinetics measurements, BrCN was added to the native metMb, the solution was purged with argon for 15 min, and then the solution of ascorbate was injected. The reduction by ascorbate was monitored at 556 nm. The reaction went to completion within 5 min. The BrCN-modified metMb was sufficiently stable for the reduction by ascorbate to go to completion. The plots of $-\ln (A_\infty - A_t)$ vs. t were linear for at least 80% completion. The observed first-order rate constants were linearly dependent on $[HA^-]_0$. The second-order rate constants decreased with an increase in acidity. It has been suggested from NMR studies that the BrCN-modified metMb has no coordinated water on the heme distal side.[12] We have confirmed this by noting that the spectra of the BrCN-modified metMb does not change over the pH range from 6 to 8. Therefore, the second-order rate constants k_1 and k_2 were obtained from the slope and the intercept of the plots of k vs. $[H^+]^{-1}$, respectively:

$$k = k_1 + k_2K_a/[H^+]^{-1} \quad (9)$$

The results are listed in Table II.

When air was introduced into the product solution (BrCN-modified deoxyMb, λ_{max} = 559 nm), the α- and β-bands (λ_{max} = 575 nm and λ_{max} = 540 nm) characteristic of oxymyoglobin (MbO_2) were detected. The complex was stable for at least a few hours. It has been reported that the BrCN-modified MbO_2 is unstable.[12] We suggest that the reaction involves the autoxidation of BrCN-modified MbO_2 to metMb, which is then converted to MbO_2.

Reductions of (PP)-, (DP)-, and (MP)Fe^{III}–Bis(pyridine) Adducts by Ascorbate in Aqueous Triton X-100. The formation constants for the proto-, deutero-, and mesohemin–bis(pyridine) adducts (eq 10) were obtained by using eq 11, where A, A_0, and

$$Fe^{III}P + 2py \rightleftharpoons^{K^{py}} Fe^{III}P(py)_2 \quad (10)$$

$$1/|A - A_0| = 1/|A_\infty - A_0| + 1/|A_\infty - A_0|K^{py}[py]_T^2 \quad (11)$$

A_∞ represent the observed absorbance, the absorbance in the absence of pyridine, and the absorbance of $Fe^{III}P(py)_2$, respectively.[25] The obtained K^{py} values are listed in Table III.

Table II. Rate Constants for Reduction by Ascorbate of the Reconstituted metMb(H_2O) and of the BrCN-Modified metMb at 25 °C, $I = 0.30$ M (NaCl), and pH 7.19–8.62

metMb(H_2O)	k_1,[a] M^{-1} s^{-1}	k_2,[b] M^{-1} s^{-1}
MP	$(2.6 \pm 0.6) \times 10^{-2}$	$(9.5 \pm 1.0) \times 10$
DP	$(7.9 \pm 2.8) \times 10^{-3}$	$(3.5 \pm 0.5) \times 10$
PP(native)[c]	$(1.2 \pm 0.2) \times 10^{-2}$	$(6.9 \pm 0.8) \times 10$
PP(reconst)	$(1.2 \pm 0.2) \times 10^{-2}$	$(5.6 \pm 1.0) \times 10$
DADP	$(3.1 \pm 1.3) \times 10^{-1}$	$(1.5 \pm 0.2) \times 10^3$
DPDP	$(3.3 \pm 2.0) \times 10^{-1}$	$(2.5 \pm 0.5) \times 10^3$
DFDP	1.2 ± 0.2	$(5.9 \pm 1.1) \times 10^3$
PPDME	$(3.0 \pm 0.5) \times 10^{-1}$	$(1.4 \pm 0.1) \times 10^4$
BrCN-modified PP[d]	3.4 ± 0.2	$(2.1 \pm 0.1) \times 10^4$

[a] For the reduction by HA^- ions. [b] For the reduction by A^{2-} ions. [c] Reference 4. [d] See text. pH 7.19–7.87.

Table III. Rate Constants for Reduction by Ascorbate of Hemin in the Presence of Pyridine in Aqueous 2% Triton X-100 at 25 °C and $I = 0.50$ M (NaCl)

hemin	pH[a]	k_0, M^{-1} s^{-1}	k^{py}, M^{-1} s^{-1}	K^{py}, M^{-2}
PP	7.34	3.0 ± 0.9	30 ± 10	140 ± 30
	7.85	2.6 ± 0.6	32 ± 5	83 ± 10
DP	7.34	5.4 ± 1.8	26 ± 6	260 ± 40
	7.85	2.1 ± 0.8	29 ± 11	110 ± 20
MP	7.34	5.0 ± 0.5	13 ± 2	260 ± 40
	7.85	3.4 ± 1.0	17 ± 4	170 ± 40

[a] In a 0.10 M Tris–HCl buffer.

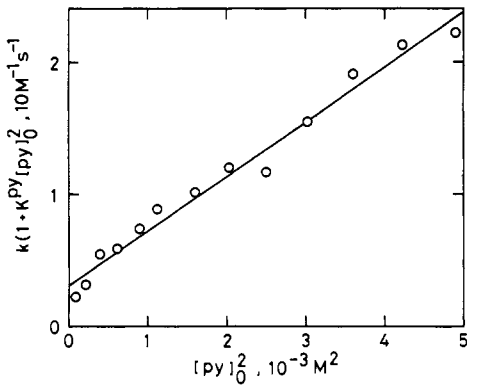

Figure 3. Plot of $k(1 + K^{py}[py]_0^2)$ vs. $[py]_0^2$ for the reduction of $Fe^{III}PP$ by ascorbate in the presence of pyridine in aqueous 2% Triton X-100 at 25 °C, I = 0.50 M (NaCl), and pH 7.34 (0.10 M Tris–HCl buffer).

The first-order plots were linear only for 70% completion, probably due to trace oxygen. Therefore, the rate constants were determined from the initial straight part. The observed first-order rate constants were linearly dependent on $[HA^-]_0$. The second-order rate constants increased with an increase in $[py]_0$. The rate constants for

$$Fe^{III}P + \text{ascorbate} \xrightarrow{k_0} \text{products} \quad (12)$$

$$Fe^{III}P(py)_2 + \text{ascorbate} \xrightarrow{k^{py}} \text{products} \quad (13)$$

were obtained from the intercept and the slope of the plots of $k(1 + K^{py}[py]_0^2)$ vs. $[py]_0^2$ (Figure 3) where

$$k = \frac{k_0 + k^{py}K^{py}[py]_0^2}{1 + K^{py}[py]^2} \quad (14)$$

The results are listed in Table III, where k_0 and k^{py} are for the reductions by HA^- and/or A^{2-} ions. The individual rate constants for HA^- and A^{2-} ions were not determined.

Reduction of BrCN-Modified Metmyoglobin by Fe(edta)$^{2-}$. The first-order plots were linear for at least 90% completion. The second-order rate constants (M^{-1} s^{-1}) were obtained at various temperatures: 2.7×10^3 (15.0 °C), 2.7×10^3 (20.0 °C), 2.9×10^3 (25.0 °C), and 3.4×10^3 (30.0 °C). The activation enthalpy and entropy changes were $\Delta H^* = 2.0 \pm 0.5$ kcal mol^{-1} and $\Delta S^* = -35 \pm 4$ cal K^{-1} mol^{-1}. A value of 28 M^{-1} s^{-1} at 25 °C was obtained as the second-order rate constant for the native metMb, which is in good agreement with that reported in the literature.[1b] When the concentration of BrCN was less than that of metMb, two reactions were observed—faster for BrCN-modified metMb and slower for the native metMb.

Discussion

It is known that the recombination of hemin and apoMb produces two protein forms, where the hemin is 180° rotationally disordered about its α-γ-meso axis in the heme pocket and that reorientation occurs by an intramolecular mechanism.[28] La Mar et al.[28] pointed out that the reequilibrated sample from a 55:45 disordered mixture contains 10% of the reversed heme and has the same oxygenation data as the native form. Although we did not check our samples on this point, the samples might be mainly in normal orientation, because the reaction mixture of hemin with apomyoglobin was kept in a refrigerator for at least 24 h and the main fraction collected by column chromatography was stored at 4 °C.

Cyanogen bromide is a useful reagent for the modification of the distal histidylimidazole as recently confirmed in NMR studies by Morishima et al.[12] They have suggested and we have confirmed that the sixth coordination site of the iron is vacant in this derivative. The reduction of metMb(H_2O) to deoxyMb must be accompanied by a structural change in the iron site (hexa- to pentacoordination). The BrCN-modified metMb is reduced 100–300 times faster than its native form. In order to evaluate the activation parameters we used $Fe(edta)^{2-}$, which is a simpler reductant than ascorbate and has been used in the reduction of the native metMb.[1b] In the $Fe(edta)^{2-}$ reductions, ΔH^* is very small for BrCN-modified metMb (2.0 ± 0.5 kcal mol^{-1}) compared with that for the native metMb (12 ± 1 kcal mol^{-1}).[1b] This strongly suggests that the geometry of the pentacoordinated BrCN-modified metMb is easily changed to the geometry of the pentacoordinated deoxymyoglobin. Importance of the geometry change in the iron site has been recently pointed out.[9] Intramolecular electron-transfer studies of ruthenium-modified metMb[9b] and cytochrome c[29] show that the activation enthalpy change for the former (ΔH^* = 7.4 ± 0.5 kcal mol^{-1}) is larger than that for the latter (ΔH^* = 3.5 ± 0.2 kcal mol^{-1}). Gray and co-workers[9b,30] estimated the enthalpic reorganizational barrier for the heme in myoglobin to be 20 kcal mol^{-1} in contrast to the lower value (7–8 kcal mol^{-1}) for that in cytochrome c. They suggested that the geometry change of the iron site in metMb accounts for the much larger reorganizational barrier. The axial ligands (His and Met) in cytochrome c are retained upon reduction of the iron center. It has been reported that the activation enthalpy change for the reduction of cytochrome c by $Fe(edta)^{2-}$ is 6.0 ± 0.3 kcal mol^{-1}.[31] This is lower than that for the reductions of the native metMb.

The spin multiplicity change upon reduction of the heme has been also an important factor.[1d,2c,8] The results of the reductions of metMb and cytochrome c are in good accord with the general observation that rate constants decrease in the order low spin/low spin > high spin/high spin >> high spin/low spin.[8a,32] A coordination number change of six to five upon low-spin to high-spin conversion is not a necessary requirement in myoglobin, as previously pointed out.[8b,9d] The study[4a] of the ascorbate reductions of metMbX derivatives where X = imidazole (Im), N-methylimidazole, H_2O, N_3^-, and CN^- has shown that the spin state is relatively unimportant, arising perhaps from the very rapid spin-exchange process.[8b] In the reduction of hemins by ascorbate in the presence of pyridine in aqueous Triton X-100 solutions, k_0 and k^{py} values differ by only 3–10 times. This is in accord with the above results on the metMb derivatives.

The self-exchange rate constant of the metMb/deoxyMb system has been estimated to be ~1 M^{-1} s^{-1}, which is much smaller than that of the metMbIm/deoxyMbIm system (3×10^4 M^{-1} s^{-1}).[3] The latter system does not require a change in the coordination number.

PPDMEmetMb(H_2O) is also reduced much faster than the native metMb(H_2O). Fe^{III}PPDME in the reconstituted metMb cannot make hydrogen bonds to Arg-45 and His-97, unlike its native form.[6] Therefore, we suggest that the heme pocket is expanded and that the hydrogen bond of the distal histidine with the coordinated water is weakened when compared with native metMb(H_2O). In fact, the pK of the coordinated water of PPDMEmetMb decreases to 7.90 from 9.16 in the native form. Weakening of the hydrogen bond may distort the iron site in PPDMEmetMb(H_2O), which would allow facile rearrangement to the pentacoordinated deoxyMb. The coordination nature of

(25) It is known that the second pyridine-binding constant for $Fe^{III}P(py)$ is much larger than the first pyridine-binding constant for $Fe^{III}P$.[24] $Fe^{III}P$ denotes the mixture of $Fe^{III}P(H_2O)_2$, $Fe^{III}(OH)(H_2O)$, and/or $Fe^{III}(OH)_2$ species. The acid dissociation constants of $Fe^{III}P(H_2O)_2$ in aqueous Triton X-100 are reported to be 4.7, 4.7, and 5.0 for $Fe^{III}PP$,[26] $Fe^{III}DP$, and $Fe^{III}MPDME$,[27] respectively. Therefore, the K^{py} consists of $[Fe^{III}P(py)_2]/[Fe^{III}P]_T[py]^2$, where $[Fe^{III}P]_T$ is the total concentration of hemin.

(26) Simplicio, J.; Schwenzer, K. *Biochemistry* **1973**, *12*, 1923.

(27) Reid, J.; Hambright, P. *Inorg. Chem.* **1978**, *17*, 2329.

(28) La Mar, G. N.; Toi, H.; Krishnamoorthi, R. *J. Am. Chem. Soc.* **1984**, *106*, 6395 and references therein.

(29) Isied, S. S.; Kuehn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722.

(30) Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145.

(31) Hodges, H. L.; Holwerda, R. A.; Gray, H. B. *J. Am. Chem. Soc.* **1974**, *96*, 3132.

(32) (a) Scheidt, W. R.; Geiger, D. K.; Haller, K. J. *J. Am. Chem. Soc.* **1982**, *104*, 495. (b) Scheidt, W. R.; Geiger, D. K.; Hayes, R. G.; Lang, G. *J. Am. Chem. Soc.* **1983**, *105*, 2625.

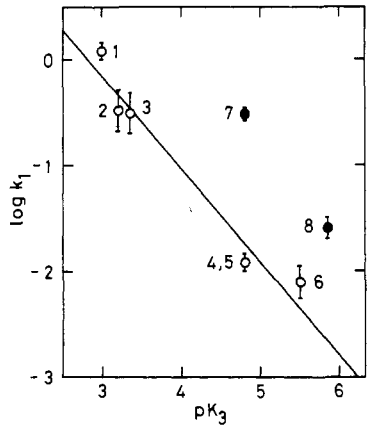

Figure 4. Plot of log k_1 vs. pK_3 for the reduction of the reconstituted metMb by ascorbate: (1) DFDPmetMb; (2) DPDPmetMb; (3) DADPmetMb; (4) native metMb; (5) reconstituted PPmetMb; (6) DPmetMb; (7) PPDMEmetMb; (8) MPmetMb.

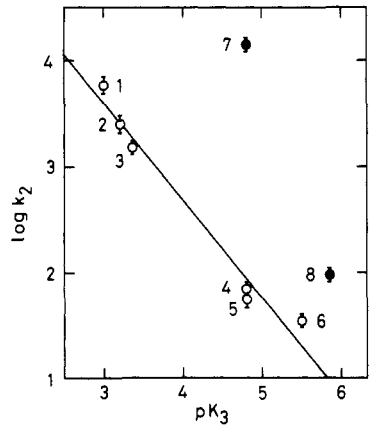

Figure 5. Plot of log k_2 vs. pK_3 for the reduction of the reconstituted metMb by ascorbate: (1) DFDPmetMb; (2) DPDPmetMb; (3) DADPmetMb; (4) native metMb; (5) reconstituted PPmetMb; (6) DPmetMb; (7) PPDMEmetMb; (8) MPmetMb.

the proximal imidazole may be also affected by the modification of the heme propionates.

The logarithm of the second-order rate constants (k_1 and k_2) for the reduction by ascorbate of the metMb(H_2O) reconstituted with 2,4-disubstituted deuterohemins (–CHO, $-COC_2H_5$, $-COCH_3$, $-CH{=}CH_2$, and –H) is linearly correlated with the pK_3 of the acid dissociation constants of the porphyrin monocation, H_3P^+ (Figures 4 and 5):

$$\log k_1 = -0.88pK_3 + 2.46 \quad (15)$$

$$\log k_2 = -0.92pK_3 + 6.34 \quad (16)$$

Mesohemin is an exception. The basicity of the free-base porphyrins (pK_3) decreases with an increase in the electron-withdrawing power of the 2,4-substituents.[24] Since the electron density on Fe(III) is considered to decrease with an increase in the electron-withdrawing power of the substituents, which is reflected on the pK values of the metMb(H_2O), it is very likely that an electron of ascorbate is more easily transferred to Fe(III) with an increase in the electron-withdrawing power. The correlation factor (0.88 or 0.92) is nearly unity, indicating that the electronic factor is predominant in changing the 2,4-substituents. We suggest that the structural change in the iron site for all of the reductions is similar, i.e. the hexacoordinated metMb(H_2O) to the pentacoordinated deoxyMb. However, the unexpected high reactivity of MPmetMb(H_2O) cannot be explained by only the electronic effect.

An X-ray crystallographic study[33] of mesomethemoglobin has shown that the ethyl group has rotated relative to the vinyl group of the native metHb, such that the ethyl β-carbon lies farther out of plane from the heme and toward the distal side. Numerous changes are also seen in the tertiary structure. On the other hand, deuteromethemoglobin has only minor structural perturbations.[34] Therefore, it is likely that the high reactivity of MPmetMb is ascribable to the structural perturbations induced by the substitution of the 2,4-positions, particularly the steric repulsion between the ethyl groups and the polypeptide side chains. The reactivity order is reasonable on the basis of the redox potential of the reconstituted metMb: $E°'$ (native metMb, 0.047 V) $\simeq$ $E°'$ (MPmetMb, 0.041 V) > $E°'$ (DPmetMb, 0.025 V).[35] We note that the rate of the reduction by ascorbate of the bis(pyridine) adduct of 2,4-disubstituted deuterohemin in aqueous Triton X-100 increases with the electron-withdrawing power of the substituents; MP < DP $\simeq$ PP (see Table III). In this system a steric interaction between hemin and Triton X-100 might not be important. Kawabe et al.[11a] have demonstrated that electronic effects, steric bulk, and specific stereochemical effects (orientation of the substituents) of the 2,4-side chains are all necessary when interpretation of the oxygenation data of the reconstituted myoglobins with 2,4-substituted deuterohemin is made. Crystal structures of $Fe^{III}PP$ complexes show that the dihedral angles between the planes of the vinyl groups and that of the porphyrin vary betwen 20 and 50°.[36,37] In contrast, the ethyl groups of $Fe^{III}MP$ complex are close to 90° out of plane of the porphyrin.[38] These results indicate that there is some degree of conjugative overlap of the p orbitals of the vinyl groups with the aromatic system of the porphyrin. This is also supported by spectroscopic studies.[39] We believe that the carbonyl groups of the acetyl, propionyl, and formyl groups interact with the porphyrin in a similar way and that these 2,4-substituents are not so out of plane of the porphyrin as in MPmetMb.[40] Therefore, no steric effect appears in these systems.

The relative ratios for k_1 and k_2 of the reconstituted metMb compared with the native metMb (Table II) are almost exactly the same although HA^- and A^{2-} do not have the same change. This indicates that electrostatics are unimportant in the system, arising perhaps from low charges of metMbs[4b] or from kinetic accessibility proposed in the reduction of cytochrome c.[41]

In conclusion, the modification of the heme distal histidine, heme propionates, and 2,4-substituents of deuterohemin affects the reduction rate of metMb by ascorbate. The geometry change in the iron site upon reduction of metMb(H_2O) to deoxyMb is an especially important factor.

Supplementary Material Available: Tables of kinetic data for the reduction of metmyoglobins by ascorbate and for the reduction of hemin–bis(pyridine) adducts by ascorbate in aqueous 2% Triton X-100 (4 pages). Ordering information is given on any current masthead page.

(33) Seybert, D. W.; Moffat, K. *J. Mol. Biol.* **1977**, *113*, 419.
(34) Seybert, D. W.; Moffat, K. *J. Mol. Biol.* **1976**, *106*, 895.
(35) Brunori, M.; Saggese, U.; Rotilio, G. C.; Antonini, E.; Wyman, J. *Biochemistry* **1971**, *10*, 1604.
(36) Koenig, D. F. *Acta Crystallogr.* **1965**, *18*, 663.
(37) Little, R. G.; Dymock, K. R.; Ibers, J. A. *J. Am. Chem. Soc.* **1975**, *97*, 4532.
(38) Hoard, J. L.; Hamor, M. J.; Hamor, T. A.; Caughey, W. S. *J. Am. Chem. Soc.* **1965**, *87*, 2312.
(39) (a) La Mar, G. N.; Walker-Jensen, F. A. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1979; Vol. IV, p 61. (b) La Mar, G. N.; Budd, D. L.; Smith, K. M.; Langry, K. C. *J. Am. Chem. Soc.* **1980**, *102*, 1822. (c) Desbois, A.; Mazza, G.; Stetzkowski, F.; Lutz, M. *Biochim. Biophys. Acta* **1984**, *785*, 161 and references therein.
(40) Resonance Raman study of DFDPmetMb has suggested that delocalization of the formyl π electrons to the C_β–CHO bond occurs: Tsubaki, M.; Nagai, K.; Kitagawa, T. *Biochemistry* **1980**, *19*, 379.
(41) Wherland, S.; Gray, H. B. In *Biological Aspects of Inorganic Chemistry*; Addison, A. W., Cullen, W. R., Dolphin, D., James, B. R., Eds.; Wiley: New York, 1977; p 289.