Kinetics and Mechanism of Reduction of Metmyoglobins by Dithionite. Role of the Heme Propionates

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The kinetics of the reduction by dithionite ions of metmyoglobins reconstituted with porphinatoiron(III) complexes containing the protoporphyrin IX dimethyl ester, the deuteroporphyrin IX dimethyl ester, and octaethylporphyrin were studied at 25 °C and pH 6.3—9.2 in aqueous solution. Esterification of the heme propionate groups accelerated the reduction rate by $SO_2^{\cdot-}$, as well as the case of octaethylporphyrin (which does not contain the propionate groups); it also affected the redox potential of metmyoglobin, but much less so the rate of the self-exchange reaction. The reaction mechanisms are discussed.

Myoglobin is an oxygen-storage heme protein found in the muscles of vertebrates. Although oxymyoglobin is quite stable under physiological conditions, it undergoes slow autoxidation to form iron(III) metmyoglobin (metMb), which is inactive to molecular oxygen. The reduction of metMb to deoxymyoglobin (deoxyMb) is therefore an important reaction used for recovering deoxyMb. The reduction kinetics of metMb have been extensively studied using both inorganic and organic reducing agents.[1] The modification of the heme environment is a useful technique for elucidating the mechanism of the electron-transfer reactions of hemoproteins. We have reported that modifying the heme distal histidine, heme propionates, and peripheral substituents of the heme affects the reduction rate of metMb and that the geometry change at the iron site upon reduction of metMb(H_2O) to deoxyMb is an especially important factor.[1,2]

The native metMb has protoporphinatoiron(III) ($Fe^{III}PP$) as a prosthetic group. The heme propionates in sperm whale metMb make a salt bridge with Arg 45 and a hydrogen bond to His 97, thereby stabilizing the structure of the heme pocket.[3] We previously suggested that the salt bridge as well as the hydrogen bond between these residues and the heme propionates are important regarding the reactivity differences in the redox reactions of sperm whale and horse heart metmyoglobins, the latter having a Lys residue in place of Arg 45 in the former.[4]

Dithionite ions are widely used as a reducing agent for the reductions of not only metal complexes, but also biological redox systems;[5] the reactive species is a $SO_2^{\cdot-}$ radical anion which is produced by a dissociation of the $S_2O_4^{2-}$ ion. In this work we report on a dithionite reduction of the reconstituted metmyoglobins with three artificial hemes containing methyl esters of propionate groups, or without propionic acid chains, which cannot make the salt bridge with Arg 45 and/or the hydrogen bond to His 97; it is therefore expected to clarify the role of the heme propionates on the reduction of metmyoglobin.

Experimental

Materials. Sperm whale skeletal muscle metmyoglobin (type II, Sigma) was purified as previously described.[4] The recombination of chloro(porphinato)iron(III) complexes containing protoporphyrin IX dimethyl ester (PPDME), deuteroporphyrin IX dimethyl ester (DPDME), and octaethylporphyrin (OEP) with apomyoglobin, as well as the purification, were carried out by a method which has already been published.[6,7] The purity of the reconstituted metmyoglobins was checked spectrophotometrically. Sodium dithionite (Fluka AG) was used without further purification. The concentration was determined spectrophotometrically by reactions with

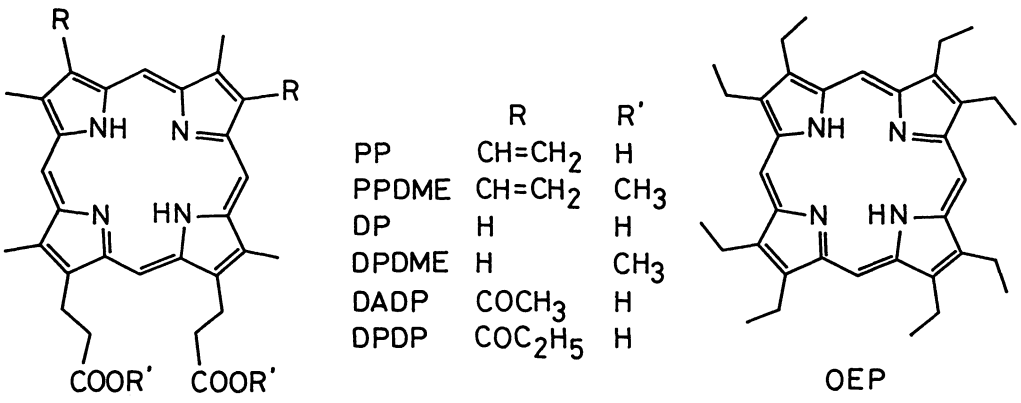

Fig. 1. Structures of porphyrins.

the hexacyanoferrate(III) ion (ε_{418}=1.01×10³ $M^{-1}cm^{-1}$; 1 M= 1 mol dm^{-3}).[8] Hexaammineruthenium(III) chloride ([Ru(NH_3)$_6$]Cl_3) was purchased from Aldrich Chemical Co. and purified by a method described in the literature.[9] The corresponding ruthenium(II) ion was prepared in situ by dithionite reduction. All other chemicals used were of guaranteed grade. All of the solutions used for measurements were prepared by using redistilled water.

Kinetic Measurements. All of the reactions were carried out in argon-saturated solutions at 25 °C. The solutions of the dithionite ion ((0.43—3.50)×10⁻³ M) and metmyoglobins ((2.0—4.0)×10⁻⁶ M) in a 0.1 M MES (2-morpholinoethanesulfonic acid) or a 0.1 M phosphate buffer at pH 6.3—9.2 and the ionic strength (I) of 0.1 M or 0.2 M (adjusted with NaCl) were mixed in Photal RA-401 stopped-flow and RA-415 rapid-scanning spectrophotometers. The reactions were followed by the decrease in absorbance at 407 nm (PPDMEmetMb(H_2O)), 393 nm (DPDMEmetMb(H_2O)), and 395 nm (OEPmetMb(H_2O)). The appearance of deoxyMb was also followed at 434 nm (PPDMEdeoxyMb), 422 nm (DPDMEdeoxyMb), and 420 nm (OEPdeoxyMb). The electron-transfer reaction between PPDMEmetMb(H_2O) and native deoxyMb produced by a dithionite ion was carried out at 25 °C, pH 6.3 (a 0.1 M MES buffer), and I=0.1 M. The reaction was followed at both 502 and 550 nm by the use of a Shimadzu UV-200S spectrophotometer. The concentrations of PPDMEmetMb(H_2O) and native deoxyMb were 3.00×10⁻⁵ M and (1.70—4.20)×10⁻⁴ M, respectively.

Equilibrium Measurements. The acid-dissociation constant for the heme-linked water molecule of DPDMEmetMb(H_2O) (1.00×10⁻⁵ M) was determined spectrophotometrically (380—395 nm) at 25 °C and I=0.1 M (a phosphate buffer). The redox potential of PPDMEmetMb(H_2O) was determined spectrophotometrically by using the [Ru(NH_3)$_6$]$^{2+}$ ion as a redox partner. Redox titration was carried out in an argon atmosphere at 25 °C, pH 6.3 (a 0.1 M MES buffer), I=0.1 M, [metMb]=4.40×10⁻⁵ M, [Ru(II)]=0—1.98×10⁻⁴ M, and [Ru(III)]=3.50×10⁻⁴ M. The spectral change was monitored in the 450—700 nm region by using a Shimadzu UV-200S spectrophotometer. The molar absorption coefficients used in this work were as follows: ε_{505}=8.80×10³ $M^{-1}cm^{-1}$ and ε_{555}=4.30×10³ $M^{-1}cm^{-1}$ for PPDMEmetMb(H_2O); ε_{505}= 6.00×10³ $M^{-1}cm^{-1}$ and ε_{555}=1.30×10⁴ $M^{-1}cm^{-1}$ for PPDMEdeoxyMb.

The pH's of the solutions were measured on a Hitachi-Horiba F-14RS pH meter.

Results and Discussion

Redox Potential of PPDMEmetMb(H_2O). The redox potential of PPDMEmetMb(H_2O) was determined spectrophotometrically in combination with the [Ru(NH_3)$_6$]$^{3+/2+}$ redox couple by a previously described method.[2] The [Ru(NH_3)$_6$]$^{2+}$ ion was prepared by a dithionite reduction of the [Ru(NH_3)$_6$]$^{3+}$ ion, since the Zn(Hg) reduction[2] of Ru(III) induced the denaturation of PPDMEmetMb(H_2O) in contrast to reconstituted metmyoglobins with hemins containing propionic acids. Four isosbestic points were observed in the 450—700 nm region during titration (Fig. 2). The equilibrium constant for the reaction

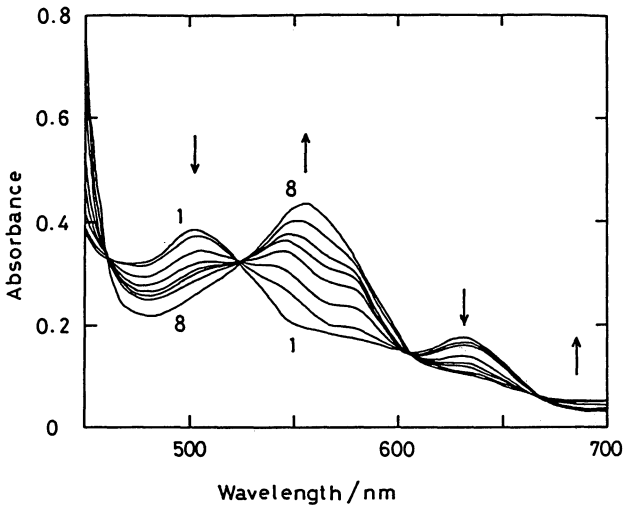

Fig. 2. Redox titration of PPDMEmetMb(H_2O) (4.40×10⁻⁵ M) in the presence of [Ru(NH_3)$_6$]$^{3+}$ ions (3.50×10⁻⁴ M) with various amounts of [Ru(NH_3)$_6$]$^{2+}$ ions at 25 °C, pH 6.3 (0.1 M MES buffer), and I=0.1 M. (1) 0, (2) 2.0×10⁻⁵, (3) 4.0×10⁻⁵, (4) 6.0×10⁻⁵, (5) 8.0×10⁻⁵, (6) 1.19×10⁻⁴, (7) 1.79×10⁻⁴, and (8) 1.98×10⁻⁴ M.

$$\text{PPDMEmetMb}(H_2O) + [Ru(NH_3)_6]^{2+} \rightleftarrows \text{PPDMEdeoxyMb} + [Ru(NH_3)_6]^{3+} \quad (1)$$

was determined from the slope of plots of [deoxyMb]/[metMb] against [Ru(II)]/[Ru(III)]. When the value of 0.05 V was used for the redox potential of the [Ru(NH_3)$_6$]$^{3+/2+}$ couple,[10] a value of 0.090±0.010 V was obtained for the redox potential of PPDMEmetMb(H_2O). The redox potential of PPDMEmetMb(H_2O) is higher than that of the native metMb(H_2O) by about 30 mV (E^0=0.059 V for the latter[11]). The shift of the E^0 value by esterification of the heme propionate groups is similar to that for cytochrome b_5.[12] The pK_a for the acid dissociation of the coordinated water molecule of PPDMEmetMb(H_2O) is 8.0, being smaller than that for native metMb(H_2O) (pK_a=9.16[1]). These results strongly suggest that the electron density of the Fe(III) center decreases upon esterification of the heme propionate groups.

Reduction of Reconstituted Metmyoglobins by Dithionite. Rapid scanning difference absorption spectra taken during the reduction of PPDMEmetMb(H_2O) by dithionite ions are shown in Fig. 3. Three isosbestic points were observed in the 380—540 nm region for each system. The plots of $-\ln(A_\infty - A_t)$ vs. time were linear for at least 90% completion of both the decay of metMb and the formation of deoxyMb, 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 half-order dependence on the initial concentrations of the dithionite ion ([$S_2O_4^{2-}$]$_0$), as is shown in Fig. 4. Therefore, the rate law for the dithionite reduction is given by

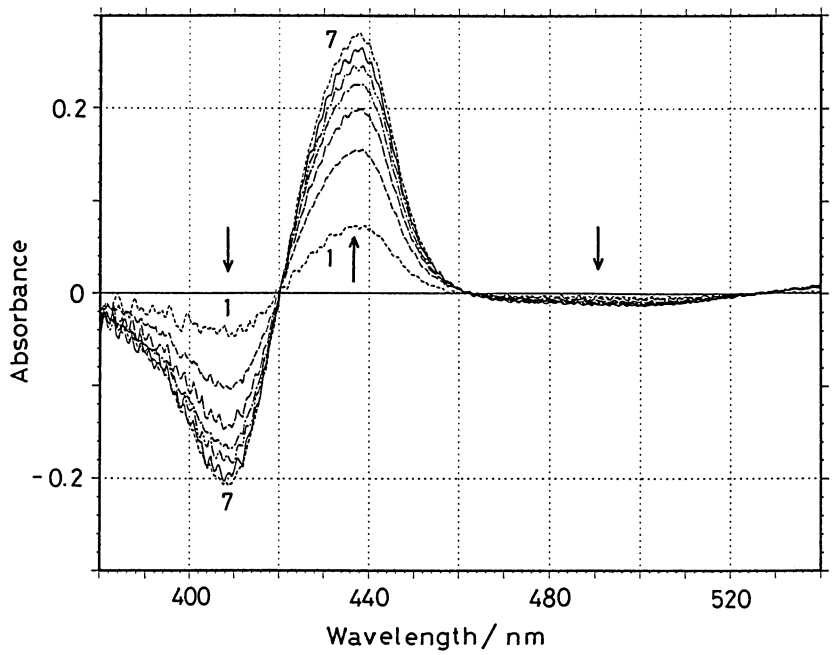

Fig. 3. Rapid-scanning spectra for the reduction of PPDME-metMb(H_2O) (3.9×10^{-6} M) by dithionite ions (1.10×10^{-3} M) at 25 °C and pH 6.7 (0.1 M phosphate buffer). (1) 18 ms, (2) 22 ms, (3) 26 ms, (4) 30 ms, (5) 34 ms, (6) 38 ms, and (7) 46 ms. The times given are for the start of scan at 380 nm.

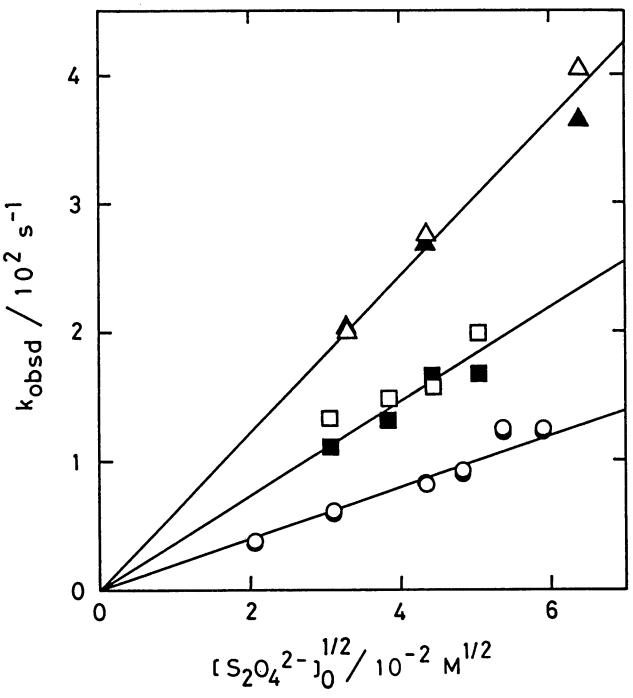

Fig. 4. Plots of k_{obsd} vs. $[S_2O_4^{2-}]_0^{1/2}$ for the dithionite reductions of reconstituted metmyoglobins at 25 °C, pH 6.3 (0.1 M MES buffer), and I=0.1 M. ○: PPDMEmetMb(H_2O) at 407 nm, ●: PPDME-metMb(H_2O) at 434 nm, □: DPDMEmetMb(H_2O) at 393 nm, ■: DPDMEmetMb(H_2O) at 422 nm, △: OEPmetMb(H_2O) at 395 nm, and ▲: OEP-metMb(H_2O) at 420 nm.

$$-d[Fe(III)]/dt = kK_D^{1/2}[S_2O_4^{2-}]_0^{1/2}[Fe(III)], \quad (2)$$

where K_D is the dissociation constant for the dithionite ion (K_D=1.4×10^{-9} M at 25 °C).[5a]

$$S_2O_4^{2-} \overset{K_D}{\rightleftarrows} 2SO_2^{\cdot-} \quad (3)$$

The second-order rate constant (k) obtained from the slopes of the straight lines in Fig. 4 decreased with an increase in pH (Fig. 5). The reduction of metMb can be accommodated by the following scheme:

$$metMb(H_2O) \overset{K_a}{\rightleftarrows} metMb(OH) + H^+ \quad (4)$$

$$metMb(H_2O) + SO_2^{\cdot-} \xrightarrow{k_{H_2O}} deoxyMb + SO_2 \quad (5)$$

$$metMb(OH) + SO_2^{\cdot-} \xrightarrow{k_{OH}} deoxyMb + SO_2 \quad (6)$$

Since the intercept in Fig. 4 is near zero, reduction by the $S_2O_4^{2-}$ species is negligible in the present systems. According to Reactions 4—6, the second-order rate constant (k) is represented as

$$k = \frac{k_{OH}K_a + k_{H_2O}[H^+]}{K_a + [H^+]}. \quad (7)$$

The k_{OH} path is at least 10-times slower than the k_{H_2O} path (k_{H_2O}=6.5×10^7 $M^{-1}s^{-1}$ and $k_{OH} \leqq 6.5\times10^6$ $M^{-1}s^{-1}$ for the PPDME system). This behavior is consistent with the results for the other dithionite reduction of metmyoglobins.[2,5] Since the electron density of the Fe(III) center increased upon the ligation of OH^-, more so than the water molecule, the rate of reduction by $SO_2^{\cdot-}$ is considered to decrease. The rate constants for Reaction 5 are listed in Table 1 along with the values of

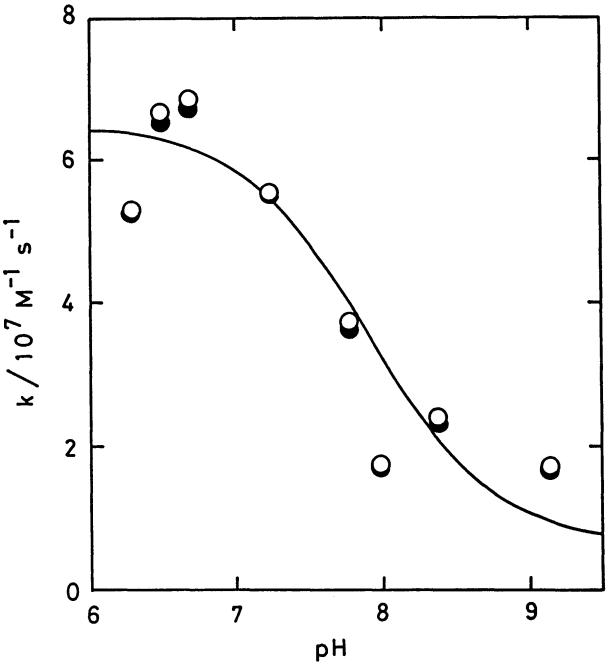

Fig. 5. pH Dependence of the second-order rate constants (k) for the reduction of PPDMEmetMb(H_2O) by $SO_2^{\cdot-}$ at 25 °C and I=0.2 M. The solid curve is on the basis of Eq. 7 by the use of pK_a=7.90, k_{H_2O}= 6.5×10^7 M^{-1} s^{-1}, and k_{OH}=6.5×10^6 M^{-1} s^{-1}. ○: 407 nm and ●: 434 nm.

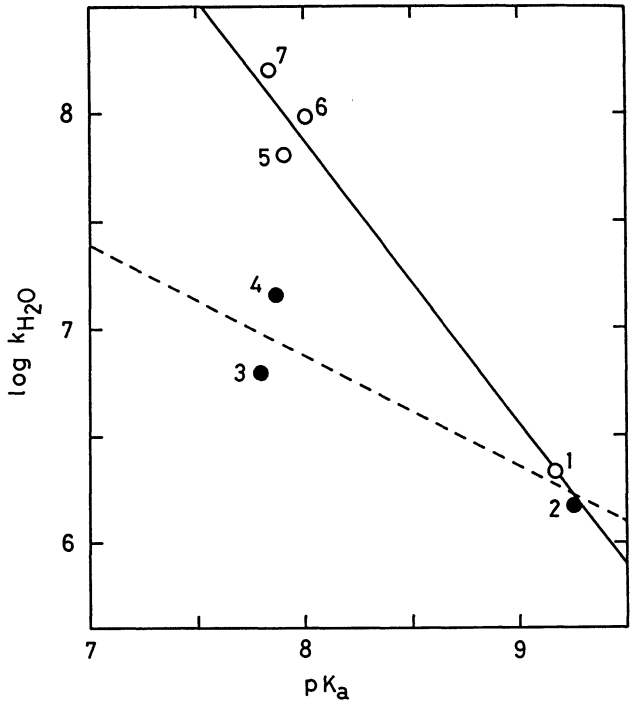

Fig. 6. Plots of log k_{H_2O} vs. pK_a of metMb(H_2O) for the reduction of metmyoglobins by $SO_2^{\cdot-}$. (1) native PPmetMb(H_2O), (2) DPmetMb(H_2O), (3) DADPmetMb(H_2O), (4) DPDPmetMb(H_2O), (5) PPDMEmetMb(H_2O), (6) DPDMEmetMb(H_2O), and (7) OEPmetMb(H_2O).

Table 1. Rate Constants of the Reduction of Metmyoglobins by $SO_2^{\cdot-}$ at 25 °C and I=0.1 M

metMb(H_2O)	k_{H_2O}/M^{-1} s^{-1}	pK_a
OEPmetMb(H_2O)	(1.6±0.1)×10^8	7.83[a]
DPDMEmetMb(H_2O)	(9.8±0.5)×10^7	8.0
PPDMEmetMb(H_2O)	(6.5±0.5)×10^7	7.90[b]
PPmetMb(H_2O)	(2.2±0.1)×10^6 [c]	9.16[d]
DPmetMb(H_2O)	(1.5±0.1)×10^6 [c]	9.25[d]
DPDPmetMb(H_2O)	(1.4±0.2)×10^7 [c]	7.87[d]
DADPmetMb(H_2O)	(6.1±0.9)×10^6 [c]	7.80[b]

a) Ref. 7. b) Ref. 1. c) Refs. 2a and 5. d) Ref. 4.

Table 2. Rate Constants of the Electron-Transfer Reaction between PPDMEmetMb(H_2O) (3.00×10^{-5} M) and Native deoxyMb at 25 °C, pH 6.3, and I=0.1 M

[deoxyMb]$_0$	k_{obsd}	k
10^{-4} M	10^{-4} s^{-1}	M^{-1} s^{-1}
1.69	1.05	0.62
2.48	1.07	0.43
3.07	1.38	0.45
3.43	1.76	0.51
4.14	1.50	0.36

pK_a regarding the dissociation of the coordinated water molecule at the heme iron site of metMb(H_2O). Plots of log k_{H_2O} vs. pK_a of metMb(H_2O) are shown in Fig. 6. The values of log k_{H_2O} increase with a decrease in the pK_a values of metMb(H_2O). Since the decrease in pK_a corresponds to that in the electron density at the Fe(III) center, the electron of $SO_2^{\cdot-}$ may be easily transferred to the Fe(III) center. The slopes of the plots of log k_{H_2O} vs. pK_a for both the dimethyl esters and OEP are quite different from those of systems containing propionic acid side chains (−1.32 for the former and −0.51 for the latter). Although the redox potential of PPDMEmetMb(H_2O) is lower than that of either DADPmetMb(H_2O) or DPDPmetMb(H_2O), the reduction of the former by $SO_2^{\cdot-}$ is faster than that for the latter. It is known that the heme environmental structures of the DP-, DADP-, and DPDP-metmyoglobins are similar to that of the native metMb(H_2O).[1] Therefore, the acceleration in the reduction of the dimethyl esters cannot be explained by only electronic effects. A steric alteration in the heme environment due to esterification must be concerned. Esterification of the heme propionate groups breaks the salt bridge between Arg 45 and the heme propionic acids and the hydrogen bond with His 97. The heme pocket is thereafter expected to expand, allowing the $SO_2^{\cdot-}$ ion to easily attack the heme iron center and/or the heme edge. Neya et al.[7] have demonstrated by an NMR study of OEP-myoglobin that OEP iron(III) serves as a prosthetic group for myoglobin with normal function, despite the structural difference from native myoglobin, especially at the heme distal side.

Estimation of Self-Exchange Rate Constant for PPDMEmetMb(H_2O)/deoxyMb System. To estimate the self-exchange rate constants for the PPDME-

Table 3. Rate Constants of the Electron-Transfer Reaction between Myoglobins at 25 °C

System	k/M^{-1} s^{-1}	E^0 (metMb)/V
S.W.metMb(H_2O)+H.H.deoxyMb[a]	1.0±0.2[b]	0.059±0.002[c]
DADPmetMb(H_2O)+native deoxyMb	0.94±0.13[d,e]	0.16±0.01[d]
PPDMEmetMb(H_2O)+native deoxyMb	0.50±0.12	0.09±0.01

a) S.W.=sperm whale and H.H.=horse heart. b) Ref. 13. c) Ref. 11. d) Ref. 2a. e) Ref. 14.

metMb(H_2O)/deoxyMb system, the reaction of PPDMEmetMb(H_2O) with native deoxyMb was examined at 25 °C, pH 6.3, and I=0.1 M. The differences in the molar absorption coefficients between metMb(H_2O) and deoxyMb are 3.1×10^3 M^{-1}cm^{-1} at 502 nm and -6.7×10^3 M^{-1}cm^{-1} at 505 nm for PPDME-myoglobins and 5.0×10^3 M^{-1}cm^{-1} at 502 nm and -9.3×10^3 M^{-1} cm^{-1} at 550 nm for native myoglobins, respectively. An increase in the absorbance at 502 nm and a decrease in the absorbance at 550 nm were observed during reactions under the condition [native deoxyMb]$_0$ > [PPDMEmetMb(H_2O)]$_0$. Therefore, the following reaction occurs:

$$\text{PPDMEmetMb}(H_2O) + \text{native deoxyMb} \rightarrow \text{PPDMEdeoxyMb} + \text{native metMb}(H_2O). \quad (8)$$

This is also expected from the redox potentials for these myoglobins (0.090 V for PPDMEmetMb(H_2O) and 0.059 V for native metMb(H_2O)). The rate constants of Reaction 8 are given in Table 2. The rate constants of the electron-transfer reactions between myoglobins are about 1 M^{-1} s^{-1}, as is given in Table 3. This value is similar to the self-exchange rate constant estimated from the Marcus theory for the electron-transfer reactions of myoglobins containing heme propionic acids with inorganic and organic redox reagents.[2,13] The slowness of the self-exchange reaction of these myoglobins may be ascribable to a geometry change at the heme iron site from hexacoordinated metMb(H_2O) to pentacoordinated deoxyMb, where the release of the water molecule must occur upon reduction.[2] Contrary to the dithionite reduction of metmyoglobins, the electron-transfer reaction between PPDMEmetMb(H_2O) and native deoxyMb was not accelerated. This is probably due to a reaction between large molecules in the latter system.

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References

1) K. Tsukahara, T. Okazawa, H. Takahashi, and Y. Yamamoto, *Inorg. Chem.*, **25**, 4756 (1986), and references therein.

2) a) K. Tsukahara, *J. Am. Chem. Soc.*, **111**, 2040 (1989); b) K. Tsukahara, *Chem. Lett.*, **1987**, 1291; c) K. Tsukahara, *J. R. Neth. Chem. Soc.*, **106**, 291 (1987).

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

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

5) a) D. O. Lambeth and G. Palmer, *J. Biol. Chem.*, **248**, 6095 (1973); b) R. P. Cox and M. R. Hollaway, *Eur. J. Biochem.*, **74**, 575 (1977); c) E. Olivas, D. J. A. de Waal, and R. G. Wilkins, *J. Biol. Chem.*, **252**, 4038 (1977).

6) M. Tamura, T. Asakura, and T. Yonetani, *Biochim. Biophys. Acta*, **295**, 467 (1973).

7) S. Neya, N. Funasaki, and K. Imai, *J. Biol. Chem.*, **263**, 8810 (1988).

8) F. Aziz and G. A. Mirza, *Talanta*, **11**, 889 (1964).

9) T. J. Meyer and H. Taube, *Inorg. Chem.*, **7**, 2369 (1968).

10) H. S. Lim, D. J. Barclay, and F. C. Anson, *Inorg. Chem.*, **11**, 1460 (1972).

11) R. J. Crutchley, W. R. Ellis, Jr., and H. B. Gray, *J. Am. Chem. Soc.*, **107**, 5002 (1985).

12) L. S. Reid, M. R. Mauk, and A. G. Mauk, *J. Am. Chem. Soc.*, **106**, 2182 (1984).

13) Z. Bradic, K. Tsukahara, P. C. Wilkins, and R. G. Wilkins, "Frontiers in Bioinorganic Chemistry," ed by A. V. Xavier, VCH Publishers, Weinheim, Germany (1986), p. 336.

14) K. Tsukahara, The 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, U. S. A., Book of Abstracts, INORG 146 (1989).