Kinetics and Mechanisms of Reduction of Metmyoglobins. Importance of the Geometry Change at the Heme Iron Site upon Reduction

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Abstract: Kinetics and thermodynamics of the reduction of BrCN-modified metmyoglobin, whose heme iron site has a pentacoordinate geometry, were studied and compared with its native metmyoglobin, the latter having a hexacoordinate geometry at the iron site. An estimated self-exchange rate constant (k_{22}) based on the Marcus theory is about 1×10^4 M^{-1} s^{-1} for the BrCN-modified myoglobin. On the other hand, the value of k_{22} for the reconstituted myoglobin with diacetyldeutero-, dipropionyldeutero-, or deuterohemin is about 1 M^{-1} s^{-1} as it is for the native myoglobin, in all of which a coordinated water molecule is present. The intrinsic enthalpic reorganizational barrier for the electron-exchange reaction of the BrCN-modified myoglobin is estimated to be 8 kcal mol^{-1}, which is much smaller than that for native myoglobin (20 kcal mol^{-1}). The importance of the geometry change at the iron site upon reduction of metmyoglobins is pointed out.

Electron-transfer reactions of hemoproteins are an important subject and have been studied extensively.[1] Kinetic investigations are concerned with several factors: (i) the spin change,[2] (ii) the geometry change at the heme iron site,[3] (iii) the extent of exposure of the heme,[4] (iv) the distance between the redox centers,[5] and (v) the orientation of the hemes.[6] Modification of the heme environment is a useful technique for elucidating the effects of such factors on the mechanism of electron-transfer reactions. We have recently reported that modification of the heme distal histidine, heme propionates, and 2,4-substituents of deuterohemin affects the reduction rate of sperm whale metmyoglobin (metMb) by ascorbate and that the geometry change at the iron site upon reduction of metMb(H_2O) to deoxyMb is an especially important factor.[7] In these cases factors iii, iv, and v are fixed.

The X-ray crystallographic study of sperm whale metMb(H_2O) has shown that the distal histidylimidazole is hydrogen bonded to the coordinated water molecule.[8] Upon reduction of metMb to deoxyMb, the geometry of the heme iron site changes from hexa- to pentacoordination. When native metMb is treated with cyanogen bromide (BrCN), the distal histidine is cyanated and the hydrogen bond with the coordinated water is broken; the coordinated water molecule dissociates and the heme iron site becomes pentacoordinated. This has been suggested in NMR studies by Morishima et al.[9a,b] The metMb modified with cyanogen bromide is, therefore, a good example for studying the effect of the geometry change upon reduction of metmyoglobins.

In this work, we shall present some evidence for the importance of the geometry change at the heme iron site, based on the kinetics of the reduction by several reducing agents, the redox potentials of metMbs, and the self-exchange rate for the pentacoordinate BrCN-modified metMb as compared to the hexacoordinate native metMb. We have also examined the reduction of metMb reconstituted with 2,4-disubstituted deuterohemin (dipropionyl (DPDP), diacetyl (DADP), or deutero (DP)) which has a coordinated water molecule and thus a hexacoordinate geometry.[7b]

Experimental Section

Reagents. Sperm whale skeletal muscle myoglobin (type II, Sigma) was purified as previously described.[10] A BrCN-modified metMb was prepared in situ by the method previously described.[7,9a,11] Recombination of 2,4-diacetyldeuterohemin (Fe^{III}DADP), 2,4-dipropionyldeuterohemin (Fe^{III}DPDP), and deuterohemin (Fe^{III}DP) with apoMb and the purification were carried out by the method which has already been published.[7]

Sodium dithionite (Fluka AG) was used without further purification. The concentration was determined spectrophotometrically by reactions with $[Fe(CN)_6]^{3-}$ (ϵ_{418} = 1.01×10^3 M^{-1} cm^{-1}).[12] $[Ru(NH_3)_6]Cl_3$ was purchased from Aldrich Chemical Co. and purified by the literature method.[13]

The complexes $[Co(sep)]Cl_3$[14] (sep = 1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]icosane) and $[Ru(Hedta)(H_2O)]\cdot 4H_2O$[15] ($edta^{4-}$ = ethylenediamine-*N,N,N′,N′*-tetraacetate) were prepared by literature methods. $[Ru(NH_3)_6]^{2+}$, $[Ru(edta)(H_2O)]^{2-}$, and $[Co(sep)]^{2+}$ ions were prepared in situ by Zn(Hg) reductions of the corresponding Ru(III) and Co(III) complexes in the presence of Na_2H_2edta which was necessary to avoid both precipitation of zinc phosphate (in phosphate buffer) and denaturation of myoglobins by Zn^{2+}.[16] All other chemicals used were of guaranteed grade.

Kinetic Measurements. All the reactions were carried out in an argon atmosphere at 15-30 °C. The solutions of reductants (0.5-10.6 mM) and metMbs (6-50 μM) in the appropriate buffer (0.1 M sodium phosphate, 0.1 M MES, or 0.05 M PIPES buffer) at the proper ionic strength (0.1 or 0.2 M) were mixed in a Union-Giken RA-401 stopped-flow spectrophotometer. The reactions were followed by the decrease in absorption at 500-510 nm for native metMb, the BrCN-modified metMb, and DPmetMb and at 413-417 nm for DADPmetMb and

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DPDPmetMb. The appearance of deoxyMb was also followed at 556–562 nm for native metMb, the BrCN-modified metMb, and DPmetMb and at 442–447 nm for DADPmetMb and DPDPmetMb. Good first-order rates were observed. For fast reactions of BrCN-modified metMb with $[Ru(NH_3)_6]^{2+}$ and $[Ru(edta)(H_2O)]^{2-}$ ions second-order conditions were employed, that is, $[metMb]_0 = [Ru(II)]_0$. The second-order plots were linear for 70% of the reactions.

Redox Potentials. Redox potentials of the myoglobins were determined spectrophotometrically by using hexaammineruthenium(II) ion as a redox partner. Redox titration was carried out in an argon atmosphere at 15.3–35.2 °C, pH 6.3–6.8 (0.1 M MES or sodium phosphate buffer), an ionic strength (I) of 0.1–0.2 M, [metMb] = 100 μM, [Ru(II)] = 0–100 μM, and [Ru(III)] = 1.0–5.3 mM. The spectral changes were monitored in the 450–700-nm region with a Hitachi 200-20 spectrophotometer. Absorption coefficients used in this work were as follows: $\epsilon_{510} = 1.18 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{560} = 4.3 \times 10^3$ M^{-1} cm^{-1} for BrCN-modified metMb[10a]; $\epsilon_{510} = 5.6 \times 10^3$ M^{-1} cm^{-1} and $\epsilon_{560} = 1.13 \times 10^4$ M^{-1} cm^{-1} for BrCN-modified deoxyMb;[11a] $\epsilon_{508} = 6.05 \times 10^3$ M^{-1} cm^{-1} and $\epsilon_{560} = 2.86 \times 10^3$ M^{-1} cm^{-1} for DPDPmetMb; $\epsilon_{508} = 3.88 \times 10^3$ M^{-1} cm^{-1} and $\epsilon_{560} = 8.14 \times 10^3$ M^{-1} cm^{-1} for DPDPdeoxyMb; $\epsilon_{510} = 6.55 \times 10^3$ M^{-1} cm^{-1} and $\epsilon_{562} = 3.30 \times 10^3$ M^{-1} cm^{-1} for DADPmetMb; $\epsilon_{510} = 3.95 \times 10^3$ M^{-1} cm^{-1} and $\epsilon_{562} = 8.22 \times 10^3$ M^{-1} cm^{-1} for DADPdeoxyMb. The pH's of the solutions were measured on a Hitachi-Horiba F-7 pH meter.

Stability of BrCN-Modified Metmyoglobin. The stability of the modified Mb has been examined by Jajczay[9c] and Morishima et al.[9a,b] It has been shown that the BrCN-modified Mb was relatively stable in a neutral region (pH 6–7) and that reversion to the native form is accelerated in the acid and alkaline pH region. We have also examined the stability of the modified metMb under our experimental conditions. When native metMb (1×10^{-4} M) was treated with two- to threefold excess of BrCN at 25 °C and pH 6.2–7.9, the absorption spectra changed for about 20 min with isosbestic points at 573, 652, and 695 nm. Further absorbance changes were gradually observed. It is found that less than 5% of native Mb was regenerated in 30 min under these conditions. Therefore, all the kinetics and the redox titrations were achieved within 30 min after native metMb was treated with BrCN. A fresh solution treated with BrCN was used in each measurement.

Results and Discussion

Morishima et al.[9a,b] have characterized the heme environmental structure and ligand binding properties of BrCN-modified Mb by NMR, IR, and absorption spectroscopic measurements. In comparison with the native form and pentacoordinate iron(III) porphyrin compounds, it has been shown that the heme environmental structure of the BrCN-modified metMb would demand the absence of a coordinated water molecule. We have also confirmed it by the fact that the spectrum of the modified metMb does not change over the pH range from 6 to 8.[7]

Redox Potentials of Metmyoglobins. Redox potentials of hemoproteins have usually been measured electrochemically by the use of mediators.[17] This method, however, could not be applied to the BrCN-modified metMb, because it took too long to attain the equilibrated potential. Instead we successfully determined the redox potential of this system spectrophotometrically in combination with the $[Ru(NH_3)_6]^{3+/2+}$ redox couple.[11a] The reaction of the BrCN-modified metMb with $[Ru(NH_3)_6]^{2+}$ ions in the presence of excess $[Ru(NH_3)_6]^{3+}$ ions was very fast and the titration was complete within 30 min, during which time the BrCN-modified myoglobin was sufficiently stable.[18] The same technique was also applied to the DPDPmetMb and DADPmetMb systems. Four isosbestic points were observed in the 450–700-nm region during the titration as is shown in Figure 1.

The equilibrium constant (K) for the reaction

$$\text{metMb} + [Ru(NH_3)_6]^{2+} \rightleftarrows \text{deoxyMb} + [Ru(NH_3)_6]^{3+} \quad (1)$$

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,[19] we obtained the redox potentials for the metmyoglobins (Table I). The values of $\Delta H°$ and $\Delta S°$ for reaction 1 are 6.0 kcal mol^{-1} and 9.9 eu, respectively. For the reduction of the BrCN-modified metMb thermodynamic parameters were obtained after these were corrected for the $[Ru(NH_3)_6]^{3+/2+}$ couple ($\Delta S°_{rc}$ = 18.5 eu,[21] $\Delta H°$ = −1.9 kcal mol^{-1}, and $\Delta S°$ = −2.6 eu[22]). The values obtained for the reaction, metMb + $^1/_2H_2 \rightleftarrows$ deoxyMb + H^+, are −7.9 kcal mol^{-1} ($\Delta H°$ = −1.9–6.0 kcal mol^{-1}) and −12.5 eu ($\Delta S°$ = −2.6–9.9 eu), respectively.

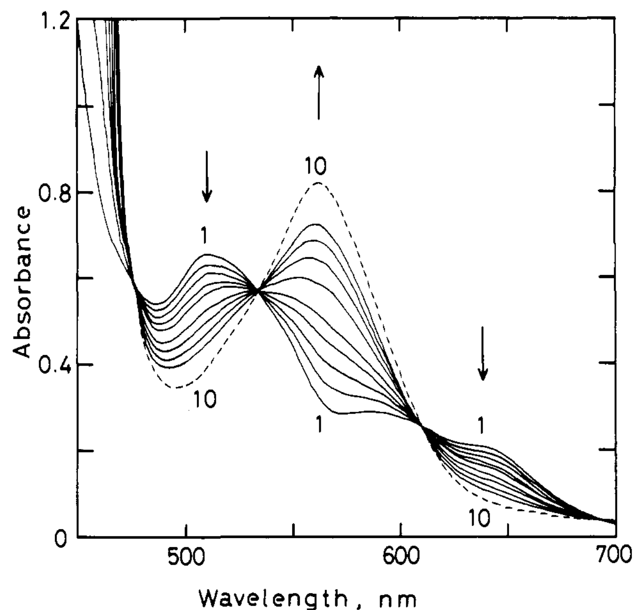

Figure 1. Redox titration of DADPmetMb(H_2O) (1.00×10^{-4} M) in the presence of $[Ru(NH_3)_6]^{3+}$ ions (1.00×10^{-3} M) with various amounts of $[Ru(NH_3)_6]^{2+}$ ions at pH 6.3 (0.1 M MES buffer), I = 0.1 M, and 25 °C: (1) 0, (2) 1.0×10^{-5}, (3) 2.0×10^{-5}, (4) 3.0×10^{-5}, (5) 4.0×10^{-5}, (6) 6.0×10^{-5}, (7) 8.0×10^{-5}, (8) 1.00×10^{-4}, (9) 1.60×10^{-4} M. The broken line is a spectrum of DADPdeoxyMb.

Table I. Redox Potentials of Metmyoglobins at 25 °C[a]

metMb	$E°$ (25 °C), V	$\Delta H°$, kcal mol^{-1}	$\Delta S°$, eu
native metMb (H_2O)[b]	0.0588 ± 0.002	−13.0 ± 0.4	−39.1 ± 1.2
BrCN-modified metMb[c]	0.18 ± 0.01	−7.9 ± 1.0	−12.5 ± 2.0
DPDPmetMb (H_2O)[d]	0.18 ± 0.01		
DADPmetMb (H_2O)[d]	0.16 ± 0.01		
DPmetMb (H_2O)[e]	0.025		

[a] Data for the reaction: metMb + $^1/_2H_2 \rightleftarrows$ deoxyMb + H^+. [b] At pH 7.0 and I = 0.1 M (phosphate buffer). Reference 3b. [c] At pH 6.8 and I = 0.2 M (0.1 M phosphate buffer). [d] At pH 6.3 and I = 0.1 M (0.1 M MES buffer). [e] At 30 °C and pH 7.1 (0.1 M phosphate buffer). Reference 20.

The redox potentials of native and reconstituted myoglobins are linearly correlated with the pK_3 of the acid dissociation constants of the porphyrin monocation (H_3P^+) as is shown in Figure 2:

$$E° = -0.065pK_3 + 0.38 \quad (2)$$

The basicity of the free-base form of porphyrins (pK_3) decreases with an increase in the electron-withdrawing power of the 2,4-substituents.[25] Since the electron density on Fe(III) is considered to decrease with an increase in the electron-withdrawing power

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(18) Absorption spectrum of the BrCN-modified metMb did not change when $[Ru(NH_3)_6]^{3+}$ added up to 5.3 mM. However, the absorption intensity decreased slightly in the presence of 10 mM of $[Ru(NH_3)_6]^{3+}$.

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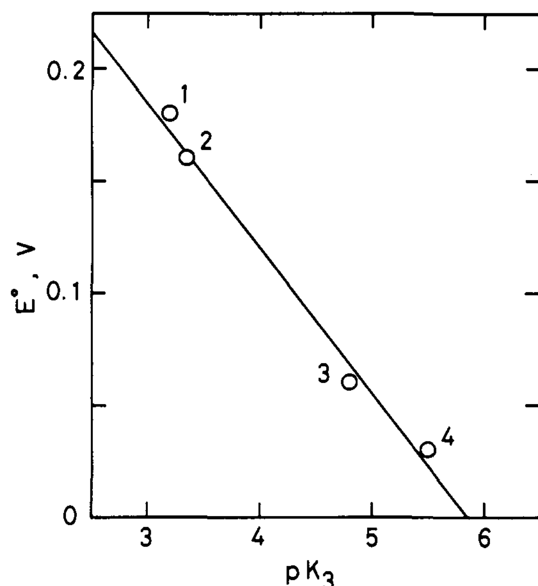


Figure 2. Plot of $E°$ of native and reconstituted myoglobins against the pK_3 of the acid dissociation constants of the porphyrin monocation: (1) DPDPmetMb(H_2O), (2) DADPmetMb(H_2O), (3) native PPmetMb(H_2O), and (4) DPmetMb(H_2O).

of the substituents, it is resonable that the heme is more easily reduced with an increase in the electron-withdrawing power. The slope of −65 mV is close to −59 mV at 25 °C, indicating that the redox potentials of these myoglobins are mainly controlled by electronic factors in changing the 2,4-substituents and therefore that the steric factor is nearly identical for each of these substituted myoglobins. We have suggested[7b] that the carbonyl groups of the acetyl and propionyl groups interact with the porphyrin π system. Resonance Raman studies of DFDPmetMb[26] and DADPmetMb[27] have shown the presence of π-conjugation of the 2- and 4-groups with the porphyrin ring. Such π-conjugation is expected to restrict free rotation of the 2,4-substituents and thus reduce steric repulsion between the substituent and the polypeptide chain. The BrCN-modified metMb has two vinyl groups at 2- and 4-positions and the electronic factor is identical with that of native metMb. The high redox potential for this derivative arises from the entropy term. The entropy difference $\Delta S°_{rc}(=S°_{red} - S°_{ox})$ between deoxyMb and metMb is negative (−11 eu)[28] for native Mb; on the other hand, for the BrCN-modified Mb it is positive (+9 eu). In the case of metal complexes in solution the values of $\Delta S°_{rc}$ increase with an increase in the charge of the complex ions.[29] The positive entropy change may be explained by a decrease in hydration at the surface of the protein upon reduction of positive charged metMb to neutral deoxyMb. Since there is little information on the thermodynamic parameters for the redox potentials of hemoproteins at present, the negative entropy change for the native Mb system cannot be easily explained. However, it may be that the geometry change at the heme iron site upon reduction is associated with a conformational change of the globin, which induces the decrease of $\Delta S°_{rc}$.

It is interesting to compare the redox potentials of myoglobins and hemoglobins with that of the BrCN-modified metMb. The high redox potential for the pentacoordinate BrCN-modified metMb is comparable with that for monomeric hemoglobins or myoglobin which lack a distal residue capable of forming a hydrogen bond to the sixth ligand; $E°$ values are 0.125 V for *Aplysia limacina* Mb,[20] 0.125 V for *Chironomous thummi* Hb,[20] and 0.153 V for *Glycera dibranchiata* Hb.[30] In the case of human hemoglobin the redox potential of the β chain (0.113 V) is higher than that of the α chain (0.052 V),[31] although both chains have distal histidines. X-ray crystallographic studies of horse metHb[32] and human deoxyHb[33] reveal that the change in the distance of iron to heme plane upon reduction is smaller in the β chain than in the α chain: 0.21 Å for metHb and 0.63 Å for deoxyHb in the β chain and 0.07 Å for metHb and 0.60 Å for deoxyHb in the α chain, respectively. A small geometry change upon reduction was also observed in *Chironomous thummi* Hb,[34] where the distances of iron to heme plane are 0.08 and 0.17 Å for metHb and deoxyHb, respectively. In the metHb a water molecule is located far from the iron center, and a pentacoordinate geometry has been suggested for the heme iron site.[34] In *Aplysia limacina* Mb,[35] the water molecule bound to the acid form of sperm whale Mb is far from the iron atom (4.2 Å), indicating almost no interaction between the two.

Table II. Rate Constants for Reduction of Native Metmyoglobin and BrCN-Modified Metmyoglobin at pH 6.8 (0.1 M Phosphate Buffer) and I = 0.2 M[a]

reductant	T, °C	k, M^{-1} s^{-1}: native metMb(H_2O)	k, M^{-1} s^{-1}: BrCN-modified metMb
SO_2^-	15.0	(1.4 ± 0.1) × 10^6	(2.0 ± 0.2) × 10^7
	20.0	(1.5 ± 0.1) × 10^6	(1.9 ± 0.2) × 10^7
	25.0	(2.2 ± 0.2) × 10^6	(1.7 ± 0.2) × 10^7
	30.0	(2.3 ± 0.2) × 10^6	(1.6 ± 0.2) × 10^7
$[Ru(NH_3)_6]^{2+}$	15.0	(2.0 ± 0.1) × 10^2	(1.4 ± 0.1) × 10^5
	20.0	(2.2 ± 0.1) × 10^2	(1.7 ± 0.2) × 10^5
	25.0	(3.0 ± 0.1) × 10^2	(1.8 ± 0.1) × 10^5
	30.0	(4.4 ± 0.2) × 10^2	(2.5 ± 0.2) × 10^5
$[Ru(edta)(H_2O)]^{2-}$	15.0	(1.4 ± 0.1) × 10^3	(2.6 ± 0.1) × 10^5
	20.0	(1.7 ± 0.1) × 10^3	(3.2 ± 0.1) × 10^5
	25.0	(2.4 ± 0.2) × 10^3	(3.8 ± 0.1) × 10^5
	30.0	(3.0 ± 0.1) × 10^3	(4.6 ± 0.2) × 10^5
$[Co(sep)]^{2+}$ [b]	15.0	(6.6 ± 0.3) × 10^2	(5.3 ± 0.1) × 10^2
	20.0	(9.0 ± 0.3) × 10^2	(7.4 ± 0.2) × 10^2
	25.0	(1.1 ± 0.1) × 10^3	(9.6 ± 0.1) × 10^2
	30.0	(1.4 ± 0.1) × 10^3	(1.3 ± 0.1) × 10^3

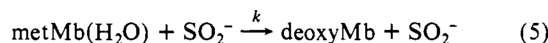
[a] Rate constants are mean values of those at two wavelengths (500 and 556 nm). [b] In 0.05 M PIPES buffer and at I = 0.1 M.

Kinetics. Reduction of native metMb by dithionite[2a,36] and $[Co(sep)]^{2+}$ [37] ions have been studied by other researchers. To evaluate the activation parameters for the reductions we examined these reactions. The rate constants at 25 °C are in agreement with the literature values (Table II). The rate law for the dithionite reduction is given in eq 3:

$$-d[Fe(III)]/dt = kK_D^{1/2}[S_2O_4^{2-}]^{1/2}[Fe(III)] \quad (3)$$

The reduction of metMb can be accommodated by the scheme:

$$S_2O_4^{2-} \rightleftarrows 2SO_2^- \quad K_D \quad (4)$$



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

It has been reported that reduction by $S_2O_4^{2-}$ also occurs in a minor path.[36c] The reduction of the BrCN-modified metMb by dithionite is also described by eq 3. From the plots of ln (k/T) vs. $1/T$ the activation parameters, $\Delta H^‡$ and $\Delta S^‡$ were obtained (Table III).

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Table III. Kinetic Parameters for Reductions of Native and BrCN-Modified Metmyoglobins at pH 6.8 (0.1 M Phosphate Buffer) and $I = 0.2$ M and Calculated Rate Constants Based on the Marcus Theory

reductant	k_{12}(obsd, 25 °C), $M^{-1}\,s^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, eu	k_{12}(calcd), $M^{-1}\,s^{-1}$
	native metMb(H_2O)[a]			
SO_2^- [b]	2.2×10^6	6.3 ± 2.0	-9 ± 2	2.7×10^6
	$(2.7–4.5) \times 10^6$ [c]			
$[Fe(edta)]^{2-}$ [d]	2.8×10 [e]			
	3.1×10 [f]	12 ± 1 [f]	-13 ± 5 [f]	5.3×10
$[Ru(edta)(H_2O)]^{2-}$ [g]	2.4×10^3	8.7 ± 1.0	-14 ± 3	9.2×10^2
$[Ru(NH_3)_6]^{2+}$ [h]	3.0×10^2	8.6 ± 1.0	-18 ± 3	7.7×10
$[Co(sep)]^{2+}$ [i,j]	1.1×10^3	8.0 ± 1.0	-18 ± 3	1.5×10^3
	3.5×10^3 [k]			
ascorbate				
HA^- [l,m]	1.2×10^{-2}			1.6×10^{-4}
A^{2-} [l,n]	6.9×10			5.5×10^2
	BrCN-modified metMb[o]			
SO_2^-	1.7×10^7	-3.4 ± 2.0	-37 ± 4	1.1×10^9
$[Fe(edta)]^{2-}$ [p]	2.9×10^3	2.0 ± 0.5	-35 ± 4	5.4×10^4
$[Ru(edta)(H_2O)]^{2-}$	3.8×10^5	5.7 ± 1.0	-14 ± 3	7.8×10^5
$[Ru(NH_3)_6]^{2+}$	1.8×10^5	5.6 ± 1.0	-16 ± 3	7.2×10^4
$[Co(sep)]^{2+}$ [i]	9.6×10^2	9.6 ± 1.0	-13 ± 2	8.9×10^5
ascorbate				
HA^- [q]	3.4			0.62
A^{2-} [q]	2.1×10^4			5.1×10^5

[a] $k_{22} = 1\ M^{-1}\,s^{-1}$ (ref 38). [b] $E°_{11} = -0.26$ V (ref 39) and $k_{11} = 1 \times 10^8\ M^{-1}\,s^{-1}$ (ref 38 and 40). [c] Reference 36. [d] $E°_{11} = 0.12$ V (ref 41) and $k_{11} = 3 \times 10^4\ M^{-1}\,s^{-1}$ estimated from the cross reaction with $[Fe(cydta)]^-$ (ref 42). [e] Reference 7b. [f] Reference 43. [g] $E°_{11} = -0.01$ V (ref 44) and $k_{11} = 6 \times 10^4\ M^{-1}\,s^{-1}$ estimated from the data for the reaction of $[Ru(edta)L]^-$ with $[Ru(edta)(H_2O)]^{2-}$ (L = acetonitrile and isonicotinamide) taken from ref 45. [h] $E°_{11} = 0.050$ V (ref 19) and $k_{11} = 4 \times 10^3\ M^{-1}\,s^{-1}$ (ref 13). [i] In 0.05 M PIPES buffer and $I = 0.1$ M. [j] $E°_{11} = -0.30$ V and $k_{11} = 5.1\ M^{-1}\,s^{-1}$ (ref 14). [k] Reference 37. [l] At pH 7.2–8.6 (0.2 M Tris buffer) and $I = 0.3$ M (ref 11). [m] $E°_{11} = 0.70$ V (ref 46) and $k_{11} = 1 \times 10^5\ M^{-1}\,s^{-1}$ (ref 47). [n] $E°_{11} = 0.05$ V (ref 46) and $k_{11} = 2 \times 10^5\ M^{-1}\,s^{-1}$ (ref 47). [o] $k_{22} = 1 \times 10^4\ M^{-1}\,s^{-1}$. [p] Reference 7b. [q] At pH 7.2–8.6 (0.2 M Tris buffer) and $I = 0.3$ M (ref 7b).

There are few studies on the temperature dependence of reaction 4.[36a,48] Various values of $\Delta H°$ and $\Delta S°$ for reaction 4 are reported: 21.3 kcal mol^{-1} and 29 eu,[36a] 8.5 kcal mol^{-1} and −15 eu,[48a] and 4.9 kcal mol^{-1} and 6.3 eu,[48b] respectively. It has also been reported that the value of K_D is dependent of ionic strength.[48b] We used the values obtained by Chien and Dickinson at $I = 0.1$ M and pH 7 (phosphate buffer) to calculate the second-order rate constant.

Reductions of native metMb and the BrCN-modified metMb by $[Co(sep)]^{2+}$, $[Ru(NH_3)_6]^{2+}$, and $[Ru(edta)(H_2O)]^{2-}$ ions obey the second-order rate law:

$$-d[Fe(III)]/dt = k[Fe(III)][reductant] \quad (6)$$

The second-order rate constants at various temperatures and the activation parameters are listed in Tables II and III.

Reduction of reconstituted metmyoglobins (DPmetMb, DPDPmetMb, and DADPmetMb) by dithionite and $[Ru(NH_3)_6]^{2+}$ ions obey the rate laws eq 3 and eq 6, respectively. The second-order rate constants are listed in Table IV. The pK values for the acid dissociation equlibrium of the coordinated water of the reconstituted metMbs are 7.87, 7.80, and 9.40 at 25 °C for DPDPmetMb(H_2O), DADPmetMb(H_2O), and DPmetMb(H_2O), respectively.[7b] The aquomet form, therefore, is present in more than 94% under the conditions of these experiments.

The BrCN-modified metMb is reduced faster than the native form except for the $[Co(sep)]^{2+}$ system. The ratio of the second-order rate constants for reduction of the BrCN-modified metMb to that for the native metMb is in the range of 10^2–10^3. This arises mainly from the activation enthalpy change: $\Delta H^{\ddagger}$ for the BrCN-modified metMb is smaller than that for the native metMb. The absence of the difference in the kinetic parameters for the $[Co(sep)]^{2+}$ system is surprising. One of the reasons may be concerned with the large reorganizational barrier for the Co(II)–Co(III) system.

Table IV. Rate Constants for Reductions of Reconstituted Metmyoglobins at 25 °C and Calculated Rate Constants Based on the Marcus Theory

metmyoglobin	reductant	k_{12}(obsd), $M^{-1}\,s^{-1}$	k_{12}(calcd),[a] $M^{-1}\,s^{-1}$
DPmetMb(H_2O)[b]	SO_2^-	$(1.5 \pm 0.1) \times 10^6$	1.7×10^6
	$[Ru(NH_3)_6]^{2+}$	$(1.9 \pm 0.2) \times 10^2$	4.3×10
DADPmetMb(H_2O)[c]	SO_2^- [d]	$(6.1 \pm 0.9) \times 10^6$	1.2×10^7
	$[Ru(NH_3)_6]^{2+}$ [e]	$(1.0 \pm 0.1) \times 10^3$	5.0×10^2
DPDPmetMb(H_2O)[c,f]	SO_2^-	$(1.4 \pm 0.2) \times 10^7$	1.7×10^7
	$[Ru(NH_3)_6]^{2+}$	$(2.0 \pm 0.1) \times 10^3$	7.4×10^2

[a] $k_{22} = 1\ M^{-1}\,s^{-1}$ for the reconstituted myoglobin. [b] At pH 6.8 (0.1 M phosphate buffer) and $I = 0.2$ M. $\lambda = 500$ and 542 nm. [c] At pH 6.3 (0.1 M MES buffer) and $I = 0.1$ M. [d] $\lambda = 510$ and 562 nm. [e] $\lambda = 417$ and 447 nm. [f] $\lambda = 413$, 432, and 442 nm.

Self-Exchange Reactions of Metmyoglobins. The self-exchange reaction of the native Mb system is expected to be slow; therefore, it is not easy to measure the rate directly. The Marcus cross relation (eq 7 and 8) has been applied to a number of electron-

$$k_{12} = (k_{11}k_{22}f_{12}K_{12})^{1/2} \quad (7)$$

$$\ln f_{12} = (\ln K_{12})^2/4 \ln (k_{11}k_{22}/10^{22}) \quad (8)$$

transfer reactions of metalloproteins.[1] The agreement of the observed and calculated exchange rates is fairly good in the case of cytochrome c, whose heme group is located at the surface of the protein. Since the heme group of myoglobin is also located at the surface of the globin, the self-exchange rate constant for the metMb/deoxyMb system might be estimated by using eq 7 and 8. From a number of redox couples including metal complexes, organic dyes, inorganic radicals, and electron-transfer proteins, the value of 1 $M^{-1}\,s^{-1}$ at 25 °C has been reported for the rate constant of the self-exchange reaction of the native metMb/deoxyMb system.[38] Work terms are neglected in the

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present case. The calculated second-order rate constants are in good agreement with those observed (Tables III and IV) using literature values for the self-exchange rate constants and the redox potentials of the reductants. The self-exchange rate constant obtained for the BrCN-modified Mb system is 10^4 M^{-1} s^{-1}, which is much higher than that for the native Mb system. The self-exchange rate constant for the reconstituted metMb's (DPmetMb(H_2O), DADPmetMb(H_2O), or DPDPmetMb(H_2O)) was estimated to be about 1 M^{-1} s^{-1} which is same as that for the native system (Table IV). These reconstituted metMb's and the native metMb have coordinated water molecules and hexacoordinated geometries. So the geometry change must occur upon reduction accompanied by dissociation of the coordinated water. On the other hand, the geometry of the BrCN-modified metMb is pentacoordinate and the reduction does not require the dissociation of the coordinated water molecule. To confirm further the slowness of the self-exchange reaction of the reconstituted metMb(H_2O), a preliminary experiment was done on the reaction of DADPmetMb(H_2O) with native deoxyMb. The reaction was followed at 543 nm under the experimental conditions: [native deoxyMb]$_0$ = 2.0 × 10^{-4} M and [DADPmetMb(H_2O)]$_0$ = 5.0 × 10^{-5} M at 25 °C, pH 6.3 (0.1 M MES buffer), and I = 0.1 M. The first-order plot was almost linear (~80%) and the second-order rate constant was ~0.9 M^{-1} s^{-1}: This value is not inconsistent with the calculated rate constant (7 M^{-1} s^{-1}) on the basis of eq 7 and 8.

Intrinsic parameters (ΔH^*_{22} and ΔS^*_{22}) for the reductions of metMbs can be estimated on the basis of the Marcus equations for free energy changes:[49]

$$\Delta G^*_{12} = (\Delta G^*_{11} + \Delta G^*_{22})/2 + \Delta G^\circ_{12}(1 + \alpha)/2 \quad (9)$$

$$\alpha = \Delta G^\circ_{12}/4(\Delta G^*_{11} + \Delta G^*_{22}) \quad (10)$$

$$\Delta H^*_{22} = [2\Delta H^*_{12} - \Delta H^\circ_{12}(1 + 2\alpha)]/(1 - 4\alpha^2) - \Delta H^*_{11} \quad (11)$$

$$\Delta S^*_{22} = [2\Delta S^*_{12} - \Delta S^\circ_{12}(1 + 2\alpha)]/(1 - 4\alpha^2) - \Delta S^*_{11} \quad (12)$$

where the Franck–Condon barrier, $\Delta G^* = \Delta G^\ddagger + RT \ln (hZ/k_BT)$, $\Delta H^* = \Delta H^\ddagger + {}^1/_2RT$, and $\Delta S^* = \Delta S^\ddagger - R \ln (hZ/k_BT) + {}^1/_2R$. In this calculation the data for reductions by $[Ru(NH_3)_6]^{2+}$ ions were used, because the kinetic and thermodynamic parameters for the self-exchange reaction of the $[Ru(NH_3)_6]^{3+/2+}$ system are well known (E° = 0.050 V at 25 °C, $\Delta H^\ddagger_{11}$ = 10.3 kcal mol^{-1}, $\Delta S^\ddagger_{11}$ = −11 eu,[13] ΔH°_{11} = −1.9 kcal mol^{-1}, and ΔS°_{11} = −2.6 eu). The intrinsic parameters, ΔH^*_{22} and ΔS^*_{22}, obtained for the native metMb(H_2O)–$[Ru(NH_3)_6]^{2+}$ system are 20 kcal mol^{-1} and 20 eu, respectively. The ΔH^*_{22} value of 20 kcal mol^{-1} is very similar to that estimated for the intramolecular electron-transfer reaction of the ruthenium-modified myoglobin.[3b] On the other hand, the values of ΔH^*_{22} and ΔS^*_{22} are 8 kcal mol^{-1} and −3 eu respectively, for the BrCN-modified metMb–$[Ru(NH_3)_6]^{2+}$ system. These data clearly show that the enthalpic reorganizational barrier for the reduction rate is a major factor in the self-exchange reaction of myoglobins compared with the entropy terms. Therefore, the entropy term is unimportant for the Franck–Condon activation in myoglobin. The enthalpic reorganizational barrier may be expressed as the sum of the solvent, $(\Delta H^*_{22})_o$, and inner sphere, $(\Delta H^*_{22})_i$, contributions (eq 13). If

$$\Delta H^*_{22} = (\Delta H^*_{22})_o + (\Delta H^*_{22})_i \quad (13)$$

the solvent reorganizational barrier for myoglobin is comparable with that for cytochrome c (2–5 kcal mol^{-1}),[1,50] the inner-sphere reorganizational contribution, $(\Delta H^*_{22})_i$, is ~15 kcal mol^{-1}, which is much larger than that for the BrCN-modified myoglobin (~3 kcal mol^{-1}). The relatively large reorganizational barrier can be explained by the movement of the heme iron, which undergoes a change in coordination number including Fe–OH_2 bond rupture. This geometry change must induce the conformational change of the globin, but the latter may not be rate determining. It has been reported that the conformational change of metMb is quite fast.[51] Recent work on the long-range electron transfer in ruthenium-modified myoglobin has shown that the electron transfer is reversible and is not controlled by conformational interconversion.[52] We can conclude that the geometry change upon reduction accompanied by a water dissociation restricts the electron-exchange process of native metMb(H_2O).

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Supplementary Material Available: Tables giving results of redox titrations with $[Ru(NH_3)_6]^{2+}$ of BrCN-modified metMb and reconstituted metMb(H_2O) and kinetic data for reductions of metmyoglobins (7 pages). Ordering information is given on any current masthead page.

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