Facilitated Photoreduction of Ruthenium Bis(4′-methylterpyridine) Pendant Myoglobin

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(Received December 4, 1995)

A synthetic heme bearing ruthenium bis(terpyridine) through a covalent bond was successfully prepared. Ruthenium bis(terpyridine) pendant myoglobin, which was synthesized by a conventional cofactor reconstitution method, was efficiently activated through a photoinduced electron-transfer reaction from the ruthenium complex to the heme center. No reaction occurred in the corresponding intermolecular reaction system.

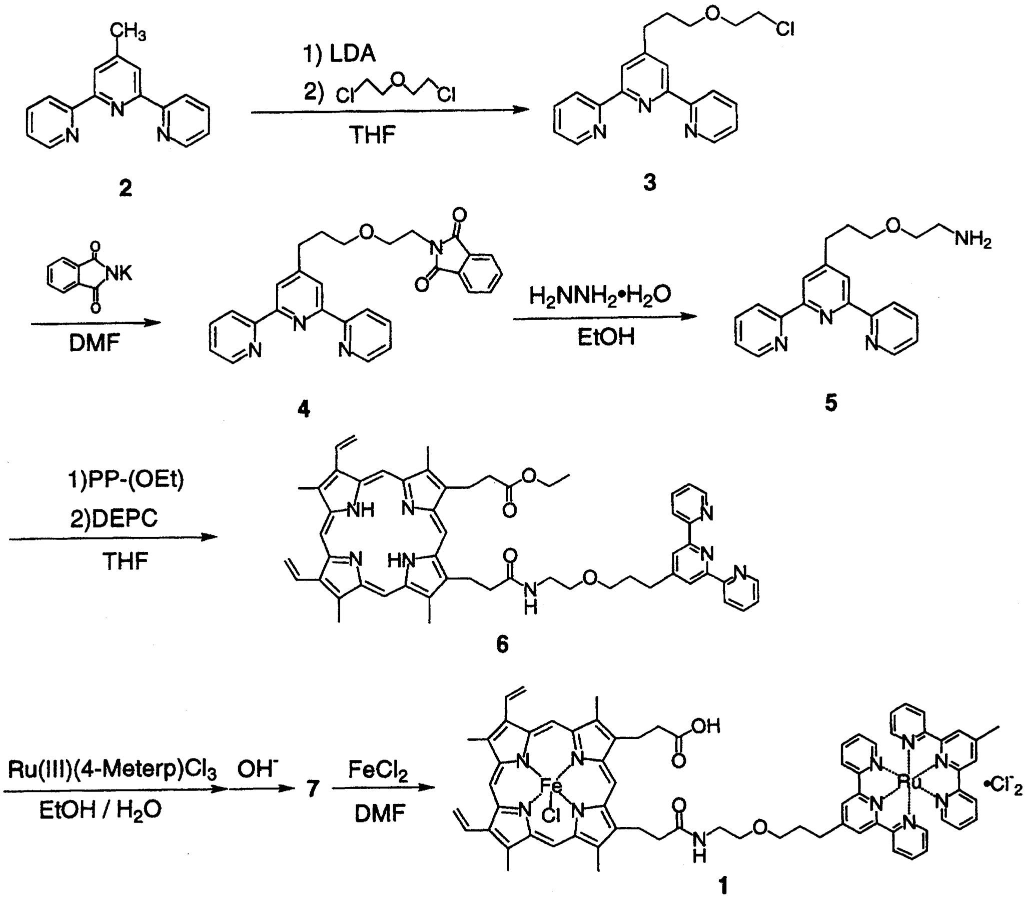
Artificially engineered biomacromolecules, activities of which can be regulated by light, are expected to be novel nanometer-sized biomaterials with high potentials in the diverse field of biotechnology. As successful examples, Willner and co-workers developed new-photoenzymes modified with photochromic molecules.[1] By using a cofactor reconstitution method, we also synthesized ruthenium tris(bipyridine) pendant myoglobin which was effectively activated through visible light irradiation.[2] The recent report of Willner on photo-reductase activity of cobalt-substituted myoglobin that was prepared according to our proposal, strongly supported the broad validity of our methodology.[3] With the aim of establishment of design strategy for photoswitchable proteins, we are now exploring photoactivation mechanisms of myoglobin, as well as the optimization of the photosensitizer and the connecting spacer. We describe herein the facilitated photoreaction process of the newly synthesized ruthenium bis(terpyridine)-pendant-myoglobin, wherein the rate-determining step shifted from the reduction of ruthenium(III) by EDTA to the other.

Results and Discussion

Synthesis and Reconstitution of Ruthenium Bisterpyridine-Pendant Myoglobin. The heme **1** covalently attaching ruthenium bis(terpyridine) was synthesized as shown in Scheme 1.[4] Lithiated 4′-methyl-2,2′ : 6′,2″-terpyridine-(4′Me-terpy) **2** was alkylated with bis(2-chloroethyl)ether, followed by Gabriel synthesis to give 4′-(6-amino-4-oxahexyl)terpyridine **5**. The amine derivative **5** was condensed with protoporphyrin IV monoethyl ester in the presence of diethylcyanophosphate (DEPC). The heme **6** thus obtained was complexed at its terpyridine site with mono(4′-methylterpy) ruthenium(III) and hydrolyzed with potassium hydroxide. Subsequent iron-insertion yielded the product **1** after purification through gel-column-chromatography (Sephadex LH-20 (CH_3CN). All new compounds were characterized by ^{1}H NMR, IR, UV-visible spectroscopies and elemental analysis. Regioisomers were not separated in this study.

Incorporation of the heme **1** into apomyoglobin (from horse heart, Sigma) was conducted by a conventional reconstitution method reported previously.[5] The reconstituted myoglobin bearing ruthenium bis(terpy) (Mb-Ru(terpy)$_2$) was characterized by axial-ligand exchange reactions (from H_2O to fluoride and azide) and reduction–oxygenation reaction. Their absorption spectra were almost identical with those of native Mb, with the exception of a distinct shoulder at 480 nm due to the metal-to-ligand charge transfer (MLCT) band and two peaks at 290 and 310 nm due to the π–π^* transition of terpyridine of ruthenium bis(terpyridine).[6] A strong band at 290 nm prevents us from evaluating the purity of the reconstituted Mb.

Photoreduction of Ru(terpy)$_2$-Mb under Anaerobic Conditions. Figure 1a shows an absorption spectral change of met-Mb-Ru(terpy)$_2$ under visible light irradiation (450 W high-pressure mercury lamp, wavelength cutoff below 450 nm) in the presence of sacrificial donor EDTA under Ar atmosphere.[7] A strong Soret band a 408 nm and a weak Q-band at 630 nm due to met-Mb (ferric heme state) are lessened and a sharp Soret band at 434 nm and a broad Q-band at 555 nm due to deoxy-Mb (ferrous heme state) are intensified with four isosbestic points at 420, 462, 523, and 606 nm. As shown in Fig. 1b, the peak of deoxy-Mb increases accompanying with the decrease of the peak of met-Mb, both of which obey first-order kinetics with respect to myoglobin concentration. The photo-generated deoxy-Mb spontaneously binds dioxygen to form a stable dioxygen complex (oxy-Mb). The reduction does not occur without light. These data clearly shows that one-electron reduction of the heme active center of Mb smoothly occurs by visible-light irradiation. More interestingly, no reaction proceeds in the simple mixture of native Mb and Ru(terpy)$_2$ (i.e., the corresponding intermolecular reaction), indicating that the proximity between a photosensitizable Ru(terpy)$_2$ and Mb through a covalent bond is essentially important for such a



Scheme 1.

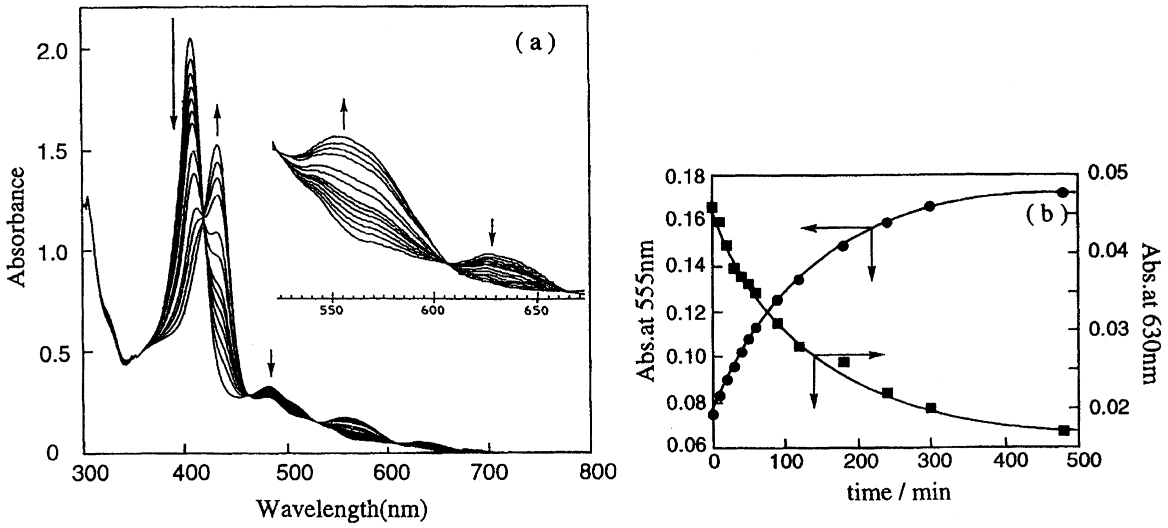


Fig. 1. (a) Absorption spectral change from Ru(terpy)$_2$-met-Mb by visible light under Ar atmosphere, (b) Time courses at 555 nm and 630 nm: Ru(terpy)$_2$-met-Mb 12 μM, EDTA 20 mM at pH 6.3, 25 °C.

facilitated photo-activation of Mb.

Mechanism of Photoreduction of Ru(terpy)$_2$-Mb. We examined the photoactivation process in detail by changing various conditions of the photoreaction. When we changed the concentration of EDTA, we observed a saturation behavior of initial rates (v_{init}) of photoreduction regarding to EDTA. Figure 2 shows the dependence of the v_{init} on EDTA concentration. At low EDTA concentrations (i.e., below 10 mM), v_{init} linearly increases, indicating that the reaction is dependent on the first order of EDTA. On the other hand, v_{init} is found to be almost independent of EDTA at its higher concentrations (i.e., above 10 mM).The present biphasic behavior suggests that the rate-determining step shifted from the process where the oxidized Ru(III) complex is reduced

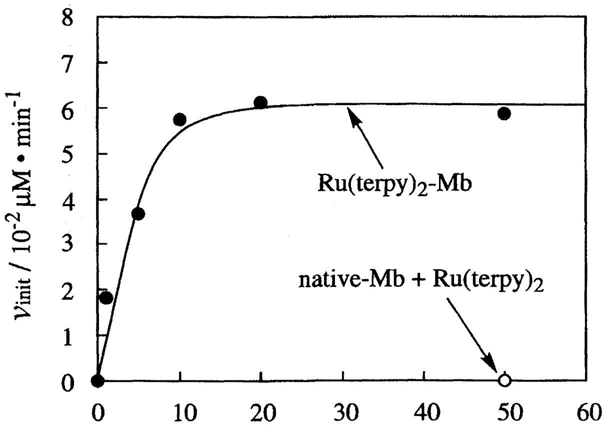


Fig. 2. EDTA dependence of photo-reduction of met-Mb: (●) Ru(terpy)$_2$-Mb, (○) intermolecular mixture of native Mb (12 μM) and Ru(Me-terpy)$_2$ (12 μM), pH 6.3, 25 °C.

by EDTA to another process where EDTA is not involved.

We next investigated pH dependence of the photoreduction of Ru(terpy)$_2$-Mb. As shown in Fig. 3, a bell-shaped curve of $v_{\rm init}$ having a maximum value at pH 6.3 was observed. Below pH 6, $v_{\rm init}$ was drastically lessened, and the rate also slowed down with pH increasing from 6.5 to 9.0. In the acidic pH range (below pH 6.0), a protonated EDTA is a dominant species, the electron donation ability of which is greatly suppressed because of loss of a lone-pair electron as a reduction source of EDTA. At higher pH (above pH 7.0), the absorption spectra of Ru(terpy)$_2$-Mb gradually changes from an aqua-met-Mb state where a coordinating axial ligand is H_2O, to a hydroxy-met-Mb state where the ligand is hydroxide anion (OH^-). The rate suppression may be ascribed to an alteration of the axial ligand of the iron heme center.

In order to confirm the axial ligand effect, we used ligand-exchanged Mbs bearing fluoride and cyanide ligand instead of H_2O. When KCN was added to the met-Mb-Ru(terpy)$_2$, the axial ligand of the heme center of Mb exchanged from H_2O to cyanide (CN^-). With increasing amount of KCN, all of the aqua-met-Mb were converted to the cyanide-ligated Mb. Consequently, the photoreduction was suppressed and finally ceased at excess amount of KCN (see Fig. 4). In the presence of fluoride anion (KF), similar suppression of the photoreduction occurred. At large excess of KF (100 mM), the reduction completely stopped. The less efficient inhibition by fluoride than that by cyanide is due to the lower binding constant of fluoride to met-Mb ($K = 40$ M^{-1} in fluoride, $K = 10^5$ M^{-1} in cyanide).8) In both cases, it was clear that the photoreduction can take place only in the form of the H_2O-coordinating Mb, but cannot in the form of the cyanide- or fluoride-coordinating Mbs.9) Such inhibition effect by cyanide and fluoride anions demonstrates that the ligand releasing process from the reduced iron(II) heme is one of the key steps in the net photoreduction of Mb. Hawkridge and co-workers also eletrochemically proposed that the axial ligand release is an essential process during the reduction of met-Mb.10) In contrast with such a thermal reduction, however, the present photoreductions contain the back electron transfer process from iron(II) heme to Ru(III)(terpy)$_2$, which may compete with the ligand releasing process. This may be a reason why the strongly-coordinating ligands *completely* inhibit the met-Mb reduction.11)

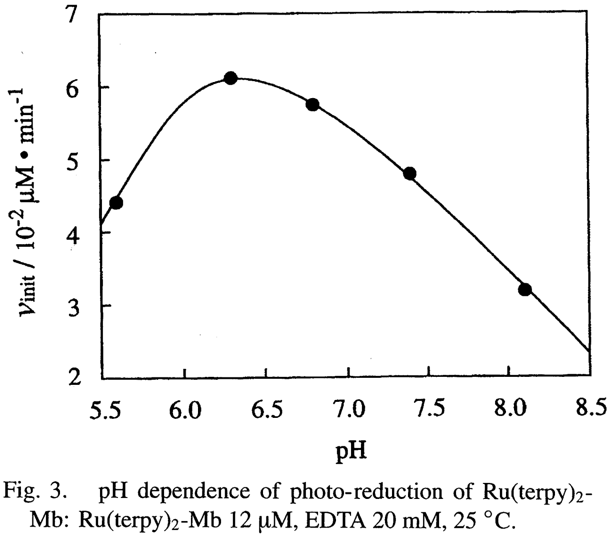


Fig. 3. pH dependence of photo-reduction of Ru(terpy)$_2$-Mb: Ru(terpy)$_2$-Mb 12 μM, EDTA 20 mM, 25 °C.

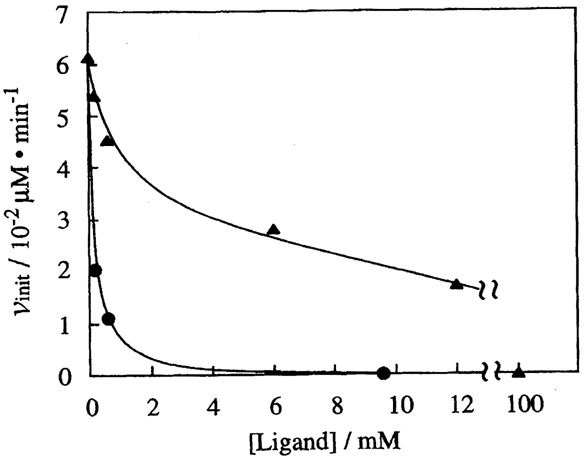


Fig. 4. Dependence of photoreduction rates on the concentrations of anion ligand: (●) KCN, (▲) KF, Ru(terpy)$_2$-Mb 12 μM, EDTA 20 mM, pH 6.3, 25 °C.

In summary, we depicted the overall photoactivation of the newly synthesized Mb bearing ruthenium bis(terpyridine) as shown in Fig. 5. Clearly, after the photoinduced electron-transfer step from the excited Ru$^{\rm II}$ to heme iron(III), there are main two process, that are the reduction of Ru(III) complex by EDTA and the release of coordinating H_2O from iron(II) heme in our photoactivatable Mb.

Experimental

Materials. 4′-Methyl-2,2′:6′2″-terpyridine (4-Meterpy) **2**,12) ruthenium(III)/mono-4-Meterpy complex (Ru(III)•(4-Meterpy)•Cl_3),13) and protoporphyrin monoethyl ester (PP–(OEt))14) were synthesized according to the literature methods. Other chemicals were used without further purification. Reconstitution of Ru(terpy)$_2$-Mb was conducted according to the method reported previously by us.5)

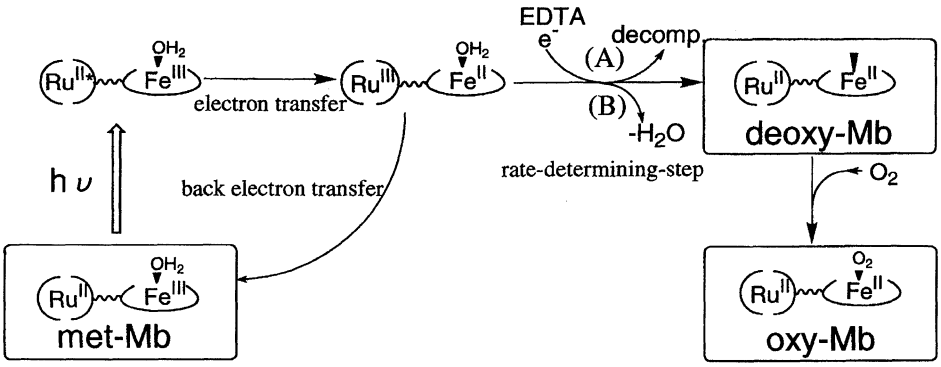


Fig. 5. Scheme of the photo-activation reaction of Ru(terpy)$_2$-Mb.

Synthesis. 4′-(6-Amino-4-oxahexyl)-2,2′ : 6′,2″-terpyridine 5. Into a solution of 4-Meterpy (1.4 g, 5.7 mmol) dissolved in 150 mL of dry THF (tetrahydrofuran, distilled over NaH), 2.0 M LDA in THF (1 M = 1 mol dm^{-3}) (lithium diisopropylamide, 3.5 mL, 8.0 mmol) was added dropwise for 15 min below 0 °C. After addition of bis(2-chloroethyl) ether (6.6 mL, 57 mmol) at 0 °C, the mixture was stirred at room temp. under N_2 atmosphere for 2 d. The reaction was quenched by 20 mL of saturated NaCl aq and it was extracted with $CHCl_3$ (100 mL×1, 20 mL×2). The organic layer was dried over Na_2SO_4 and concentrated. Excess amount of bis(2-chloroethyl) ether was removed by distillation under reduced pressure (50 °C/0.06 Torr, 1 Torr = 133.322 Pa) to give the crude 4′-(6-chloro-4-oxahexyl)-2,2′ : 6′,2″-terpyridine **3**. The chloro derivative **3** (1.82 g, 3.9 mmol) and potassium phthalimide (1.05 g, 11.4 mmol) were dissolved in 30 mL-dry DMF (*N*,*N*-dimethylformamide, distilled over NaH). After the reaction mixture was stirred at 80 °C for 36 h under N_2 atmosphere, the reaction was stopped by addition of H_2O (30 mL). The aqueous layer was extracted with $CHCl_3$ (20 mL×3) and the combined organic layer was washed with 0.2 M-NaOH aq (15 mL), and with H_2O (15 mL). The organic layer was evaporated and the oily residue was purified through chromatography (neutral alumina, ϕ = 5 cm×20 cm, eluent : hexane : ethyl acetate = 3 : 1 (v : v)) to obtain the phthalimide derivative **4**, which was further purified with recrystallization from EtOH. 0.61 g (20% yield from 4-Meterpy). Mp = 118 °C, ^{1}H NMR ($CDCl_3$, 250 MHz) δ = 2.00 (2H, m, $-CH_2-$), 2.81 (2H, t, Ar–CH_2–), 3.53 (2H, t, O–CH_2–), 3.70 (2H, t, O–CH_2–), 3.92 (2H, t, N–CH_2–), 7.84 (4H, m, phthalimide Ar–H), 7.33, 7.69, 8.27, 8.60, 8.69 (2H, each, m, m, s, m, m, terpyridine Ar–H).

The above-obtained compound **4**, (0.50 g, 1.1 mmol) and hydrazine monohydrate (0.5 mL, 10.6 mmol) were dissolved in EtOH (30 mL) and the mixture was refluxed for 4 h. After cooling, it was poured into 100 mL of saturated NaCl aq solution. The aqueous solution was made basic (pH 12) and extracted with CH_2Cl_2 (50 mL×3). The organic layer was dried over Na_2SO_4 and evaporated in vacuo to give pale yellow oil **5**, (0.34 g, 94%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ = 2.07 (2H, m, $-CH_2-$), 2.87 (4H, m, Ar–CH_2– and N–CH_2–), 3.48 (4H, m, O–CH_2–), 7.33, 7.85, 8.33, 8.62, 8.69 (2H each, m, m, s, m, m, Ar–H).

3,8,12,18-Tetramethyl-2,7-divinyl-17-[2-(ethoxycarbonyl)ethyl]-13-[2- (*N*-{2-[3-(2,2′ : 6′,2″-terpyridin- 4′-yl)propoxy]ethyl}carbamoyl)ethyl]porphine 6. To a dry THF solution containing the amino derivative of terpyridine **5** (0.33 g, 1 mmol), PP–(OEt), (0.49 g, 0.83 mmol) and triethylamine (0.24 mL, 1.7 mmol), was added diethylcyanophosphate (DEPC, 0.27 g, 1.7 mmol) with ice-cooling. The reaction mixture was stirred for 24 h at room temperature under N_2 atmosphere. THF was evaporated off and the residue dissolved in $CHCl_3$ was washed with 5%-$NaHCO_3$ aq (20 mL×2), dried over $MgSO_4$ and concentrated in vacuo. The oily residue was purified by reprecipitation ($CHCl_3$/MeOH), followed by washing with MeOH to give the dark purple solid **6** (0.56 g, 74%). IR (KBr, cm^{-1}) 3300 (ν_{NH}), 1730 (ν_{CO}), 1650 (ν_{CO}). ^{1}H NMR ($CDCl_3$, 250 MHz) δ = −3.92 (2H, s, NH), 0.64 (2H, m, Ar–CH_2–), 7.02 (3H, t, $-CH_3$), 2.09 (2H, m, $-CH_2-$), 2.83 (2H, m, $-CH_2-$), 3.15 (4H, m, $-CH_2-O-CH_2-$), 3.18 (4H each, m, 13, 14, CH_2), 3.40—3.68 (12H, s, each, 2, 7, 12, 18, CH_3) 4.02 (2H, q, CH_2), 4.23, 4.39 (2H each, t, each, 13′, 17′, CH_2) 6, 13, 6, 31 (2H each, d each, 3.8, $=CH_2$), 6.66 (1H, m, NH) 7.08, 7.15, 7.64, 8.22, 8.40 (2H each, m, s, m, m, m, Ar–H), 8.17 (2H, m, –CH=) 9.92—10.08 (4H, m, *meso*-H).

{4′- [3- (2- {2- [3, 8, 12, 18- Tetramethyl- 2, 7- divinyl- 17- (2-carboxyethyl)porphine-13-yl]ethylcarbonylamino}ethoxy)propyl]-2,2′ : 6′,2″-terpyridin} (4′-methyl-2,2′ : 6′,2″-terpyridine)-ruthenium(II)·Dichloride 7. The preceding compound **6** (0.30 g, 0.33 mmol) and Ru(III)(4-Meterpy)·Cl_3 (0.15 g, 0.33 mmol) dissolved in EtOH–H_2O (50 mL–5 mL) were refluxed for 3 h under N_2 atmosphere. Thirty milligrams of Ru(III)(4-Meterpy)·Cl_3 was added again and the reflux was continued for more 3 h. Insoluble impurities were removed by hot-filtration and the filtrate was concentrated to obtain dark-red solid, which was hydrolyzed in aqueous methanol (MeOH : H_2O = 1 : 1, (v : v)) containing, 0.7 mL of 1 M-NaOHaq. After acidification followed by concentration, the residue was purified through gel chromatography (Toyo-pearl HW-50 F, eluent MeOH). The first fraction was collected, dried in vacuo, and then a water-insoluble staff was filtered off to yield reddish purple solid **7** (0.15 g, 37%). IR (KBr, cm^{-1}) 3300 (ν_{NH}), 1710 (ν_{CO}), 1650 (ν_{NH}). Found: C, 60.59, H, 4.88, N, 10.86%. Calcd for $C_{70}H_{67}N_{11}O_4Cl_2Ru\cdot 4H_2O$: C, 60.39, H, 5.35, N, 11.07%.

Iron(II) Complex 1 Derived from the Ruthenium(II) Complex 7. The free base **7** (150 mg, 0.12 mmol) and $FeCl_2\cdot 4H_2O$ (229 mg, 1.2 mmol) dissolved in 20 mL of dry DMF was heated at 65 °C with stirring for 6 h under N_2 atmosphere. DMF was evaporated off and an acidic brine (pH 3, 50 mL) was added to the residual solid. The supernatant was removed and the precipitated solid was purified through gel chromatography (Sephadex LH-20, eluent: acetonitrile) to yield a black product **1** (130 mg, 81%). IR (KBr, cm^{-1}) 1710 (ν_{CO}), 1650 (ν_{CO}). UV-visible spectrum (methanol, nm) 271, 305, 396 (Soret), 480, 627. Found: C, 51.90, H, 4.29, N, 9.34%. Calcd for $C_{70}H_{65}N_{11}O_{12}Cl_{13}RuFe\cdot 2NaCl$: C, 51.43, H, 4.13, N, 9.42%.

Photoreduction of Ru(terpy)$_2$-Mb. 3 mL of the aqueous solution of Ru(terpy)$_2$-Mb in a specially prepared quartz cell was deaerated through a normal freeze-pump-and-thaw technique. Photoirradiation was conducted by a high-pressure Hg lamp with a filter of wavelength cut off below 450 nm in a thermostat.

We thank Mr. Hideki Horiuchi for his skillful preparation of special quartz cells for UV-visible spectroscopy. This research was partially supported by the Foundation Advanced Technology Institute.

References

1) a) I. Willner, S. Rubin, and A. Riklin, *J. Am. Chem. Soc.*, **113**, 3321 (1991); b) I. Willner, S. Rubin, J. Wonner, F. Effenberger, and P. Bäuerle, *J. Am. Chem. Soc.*, **114**, 3150 (1992); c) I. Willner, S. Rubin, and Y. Cohen, *J. Am. Chem. Soc.*, **115**, 4937 (1993); d) I. Willner and E. Zahavy, *Angew. Chem., Int. Ed. Engl.*, **33**, 581 (1994).

2) a) I. Hamachi, S. Tanaka, and S. Shinkai, *Chem. Lett.*, **1993**, 1417; b) I. Hamachi, S. Tanaka, and S. Shinkai, *J. Am. Chem. Soc.*, **115**, 10458 (1993).

3) I. Willner, E. Zahavy, and V. H-Shabtai, *J. Am. Chem. Soc.*, **117**, 542 (1995).

4) Compared to Ru tris(bipyridine)-heme which was previously prepared by us, the present Ru bis(terpyridine)-heme derivative is expected to be conveniently modified with controlled sites and numbers because stereogenic problems of 2,2′-bipyridine become simple in the terpyridine systems. Such functionallized terpyridine derivatives are now synthesized under progress.

5) a) A. W. Axup, M. Albin, S. L. Mayo, R. J. Crutchley, and H. B. Gray, *J. Am. Chem. Soc.*, **110**, 435 (1988); b) I. Hamachi, K. Nakamura, A. Fujita, and T. Kunitake, *J. Am. Chem. Soc.*, **115**, 4966 (1993); c) I. Hamachi, Y. Tajiri, and S. Shinkai, *J. Am. Chem. Soc.*, **116**, 7437 (1994).

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

7) A half-life time of the generated oxy-Mb was estimated to be 2.8 h.

8) R. P. Cox and M. R. Hollaway, *Eur. J. Biochem.*, **74**, 575 (1977).

9) The redox potential change by the axial ligand alteration is also considered to be one of the important factor.

10) B. C. King, F. M. Hawkridge, and B. M. Hoffman, *J. Am. Chem. Soc.*, **114**, 10603 (1992).

11) In addition to this steady-state photoreaction, detailed laser-flash photolysis studies both in Ru(bpy)$_3$-Mb and Ru(terpy)$_2$-Mb are now under way in our laboratory.

12) K. T. Potts, D. A. Usifer, Guadalupe, and H. D. Abruna, *J. Am. Chem. Soc.*, **109**, 3961 (1987).

13) P. A. Adcock, F. R. Keene, R. S. Smythe, and M. R. Snow, *Inorg. Chem.*, **23**, 2336 (1984).

14) E. Tsuchida, H. Nishide, Y. Sato, and M. Kaneda, *Bull. Chem. Soc. Jpn.*, **55**, 1890 (1982).