Artificial Protein−Protein Complexation between a Reconstituted Myoglobin and Cytochrome *c*

Takashi Hayashi,*,† Yutaka Hitomi, and Hisanobu Ogoshi*,‡

Contribution from the Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Received July 17, 1997

Abstract: Artificial prosthetic porphyrins, **1·Fe** and **1·Zn**, in which two isophthalamide units having four carboxylates were bound to the terminal of each peripheral propionate side chain in protoporphyrin IX, were inserted into horse heart apomyoglobin to give novel myoglobins, rMb(**1·Fe**) and rMb(**1·Zn**), respectively. The resultant reconstituted myoglobins were designed to bind cationic cytochrome *c* on the protein surface via electrostatic interaction. The isoelectric point for rMb(**1·Fe**) was determined to be 5.5, which is about 2 pH units lower than that of native myoglobin. The p*I* value suggests that eight carboxylates of prosthetic group are located on the surface of the myoglobin. A construction of a myoglobin−cytochrome *c* complex was probed by paramagnetic ^{1}H NMR and flash photolysis studies. The behavior of ^{1}H NMR paramagnetic shifts in the rMb(**1·FeCN**)−cytochrome *c* complex is comparable with that in the native pairing of cytochrome *c*−cytochrome *c* peroxidase. Laser flash photolysis shows that a long-range ET from photoexcited rMb(**1·Zn**) to cytochrome *c* occurs within the protein−protein complex. The time-dependence of the transient spectra at 460 nm identified as the triplet excited state of rMb(**1·Zn**) leads to rate constant of forward ET and affinity of the protein−protein complex; $k_{intra} = (2.2 \pm 0.1) \times 10^3$ s^{-1} and $K_a = (6.5 \pm 3.0) \times 10^4$ M^{-1} at 10 mM ionic strength and $k_{intra} = (2.3 \pm 0.2) \times 10^3$ s^{-1} and $K_a = (1.5 \pm 0.6) \times 10^4$ M^{-1} at 20 mM ionic strength and pH 7.0. The binding affinity for cytochrome *c* decreases with increasing the ionic strength, indicating that the protein−protein complex is formed by electrostatic interaction. This work demonstrates that the artificial functional groups bound to the terminal of porphyrin in the reconstituted myoglobin can act as an effective recognition domain for a protein at the surface of the myoglobin.

Introduction

Protein−protein recognition at a special binding domain plays an essential role on the formation of organized system to initiate a biological function. To clarify the binding mechanism, a number of synthetic host−guest models have been reported, some of them giving a valuable insight into the nature of molecular recognition via intermolecular weak interactions.[1] The next stage in this study should be the construction of supramolecular assemblies to understand the highly ordered biological systems.[2−5] We have recently reported a new myoglobin reconstituted with a chemically modified prosthetic group having multiple substituted carboxylates at the terminal of two propionates to give a novel complex between the myoglobin and cationic methyl viologen.[6] In this system, we found photoinduced singlet electron transfer (ET) in the myoglobin−methyl viologen complex via anionic binding site on the protein surface.

Here, we report a new architecture of a stable complex between cytochrome *c* and a reconstituted myoglobin, in which the prosthetic porphyrins **1·Fe** or **1·Zn** possesses a specific interaction domain for the basic patch of cytochrome *c* (Scheme 1).[7,8] The present nonmutagenic approach by the combination of chemical modification of heme and reconstitution technique can be exploited in the design of the assembly of proteins to evaluate the importance of protein−protein binding, such as cytochrome *c*−cytochrome *c* peroxidase or cytochrome *c*−cytochrome *c* oxidase, in the ET process.

Results and Discussion

Preparation and Characterization of Reconstituted Myoglobin. The synthetic routes to the metalloporphyrins **1·Fe** and **1·Zn** are shown in Scheme 2. Bis(*p*-nitrophenyl)ester **5** prepared from protoporphyrin IX (**2**) and *p*-nitrophenyl trifluoroacetate was coupled with 5-aminoisophthalic acid derivative **4** having four ethoxycarbonyl groups to allow the precursor **6**

† Current address: Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan.

‡ Current address: Fukui National College of Technology, Sabae, Fukui 916-8507, Japan.

(1) For recent reviews, see: Hamilton, A. D., Ed.; Molecular Recognition (Tetrahedron Symposia No. 56). *Tetrahedron* **1995**, 51.

(2) Vögtle, F. *Supramolecular Chemistry*; Wiley: Chichester, 1991.

(3) Balzani, V.; Scandola, F. *Supramolecular Photochemistry*; Ellis Horwood: Chichester, 1991.

(4) Lehn, J.-M. *Supramolecular Chemistry*; VCH: Weinheim, 1995.

(5) Sessler, J. L.; Wang, B.; Springs, S. L.; Brown, C. T. In *Comprehensive Supramolecular Chemistry*; Murakami, Y., Ed.; Pergamon: Oxford, 1996; Vol. 2, pp 311−336.

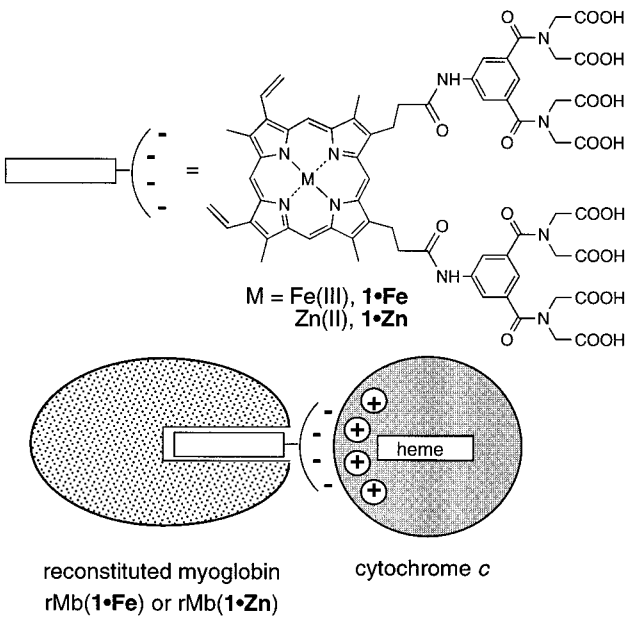
(6) (a) Hayashi, T.; Takimura, T.; Ogoshi, H. *J. Am. Chem. Soc.* **1995**, *117*, 11606−11607. (b) Hayashi, T.; Ogoshi, H. *Chem. Soc. Rev.* **1997**, *26*, 355−364.

(7) For recent examples of artificial assembly system of hemoprotein, cf.: (a) Zhou, J. S.; Granada, E. S. V.; Leontis, N. B.; Rodgers, M. A. J. *J. Am. Chem. Soc.* **1990**, *112*, 5074−5080. (b) Lahiri, J.; Fate, G. D.; Ungashe, S. B.; Groves, J. T. *J. Am. Chem. Soc.* **1996**, *118*, 2347−2358. (c) Tsukahara, K.; Kimura, C. *J. Electroanal. Chem.* **1997**, *438*, 67−70. (d) Hamuro, Y.; Calama, M. C.; Park, H. S.; Hamilton, A. D. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2680−2683.

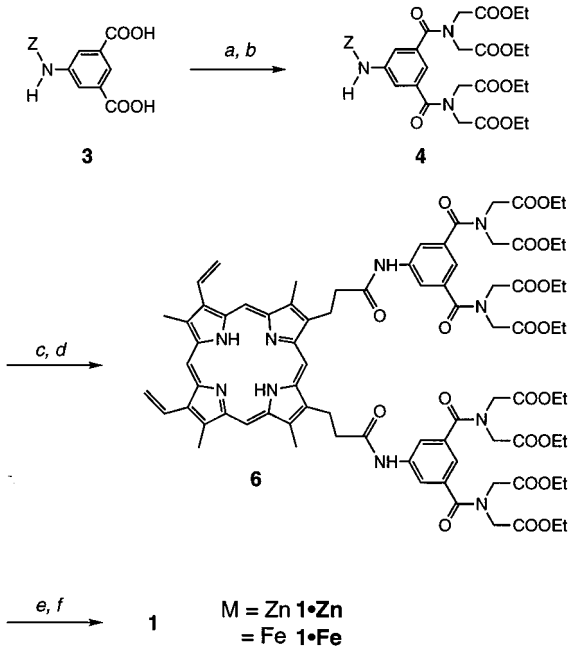
(8) The myoglobins reconstituted with artificial prosthetic groups **1·Fe** and **1·Zn** based on native protoporphyrin (**2**) have improved in their stability compared to our previous reconstituted myoglobin.[6a]

S0002-7863(97)02395-0 CCC: $15.00
Published on Web 05/12/1998

Scheme 1



Scheme 2[a]



[a] Key: (a) $SOCl_2$, (b) $TsOH{\cdot}HN(CH_2CO_2Et)_2/Et_3N$, (c) HBr/AcOH, (d) protoporphyrin bis(*p*-nitrophenyl)ester (**5**)/pyridine, (e) $FeCl_2$ or $Zn(OAc)_2$, (f) KOH/THF/MeOH.

in 94% yield.[9] Metalation of **6** and the following basic hydrolysis proceeded smoothly to give the desired iron and zinc porphyrins **1·Fe** and **1·Zn** possessing eight carboxylic acids at the terminal of two peripheral propionate side chains. These metalloporphyrins were readily inserted into horse heart apomyoglobin by usual method to give the reconstituted metmyoglobin rMb(**1·Fe**) and zinc myoglobin rMb(**1·Zn**), respectively.[6a,10,11]

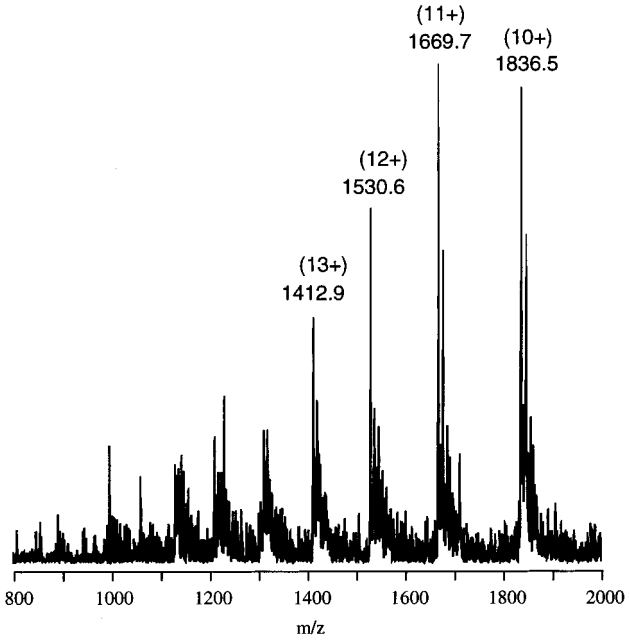


Figure 1. Electrospray-ionization mass spectrum of rMb(**1·Fe**) at pH 7.0. The peaks are labeled with the protonation state, *n*+, with the molecular masses determinated from the measured *m*/*z* values.

The electronic absorption spectra of rMb(**1·Fe**) and rMb(**1·Zn**) were identical to those of reference myoglobins, native metmyoglobin Mb(**2·Fe**), and zinc myoglobin rMb(**2·Zn**), respectively. The mass spectrum of rMb(**1·Fe**) by electrospray ionization at pH = 7.0 shows a distribution of peaks with protonation states ranging from 13+ to 10+ (Figure 1). The average molecular mass measured from these peaks is 18 366.7 ± 2 and corresponds to the calculated mass of rMb(**1·Fe**) (18 343). Furthermore, the paramagnetic ^{1}H NMR spectrum of cyanometmyoglobin rMb(**1·FeCN**) shows the characteristic shifts of peripheral methyl protons in hemin and Ile99 protons similar to those of native Mb(**2·FeCN**).[12] These results indicate that the synthetic metalloporphyrins are evidently incorporated into the normal position of heme cavity.

Isoelectric focusing was carried out for rMb(**1·Fe**) and native Mb(**2·Fe**) as shown in Figure 2. The isoelectric point, p*I*, of the metaquo form of rMb(**1·Fe**) is 5.5, which is about 2 pH units lower than that of native Mb(**2·Fe**).[13] It is clear that the rMb(**1·Fe**) changes from a neutral protein to an acidic one due to the formation of anionic interface located on the protein surface.

Protein−Protein Complexation Monitored by ^{1}H NMR Spectroscopy. The results of ^{1}H NMR experiments on a mixture of rMb(**1·FeCN**) and ferricytochrome *c* (horse heart, Type IV) in phosphate buffer (pH 7.4, μ = 10 mM) are illustrated in Figure 3. In the presence of rMb(**1·FeCN**), the peripheral methyl substituents at the 3- and 8-positions of hemin in cytochrome *c* clearly shifted to lower and upper fields, respectively, whereas the chemical shift of $C\epsilon H_3$ protons of Met80 only deviated within 0.02 ppm. The observed changes in chemical shifts of cytochrome *c* upon addition of rMb(**1·FeCN**) are comparable with those in cytochrome *c*−cytochrome *c* peroxidase complexation described in previous literature.[14] Thus, the result supports that the artificial interface

(9) Collman, J. P.; Denisevich, P.; Konai, Y.; Marrocco, M.; Koval, C.; Anson, F. C. *J. Am. Chem. Soc*. **1980**, *102*, 6027−6036.

(10) Teale, F. W. *Biochim. Biophys. Acta* **1959**, *35*, 543.

(11) For examples of reconstituted myoglobin, cf.: (a) Suzuki, A.; Okuda, K.; Kawagoe, K.; Toi, H.; Aoyama, Y.; Ogoshi, H. *Chem. Lett.* **1985**, 1169−1172. (b) Hamachi, I.; Tajiri, Y.; Shinkai, S. *J. Am. Chem. Soc.* **1994**, *116*, 7437−7438. (c) Willner, I.; Zahavy, E.; Heleg-Shabtai, V. *J. Am. Chem. Soc*. **1995**, *117*, 542−543. (d) Hayashi, T.; Hitomi, Y.; Suzuki, A.; Takimura, T.; Ogoshi, H. *Chem. Lett*. **1995**, 911−912. (e) Hayashi, T.; Takimura, T.; Aoyama, Y.; Hitomi, Y.; Suzuki, A.; Ogoshi, H. *Inorg. Chim. Acta* In press.

(12) La Mar, G. N.; Davis, N. L.; Parish, D. W.; Smith, K. M. *J. Mol. Biol*. **1983**, *168*, 887−896.

(13) Varadarajan, R.; Lambright, D. G.; Boxer, S. G. *Biochemistry* **1989**, *28*, 3771−3781.

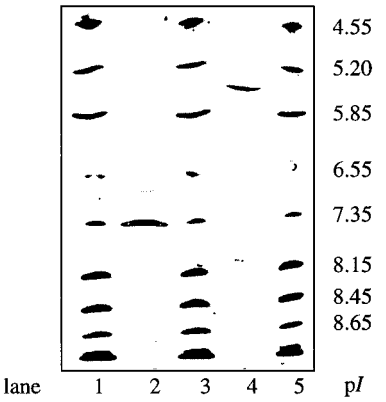


Figure 2. Results of isoelectric focusing with PhastGel IEF 3-9 (Pharmacia). p*I* along the gel was determined by measuring the position of protein standards (p*I* calibration kit 3-10, Pharmacia): (lanes 1, 3, and 5) marker proteins; (lane 2) native metmyoglobin Mb(**2·Fe**); (lane 4) rMb(**1·Fe**).

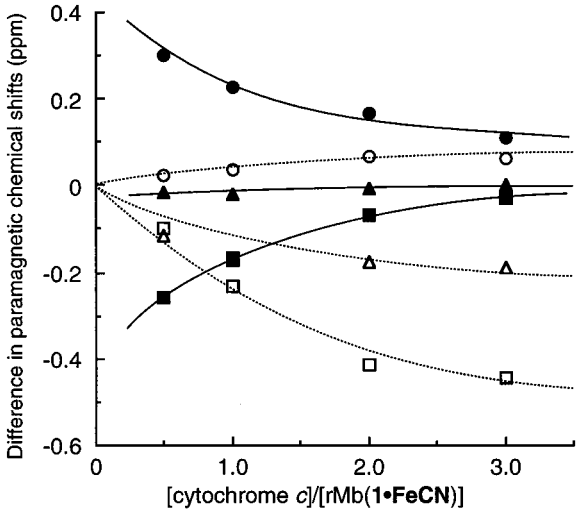


Figure 3. Changes in ^{1}H NMR chemical shifts of selective protons in rMb(**1·FeCN**) and cytochrome *c* at different concentration of cytochrome *c*; [rMb(**1·Fe**)]$_0$ = 0.49 mM in phosphate buffer containing 1.0 mM KCN (μ = 10 mM, pH 7.4) at 20 °C. The solid lines represent the changes in chemical shifts of cytochrome *c*; heme 3-CH_3 (●), Met80 CϵH$_3$ (▲), and heme 8-CH_3 (■). The broken lines represent the changes in chemical shifts of rMb(**1·FeCN**); heme 1-CH_3 (○), Ile99 CγH (△), and heme 5-CH_3 (□).

of rMb(**1·Fe**) interacts with the several Lys residues in the proximity of the exposed heme edge in cytochrome *c*. Furthermore, the significant shifts of 5-CH_3 of hemin and Ile99 protons in rMb(**1·FeCN**) were also observed due to the complex formation with cytochrome *c*.

Photoinduced ET in rMb(1·Zn)−Cytochrome *c* Complex. Flash photolysis of rMb(**1·Zn**) using a pulsed Nd:YAG laser gave an exponential decay profile at 460 nm identified as the triplet excited state of zinc porphyrin with a rate constant of 59 $\pm$ 1 s^{-1}, which is almost same as that for rMb(**2·Zn**),[15] in argon-saturated solution at pH 7.0 (Figure 4a). Upon addition of ferricytochrome *c*, the triplet state of rMb(**1·Zn**) was rapidly quenched at the ionic strength of 10 mM (Figure 4b). In contrast, the same behavior was not obtained in a mixture of rMb(**1·Zn**) and ferrocytochrome *c*. The decay rate of triplet

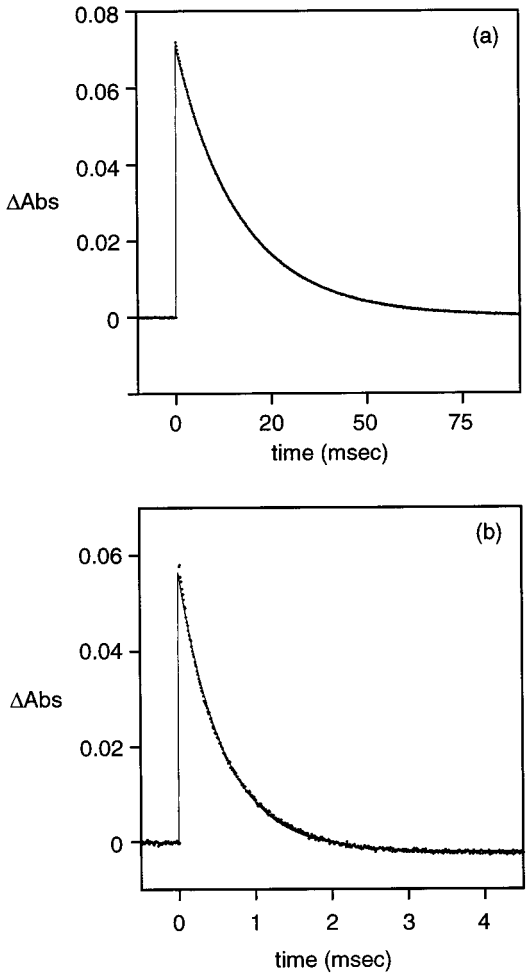


Figure 4. Transient absorbance changes: (a) decay of the triplet state of rMb(**1·Zn**) in the absence of cytochrome *c*, monitored at 460 nm, (b) decay of the triplet state of rMb(**1·Zn**) in the presence of cytochrome *c*, monitored at 460 nm. Conditions: [rMb(**1·Zn**)] = 4.3 × 10^{-6} M, [cytochrome *c*] = 1.6 × 10^{-5} M in phosphate buffer (pH 7.0, μ = 10 mM) at 20 °C. Experimental data points are represented by dots; the solid lines are fitting curves using a single-exponential function.

state in the presence of ferricytochrome *c* decreased with the increase of ionic strength, indicating that the protein−protein complex is efficiently formed by electrostatic interaction between eight carboxylates at the surface of rMb(**1·Zn**) and the special lysine residues of cytochrome *c*. Furthermore, a short-lived transient band was observed at 680 nm, which is representative of the zinc porphyrin cation radical species as an intermediate of ET (Figure 5).[6,16] The transient band at 680 nm increased with the decay of triplet state of rMb(**1·Zn**) and subsequently decreased in a longer time scale. The formation and decay of another charge separated species, ferrocytochrome *c*, were also detected at 550 nm, which show same profile as those shown in Figure 5, with the formation and decay of cation radical species of rMb(**1·Zn**). Thus, it is apparent that the

(14) (a) Moench, S. J.; Chroni, S.; Lou, B.-S.; Erman, J. E.; Satterlee, J. D. *Biochemistry* **1992**, *31*, 3661−3670. (b) Yi, Q.; Erman, J. E.; Satterlee, J. D. *Biochemistry* **1993**, *32*, 10988−10994.

(15) Zemel, H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1981**, *103*, 1192−1201.

(16) Zhou, J. S.; Rodgers, M. A. J. *J. Am. Chem. Soc.* **1991**, *113*, 7728−7734.

(17) Estimated distance between a zinc atom of rMb(**1·Zn**) and an iron atom in cytochrome *c* is ca. 18 Å by computer modeling which emerged from plausible interactions between carboxylate interface of rMb(**1·Zn**) and special Lys residues of cytochrome *c*. The driving force for the rMb(**1·Zn**)^{3*} → ferricytochrome *c* is estimated to be ca. 1.1 eV by use of the following papers: Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. Q. *Rev. Biophys.* **1986**, *18*, 261−322. Cheng, J.; Zhou, J. S.; Kostić, N. M. *Inorg. Chem.* **1994**, *33*, 1600−1606.

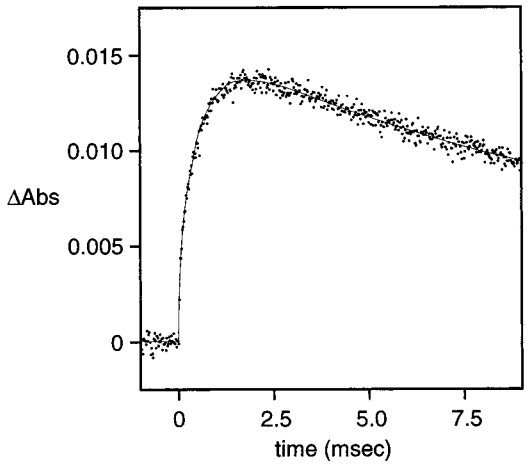


Figure 5. Transient absorbance changes: appearance and disappearance of the cation radical of rMb(**1·Zn**), monitored at 680 nm. Conditions: [rMb(**1·Zn**)] = 4.3×10^{-6} M, [cytochrome *c*] = 1.6×10^{-5} M in phosphate buffer (pH 7.0, μ = 10 mM) at 20 °C. Experimental data points are represented by dots; the solid line is a fitting curve using a double-exponential function.

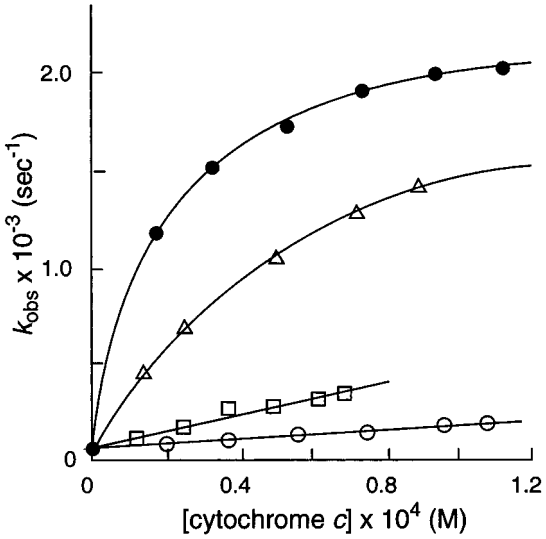


Figure 6. Decay rate of the triplet-state rMb(**1·Zn**) as a function of cytochrome *c* concentration, in phosphate buffer at pH 7.0 and 20 °C. Ionic strengths are 10 mM (●), 20 mM (△), 50 mM (□), and 100 mM (○), respectively. Transient absorptions were monitored at 460 nm by using the laser flash photolysis with the 532-nm output of a Q-switched Nd:YAG laser (6-ns fwhm). Initial concentration of rMb(**1·Zn**) is 4.3×10^{-6} M. The solid lines at 10 and 20 mM ionic strengths are a fitting curve using eq 1.

photoinduced ET from rMb(**1·Zn**) to ferricytochrome *c* occurs within the protein−protein complex linked by specific interface.[17]

The decay curve of triplet state could be analyzed by a single-exponential function to give a rate constant k_{obs} (correlation coefficient $r > 0.98$).[18,19] At lower ionic strength, the resulting rate constant k_{obs} increases nonlinearly with the cytochrome *c* concentration and levels off at higher concentration as shown in Figure 6. In contrast, in the case of rMb(**2·Zn**), linear dependence of k_{obs} on cytochrome *c* concentration was observed due to the diffusional quenching of the triplet state, and ferrocytochrome *c* was not detected at 550 nm.[20] The linearity was also obtained in rMb(**1·Zn**)−cytochrome *c* system at the ionic strengths of 50 mM and higher. The experimental data at the ionic strengths of 10 and 20 mM in Figure 6 were fitted to the following equation by an iterative, nonlinear least-squares routine to yield a rate constant of intracomplex ET and binding affinity between rMb(**1·Zn**) and ferricytochrome *c*:

$$k_{obs} = k_0 + k_{intra}f$$
$$= k_0 + k_{intra}[M + C + 1/K_a - \{(M + C + 1/K_a)^2 - 4MC\}^{1/2}]/2M \quad (1)$$

where k_0, k_{intra}, and f represent the rate constants of triplet-state decay and forward ET within the complex and the fraction of complexed rMb(**1·Zn**), and M and C represent the total concentration of rMb(**1·Zn**) and cytochrome *c*, respectively.[18] From this equation, the values of k_{intra} and K_a are determined to be $(2.2 \pm 0.1) \times 10^3$ s^{-1} and $(6.5 \pm 3.0) \times 10^4$ M^{-1} at 10 mM ionic strength, $(2.3 \pm 0.2) \times 10^3$ s^{-1} and $(1.5 \pm 0.6) \times 10^4$ M^{-1} at 20 mM ionic strength, respectively. These results clearly indicate that the intracomplex ET rate is independent of the ionic strength, whereas the binding affinity decreases with increasing ionic strength.[21] Furthermore, the formation of cation radical species of rMb(**1·Zn**) at 680 nm is fitted by a two-exponential function to have a rate constant and binding affinity of $(2.2 \pm 0.1) \times 10^3$ s^{-1} and $(6.9 \pm 3.2) \times 10^4$ M^{-1}, respectively, at 10 mM ionic strength. These values are exactly identical with those determined from the analysis of triplet decay profile, and this agreement supports the reliability of experimental data. Compared to the kinetic data in native protein−protein pairing,[18,22] the present value shows the reasonable rate constant of intracomplex ET, and the binding affinity derived from kinetic study reveals that rMb(**1·Zn**) forms the stable complex with cytochrome *c*.

Conclusion

This work indicates that the reconstitution of myoglobin with a chemically modified prosthetic group such as **1** gives a highly anionic protein which can form a stable complex with cationic cytochrome *c* on the protein surface. Furthermore, the present system demonstrates the first example of a long-range ET reaction within the noncovalently linked protein−protein complex via synthetic binding domain bound to the prosthetic group. Construction of a variety of artificial protein−protein complexes via electrostatic contacts and exploration of their properties are currently in progress.

Experimental Section

General Procedure. ^{1}H NMR spectra were obtained using a JEOL A-500 spectrometer and chemical shifts are reported relative to Me_4Si or DSS at 0 ppm. Mass spectra were measured in FAB mode and ESI

(18) Hoffman and co-workers have reported the photoinduced ET within the native protein pairing of zinc myoglobin and cytochrome b_5. In this system, a single-exponential decay was observed due to the fast dissociation of the complex. Our system could be followed by the same kinetic model as their case. Nocek, J. M.; Sishta, B. P.; Cameron, J. C.; Mauk, A. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1997**, *119*, 2146−2155.

(19) Tsukahara, K.; Asami, S. *Chem. Lett.* **1991**, 1337−1340.

(20) Decay profile of triplet state of rMb(**2·Zn**) in the presence of cytochrome *c* gave a second-order rate constant of $k_q = (8.0 \pm 0.3) \times 10^5$ M^{-1} s^{-1}. Aono, S.; Nemoto, S.; Ohtaka, A.; Okura, I. *J. Mol. Catal. A* **1995**, *95*, 193−196.

(21) Figure 6 also reveals that the binding affinity remarkably decreases at higher ionic strength, although it is difficult to obtain accurate parameters at higher ionic strength due to the insufficient curvature of the plots.

(22) For recent reviews, see: (a) Nocek, J. M.; Zhou, J. S.; Forest, S. D.; Priyadarshy, S.; Beratan, D. N.; Onuchic, J. N.; Hoffman, B. M. *Chem. Rev.* **1996**, *96*, 2459−2489. (b) Durham, B.; Millett, F. S. In *Comprehensive Supramolecular Chemistry*; Suslick, K. S., Ed.; Pergamon: Oxford, 1996; Vol. 5, pp 219−247. (c) McLendon, G.; Hake, R. *Chem. Rev.* **1992**, *92*, 481−490. references are cited therein.

mode with a JEOL JMS-SX102A spectrometer and a JEOL JMS-700 MStation spectrometer, respectively. UV−vis spectra were recorded on a HITACHI U-3410 spectrophotometer and a Hewlett-Packard 8452A diode array spectrophotometer. Protoporphyrin IX was purchased from Porphyrin Products, Inc. Bis(*p*-nitrophenyl)ester of protoporphyrin IX (**5**) was prepared by previous literature method.[9]

5-(Benzyloxycarbonylamino)isophthalic Acid (3). Benzyloxycarbonyl chloride (17.1 mL, 120 mmol) and 2 N NaOH (aq) (60.0 mL, 120 mmol) were added dropwise to a stirred solution of 5-aminoisophthalic acid (18.1 g, 100 mmol) in 2 N NaOH (aq) (100 mL, 200 mmol) at 0 °C over 0.5 h. The solution was allowed to warm to room temperature and stirred for 6 h. The reaction mixture was then washed with Et_2O (4 × 100 mL) to remove the excess of benzyloxycarbonyl chloride, and the resulted aqueous solution was treated with a concentrated HCl (aq), adjusting to pH 2. The precipitated material was filtered, washed with Et_2O, and dried in vacuo for several hours to obtain **3** (24.7 g, 80%) as a white solid: mp 297−298 °C; ^{1}H NMR (acetone-d_6) δ = 9.14 (1H, brs), 8.52 (1H, d, J = 1.5 Hz), 8.35 (2H, t, J = 1.5 Hz), 7.32−7.46 (5H, m), 5.22 (2H, s).

Bis(*N*,*N*-diethoxycarbonylmethyl)-5-(benzyloxycarbonylamino)-isophthalamide (4). One drop of DMF was added to a solution of **3** (0.76 g, 2.4 mmol) in thionyl chloride (3 mL), and the mixture was then refluxed for 2 h. After the solution was cooled to room temperature and the solvent was removed, the residue was taken up in benzene and evaporated twice. A solution of the obtained acyl chloride and diethyl iminodiacetate *p*-toluenesulfonate salt (1.9 g, 5.2 mmol)[23] in dry CH_2Cl_2 (25 mL) was cooled to 0 °C, and triethylamine (2 mL, 14 mmol) was then added dropwise to the mixture. After the reaction mixture was stirred overnight at room temperature, the solution was washed successively with 1 N HCl (aq), saturated NaCl (aq), and H_2O and then dried over anhydrous Na_2SO_4 and evaporated to dryness. Recrystallization from MeOH gave **4** (1.2 g, 77%) as a white solid: ^{1}H NMR (acetone-d_6) δ = 9.10 (1H, brs), 7.72 (2H, d, J = 1.3 Hz), 7.30−7.43 (5H, m), 7.07 (1H, t, J = 1.3 Hz), 5.19 (2H, s), 4.29 (4H, s), 4.19 (4H, s), 4.17 (4H, q, J = 7.0 Hz), 4.16 (4H, q, J = 7.0 Hz), 1.25 (6H, t, J = 7.0 Hz), 1.19 (6H, t, J = 7.0 Hz).

Protoporphyrin 13^3,17^3-Bis(5-amidobis(*N*,*N*-diethoxycarbonylmethyl)isophthalamide) (6). The removal of the benzyloxycarbonyl protection group was carried out by treatment of **4** (130 mg, 0.2 mmol) with 30% HBr in AcOH (3 mL) for 0.5 h. After completion of the reaction, the solvent was removed under reduced pressure. A solution of the residual HBr salt and **5** (40 mg, 0.05 mmol) in dry pyridine (5 mL) was stirred at 70 °C for 8 h. The solution was condensed by evaporator, and the residue was dissolved in CH_2Cl_2. The organic solution was washed successively with saturated Na_2CO_3 (aq), 1 N HCl (aq), saturated Na_2CO_3 (aq), saturated NaCl (aq), and H_2O and then dried over anhydrous Na_2SO_4 and evaporated to dryness. The main product was separated by column chromatography (SiO_2, $CHCl_3$/MeOH = 100:1). The solvents were evaporated off, and the residue was dried thoroughly to yield **6** (73 mg, 94%) as a reddish purple solid: ^{1}H NMR (DMSO-d_6) δ = 9.71 (1H, s), 9.60 (1H, s), 9.51 (1H, s), 9.49 (1H, brs), 9.48 (1H, brs), 9.44 (1H, s), 8.14 (1H, dd, J = 18.0 Hz, J = 11.6 Hz), 8.03 (1H, dd, J = 18.0 Hz, J = 11.6 Hz), 7.68 (4H, d, J = 1.2 Hz), 6.95 (2H, t, J = 1.2 Hz), 6.25 (1H, d, J = 18.0 Hz), 6.19 (1H, d, J = 18.0 Hz), 6.10 (1H, d, J = 11.6 Hz), 6.04 (1H, d, J = 11.6 Hz), 4.25 (2H, t, J = 7.0 Hz), 4.23 (2H, t, J = 7,0 Hz), 4.17 (8H, s), 4.10 (8H, q, J = 7.3 Hz), 3.94 (8H, s), 3.86 (8H, t, J = 9.8 Hz), 3.42 (3H, s), 3.40 (3H, s), 3.38 (3H, s), 3.35 (3H, s), 3.28 (2H, t, J = 7.0 Hz), 3.27 (2H, t, J = 7.0 Hz), 1.17 (12H, q, J = 7.3 Hz), 0.91 (12 H, q, J = 7.3 Hz), −5.40 (1H, brs); HRFAB MS calcd for $C_{82}H_{96}O_{22}N_{10}$ 1573.6777 (MW + 1), found 1573.6758; UV−vis ($CHCl_3$) λ_{max} (rel intensity) 409.7 (1.0), 506.6 (0.085), 543.1 (0.067), 576.4 (0.043), 629.8 (0.028) nm.

Zinc Complex (1·Zn). Zinc was inserted into **6** (30 mg) in quantitative yield using zinc acetate method:[24] ^{1}H NMR (DMSO-d_6) δ = 10.31 (2H, brs), 10.24 (1H, s), 10.16 (1H, s), 10.15 (1H, s), 10.13 (1H, s), 8.50 (1H, dd, J = 17.7 Hz, J = 11.6 Hz), 8.47 (1H, dd, J = 17.7 Hz, J = 11.6 Hz), 7.69 (4H, d, J = 0.9 Hz), 6.79 (2H, t, J = 0.9 Hz), 6.25 (1H, dd, J = 17.7 Hz, J = 1.6 Hz), 6.19 (1H, dd, J = 17.7 Hz, J = 1.6 Hz), 6.13 (1H, dd, J = 11.6 Hz, J = 1.6 Hz), 6.12 (1H, dd, J = 11.6 Hz, J = 1.6 Hz), 4.42 (4H, t, J = 6.9 Hz), 4.17 (8H, s), 4.10 (8H, q, J = 7.1 Hz), 4.03 (8H, s), 4.01 (8H, q, J = 7.1 Hz), 3.72 (3H, s), 3.71 (3H, s), 3.62 (3H, s), 3.60 (3H, s), 3.33 (4H, t, J = 6.9 Hz), 1.18 (12H, q, J = 7.1 Hz), 1.04 (12H, q, J = 7.1 Hz); UV−vis ($CHCl_3$) λ_{abs} (rel intensity) 417.0 (1.0), 545.7 (0.064), 582.8 (0.074) nm.

The zinc porphyrin was dissolved in 9 mL of a solution containing THF (10 mL), MeOH (10 mL), and 0.2 N aqueous KOH solution (10 mL). The solution was stirred at room temperature for 8 h. Solid CO_2 was then added, the solution was evaporated to dryness, and the residue was taken up in MeOH. The solution was filtered over Celite and passed through Sephadex LH-20. The solvent was evaporated and the residue was dried to obtained **1·Zn** as a pink solid in 88% yield: ^{1}H NMR (D_2O) δ = 10.36 (1H, s), 10.22 (1H, s), 10.21 (1H, s), 10.09 (1H, s), 8.43 (1H, dd, J = 17.4 Hz, J = 11.6 Hz), 8.42 (1H, dd, J = 17.4 Hz, J = 11.6 Hz), 7.09 (2H, s), 7.05 (2H, s), 7.03 (2H, s), 6.40 (1H, d, J = 17.4 Hz), 6.36 (1H, d, J = 17.4 Hz), 6.14 (1H, d, J = 11.6 Hz), 6.12 (1H, d, J = 11.6 Hz), 4.44 (4H, t, J = 6.4 Hz), 3.71 (4H, s), 3.69 (8H, s), 3.68 (4H, s), 3.45 (3H, s), 3.44 (3H, s), 3.42 (6H, s), 3.29 (4H, t, J = 6.4 Hz); UV−vis (10 mM KPi, pH 7.0) λ_{max} (relative intensity) 414.2 (1.0), 545.0 (0.072), 582.5 (0.064) nm.

Iron(III) Complex (1·Fe). To the solution of ferrous chloride hydrate (40 mg) in dry acetonitrile (7 mL), a solution of **6** (30 mg) in nitrogen-purged $CHCl_3$ (3 mL) was added dropwise with vigorous stirring at 50 °C under a stream of nitrogen. After complete addition, the mixture was stirred for 10 min under nitrogen before being exposed to air. The resulting brown solution was then diluted with CH_2Cl_2 and washed with an aqueous HCl solution and then with H_2O. The organic phase was evaporated off, and the residue was purified by chromatography (SiO_2, $CHCl_3$/MeOH = 20:1). The iron porphyrin was hydrolyzed and purified as above to obtain **1·Fe** as a brown solid in 75% yield: UV−vis (10 mM KPi, pH 7.0) λ_{max} (rel intensity) 400.4 (1.0), 496.7 (0.048), 622.5 (0.014) nm.

Preparation of Reconstituted Myoglobins. Apomyoglobin was prepared from metmyoglobin (horse heart, Sigma) by Teale's 2-butanone method.[10] The solution of apomyoglobin was mixed with **1·Fe** or **1·Zn** and allowed to stand over 12 h at 4 °C. The mixture was passed through Sephadex G-25 and CM-cellulose columns and then lyophilized.

Isoelectric Focusing Electrophoresis. Isoelectric focusing was curried out on an automated electrophoresis system (PhastSystem, Pharmacia) with a Precast gel (PhastGel IEF 3-9, Pharmacia). The quantities of proteins were detected by Coomassie blue staining. pI along the gel was determined by measuring the positions of protein standards (pI calibration kit 3-10, Pharmacia) having known isoelectric points.

NMR Titration. Solutions of metcyanomyoglobin and ferricytochrome *c* were prepared in D_2O phosphate buffer containing 1.0 mM KCN (μ = 10 mM, pH 7.4). Aliquots of cytochrome *c* solution were titrated into the metcyanomyoglobin solution, and the NMR spectra were recorded on a JEOL A500 NMR spectrometer. The temperature was maintained at 20 °C.

Laser Flash Photolysis Studies. The nanosecond laser photolysis studies were carried out by a Q-switched Nd:YAG laser, which delivered 6-ns pulses at 532 nm. The incident energy was about 10 mJ. The probe source was a continuous 150-W xenon arc lamp passed through a monochromator. Decay of the triplet state of reconstituted zinc myoglobin was monitored at 460 nm. Appearance and disappearance of the zinc porphyrin cation radical of reconstituted myoglobin were monitored at 680 nm. Signals were detected in transmission using a photo multiplier (Hamamatsu Photonics, R2949), and the transient signals were digitized using Tektronix TDS 320 oscilloscope. Signals were averaged 1000−5120 times. The data were transferred to a NEC PC9821Ae computer for further data analysis. No smoothing artifact affected the results of the following data analysis. The sample solution in a 10-mm quartz cell was prepared in a glovebox. The concentration of reconstituted zinc myoglobin was about 4.3 × 10^{-6} M. The temperature was maintained at 20 °C. UV−vis spectra of a mixture of the reconstitued myoglobin and cytochrome *c* were always measured

(23) Ueda, K. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 1879.

(24) Fuhrhop, J.-H.; Smith, K. M. In *Porphyrins and Metalloporphyrins*; Smith, K. M., Ed.; Elsevier: Amsterdam, 1975; p 772.

before and after flash photolysis studies in order to check that no significant photodegradation of the protein samples was occurring.

Acknowledgment. We are grateful to Prof. I. Morishima, Dr. K. Ishimori, Dr. S. Takahashi, and Dr. T. Uchida, Kyoto University, for the arrangement of laser flash photolysis equipment. We thank Mr. K. Matsuura, JEOL Ltd., for measuring the electrospray-ionization mass spectra. T.H. thanks Prof. Y. Hisaeda, Kyushu University, for warmful encouragement to the preparation of this article. This work was partially supported by Foundation Advanced Technology Institute, Japan. Y.H. was supported by Research Fellowships of Japan Society for the Promotion of Science for Young Scientists.

Supporting Information Available: Experimental details (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA9723951