Anisotropic Incorporation of Lipid-Anchored Myoglobin into a Phospholipid Bilayer Membrane

Itaru Hamachi, Kentaro Nakamura, Akio Fujita, and Toyoki Kunitake*

Contribution No. 957 from the Department of Chemical Science and Technology, Faculty of Engineering, Kyushu University, Fukuoka 812, Japan

Received September 14, 1992

Abstract: Directionally controlled anchoring of myoglobin, a water-soluble globular protein, on a dipalmitoylphosphatidylcholine (DPPC) bilayer has been successfully conducted. Lipid-anchored myoglobin was obtained from a monoalkylated heme derivative and apomyoglobin. It was indicated by gel filtration and ultrafiltration studies that the lipid-anchored myoglobin Mb(**1a**), but not native myoglobin and Mb(**2a**), is bound to the DPPC bilayer membrane in aqueous dispersion. A cast film of the phospholipid and the anchored myoglobin displayed anisotropic ESR signals, which depend on the disposition of the cast film in the magnetic field. These results suggest that myoglobin molecules are placed on the lipid bilayer in a fixed orientation by inserting the anchor alkyl chain into the bilayer. The ESR anisotropy was not observed without the anchor.

Introduction

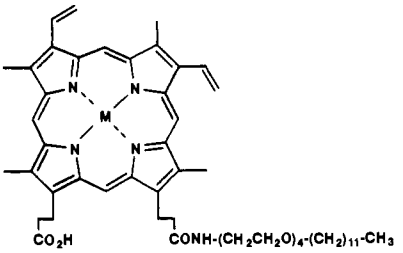
Artificially designed organization of protein molecules attracts much attention because of its unquestionable importance in many of the protein-based technologies.[1]

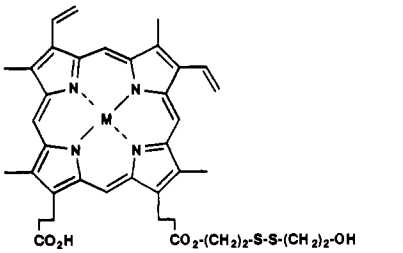
We found recently that self-organized lipid-protein layers are formed by simple casting of aqueous dispersions of myoglobin and a phosphate-bearing synthetic bilayer membrane.[2-4] The protein orientation is regulated through electrostatic interactions between myoglobin and the phosphate bilayer. This protein–membrane interaction also led to functional conversion of myoglobin so that an electron-transport chain from NADH to dioxygen could be assembled on synthetic bilayer matrices.[5] It is desirable to explore the use of other noncovalent forces, along with the electrostatic force, in order to create a more general methodology for orientational control of (originally) globular proteins. The presence of signal peptides in membrane transport of proteins[6] and the role of protein anchoring in signal transduction[7,8] gave us an important clue in our objective.

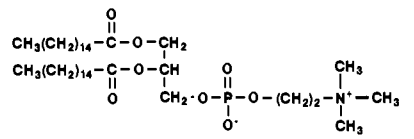
Results

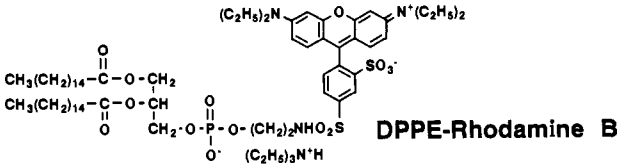
Reconstitution of Anchored Myoglobins. It is established that the heme unit of myoglobin (Mb) can be easily replaced with various iron–protoporphyrin IX (Fe–PP) derivatives by simple mixing of the latter with apo-Mb.[9] We prepared a long-chain derivative of PP monoamide (**1a**) and a hydrophilic PP monoester (**2a**) and incorporated them into the heme pocket by slightly modifying a procedure described in the literature.[9,10] Reactions

Chart I









of apo-Mb with protoporphyrin derivatives **1a** and **2a** were monitored with visible absorption changes. Figure 1 shows spectrophotometric titration of porphyrin **1a** with successive additions of small amounts of apo-Mb. In aqueous buffer, porphyrin **1a** forms aggregates and shows broad absorption maxima at 360 and 392 nm due to stacking (Soret band). When apo-Mb is added, intensities of these broad peaks decrease gradually and a sharp peak at 408 nm, which is identical to the Soret band of native met-Mb,[11] appears and intensifies. Such

(1) Ahlers, M.; Muller, W.; Reichert, A.; Ringsdorf, H.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1269 and references cited therein.
(2) Hamachi, I.; Noda, S.; Kunitake, T. *J. Am. Chem. Soc.* **1990**, *112*, 6745.
(3) Hamachi, I.; Honda, T.; Noda, S.; Kunitake, T. *Chem. Lett.* **1991**, 1121.
(4) Hamachi, I.; Fujimura, H.; Kunitake, T. *Chem. Lett.* **1991**, 2227.
(5) Hamachi, I.; Noda, S.; Kunitake, T. *J. Am. Chem. Soc.* **1991**, *113*, 9625.
(6) Stryer, L. *Biochemistry*, 3rd ed.; Freeman: New York, 1988; p 767.
(7) (a) Towler, D. A.; Gordon, J. I.; Adams, S. P.; Glaser, L. *Annu. Rev. Biochem.* **1988**, *57*, 69. (b) Ferguson, M. A. J.; Williams, A. F. *Annu. Rev. Biochem.* **1988**, *57*, 285.
(8) James, G.; Olson, E. N. *Biochemistry* **1990**, *29*, 2623.
(9) (a) Asakura, T.; Yonetani, T. *J. Biol. Chem.* **1969**, *262*, 6725. (b) Asakura, T. In *Methods in Enzymology*; Fleiser, S., Packer, L., Eds.; Academic Press: New York, 1978; Part C, p 446. (c) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543. (d) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435.
(10) Tamura, M.; Asakura, T.; Yonetani, T. *Biochim. Biophys. Acta* **1973**, *295*, 467.

0002-7863/93/1515-4966$04.00/0

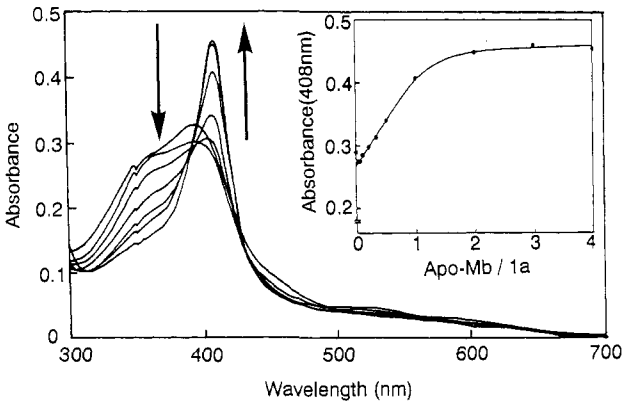


Figure 1. UV–visible spectral changes of protoporphyrin derivative **1a** by addition of apo-Mb (0–4.0 equiv): 3.3 μM **1a** in 10 mM phosphate buffer, pH 7.0, 25 °C. Inset: titration curve of **1a** with apo-Mb monitored at 408 nm.

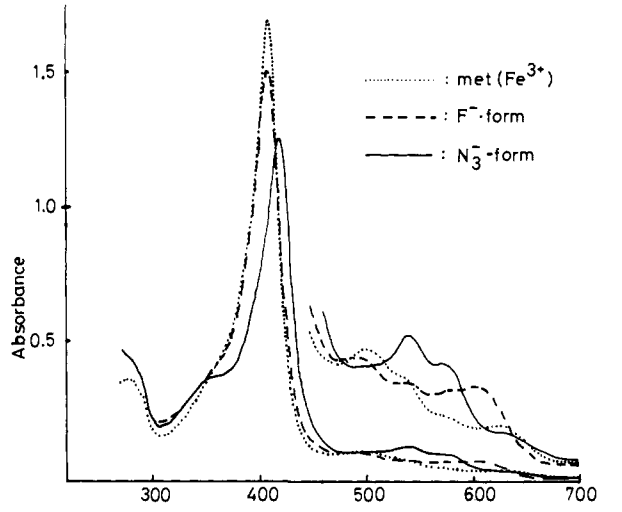


Figure 2. UV–visible spectra of met-Mb(**1a**) with various ligands: (···), met-Mb(**1a**) (11 μM) alone; (—), met-Mb(**1a**) (11 μM) + NaN_3 (30 mM); (- - -), met-Mb(**1a**) (11 μM) + KF (20 mM).

absorption spectral changes are saturated at about a 1/1 ratio of apo-Mb to porphyrin **1a** (see the inset in Figure 1), indicating formation of the 1/1 complex. A similar behavior was observed in the combination of apo-Mb with porphyrin **2a**.

Figure 2 shows UV–visible spectra of the reconstituted Mb from **1a** (Mb(**1a**)) after purification. Absorption maxima at 408 nm (sharp Soret band) and at 500, 540, and 630 nm (weak Q-bands) are consistent with the literature values of the reconstituted Mb from Fe–PP monomethyl ester (Mb(PP monoester)).[10] The ratio of Soret absorbance to protein absorbance (at 280 nm) is 4.5 for Mb(**1a**) and 3.9 for Mb(**2a**), which are comparable to the value of 4.8 for native Mb and are greater than 3.22 for Mb(PP monoester). This indicates that both of the Mb's that are reconstituted from **1a** and **2a** are pure enough to use in the subsequent studies. By addition of azide, fluoride, and cyanide, the sixth ligand of iron–heme of met–Mb is readily exchanged from H_2O to one of these anions. This ligand binding as monitored by UV–visible spectroscopy is useful for probing the heme environment of Mb. Spectral changes due to ligand binding (λ_{max} 419, 540, and 575 nm in the azide form and 407, 490, and 606 nm in the fluoride form, as shown in Figure 2) are similar to those of Mb(PP monoester) (418, 540, and 572 nm in the azide form and 404, 488, and 604 nm in the fluoride form).[10] Spectral changes upon ligand binding were the same in Mb(**2a**) (λ_{max} 420, 540, and 575 nm in the azide form and 405, 493, and 605 nm in the fluoride form). It is thus suggested that the heme environment of the reconstituted Mb's is almost identical with that of Mb(PP monoester). Accelerated autoxidation of Mb(**1a**) (the half-life of the dioxygen complex is a few minutes) is consistent with that of Mb(PP monoester) reported in the literature.[10] These data demonstrate that alkylated Mb derivatives are successfully synthesized by the heme-substitution method.

(11) (a) Gibson, Q. H.; Smith, M. H. *J. Physiol.* **1957**, *136*, 27. (b) Chang, C. K.; Traylor, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2647.

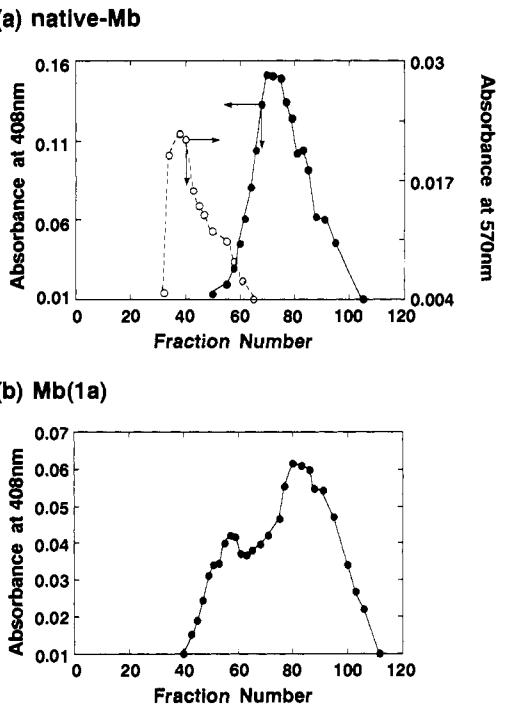


Figure 3. Gel filtration profiles of DPPC bilayer dispersion mixed with modified myoglobins. The dotted line indicates eluted fractions of DPPC bilayer dispersion monitored with DPPE–rhodamine B (absorbance at 570 nm): (a) DPPC with native Mb; (b) DPPC with Mb(**1a**).

Binding of Modified Myoglobins with Aqueous Bilayer Dispersion. We have reported that zwitterionic phosphatidylcholine can not orient Mb molecules in specific manners since strong electrostatic interactions do not operate between Mb and the zwitterionic bilayer membrane.[3,12] Because of this neutrality in electrostatic interaction, zwitterionic bilayers are most suitable for evaluating the hydrophobic effect of the anchor chain for protein orientation.

The binding of the alkylated Mb with phospholipid bilayers was first examined in aqueous systems by gel filtration chromatography and ultrafiltration binding assay.

Modified myoglobins were mixed with aqueous dipalmitoylphosphatidylcholine (DPPC) dispersions and subjected to Sepharose 4B gel column chromatography. Typical profiles of the gel filtration are summarized in Figure 3. DPPC dispersions were eluted at fraction numbers of 32–65, as monitored by the absorbance of membrane-bound DPPE–rhodamine B. An elution curve of native Mb showed a single band with fraction numbers of 50–105, and native Mb does not appear to elute together with the DPPC fraction (Figure 3a). Similar elution behavior was obtained for Mb(**2a**). Thus, we conclude that association of native Mb and Mb(**2a**) with the DPPC bilayer is negligible in the aqueous system. On the other hand, two bands were observed in the case of Mb(**1a**) (Figure 3b). The first fraction (fraction numbers of 40–65) contained both Mb(**1a**) and the DPPC dispersion, indicating that these two components were bound. The second fraction (fraction numbers of 65–110) contained only Mb(**1a**) and not the DPPC components, corresponding to unbound Mb-

(12) Hamachi, I.; Noda, S.; Kunitake, T. Manuscript in preparation.
(13) (a) Blasie, J. K.; Erecinska, M.; Samuels, S.; Leigh, J. S. *Biochim. Biophys. Acta* **1978**, *501*, 33. (b) Erecinska, M.; Wilson, D. F.; Blasie, J. K. *Ibid.* **1978**, *501*, 53.

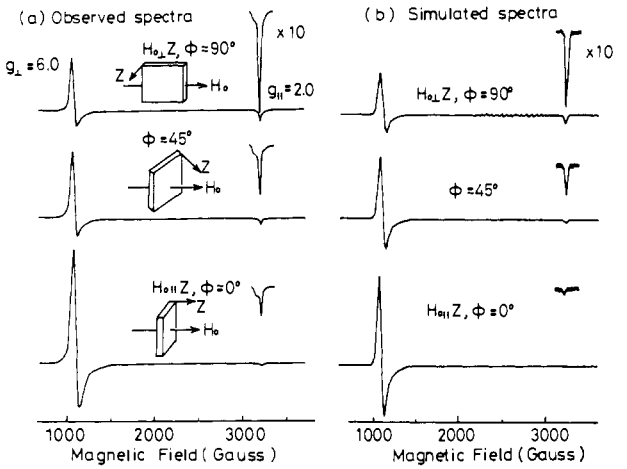


Figure 4. Electron spin resonance (ESR) spectra of a cast film of DPPC containing Mb(**1a**) at 4 K. Mb(**1a**)/DPPC = 1/700: (a) observed spectra; (b) simulated spectra with parameters of $\theta = 80°$ and $\sigma = 30°$. Detailed conditions were described previously.[2,5]

(**1a**). It is clear that Mb(**1a**) is partially bound to the DPPC dispersion. The peak separation in Figure 3b is less than that in Figure 3a. This is explained by partial release of membrane-bound Mb(**1a**) by dilution during the gel filtration process.

The binding of the anchored Mb(**1a**) to the DPPC dispersion was also supported by the ultrafiltration experiment.[5] When an ultrafiltration membrane of cutoff molecular weight of 100 000 was used, free Mb molecules (MW 18 000) should be easily filtered off. Bilayer-bound Mb can not pass through the filter because molecular weights of bilayer aggregates are greater than 10^6. The fraction of the bilayer-bound Mb (i.e., not passing through the filter), thus estimated was 53, 13, and 14% for Mb(**1a**), Mb(**2a**), and native Mb, respectively. However, a control experiment without the DPPC bilayer showed that 15–20% of Mb's were adsorbed nonspecifically to the ultrafiltration membrane. These results imply that the lipid-anchored Mb(**1a**) is bound to the DPPC bilayer but that Mb(**2a**) and native Mb are not.

Orientation of Myoglobin Molecules in Cast Multibilayer Film. Different membrane affinities of Mb's are reflected in their orientations in cast films of DPPC. Mb-containing cast films of DPPC were prepared according to the method reported previously.[2-5] Absorption spectra of the films cast on glass plates are almost the same as those in aqueous solution (λ_{max} 409 (Soret band), 500, 540, and 630 nm (Q-bands)), although there are observed strong overlapping peaks due to scattering from bilayer membranes in the region below 450 nm. Therefore, we conclude that the alkylated Mb's are immobilized in the cast film without detectable denaturation.[14]

Low-temperature ESR spectra of the alkylated Mb(**1a**) in a DPPC cast film are shown in Figure 4a. The g values are identical to those of native met-Mb (g = 6.0 and 2.0),[15] which means that the active site of Mb(**1a**) is in the ferric high-spin state. Macroscopic anisotropy was clearly seen when the angle (ϕ) between the normal of the film plane and the applied magnetic field was varied. The intensity of the $g_\parallel$ component (g_z = 2.0) is maximal at $\phi = 90°$ and is gradually weakened to a minimum value by bringing the ϕ value to 0°. The $g_\perp$ component ($g_x = g_y$ = 6.0) changes its intensity in the opposite direction. This strong angular dependence suggests that the heme plane of Mb(**1a**) is oriented in a specific manner against the bilayer plane. The simulated spectra (Figure 4b) are in practical coincidence with the observed spectra at a tilt angle of the heme plane of 80° and a standard deviation of 30°.[5,16] This perpendicular heme orientation relative to the membrane plane is reasonably explained by assuming that the hydrophobic alkyl chain of Mb(**1a**) is anchored to the hydrophobic region of the DPPC bilayer membrane. Figure 5 illustrates this situation schematically.

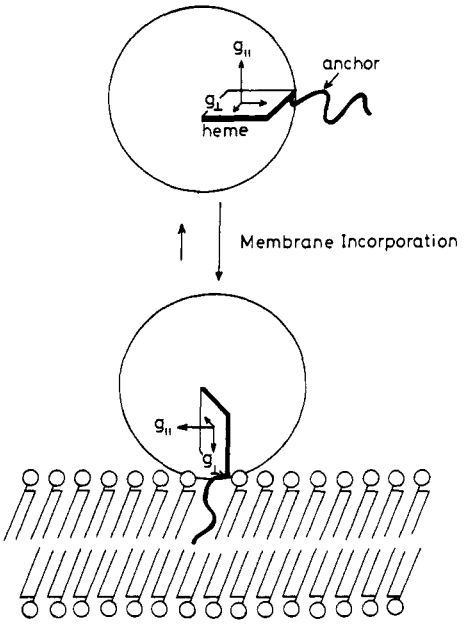


Figure 5. Schematic illustration of anisotropic incorporation of lipid-anchored Mb(**1a**) into the DPPC bilayer membrane.

On the other hand, ESR spectra of Mb(**2a**) in which the g values are the same as those of Mb(**1a**) are completely isotropic. Native Mb similarly does not display angular dependence in a cast film of DPPC. This lack of orientation is consistent with the absence of membrane affinity of aqueous Mb's, as described above, and points to the essential role played by the alkyl chain anchor in Mb(**1a**). The interaction of the native protein surface and the hydrophilic alkyl chain of Mb(**2a**) with the zwitterionic DPPC surface cannot produce specific orientations by themselves.

Discussion

An increasing number of unique proteins have been found that are covalently modified with fatty acids (myristic acid or palmitic acid) or phosphatidylinositol at specific positions.[7,8] They play crucial roles in the intracellular signaling pathway in living cells. It is suspected that the long chains act as hydrophobic anchors and optimize protein orientations to regulate protein–protein interactions on the biomembrane.[8] The present result is the first direct evidence for protein orientation by a long-chain anchor.

Lipid monolayers on the surface of water have been used for protein orientation. Kornberg and co-workers used an antigen-functionalized lipid as a specific host of an antibody protein.[17] They also reported that a monolayer of an adenosine triphosphate-(ATP)-modified lipid facilitated formation of a two-dimensional crystal of ribonucleotide reductase.[18] Blankenburg et al. showed that streptavidin protein was bound to biotinylated lipids at the air–water interface with fixed orientation.[19] Preorganized ligands (i.e., antigens, ATP, and biotin) in monolayers are undoubtedly effective for the vectorial orientation of proteins. Without an added ligand, cytochrome c was vectorially oriented by electrostatic or covalent interactions at the surface of Langmuir–Blodgett films.[20] Some proteins (e.g., ATPase) were oriented on a pure

(14) The free heme **1a** in a DPPC cast film gives a broad Soret band at around 406 nm in the absorption spectrum and broad ESR signals at g = 6.0 and 2.0 without angular dependence.

(15) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 3926.

(16) Ishikawa, Y.; Kunitake, T. *J. Am. Chem. Soc.* **1991**, *113*, 621.

(17) Uzgiris, E. E.; Kornberg, R. D. *Nature (London)* **1983**, *301*, 125.

(18) Ribi, H. O.; Reichard, P.; Kornberg, R. D. *Biochemistry* **1987**, *26*, 7974.

(19) Blankenburg, R.; Meller, P.; Ringsdorf, H.; Salesse, C. *Biochemistry* **1989**, *28*, 8214.

(20) (a) Pachence, J. M.; Blasie, J. K. *Biophys. J.* **1988**, *52*, 735. (b) Panchence, J. M.; Fischetti, R.; Blasie, J. K. *Ibid.* **1989**, *56*, 327.

Hg surface by self-assembly.[21] The latter method, however, is limited to proteins that have characters of anisotropic shape and/or asymmetrical charge distribution.

The present study establishes that insertion of the alkyl chain anchor into a bilayer membrane leads to effective protein orientation. Increased membrane affinity of the anchored protein in aqueous dispersions is related to its anisotropic orientation in immobilized cast films. In contrast to the use of specific ligands, alkyl chains would be specifically attached to various amino acid residues of protein surfaces. Therefore, versatile vectorial orientations may be achieved by this method.

In summary, the anchoring approach should be applicable to the spatially-defined orientation of globular proteins in general. Together with our previous approach, based on the electrostatic attraction, orientational control of globular proteins at the bilayer surface is now much facilitated.

Experimental Section

DPPE–rhodamine B was purchased from Molecular Probes, Inc. and used without purification. Sepharose 4B and Sephadex G-25 were purchased from Pharmacia. Protoporphyrin derivatives were synthesized as follows. Protoporphyrin monoester **4** was converted to PP monoester monoamide **5** by an appropriate amine (**3**) in the presence of diethyl cyano phosphate. This was hydrolyzed with aqueous NaOH, followed by iron chelation to give **1a**. **2b** was prepared from protoporphyrin disodium salt and 2-hydroxyethyl disulfide in the presence of PCl_5. **2a** was synthesized by iron complexation of **2b** in $FeCl_2$/DMF. In our experiments, isomers of **1a** and **2a** were not separated.

3,6,9,12-Tetraoxatetraeicosylamine (3). Tosyl chloride (6.4 g, 34 mmol) dissolved in 20 mL of pyridine was added dropwise to tetraethylene glycol monododecyl ether (Nikko Chemicals, 10.2 g, 28 mmol) in 20 mL of pyridine over 1 h with ice-cooling, and the solution was stirred at 4 °C for 24 h. The reaction mixture was poured into 200 mL of ice-cooled water, acidified to pH 2 by addition of concentrated HCl, and extracted with $CHCl_3$ (150 mL × 2). The combined $CHCl_3$ layers were dried over anhydrous Na_2SO_4, the solvent was removed to give the crude tosyl ester as a colorless oil: yield 14 g (96%). The crude tosyl ester (14.0 g, 27 mmol) and potassium phthalimide (6.0 g, 32 mmol) were suspended in 100 mL of dry dimethylformamide (DMF, heated at 60° over CaH_2 and distilled), and the mixture was stirred at 60 °C for 72 h. The precipitates were removed, the filtrate was concentrated, and the oily residue was applied to a short column (silica gel, 10 cm ϕ × 3 cm, $CHCl_3$) to remove the brown impurity and obtain crude tetraethylene glycol monododecyl monophthalimide as a pale yellow oil: yield 8.4 g (63%). The monophthalimide (4.0 g, 8.1 mmol) and hydrazine monohydrate (2 mL) were dissolved in 200 mL of ethanol and refluxed for 2.5 h. After a white precipitate was filtered off, 50 mL of saturated aqueous NaCl was added to the filtrate, and the mixture was stirred for 30 min. The mixture was extracted with CH_2Cl_2 (200 mL × 2) after the solution was adjusted to pH 11 with 1 N NaOH. Combined CH_2Cl_2 layers were dried over anhydrous Na_2SO_4 and evaporated to give the desired product **3** as a pale yellow oil: yield 2.0 g (68%); ^{1}H NMR (60 MHz, $CDCl_3$) δ 0.88 (t, 3 H, CH_3), 1.25 (m, 20 H, CCH_2C), 2.75 (t, 2 H, CH_2N), 3.60 (m, 16 H, CH_2O).

2,7,12,18-Tetramethyl-3,8-divinyl-13-(carboxyethyl)-17-((ethoxycarbonyl)ethyl)porphyrin (4). The PP monoethyl ester was prepared from protoporphyrin disodium salt (Aldrich) and PCl_5 in $CHCl_3$/ethanol, according to the reported method.[22]

2,7,12,18-Tetramethyl-3,8-divinyl-13-(((3′,6′,9′,12′-tetraoxatetraeicosyl)carbamoyl)ethyl)-17-((ethoxycarbonyl)ethyl)porphyrin (5). PP monoethyl ester **4** (0.20 g, 0.34 mmol) and the preceding alkylamine **3** (0.62 g, 1.8 mmol) were dissolved in 100 mL of dry (refluxed over NaH) THF. Diethoxyphosphinyl cyanide (Aldrich, 90%, 0.5 g, 3.0 mmol) was added with ice-cooling. After the solution was stirred for 96 h at room temperature, the solvent was removed. The dark purple oil was purified with column chromatography (silica gel, 3 cm ϕ × 20 cm, $CHCl_3/CH_3OH$ = 20/1, (v/v)) to obtain a purple black solid **5**: yield 0.19 g (60%); mp 202–210 °C; IR (cm^{-1}, KBr) ν_{N-H} 3320, ν_{C-H} 2930, 2850, $\nu_{C=O}$ 1730, $\nu_{C=O}$ 1655, ν_{C-O-C} 1110; ^{1}H NMR (60 MHz, $CDCl_3$) δ −2.0 (s, 2 H, NH), 0.6–1.50 (m, 26 H, CCH_2C, $-CCH_3$), 2.45–3.40 (m, 20 H, OCH_2–, 3.60 (s × 2, 12 H, $-CH_3$), 4.00 (m, 4 H, NCH_2, OCH_2), 4.37 (t, 4 H, $-CH_2-$), 6.26 (dd, 4 H, $-C=CH_2$), 8.26 (dd, 2 H, CH=C), 10.00 (s × 4, 4 H, CH). Anal. Calcd for $C_{56}H_{79}N_5O_7 \cdot {}^1/_2H_2O$: C, 71.31; H, 8.55; N, 7.42. Found: C, 71.21; H, 8.45; N, 7.53.

2,7,12,18-Tetramethyl-3,8-divinyl-13-(((3′,6′,9′,12′-tetraoxatetraeicosyl)carbamoyl)ethyl)-17-(carboxyethyl)porphyrin (1b). The preceding product **5** (190 mg, 0.20 mmol) was dissolved in THF/MeOH/1 N NaOH (30 mL/20 mL/5 mL), and the mixture was stirred at room temperature for 24 h. After 30 mL of H_2O was added, the solution was acidified with 1 N HCl to pH 2. The precipitated black solid was collected by filtration, washed with H_2O (50 mL), and dried in vacuo to give a black fine powder: 94% (170 mg) yield; mp 191–204 °C; IR (cm^{-1}, KBr) ν_{O-H} 3400, ν_{N-H} 3320, ν_{C-H} 2930, 2850, $\nu_{C=O}$ 1705, $\nu_{C=O}$ 1640, ν_{C-O-C} 1110; ^{1}H NMR (60 MHz, $CDCl_3$) δ 0.6–1.40 (m, 23 H, CCH_2C, CCH_3), 2.50–3.25 (m, 20 H, OCH_2–), 3.60 (s, 12 H, $-CH_3$), 3.80–4.40 (m, 6 H, NCH_2, $-CH_2-$), 6.26 (dd, 4 H, $-CH=CH_2$), 8.00 (m, 2 H, CH=C), 9.80 (s × 2, 4 H, CH). Anal. Calcd for $C_{54}H_{75}N_5O_7 \cdot {}^1/_2H_2O$: C, 70.87; H, 8.37; N, 7.65. Found: C, 70.73; H, 8.29; N, 7.61.

[2,7,12,18-Tetramethyl-3,8-divinyl-13-(((3′,6′,9′,12′-tetraoxatetraeicosyl)carbamoyl)ethyl)-17-(carboxyethyl)porphyrinate]iron(III) Chloride (1a). The preceding product **1b** (100 mg, 0.11 mmol) and ferrous chloride (150 mg, 1.18 mmol) were dissolved in 10 mL of dry DMF under a N_2 atmosphere and allowed to react at 50–60 °C with stirring for 5 h. After DMF was evaporated, 100 mL of H_2O was added. The black precipitate was collected by filtration and washed with 0.01 N HCl (10 mL) and MeOH (0.5 mL) and dried to give a black fine powder **1a**: yield 80 mg (73%); mp > 300 °C; IR (cm^{-1}, KBr) ν_{O-H} 3400, ν_{C-H} 2930, 2850, $\nu_{C=O}$ 1720, $\nu_{C=O}$ 1630, ν_{C-O-C} 1110. Anal. Calcd for $C_{54}H_{73}N_5O_7FeCl \cdot 3H_2O$: C, 61.80; H, 7.59; N, 6.64. Found: C, 61.37; H, 7.07; N, 6.64.

2,7,12,18-Tetramethyl-3,8-divinyl-17-(carboxyethyl)-13-((((6′-hydroxy-3′,4′-dithiahexyl)oxy)carbonyl)ethyl)porphyrin (2b). Protoporphyrin disodium salt (Aldrich, 1.0 g, 1.65 mmol) and PCl_5 (0.5 g, 2.4 mmol) were suspended in 100 mL of $CHCl_3$, and the mixture was stirred at 30–40 °C for 1 h. Then, hydroxyethyl disulfide (Janssen Chemicals, 6 mL) in 10 mL of THF was added. After the solution was stirred for 30 min, hydroxyethyl disulfide (6 mL) in 10 mL of THF was added again. The mixture was heated at 40 °C for an additional 2.5 h, cooled to 0 °C, and quenched with 10 mL of H_2O. It was neutralized with triethylamine (pH 6–7) and extracted with $CHCl_3$ (150 mL × 2), and the combined organic layers were dried over anhydrous Na_2SO_4. After solvent evaporation, the oil residue was purified with column chromatography (silica gel, 5 cm ϕ × 20 cm, $CHCl_3$/MeOH = 20/1–10/1 (v/v)). The first major band (R_f = 0.65 (TLC (Merck, silica gel), $CHCl_3$/MeOH = 10/1)) was a diester derivative of protoporphyrin. The second main band (R_f = 0.56 (TLC (Merck, silica gel), $CHCl_3$/MeOH = 10/1)) was collected and purified further by reprecipitation from MeOH to give a black fine powder: 24% (0.27 g) yield; mp > 300 °C; IR (cm^{-1}, KBr) ν_{O-H} 3400, ν_{N-H} 3320, ν_{C-H} 2920, 2850, $\nu_{C=O}$ 1730. Anal. Calcd for $C_{38}H_{42}N_4O_5S_2 \cdot H_2O$: C, 63.66; H, 6.19; N, 7.81. Found: C, 63.76; H, 6.07; N, 7.70.

[2,7,12,18-Tetramethyl-3,8-divinyl-17-(carboxyethyl)-13-((((6′-hydroxy-3′,4′-dithiahexyl)oxy)carbonyl)ethyl)porphyrinate]iron(III) Chloride (2a). Iron chelation of free base porphyrin **2b** was conducted by a procedure similar to that for **1a**, giving a black fine powder: 90% yield; mp > 300 °C; IR (cm^{-1}, KBr) ν_{O-H} 3400, ν_{C-H} 2920, 2850, $\nu_{C=O}$ 1730. Anal. Calcd for $C_{38}H_{40}N_4O_5S_2 \cdot 3H_2O$: C, 54.19; H, 5.51; N, 6.65. Found: C, 54.21; H, 4.98; N, 6.51.

Reconstitution of Modified Myoglobins. Preparation of apomyoglobin from metmyoglobin (horse heart from Sigma) was conducted by the standard method.[9] Protoporphyrin derivatives **1a** and **2a** were incorporated into the heme pocket of apomyoglobin by slightly modifying a procedure described in the literature.[9c] Iron porphyrins **1a** and **2a** dissolved in pyridine (1 mg/0.1 mL) were added dropwise to an aqueous apo-Mb solution with ice-cooling (6 mL, apo-Mb 0.14 mM, phosphate buffer, pH 7.0). The resulting mixtures were incubated at 4 °C overnight and centrifuged (10 000 rpm, 15 min), and the supernatants were purified by dialysis followed by gel chromatography (Sephadex G-25).

Gel Filtration. To a sonicated aqueous dispersion of the DPPC bilayer (10 mg of DPPC/1 mL of 10 mM Tris-HCl (pH 7.5)) was added 500 μL of a Mb solution (0.54 mM in 10 mM phosphate buffer (pH 6.0)). The mixture was applied to a gel column (Sepharose 4B, 2.7 cm ϕ × 10 cm, equilibrated with 10 mM Tris-HCl (pH 7.5)). Every 1.0 mL of eluents was fractionated and analyzed by UV–visible spectrometry. Fractions containing the DPPC bilayer membrane with DPPE–rhodamine

(21) (a) Yoshimura, H.; Endo, S.; Matsumoto, M.; Nagayama, K.; Kagawa, Y. *J. Biochem.* **1989**, *106*, 958. (b) Yoshimura, H.; Matsumoto, M.; Endo, S.; Nagayama, K. *Ultramicroscopy* **1990**, *32*, 265.

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

B (DPPE–rhodamine B/DPPC = 1/100, (mol/mol)) were monitored at 570 nm, and those containing Mb, at 408 nm.

Preparation of Cast Films Containing Modified Myoglobins. Cast films of dipalmitoylphosphatidylcholine (DPPC, Sigma) were prepared by the same procedure as reported earlier.[5] To a sonicated aqueous dispersion of the DPPC bilayer was added an appropriate amount of modified Mb at room temperature (modified Mb/DPPC = 1/700, (mol/mol)), and the mixture was gently shaken. The mixture was spread onto solid supports (glass plate or Fluoropore membrane filter (Sumitomo Electric, FP 010, pore size 10 μm)) and allowed to dry for a few days to give a red-brown flexible composite film.

Measurements. Electron spin resonance (ESR) spectral measurements (JEOL JES-2X X-band spectrometer) and their simulations were conducted by the procedure already reported by us.[5,15] Reduction–oxidation reactions of modified Mb's were performed by addition of aqueous $Na_2S_2O_4$ under a N_2 atmosphere and O_2 bubbling and monitored by UV–visible spectroscopy (Hitachi 220 A or Shimadzu UV-2200 spectrophotometer).[5] Ligand-binding experiments (N_3^-, F^-, and CN^-) and titration experiments of **1a** and **2a** with apo-Mb were also measured by UV–visible spectroscopy. Ultrafiltration experiments of an aqueous mixture of modified Mb's and the DPPC bilayer were conducted according to our method described previously[5]. Conditions: Mb's 9 μM, DPPC bilayer 1.0 mM in 10 mM Tris-HCl, pH 7.5. Ultrafiltration membrane: MOLCUT II, UFP1 THK24, cutoff molecular weight 100 000.

Acknowledgment. We are grateful to Professors Tsuneo Omura and Akio Ito (Kyushu University) for stimulating discussions. We also thanks Professor Hisanobu Ogoshi and Dr. Akihiro Suzuki (Kyoto University) for their help in the Mb reconstitution.