Synthesis of Ring-Fluorinated Porphyrins and Reconstitutional Myoglobins with Their Iron Complexes

Akihiro Suzuki,* Tadashi Tomizawa, Takashi Hayashi,† Tadashi Mizutani,† and Hisanobu Ogoshi*,†

Department of Materials Engineering, Nagaoka College of Technology, Nishikatakai, 888, Nagaoka 940

†Department of Synthetic Chemistry and Biological Chemistry, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-01

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The total syntheses of monofluoroporphyrin derivatives from monofluoropyrroles and the reconstitution of synthetic heme into sperm-whale myoglobin are described. The heme orientation in a cavity of the protein was determined by ^{1}H and ^{19}F NMR studies. It is noted that a fluoro substituent was found to be a useful probe for studying the heme-protein interaction.

Site-specific fluorine introduction to a substrate in an enzymatic reaction, using a coenzyme and protein, has been a unique method for elucidating the function–structure relationship in biological systems.[1] During a study of fluorinated porphyrins, two new aspects of fluoroporphyrin chemistry were noted: (1) For perfluoroalkyl groups, electronic effects perturb the electronic states of porphyrin, and a number of properties, such as the redox potentials and electronic spectra, are modified. (2) For both perfluoroalkyl and fluoro substituents, ^{19}F NMR serves as a probe which reflects the particular local environment around the fluorine nuclei.[2,3] ^{19}F resonances of fluoro and perfluoroalkyl groups introduced to prosthetic groups and proteins appear over a wide magnetic-field region with high susceptibility to the environment around the nuclear probe.

Kaesler and LeGoff prepared the first porphyrins having perfluoroalkyl side chains, where perfluoroalkyl groups are not linked directly to the porphyrin ring.[4] Their attempts to prepare a perfluoroalkyl-substituted porphyrin with the 3- and 4-(trifluoromethyl)pyrrole were unsuccessful due to the deactivation of cyclotetramerization.[5] We have reported on the first synthesis of 3-trifluoromethylpyrroles by a modified Knorr condensation[6] and an addition reaction of *p*-toluenesulfonylmethyl isocyanate to β-perfluoroalkyl α,β-unsaturated ketones.[7] The electron-deficient tetrakis(trifluoromethyl)etioporphyrin was obtained by the cyclization of trifluoromethylpyrrole,[8] and their particular physicochemical properties due to strong electron-withdrawing CF_3 groups were characterized by electrochemical measurements, as well as MCD and ESR studies.[9,10] In our previous work, paramagnetic ^{19}F NMR resonances were found to be useful to define both the low-spin ($s = 1/2$) state and the high-spin ($s = 5/2$) state of porphyrinatoiron(III) complexes and reconstituted myoglobin with fluorinated hemin (see Fig. 1).[2,11] Snow and Smith[12] have emphasized that a strong perturbation by the CF_3 group can be expected in the molecular orbital of the porphyrin framework, and brings about a marked effect on the functions of the reconstituted hemoglobin and myoglobin. They have reported on the synthesis of a (monofluoromethyl)- and (difluoromethyl)porphyrin designed to produce reconstituted hemoproteins, where a perturbation of the substituents is estimated to be small. Recently, DiMango and coworkers have reported 5, 10, 15, 20-tetrakis(perfluoro)alkylporphyrins and their anomalous properties.[13] The chemistry of fluoroporphyrins has currently attracted the interest of organic, bioorganic, and bioinorganic chemists.

This paper reports on a site-specific synthesis of monofluoroporphyrins and the reconstitution of stable myoglobins with their iron complexes. The heme orientation in the protein cavity is discussed based on the paramagnetic ^{1}H and ^{19}F NMR spectra of the low-spin state; notably, the utility of ^{19}F NMR in studies of the heme-protein interaction is demonstrated, as compared to ^{1}H NMR spectroscopy.

Experimental

The melting points were measured using a Sibata apparatus, and are uncorrected. The IR spectra were obtained using KBr disks on a Hitachi 260-10 spectrometer. The electronic absorption spectra were recorded on a Shimadzu UV-2500PC spectrometer. The NMR spectra were measured with a JEOL α 500 or a JMX-PMX 60 spectrometer. The chemical shifts were recorded in ppm downfield from an internal reference: ($(CH_3)_4Si$ for ^{1}H nuclei) and an external reference: ($CFCl_3$ for ^{19}F nuclei). The redox potential was determined by cyclic voltammetry using a Yanagimoto P-1100 polarographic analyzer. High-resolution mass spectra were obtained with a JEOL JMS-SX102A spectrometer. Reconstitution of apomyoglobin with hemin was performed by treating metmyoglobin with HCl/ethyl methyl ketone.[14] The hemin was dissolved in a minimal volume of pyridine and diluted with deionized water immediately before the reconstitution. The chilled hemin solution was added dropwise to a chilled solution of apomyoglobin in a 50 mM potassium phosphate buffer, pH 7.0. Excess hemin and pyridine were removed by passing through a Sephadex G-25 column. The reconstituted metmyoglobin was purified on a CM-52 column. All of the above-mentioned procedures were carried out at 4 °C. Sperm-whale myo-

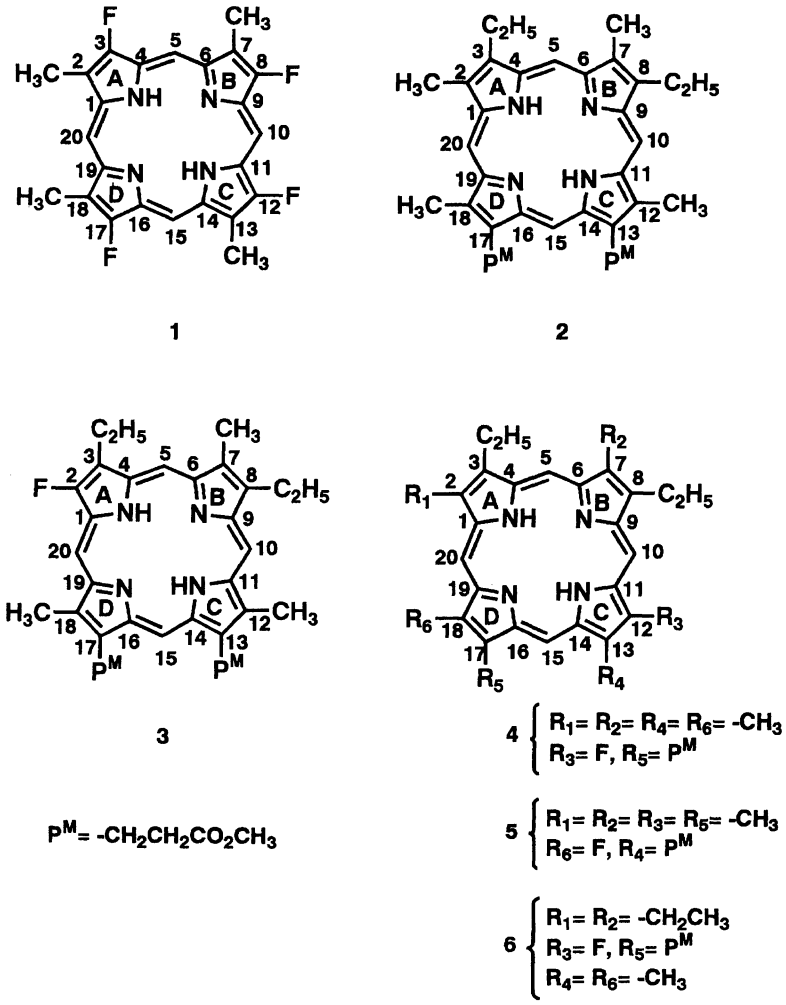


Fig. 1. Structures of ring-fluorinated porphyrins.

globin was purchased from Sigma Chemical Company and used without further purification.

3,4′-Diethyl-4-fluoro-3′,5-dimethyl-2,2′-dipyrromethene Hydrobromide (7). 3-Ethyl-4-fluoro-5-methylpyrrole-2-carboxylic acid (**13**): The high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_8H_{10}NO_2F$: M, 171.0696. Found: *m/z* 171.0687 (221 mg, 1.29 mmol), was decarboxylated in a glass vessel of Kugelrohr at 180 °C/3 mmHg (1 mmHg = 133.322 Pa) under a nitrogen atmosphere to give a light green-colored liquid, 3-ethyl-4-fluoro-5-methylpyrrole (**12**) (133 mg, 78.3%). The product was used immediately without further purification. 3-ethyl-4-fluoro-5-methylpyrrole (133 mg, 1.04 mmol) and 4-ethyl-2-formyl-3-methylpyrrole (143 mg, 1.04 mmol) in MeOH (1.1 ml) were treated with 47% HBr (0.23 ml), and the mixed solution was kept at 0 °C for 1 h. The product was filtered, washed with ether. 3,4′-Diethyl-4-fluoro-3′,5-dimethyl-2,2′-dipyrromethene hydrobromide (**7**) (268 mg, 78.3%) was obtained as an orange-yellow powder. Mp 158—159 °C; MS *m/z* 246 (M^+); ^1H NMR ($CDCl_3$, TMS) δ = 13.22 (br s, 2H, NH), 7.68 (d-s, 1H, –CH=), 7.12 (s, 1H, pyrrole-H), 2.71 (q, 2H, J = 7 Hz, CH_2CH_3), 2.70 (s, 3H, CH_3), 2.48 (q, 2H, J = 7 Hz, CH_2CH_3), 2.30 (s, 3H, CH_3), 1.30 (t, 3H, J = 7 Hz, CH_2CH_3), 1.20 (t, 3H, J = 7 Hz, CH_2CH_3); IR (KBr) 3100 (s), 1630 (s), 1185 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{15}H_{20}N_2F$: (M^+–Br), 247.1611. Found: *m/z* 247.1605.

13,17-Bis[2-(methoxycarbonyl)ethyl]-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrin (3a). A solution of 3,4′-diethyl-4-fluoro-3′,5-dimethyl-2,2′-dipyrromethene hydrobromide (**7**) (129 mg, 39 μmol) and 3,3′-bis[2-(methoxycarbonyl)ethyl]-5′-bromo-5-bromomethyl-4,4′-dimethyl-2,2′-dipyrromethene hydrobromide (236 mg, 39 μmol) in dry CH_2Cl_2 (95 ml) was treated with anhydrous $SnCl_4$ (4.5 ml) for 1 h in the dark. The solvent was removed under reduced pressure; after adding dry MeOH (51 ml) and 47% HBr (10.2 ml), the solution was stirred for 2 h. The reaction mixture was poured into water, and extracted with $CHCl_3$; the combined $CHCl_3$ extract was then washed with 10% HBr solution (70 ml × 3), and the organic layer was dried over Na_2SO_4. After the solvent was evaporated, the residue was dissolved in DMSO (70 ml) and pyridine (1.7 ml), and then stirred for 48 h at room temperature in the dark. After removing the solvent, purification by chromatography on Al_2O_3 gel and SiO_2 gel with $CHCl_3$ respectively, and recrystallization from $CHCl_3$ and hexane gave 65 mg (27.5%) of porphyrin **3a** as red purple crystals. Mp 194—195 °C; MS *m/z* 598 (M^+); ^1H NMR ($CDCl_3$, TMS) δ = 10.01, 10.08 (s, 4H, *meso*-H), 4.36, 4.49 (t, 4H, J = 7 Hz, two $CH_2CH_2CO_2CH_3$), 4.00, 4.15 (q, 4H, J = 7 Hz, two CH_2CH_3), 3.60, 3.62, 3.64, 3.68, 3.71 (s, 15H, five CH_3), 3.29, 3.30 (t, 4H, J = 7 Hz, two $CH_2CH_2CO_2CH_3$), 1.95 (t, 3H, J = 7 Hz, CH_2CH_3), 1.90 (t, 3H, J = 7 Hz, CH_2CH_3), −3.93 (br s, 2H, NH); ^{19}F NMR ($CDCl_3$, $CFCl_3$) δ = −147.0; IR (KBr) 3300 (s), 1738 (s), 1200 cm^{-1} (s); Vis ($CHCl_3$) λ_{max} nm (log ϵ) 398 (5.29),

497 (4.25), 533 (4.11), 564 (4.06), 617 (3.93); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{35}H_{39}N_4O_4F$: M, 598.2955. Found : *m/z* 598.2967.

13,17-Bis[2-(methoxycarbonyl)ethyl]-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrinatoiron(III) Chloride (3b). To a solution of porphyrin-free base **3a** (20 mg, 33.4 μmol) in a mixture of acetic acid (52 ml) and pyridine (1.0 ml) was added iron(II) sulfate (104 mg) and sodium chloride (104 mg). The reaction mixture was refluxed for 2 h and poured into water. The aqueous layer was extracted with $CHCl_3$. The $CHCl_3$ extract was treated with a 5%-HCl solution, and washed with aqueous saturated NaCl to neutral. The extracted solution was dried over Na_2SO_4 and concentrated under reduced pressure. The crude hemin was purified by chromatography on SiO_2 gel with $CHCl_3$. Evaporation of solvent and recrystallization from $CHCl_3$ and hexane gave 17.0 mg (74.1%) of **3b** as brown crystals. Mp 231—233 °C; MS *m/z* 652 (M^+-Cl); IR (KBr) 1735 (s), 1140 cm^{-1} (s); Vis ($CHCl_3$) λ_{max} nm (log ε) 376 (4.90), 504 (4.03), 532 (4.20), 575 (3.84), 632 (3.97); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{35}H_{37}N_4O_4FFe$: M, 652.2148. Found : *m/z* 652.2203.

13,17-Bis(2-carboxyethyl)-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrinatoiron(III) Chloride (3c). Porphyrin dimethyl ester **3b** (11.0 mg, 16.0 mmol) was dissolved in 1% aqueous KOH and MeOH (50 ml). After the mixture was refluxed for 2 h the alcohol was distilled off. The residue was allowed to cool to room temperature and carefully acidified with 2 M HCl (1M = 1 mol dm^{-3}). The product was collected and washed with H_2O. The hemin **3c** (6 mg, 56.6%) was obtained as brown crystals. IR (KBr) 1710 (m), 1150 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{33}H_{33}N_4O_4FFe$: M, 624.1836. Found : *m/z* 624.1862.

4-Fluoro-3′-[2-(methoxycarbonyl)ethyl]-3,4′,5-trimethyl-2,2′-dipyrromethene Hydrobromide (8). 4-Fluoro-3,5-dimethylpyrrole-2-carboxylic acid (**15**): The high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_7H_8NO_2F$: M, 157.0539. Found : *m/z* 157.0538. (95 mg, 0.61 mmol), was decarboxylated in a glass vessel of Kugelrohr at 180 °C/4 mmHg under a N_2 atmosphere to give a pink solid of 3-fluoro-2,4-dimethylpyrrole (**14**) (43 mg, 63.2%). 3-Fluoro-2,4-dimethylpyrrole (**14**) (43 mg, 0.38 mmol) and 2-formyl-3-[2-(methoxycarbonyl)ethyl]-4-methylpyrrole (74 mg, 0.38 mmol) in MeOH (0.4 ml) were treated with 47% hydrobromic acid (0.08 ml); the solution was kept at 0 °C for 1 h. The product was collected and washed with ether. Dipyrromethene hydrobromide **8** (90 mg, 63.8%) was obtained as a red-brown powder. Mp 150 °C (decomp); ^1H NMR ($CDCl_3$, TMS) δ = 7.68 (s, 1H, ring-H), 7.47 (s, 1H, –CH=), 3.68 (s, 3H, $CH_2CH_2CO_2CH_3$), 3.06 (t, *J* = 7 Hz, 2H, $CH_2CH_2CO_2CH_3$), 2.93 (t, *J* = 7 Hz, 2H, $CH_2CH_2CO_2CH_3$), 2.11, 2.38, 2.72 (s, 9H, three CH_3); IR (KBr) 3450 (w), 1725 (s), 1625(s), 1250 (s), 1155 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{16}H_{20}N_2O_2F$: (M^+-Br), 291.15009. Found : *m/z* 291.1490.

3,8-Diethyl-12-fluoro-17-[2-(methoxycarbonyl)ethyl]-2,7,13,18-tetramethylporphyrin (4a). This compound was prepared in a similar manner to that for **3a**. Yield 53.1%; mp 229—230°C; ^1H NMR ($CDCl_3$, TMS) δ = 10.05, 10.04, 10.03, 9.92 (s, 4H, *meso*-H), 4.40 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 4.11, 3.99 (q, *J* = 8 Hz, 4H, two CH_2CH_3), 3.65 (s, 6H, two CH_3), 3.64, 3.53, 3.48 (s, 9H, three CH_3), 3.23 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 1.85 (t, *J* = 8 Hz, 3H, CH_2CH_3), 1.83 (t, *J* = 8 Hz, 3H, CH_2CH_3), −4.04 (br s, 2H, two NH); IR (KBr) 3300 (w), 1730 (s), 1200 cm^{-1} (m); Vis ($CHCl_3$) λ_{max} nm (log ε) 396 (5.11), 497 (4.03), 531 (3.78), 565 (3.67), 618 (3.57); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{32}H_{35}N_4O_2F$: M, 526.2744. Found : *m/z* 526.2722.

3,8-Diethyl-12-fluoro-17-[2-(methoxycarbonyl)ethyl]-2,7,13,18-tetramethylporphyrinatoiron(III) Chloride (4b). This compound was prepared in a similar manner to that for **3b**. Yield 59.9%; mp >280 °C; IR (KBr) 1730 (s), 1150 cm^{-1} (s); Vis ($CHCl_3$) λ_{max} nm (log ε) 376 (4.82), 501 (3.79), 536 (3.79), 636 (3.50); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{32}H_{33}N_4O_2FFe$: M, 580.1937. Found : *m/z* 580.1913.

17-(2-carboxyethyl)-3,8-diethyl-12-fluoro-2,7,13,18-tetramethylporphyrinatoiron(III) Chloride (4c). This compound was prepared in a similar manner to that for **3c**. Yield 90.6%; IR (KBr) 1710 (m), 1150 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{31}H_{31}N_4O_2FFe$: M, 566.1780. Found : *m/z* 566.1816.

3,8-Diethyl-18-fluoro-13-[2-(methoxycarbonyl)ethyl]-2,7,12,17-tetramethylporphyrin (5a). This compound was prepared in a similar manner to that for **3a**. Yield 16.0%; mp 221 °C; IR (KBr) 3300 (w), 1730 (s), 1200 cm^{-1} (s); ^1H NMR ($CDCl_3$, TMS) δ = 10.08 (s, 2H, two *meso*-H), 10.07, 9.96 (s, 2H, *meso*-H), 4.44 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 4.12, 4.00 (q, *J* = 8 Hz, 4H, two CH_2CH_3), 3.69, 3.66, 3.65, 3.55, 3.49 (s, 15H, five CH_3), 3.26 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 1.86 (t, *J* = 8 Hz, 3H, CH_2CH_3), 1.84 (t, *J* = 8 Hz, 3H, CH_2CH_3), −3.98 (br s, 2H, two NH); Vis ($CHCl_3$) λ_{max} nm (log ε) 396 (5.20), 497 (4.13), 532 (3.88), 565 (3.80), 617 (3.69); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{32}H_{35}N_4O_2F$: M, 526.2744. Found : *m/z* 526.2698.

3,8-Diethyl-18-fluoro-13-[2-(methoxycarbonyl)ethyl]-2,7,12,17-tetramethylporphyrinatoiron(III) Chloride (5b). This compound was prepared in a similar manner to that for **3b**. Yield 77.8%; mp >280 °C; IR (KBr) 1730 (s), 1150 cm^{-1} (s); Vis ($CHCl_3$) λ_{max} nm (log ε) 377 (4.86), 502 (3.83), 533 (3.80), 635 (3.51); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{32}H_{33}N_4O_2FFe$: M, 580.1937. Found : *m/z* 580.1906.

13-(2-Carboxyethyl)-3,8-diethyl-18-fluoro-2,7,12,17-tetramethylporphyrinatoiron(III) Chloride (5c). This compound was prepared in a similar manner to that for **3c**. Yield 95.9%; IR (KBr) 1710 (m), 1150 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{31}H_{31}N_4O_2FFe$: M, 566.1780. Found : *m/z* 566.1802.

2,3,7,8-Tetraethyl-12-fluoro-13,18-dimethyl-17-[2-(methoxycarbonyl)ethyl]porphyrin (6a). This compound was prepared in a similar manner to that for **3a**. Yield 44.4%; mp 180—181 °C; ^1H NMR ($CDCl_3$, TMS) δ = 10.12, 10.10, 10.09, 9.97 (s, 4H, *meso*-H), 4.45 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 4.16, 4.14 (q, *J* = 8 Hz, 4H, two CH_2CH_3), 4.03 (q, *J* = 8 Hz, two CH_2CH_3), 3.70, 3.66, 3.50 (s, 9H, three CH_3), 3.27 (t, *J* = 8 Hz, 2H, $CH_2CH_2CO_2CH_3$), 1.93 (t, *J* = 8 Hz, 6H, two CH_2CH_3), 1.90 (t, *J* = 8 Hz, 3H, CH_2CH_3), 1.89 (t, *J* = 8 Hz, 3H, CH_2CH_3), −3.93 (br s, 2H, two NH); IR (KBr) 3300 (m), 1730 (s), 1200 cm^{-1} (m); Vis ($CHCl_3$) λ_{max} nm (log ε) 397 (5.13), 497 (4.05), 532 (3.81), 564 (3.73), 617 (3.60); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{34}H_{39}N_4O_2F$: M, 554.3057. Found : *m/z* 554.3031.

2,3,7,8-Tetraethyl-12-fluoro-17-[2-(methoxycarbonyl)ethyl]-13,18-dimethylporphyrinatoiron(III) Chloride (6b). This compound was prepared in a similar manner to that for **3b**. Yield 87.1%;

mp >280 °C. IR (KBr) 1735 (s), 1150 cm^{-1} (s); Vis ($CHCl_3$) λ_{max} nm (log ϵ) 377 (4.88), 503 (3.79), 529 (3.77), 631 (3.47); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{34}H_{37}N_4O_2FFe$: M, 608.2250. Found : *m/z* 608.2289.

17-(2-Carboxyethyl)-2,3,7,8-tetraethyl-12-fluoro-13,18-dimethylporphyrinatoiron(III) Chloride (6c). This compound was prepared in a similar manner to that for **3c**. Yield 68.2%; IR (KBr) 1710 (m), 1150 cm^{-1} (s); high-resolution mass spectrum (HRFAB MS) (*m*-nitrobenzyl alcohol matrix) *m/z* calcd for $C_{33}H_{35}N_4O_2FFe$: M, 594.2093. Found : *m/z* 594.2064.

Results and Discussion

Synthesis of Ring-Fluorinated Porphyrins and Hemins. The framework of monofluoroporphyrin is divided to two kinds of dipyrromethenes on the basis of a facile retro-synthetic consideration. Cyclization of the tetrapyrrole ring has been performed by the coupling of two dipyrromethenes consisting of the A-B pyrrole rings and the C-D rings. There are two key pyrroles, 3-ethyl-4-fluoro-5-methylpyrrole-2-carboxylic acid (**13**) and 4-fluoro-3,5-dimethylpyrrole-2-carboxylic acid (**15**), to accommodate a fluorine at the site-directed β-position of the pyrrole ring of porphyrins. Mesoporphyrin IX (**2**) has been chosen as a standard where two vinyl groups at the 3- and 8-positions are replaced by two ethyl groups. The coupling reactions of these two pyrroles, **13** and **15**, give rise to two (monofluoro)dipyrromethene derivatives, (**7**) and (**8**), respectively. The combination of dipyrromethenes and monofluorodipyrromethene leads to the synthesis of several site-specific fluoroporphyrins.

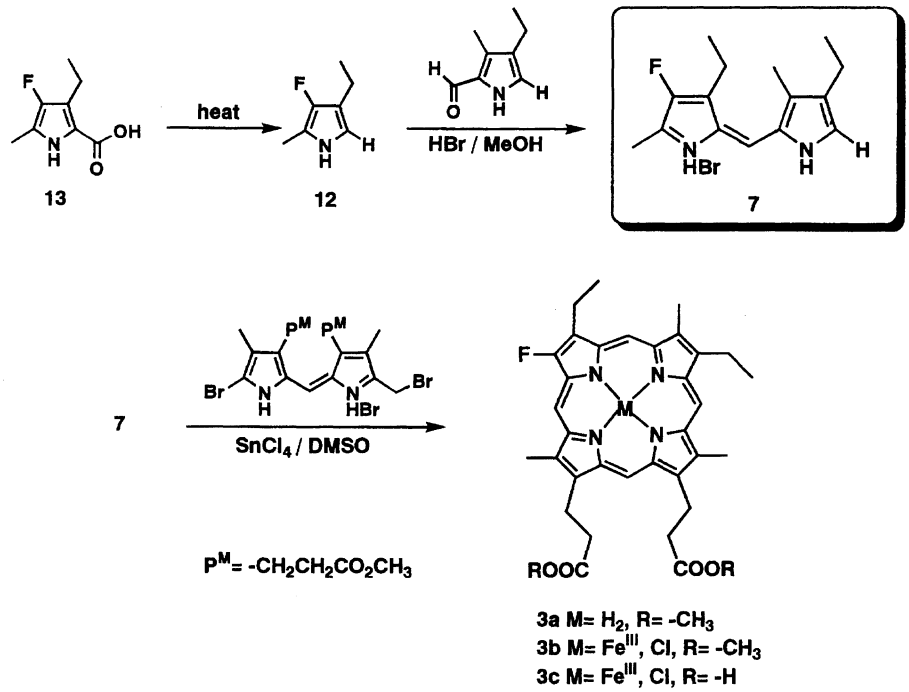
The oxidative cyclization[15] of two biladiene-*a,c* hydrobromides was carried out with anhydrous $SnCl_4$ to afford 2-fluoro-2-demethylmesoporphyrin IX (**3**), as a mesoporphyrin IX analogue (see Scheme 1). Similar reactions of monofluoro dipyrromethene and different types of tetraalkyldipyrromethenes, (**9**) and (**10**), gave the desired ring-fluorinated porphyrins, (**4**) and (**5**), in moderate yields, respectively, as shown in Schemes 2 and 3.

The porphyrin (**6**) was obtained by the oxidative cyclization of tetraethyldipyrromethene and monofluorodipyrromethene (**8**) (see Scheme 4). Symmetrical tetraethyl groups of the A and B rings have been designed so as to decrease the complexity in the heme orientation in the hydrophobic protein cavity. Iron(III) complexes of fluoroporphyrins **3**—**6** were prepared by treating the free base with $FeSO_4(7H_2O)$, NaCl and pyridine in acetic acid. The structures of the porphyrins and their iron(III) complexes were characterized by ^{1}H and ^{19}F NMR and the absorption spectra.

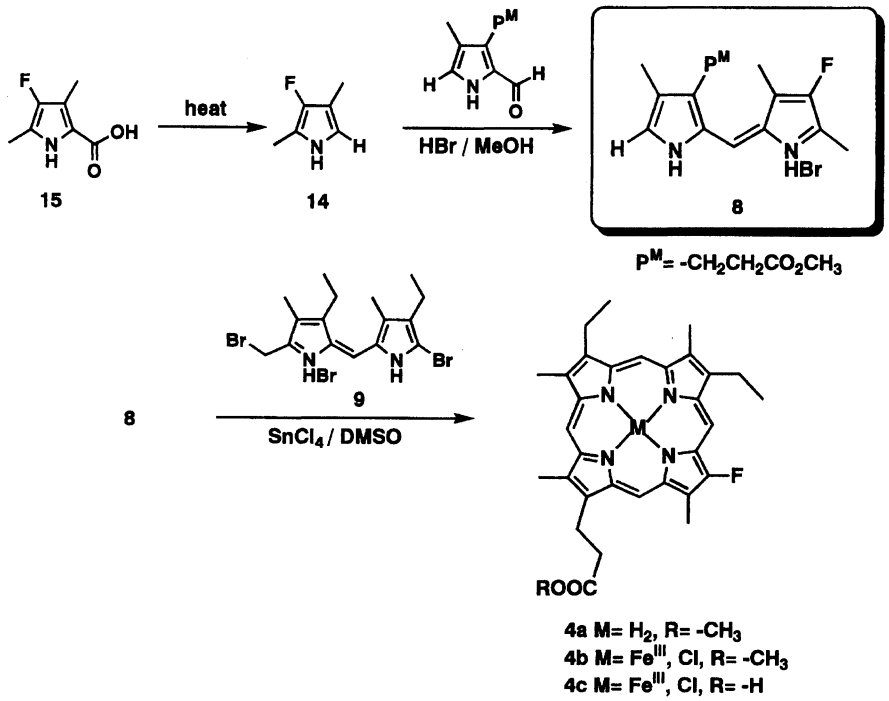
Electronic Absorption Spectra of Synthetic Hemins and Reconstituted Myoglobins. The absorption maxima of Soret region, Q bands and charge-transfer band show a small difference among synthetic hemins, as shown in Table 1.

The electronic effects of the fluoro substituent on the electronic spectra were very small. For example, the Soret band of **3** appears at 398 nm, while that of the mesoporphyrin IX dimethyl ester appears at 400 nm; the Q-bands of both porphyrins appear at almost the same wavelength. This is readily explicable on the basis of electronic effects of the fluoro substituent for aromatic compounds. Similar trends have been observed for the met-, deoxy-, and oxymyoglobins (see Table 2). There are no significant differences among the myoglobins in met-, deoxy- and oxy-forms.

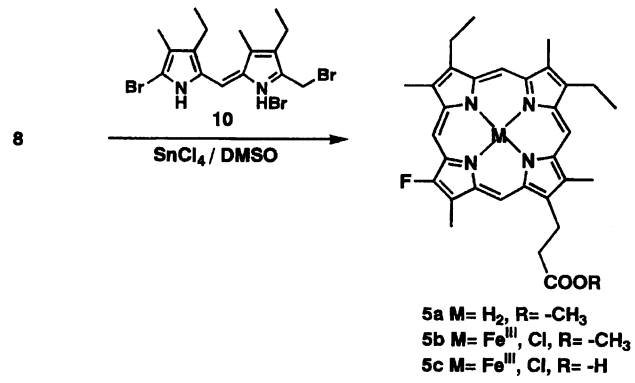
Cyclic Voltammetric Measurement. Table 3 shows cyclic-voltammetry measurements of the porphyrins. Monofluoroporphyrin **3** shows a slight anodic shift for the first re-



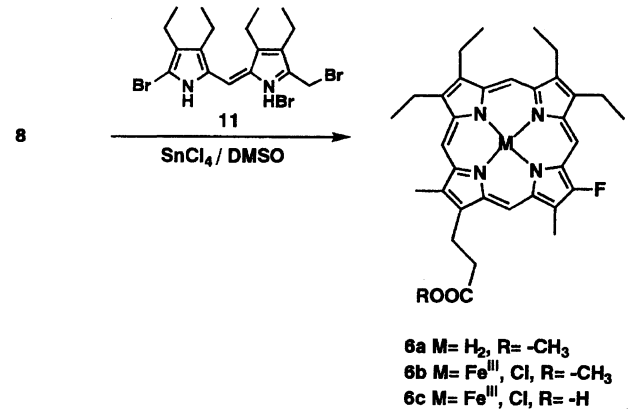
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

duction potential compared to those of the reference, *meso*-porphyrin IX **2** and native protoporphyrin IX diesters. The replacement of an electron-donating methyl group with a weak electron-withdrawing fluoro substituent gives rise to a slight anodic shift. Tetrafluorotetramethylporphyrin (**1**) is readily reduced relative to the octaalkylporphyrin due to four fluoro substitution. The potential of the redox couple of Fe(III)/Fe(II) of the iron complex of **3** is close to that of the iron complexes of **2**. Since the perturbation of monofluoro substitution of porphyrin is very small, chemical functionalization, like ring-fluorinated heme, seems to be useful as a mimicking prosthetic heme in reconstitutional studies concerning heme proteins and heme enzymes.

Table 1. Absorption Maxima of Ring-Fluorinated Hemins[a)]

(λ_{max}, nm), (log ϵ)

Hemin	Soret	III	II	I
3b	376 (4.90)	504 (4.03)	532 (4.20)	632 (3.97)
4b	376 (4.82)	501 (3.79)	536 (3.79)	636 (3.50)
5b	377 (4.86)	502 (3.83)	533 (3.80)	635 (3.51)
6b	377 (4.88)	503 (3.79)	529 (3.77)	631 (3.47)

a) The spectra were measured in $CHCl_3$.

Table 2. Absorption Maxima of Reconstituted Sperm Whale Myoglobins[a)]

(λ_{max}, nm)

Myoglobin	Fe(III)			Fe(II)		Fe(II)–O_2		
	Soret	$\alpha\beta$	CT	Soret		Soret	β	α
rMb(**3c**)	392	489	618	419	542	400	530	564
rMb(**4c**)	391	490	619	418	543	394	528	563
rMb(**5c**)	391	490	620	419	541	394	529	564
rMb(**6c**)	393	499	619	419	542	398	530	563

a) The spectra were measured in 50 mmol dm^{-3} potassium phosphate buffer (pH 7.0) at 25 °C.

Heme Orientation by ^{1}H and ^{19}F NMR Studies.

Table 3. Half-Wave Potentials of Porphyrin Free Bases and Their Metal Complexes[a)]

Compound	M	Ring oxidation $E_{1/2}$ (V vs. SCE)		Ring reduction $E_{1/2}$ (V vs. SCE)	
		Second	First	Second	First
(3)	2H	1.34	1.01	−1.30	
	Zn(II)	1.09	0.74	−1.49	
	Fe(III)Cl	1.44	1.13	−0.88	−1.16
		Fe(III)/Fe(II)		−0.31	
(1)	2H			−1.17	ca.−1.6
Porphyrin	2H			−1.18	−1.59
Mesoporphyrin IX Dimethyl ester (2)	2H	1.34	0.91	−1.37	−1.74
	Zn(II)	1.03	0.69	−1.55	
	Fe(III)Cl	1.37	1.03		
		Fe(III)/Fe(II)		−0.37	

a) Measured in CH_2Cl_2 (0.1 M TBPA).

Paramagnetic ^{1}H NMR spectroscopy has provided a powerful and convenient tool for discriminating the heme orientations in the cavity of hemoprotein. La Mar and coworkers have made confident assignments of the peripheral substituents with the aid of synthetic hemes. Especially the proton resonances of the four methyl groups are observable at a region apart from the tremendous numbers of various protons from the protein. In combination with the synthetic method of site-specific deuteration of the respective methyl groups of protoheme one can make a more reliable assignment. The isotropic chemical shifts of the peripheral methyl groups reflect the structure near to be proximity of the heme. Figure 2 gives a schematic representation of the normal and reverse heme orientations in the heme cavity from a top view of the proximal site. The positions of the heme side chains are numbered from the A ring to the D ring clockwise according to the IUPAC-IUB nomenclature. Synthetic hemes **3**—**6** and native protoheme have no C_2 symmetry for the C_5–C_{15} axis. Therefore, the asymmetric structure of the heme gives rise to two forms in the chiral heme cavity. At the initial state of reconstituted myoglobin, an equal population of the normal and reverse forms seems to be expected. The ratio of the two orientations may mainly depend on the thermodynamic stability. In the case of native protoheme, the major orientation at the equilibrium state is referred to as normal. The definition of the normal orientation for synthetic hemes is that the 2- and 7-methyl groups and the porphyrin framework of a synthetic heme in the heme cavity can be superposed on those of the native protoheme (2-CH_3 and 7-CH_3). The reverse heme orientation in the heme pocket is such that the normal oriented heme is rotated by 180 ° about an axis through the C_5-to-C_{15} *meso* carbons. The heme disorder for orientation is governed by interactions between the peripheral heme side chains and the amino acid residues. La Mar and Smith have stated that the vinyl contacts are much more important than the propionate salt bridge in determining the orientational preference in the heme cavity, base on a ^{1}H NMR study of the protoheme IX derivatives with each propionate replaced

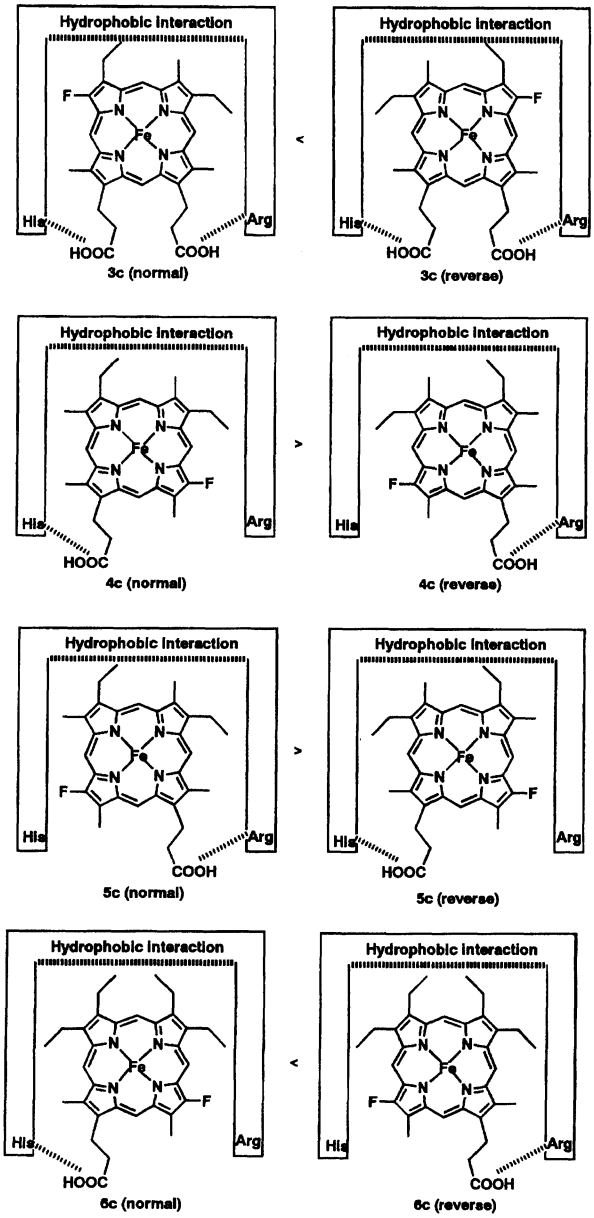


Fig. 2. Schematic representation of two orientational isomers of hemin **3**—**6** in heme pocket. According to X-ray structural analysis, Arg (CD3) and His (FG2) contact with 13- and 17-propionates of hemin, respectively.

with a methyl group.[16] We have reported on crystallographic studies of reconstituted myoglobins with chemically modified hemes at the 3- and 8-positions in order to clarify the hydrophobic interaction between the heme and the protein. The heme orientations were determined by subtracting the electron densities of the peripheral 2-, 3-, 7-, and 8-positions of the hemes in myoglobin from native myoglobin. The amino acid residues of Phe 43 (CD1), Val 68 (E11) and Ile 99 (FG4) play an important role in governing the heme disorder.[17] The hydrophobic interaction of the peripheral side chains with amino acid residues and the coplanarity of the 8-viny group with the porphyrin plane have been evidenced by ^{1}H NMR and crystallographic investigations. Furthermore,

interactions between the 13- and 17-propionates of the heme and polar residues of proteins like Arg and Gln through a hydrogen-bonding network involving water molecules help to stabilize the structure of the protein, and to decrease the autoxidation rate of oxymyoglobin.[13] NMR and thermodynamic studies on reconstituted myoglobins with a monopropionate *meso*-type heme have revealed that the contribution of the hydrophobic interaction is more effective in stabilizing the normal orientation than that of the polar interaction of the two propionates.[18] La Mar and co-workers have reported on NMR studies of myoglobins reconstituted with the monopropionate protoheme and 2, 3, 7, 8, 12, 13, 18-heptamethyl-17-propionate heme, and have discussed the contribution of the propionate to the heme orientation.[16,19] It was found that the hydrophobic interaction of 2-, 3-, 7-, 8-substituents of the A and B ring of the synthetic heme, arranged like protoheme (2-CH_3, 3-vinyl, 7-CH_3, and 8-vinyl), is more effective than polar interactions of the 13-propionate-Arg (CD3) and the 17-propionate linked to interior His (FG3). Independently we reported the studies on ^{1}H NMR, autoxidation and oxygen affinity of myoglobin reconstituted with the chemically modified mesohemes.[20]

Orientation of 3c. The introduction of a 2-fluoro substituent in the place of the 2-CH_3 of mesoheme stabilizes the reverse orientation at equilibrium. Monitoring of the change in the ratio of the normal-to-reverse forms shortly after reconstitution by ^{1}H and ^{19}F NMR has shown the existence of a small fraction of the normal form during the initial stage, and its decrease with time. For ^{1}H NMR of rMb (**3c**), signals at 29 and 22 ppm have been assigned to 18-CH_3 and 7-CH_3 respectively, which take the 3- and 12-position of the normally oriented heme. A small signal at 26 ppm has been assigned to the 12-CH_3 proton of the normal form, which disappears at equilibrium (see Fig. 3), because the 12-CH_3 group takes the 18-position upon rotation, the signal of which appears at a higher magnetic field than 10 ppm. La Mar and co-workers have reported on an exact assignment of 2-, 7-, 12-, and 18-CH_3 of protoheme in metcyano Mb using a site-specific deuterated methyl group. According to their studies, the signals of 2- and 3-CH_3 (A ring) and 12- and 13-CH_3 (C ring) are observed at a lower magnetic field than those set of the signals of 7- and 8-CH_3 (B ring) and 17- and 18-CH_3 (D ring). A methyl group of the C ring appears at a lower magnetic field than that of the A ring. A paramagnetic shift, indirectly caused by the coordinated imidazole (His 98 (E 8)) to the heme, is considered to give a large difference in those chemical shifts in the heme cavity.[16,20]

The ^{19}F NMR spectra of the low-spin iron(III) complex and high-spin complex show signals at −104 and 151 ppm from $CFCl_3$, respectively.[11] The ^{19}F NMR spectrum of rMb (**3c**)(CN) at the low-spin state reveals a large peak at −124 ppm and a small one at −96.5 ppm (see Fig. 4). The smaller signal assigned to the normal oriented heme disappeared at equilibrium due to a change from the normal to reversed heme. The 2-Fluoro signal of **3** takes the 8-position in the reversed mode. The methyl signals in a lower magnetic field in ^{1}H NMR (Fig. 3) also indicate that the reverse orientation

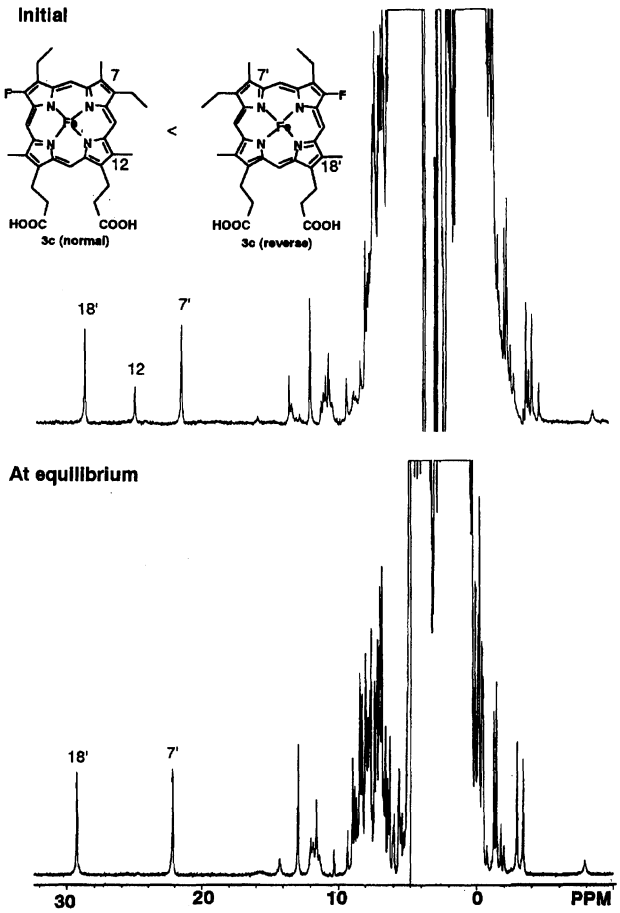


Fig. 3. Low-field portion of 500 MHz ^{1}H NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0. Tentative assignments of methyl groups are shown in the spectrum. Substituents in the reverse oriented heme are designated by primes such as 7′ and 18′.

dominates at equilibrium. We can tentatively assign the signal at 29.2 ppm to 18-Me in the reversed form, and that at 22.1 ppm to 7-Me in the reversed form. It is noted that pyrrole β-hydrogen of the low- and high-spin iron(III) complexes of 5, 10, 15, 20-tetraphenylporphyrin appear at −19 and 60 ppm. The population of native protoheme in the normal mode is higher than that of mesoheme due to a particular stabilization of the 3- and 8-vinyl groups. The fraction of the normal orientation of mesoheme (2- and 7-methyl, 3- and 8-ethyl) in the heme cavity is seven-times that of the reverse form. It is noted that the replacement of 2-CH_3 of *meso*heme with a fluoro substituent results in a dramatic change in the heme orientation compared to *meso*heme. We have reported that the introduction of a bulky alkyl group, such as an isopropyl group, to the 8-position stabilizes the reversed form.[17] This implies that, in rMb (**3c**)(CN), the repulsive interaction of the 8-ethyl-group of heme with Ile 99 and Phe 43 decreases the stability of the normal orientation. A positional change of the smaller 2-F of **3** from the 2-position in the normal form to the 8-position of the reverse form reduces the repulsive interaction, resulting in a marked stabilization of the reversed

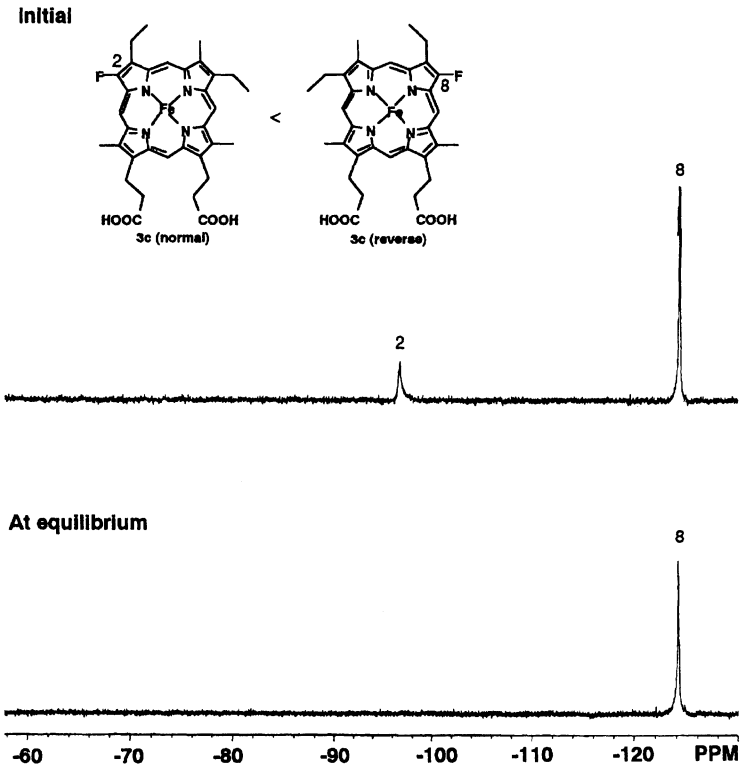


Fig. 4. ^{19}F NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

mode in the heme cavity. Changes in the C_γ–H signals of Ile 99 (FG 43) at around −3 ppm (Fig. 3) also support the above-mentioned conclusion. With time those signals due to Ile of the minor component disappear at equilibrium.

Orientation of 4c, 5c and 6c. In complexes **4c** and **5c**, the structure of the A and B rings is the same as that of the *meso*-type porphyrin, having 2-CH_3, 3-C_2H_5, 7-CH_3, and 8-C_2H_5 groups; however the C and D rings are rotated by 180° about the C_5–C_{15} axis. The respective C ring of **4c** and the D ring of **5c** have no propionate group to serve as a polar interaction site with the protein. The rMb (**4c**) reveals a larger fluoro signal at −81 ppm and a smaller one at −112 ppm (Fig. 6). If the ^{19}F NMR signals at higher and lower magnetic field are assignable to the fluoro substituent at the 18- and 12-position in the heme cavity, respectively, one may estimate the heme disorder for **4c** and **5c** in the protein. The ratio of the normal-to-reverse form of **4c** was 4.6 based on the peak area of ^{19}F NMR, while it was 4.7 based on the peak area of ^1H NMR, indicating that both NMR spectra give consistent results. The normal orientation is a more favorable mode than the reverse form in the protein (see Figs. 5, 6, 7, and 8). In the ^{19}F NMR of rMb (**5c**), a larger peak at −109 ppm and a very small peak at −80 ppm indicate the marked preferable normal orientation shortly after reconstitution. In the case of **4c**, a salt bridge formation of 17-propionate at the 13-position with Arg (CD3) of myoglobin during an earlier stage results in a considerable population in the reverse form shortly after reconstitution. In both myoglobins, the hydrophobic interaction of particularly ordered side chains of the A and B rings is more effective than a polar interaction of

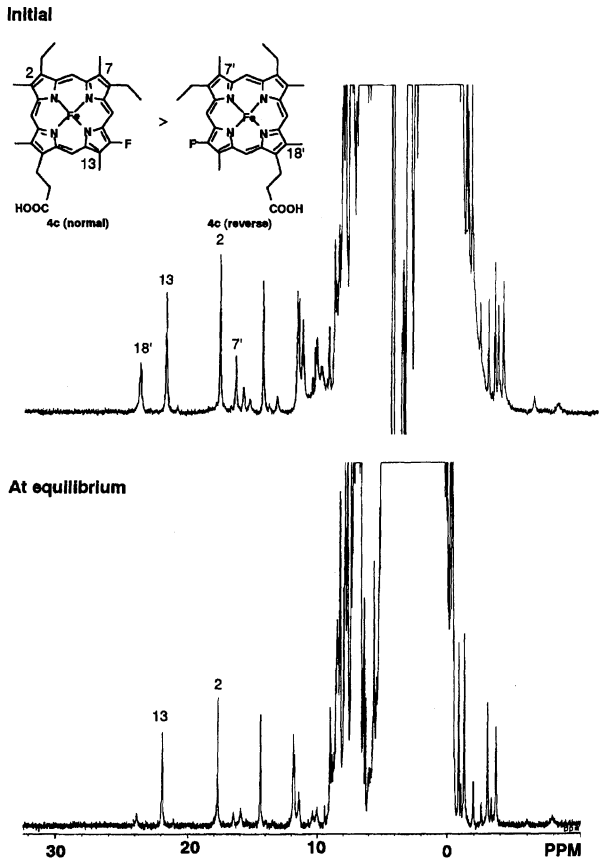


Fig. 5. Low-field portion of 500 MHz ^1H NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

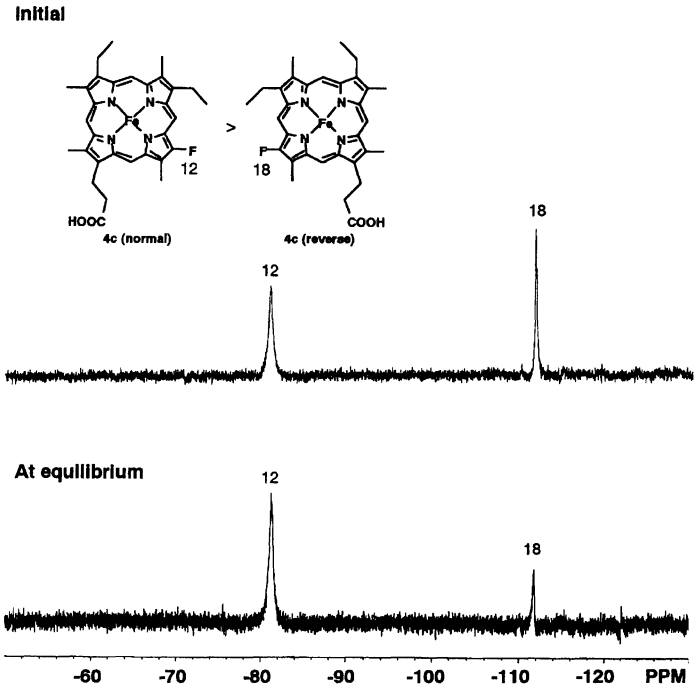


Fig. 6. ^{19}F NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

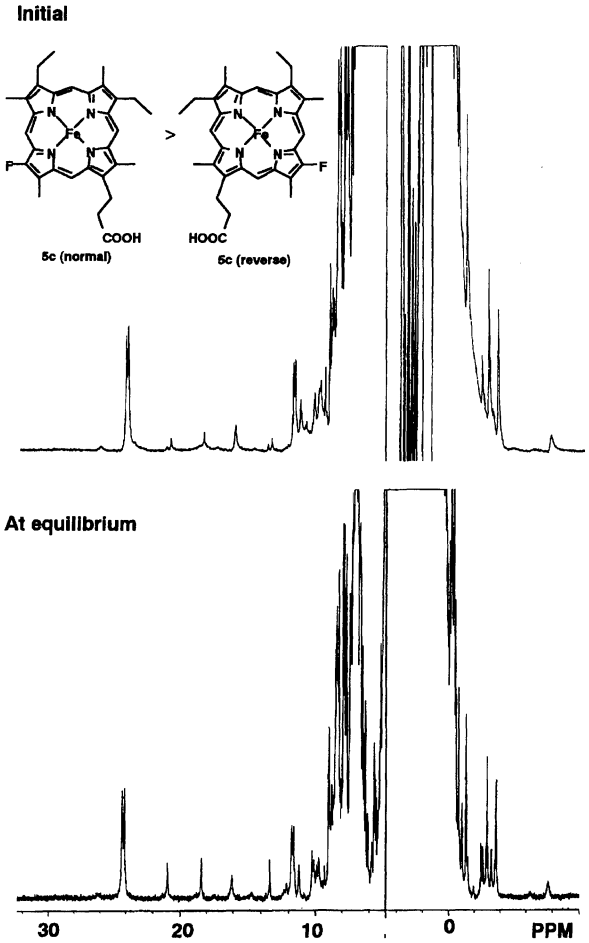


Fig. 7. Low-field portion of 500 MHz ^{1}H NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

the hemin propionate with amino acid residues to stabilize the normal orientation at equilibrium.

Heme **6c** has a symmetric structure for the substituents of the A and B rings, having tetraethyl groups and β-positions of the C ring substituted with fluoro substituent and a methyl group. The ratio of the major-to-minor modes has been estimated to be 1.4 based on the integration of signals of −83 and −112 ppm in ^{19}F NMR of rMb (**6c**). The polar interaction of a 13-propionate with Arg (CD3) constitutes a slightly stable form in the protein (see Figs. 9 and 10).

Consequently, the 13- and 17-propionate groups are not

Table 4. ^{1}H NMR Chemical Shifts of Reconstituted Sperm Whale Metcyanomyoglobins in H_2O–D_2O, pH 7.0 at Initial and Equilibrium[a)]

	Methyl (at initial)[b)]		Ile 99 (at equilibrium)[c)]	
Myoglobin	Normal	Reverse	Major	Minor
rMb(**3c**)	25.5	29.2	−2.9	—
		22.1	−3.4	—
			−7.9	—
rMb(**4c**)	21.8	23.8	−3.1	−2.6
	17.7	16.4	−3.7	−3.3
			−7.9	−6.0
rMb(**5c**)	24.2	26.0	−3.0	−2.5
	24.0	23.8[d)]	−3.7	−3.3
			−7.7	−6.3
rMb(**6c**)	18.2	23.3	−2.5	−2.4
			−3.0	−2.9
			−5.4	−5.0

a) Shifts in ppm from DSS in D_2O solution at 25 °C. b) Peripheral methyl protons of hemin (2-, 7-, 12-, 13-, 17-, and 18-Me). c) Cγ- and δ-protons of Ile 99. d) Minor component was not completely detected by ^{1}H NMR.

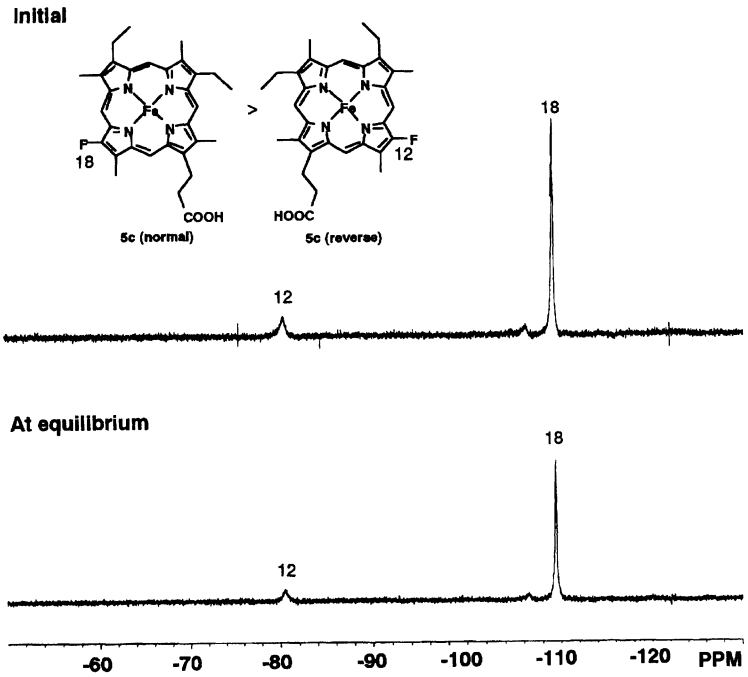


Fig. 8. ^{19}F NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

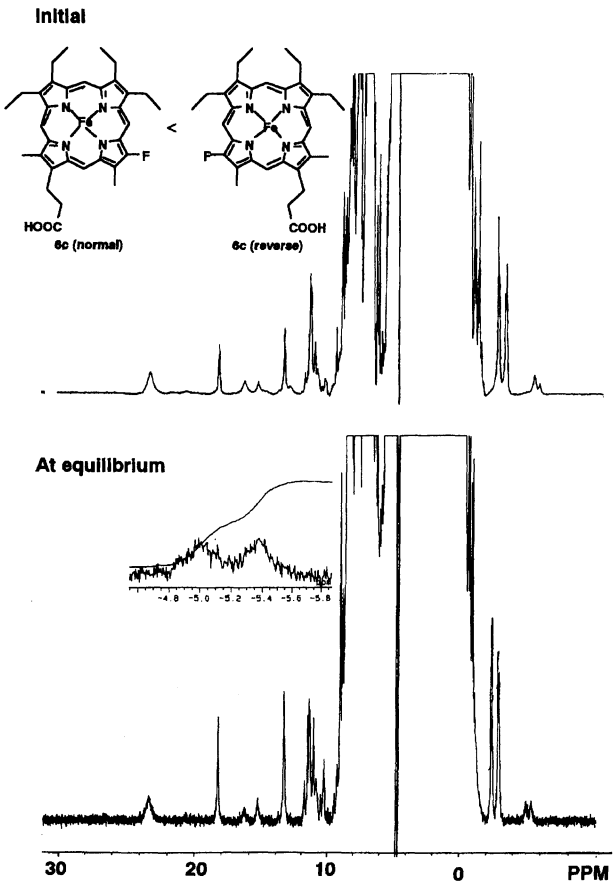


Fig. 9. Low-field portion of 500 MHz ^{1}H NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

effective in determining the heme orientation as vinyl groups of the protoheme and ethyl groups of mesoheme, as pointed out by La Mar. Table 4 lists the prominent CH_3 signals and the methyl signals of the residue of Ile 99 of reconstituted myoglobins. The signal of C_γ–H at a higher magnetic field due to the ring current of the porphyrin macrocycle indicates the existence of the two orientation.[18]

In conclusion, the present work has demonstrated that (1) The site-specific fluorination of the prosthetic hemes was achieved by a stepwise synthesis from the monofluoropyr-

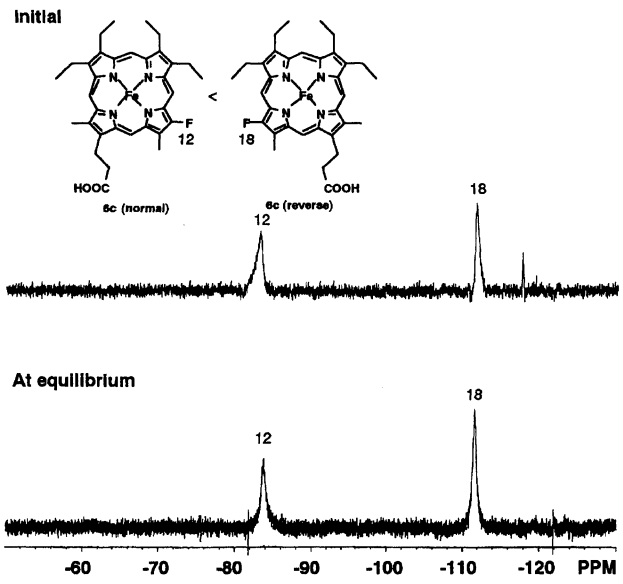


Fig. 10. ^{19}F NMR spectra of reconstituted metmyoglobin with KCN (low spin) in 50 mmol dm^{-3} potassium phosphate buffer at 25 °C, pH 7.0.

roles; (2) ^{19}F NMR studies on iron(III) complexes of the ring-fluorinated porphyrins enable us to discriminate the low- and high-spin states based on the signal positions; and (3) the thus-obtained fluoroheme briefly allows us to determine the heme orientation in proteins and to understand the interaction between heme and protein.

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