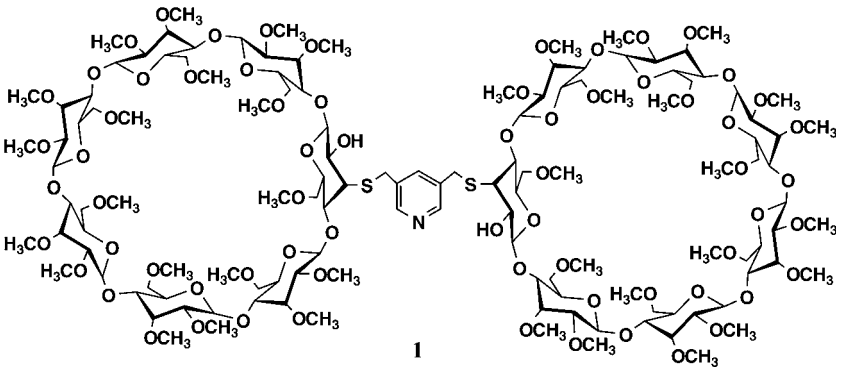
Supramolecular Chemistry

Dioxygen Binding to a Simple Myoglobin Model in Aqueous Solution**

Koji Kano, Hiroaki Kitagishi, Masahito Kodera, and Shun Hirota*

Herein we report reversible dioxygen binding to a simple model system of myoglobin composed of a per-*O*-methylated β-cyclodextrin (CD) dimer having a pyridine linker (**1**) and [tetrakis(4-sulfonatophenyl)porphinato]iron(II) ([Fe^{II}(tpps)]) in aqueous solution. Myoglobin (Mb) and hemoglobin (Hb) form stable O_2 adducts in aqueous solution at a neutral pH value. Globin plays an essential role in stabilizing the O_2 adduct in biological systems. Without this protein, oxyheme is immediately oxidized to a Met form by the action of a proton or hydroxide ion.[1] Heme is protected from acid- or base-induced autoxidation by the globin that surrounds the iron porphyrin (FePor). Many model systems of Mb and Hb were examined after the development of the picket-fence porphyrin by Collman et al.[2,3] In most model systems O_2 binding was achieved in organic solvents, where a trace amount of water had to be removed from the system. The concept proposed by Collman's research group has been expanded into the binding of O_2 to hydrophobic picket-fence Fe^{II} porphyrins, which are included in vesicles[4] or albumin[5] in aqueous media. Although dendrimers with Fe^{II}Por cores were expected to mimic the function of Mb or Hb, no stable O_2 adducts were formed in these systems in aqueous solution.[6,7] Since the O_2 adduct of the dendrimers is efficiently formed in absolute toluene,[6] it is evident that construction of a hydrophobic pocket for placing the iron(II) center of an FePor is the essential factor for realizing O_2 binding in aqueous solution. In the present model system, we used the striking ability of per-*O*-methylated β-CD to include water-soluble tetraarylporphyrins and yield extremely stable 1:2 (Por:CD) inclusion complexes in aqueous solution.[8] Two per-*O*-methylated β-CD moieties were linked by a bridge involving a pyridine ligand. Such a CD dimer (**1**) worked well as a simple Mb model in aqueous solution.



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The synthesis of **1** (Scheme 1) was carried out by using the method reported by Lawrence et al.[9] The changes in the absorption spectrum of [Fe^{III}(tpps)] in phosphate buffer of pH 5.0 were measured as a function of the concentration of **1** (Figure 1) to examine whether **1** forms a suitable complex in which two CD moieties include the sulfonatophenyl groups at the 5 and 15 positions of [M(tpps)] (M = metal ion) and the pyridine ligand coordinates to the M^{II} or M^{III} site of the porphyrin. No μ-oxo dimer was formed at pH 5.0 and the predominant species involving [Fe^{III}(tpps)] was the diaqua complex [$Fe^{III}(H_2O)_2$(tpps)].[10] The Soret band of [$Fe^{III}(H_2O)_2$(tpps)] at 388 nm shifted to 421 nm upon addition of **1**. The spectral changes were sharply saturated after the addition of one equivalent of **1**, which indicated the formation of a very stable 1:1 complex between [$Fe^{III}(H_2O)_2$(tpps)] and **1**. Analysis of the titration curve provided a binding constant

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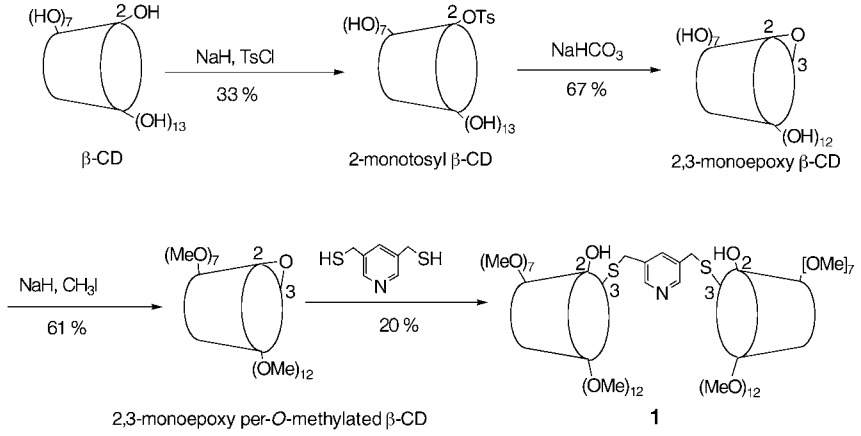


Scheme 1. Synthetic route of **1**. Ts = toluene-4-sulfonyl.

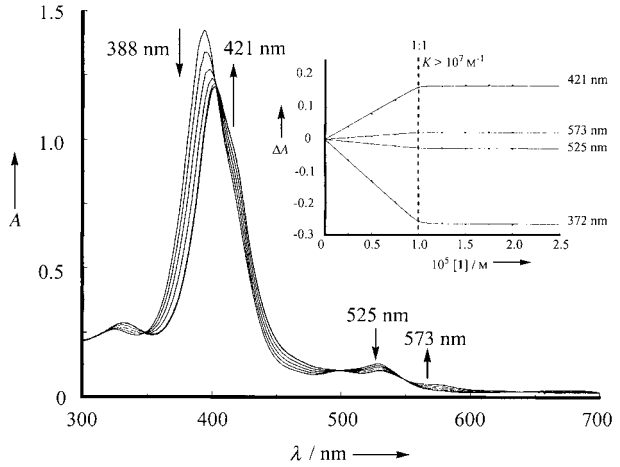


Figure 1. UV/Vis spectral changes of [Fe^{III}(tpps)] (1×10^{-5} M) as a function of [**1**] in 0.05 M phosphate buffer at pH 5.0 and 25 °C. The titration curves were analyzed by the nonlinear least-squares method to estimate *K*.

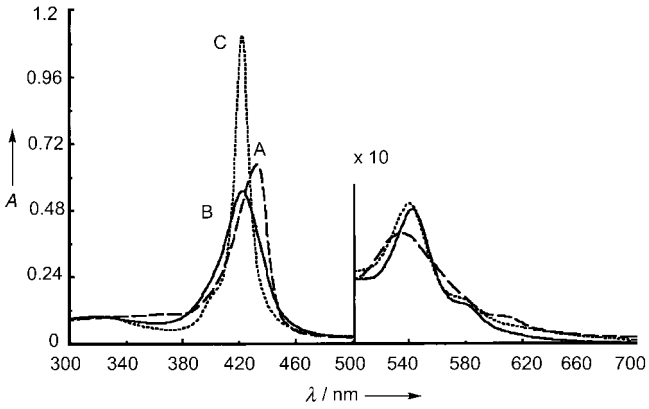
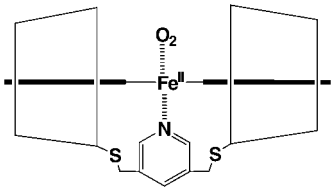


Figure 2. UV/Vis absorption spectra of [Fe^{II}(tpps)(**1**)] (5×10^{-6} M) in the deoxy form (A) as well as the O_2-coordinated (B) and CO-coordinated forms (C) in 0.05 M phosphate buffer at pH 7.0 and 25 °C. The method of sample preparation is given in the text.

(K) for complexation of [$Fe^{III}(H_2O)_2$(tpps)] with **1** of $> 10^7\,M^{-1}$, although the exact K value could not be determined. The ^{1}H NMR spectrum of the [Fe^{III}(tpps)]/**1** system in D_2O at pD 4.4 showed signals arising from the pyrrole β-protons at $\delta = 51$, 53, and 55 ppm (see the Supporting Information), which suggests the formation of a complex in a five-coordinate admixed intermediate spin state.[11] Such a complex should be pyridine-coordinated [Fe^{III}(tpps)(**1**)] which has the desired structure. The pH-dependent UV/Vis absorption spectral changes suggest that the pyridine nitrogen–Fe^{III} coordination bond of the [Fe^{III}(tpps)(**1**)] complex dissociates to give [$Fe^{III}(H_2O)_2$(tpps)(**1**)] below pH 3 and [Fe^{III}(OH)(tpps)(**1**)] above pH 7. The ^{1}H NMR signals resulting from the pyrrole β-protons at pD 0.5 appeared at $\delta = 71$ and 66 ppm as two singlets, which supports the formation of [$Fe^{III}(H_2O)_2$(tpps)(**1**)] in a six-coordinate admixed intermediate spin state. Meanwhile, the pyrrole β-protons were observed at $\delta = 84$ ppm as a broad singlet at pD 8.7, which is ascribed to [Fe^{III}(OH)(tpps)(**1**)] in a five-coordinate high-spin state. In addition, [Zn^{II}(tpps)] was used as a divalent metalloporphyrin in place of [Fe^{II}(tpps)]. The structure of the complex of [Zn^{II}(tpps)] and **1** was estimated by UV/Vis spectroscopy. As in the case of [Fe^{III}(tpps)], an extremely stable 1:1 complex was formed and the pyridine moiety of **1** coordinated to the zinc ion above pH 3 (see the Supporting Information). From these findings, it can be expected that **1** forms a 1:1 complex with [Fe^{II}(tpps)], in which the pyridine moiety of **1** can act as a proximal ligand.

Two equivalents of $Na_2S_2O_4$ were added to an aqueous solution of a mixture of [Fe^{III}(tpps)] and **1** in an argon atmosphere to prepare a stock solution of [Fe^{II}(tpps)] complexed with **1**, which was diluted by N_2-saturated phosphate buffer at pH 7.0 for measurement of the absorption spectrum (Figure 2). The Soret band of [Fe^{II}(tpps)] included by **1** appeared at 433 nm ($\varepsilon_{max} = 129\,600$), which corresponds to the band from a five-coordinate high-spin Fe^{II}Por species.[12] Careful introduction of O_2 into a solution of [Fe^{II}(tpps)(**1**)] caused a change in the absorption spectrum and a new Soret band appeared at 423 nm ($\varepsilon_{max} = 110\,400$). Replacing O_2 by CO resulted in a sharp Soret band appearing at 422 nm ($\varepsilon_{max} = 222\,600$) as a consequence of coordination of CO to [Fe^{II}(tpps)]. These spectral changes strongly suggest that the Soret band at 423 nm which appeared when the complex was formed in the O_2 atmosphere should be ascribed to [$Fe^{II}(O_2)$(tpps)(**1**)].

The formation of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex was confirmed by ^{1}H NMR and resonance Raman spectroscopy. The ^{1}H NMR signals of the [Fe^{II}(tpps)(**1**)] complex in anaerobic D_2O were broad because of the paramagnetic nature of the sample. These broad signals appeared at $\delta = 40$–



60 ppm (with TSP as an external standard) and can be ascribed to a five-coordinate high-spin [Fe^{II}(tpps)] ($S=2$) complex.[13] This solution was charged with O_2, and all NMR signals between $\delta=0$ and 10 ppm were sharpened, which indicated the formation of a six-coordinate low-spin state ($S=0$). In other words, the ^{1}H NMR spectrum supports the formation of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex. The sample obtained by replacing the O_2 atmosphere with CO was also diamagnetic.

The formation of the Fe^{II}–O bond was verified by resonance Raman spectroscopy (Figure 3). The sample was prepared by introducing $^{18}O_2$ into a phosphate buffer solution

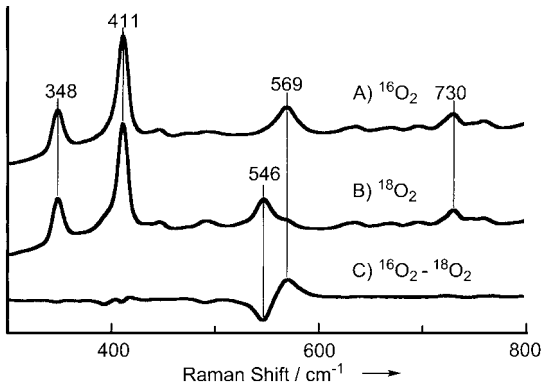


Figure 3. Resonance Raman spectra of the $^{16}O_2$ (A) and $^{18}O_2$ (B) adducts of the [Fe^{II}(tpps)(**1**)] complex (5×10^{-5} M) and the difference spectrum (C) between spectra A and B. Experimental conditions: 0.05 M phosphate buffer at pH 7.0; room temperature; 413.1 nm excitation; 12 mW power.

of the [Fe^{II}(tpps)(**1**)] complex at pH 7.0 and room temperature. A Raman signal was observed at 546 cm^{-1}, which was characterized as the stretching band of the Fe^{II}–^{18}O bond.[14] When the measurement was repeated after the sample had stood in air for a while, the band shifted to 569 cm^{-1}, which is ascribed to the Fe^{II}–^{16}O bond. On the basis of these data, it is concluded that a Fe^{II}–O bond is formed. In the case of horse heart Mb, the stretching bands of the Fe^{II}–^{18}O and Fe^{II}–^{16}O bonds appear at 545 and 571 cm^{-1}, respectively,[14] which is in good agreement with the bands measured in the present system.

The rates of autoxidation of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex were determined by following the absorbance at the Soret band, and the results are summarized in Table 1. The [$Fe^{II}(O_2)$(tpps)(**1**)] ($\lambda_{max}=423$ nm) was gradually decomposed to [Fe^{III}(OH)(tpps)(**1**)] ($\lambda_{max}=418$ nm) and the half-life ($t_{1/2}$) of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex was 30.1 h in phosphate buffer at pH 7.0. Interestingly, [$Fe^{II}(O_2)$(tpps)(**1**)] was still stable in aqueous acidic solution at pH 4.0 ($t_{1/2}=13.1$ h). The O_2 adduct of beef heart Mb is much more unstable in the lower pH region ($t_{1/2}=11$ h at pH 7 and 0.5 h at pH 5).[15] Such novel stabilization of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex might be achieved by blocking the Fe^{II} center with two per-*O*-methylated β-CD moieties. Most water molecules seem to be excluded from the cleft of the [$Fe^{II}(O_2)$(tpps)(**1**)] complex, which was more stable in phosphate buffer at pH 6.0 than in succinate buffer at the same pH value. This observation might be ascribed to a slow anion-catalyzed autoxidation[1] caused by weak coordination of the CO_2^- group of the succinate anion to the iron center, since it is known that inorganic anions accelerate the autoxidation of oxyMb.[1]

Table 1: First-order rate constants (k) and half-lives ($t_{1/2}$) for the autoxidation of [$Fe^{II}(O_2)$(tpps)(**1**)] at 25 °C.

pH	k [h^{-1}]	$t_{1/2}$ [h]
4.0[a]	0.053	13.1
5.0[a]	0.055	12.6
6.0[a]	0.041	16.9
6.0[b]	0.020	34.7
7.0[b]	0.023	30.1
8.0[b]	0.024	28.9

[a] In 5 mM succinate buffer. [b] In 5 mM phosphate buffer.

The affinity of the present system for dioxygen was determined from the changes in the absorption spectrum of the [Fe^{II}(tpps)(**1**)] complex as a function of the partial pressure of O_2.[6b] The O_2 affinity ($P_{1/2}$) in 0.05 M phosphate buffer at pH 7.0 and 25 °C was 17.5 ± 1.7 Torr, which is larger than that of sperm whale Mb (0.29 Torr) and is somewhat smaller than the $P_{1/2}$ value of human Hb in the T state (26 Torr).[3]

The bound O_2 could be released from [$Fe^{II}(O_2)$(tpps)(**1**)] by introducing N_2 into the system. The present system showed good reversibility in the O_2 binding–releasing cycles, although gradual autoxidation occurred (see the Supporting Information).

In the present study, we found that **1** is an excellent model of Mb that binds O_2 reversibly in aqueous solution. Recently, a CD/Fe^{II}Por ensemble having a complex structure was prepared as a hemoprotein model which binds dioxygen, although no details of O_2 binding were investigated.[16] The present study introduced a new system to the family of metalloprotein models composed of CDs and metal complexes.[17]

Experimental Section

2,3-Monoepoxy-β-cyclodextrin,[18] 3,5-dimercaptomethylpyridine,[9a] and [Fe^{III}(tpps)][19] were synthesized according to the procedures reported in the literature.

2,3-Monoepoxy per-*O*-methylated β-cyclodextrin: 2,3-Monoepoxy-β-CD (2.00 g, 1.45 mmol) was added to a mixture of dry DMF (80 mL) and THF (30 mL) under an Ar atmosphere. After the mixture became translucent, the solution was cooled to 0 °C. NaH (1.39 g, 58.0 mmol) was added to the solution, and the mixture was stirred for 1 h at 0 °C. CH_3I (3.6 mL, 58.0 mmol) was added dropwise over a period of 20 min, and then the mixture was warmed to room temperature. After stirring the reaction mixture overnight, methanol (4 mL) was added to quench the reaction. The solvent was removed under reduced pressure, and the residue was extracted with $CHCl_3$ (100 mL) and H_2O (100 mL). The combined organic layer was separated and washed with aqueous $Na_2S_2O_3$ (100 mL) and then with H_2O (100 mL). The $CHCl_3$ layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with gradient elution from $CHCl_3$ to $CHCl_3$/acetone (5:2 (v/v)) to give 2,3-monoepoxy per-*O*-methylated β-CD as a colorless solid (1.50 g, 61 %). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=5.19$–5.00 (m, 7H), 3.92–3.19 ppm (m, 99H); MS (MALDI-TOF, α-cyano-4-hydroxycinnamic acid (α-CHCA)) m/z 1406.6 (calcd for $[M+Na]^+$: 1406.46). Elemental analysis (%) calcd for $C_{61}H_{106}O_{34}\cdot0.5\,CHCl_3$: C 51.18, H 7.44, O 37.69; found: C 51.15, H 7.42, O 38.26.

1: A solution of 2,5-dimercaptomethylpyridine (50 mg, 0.29 mmol) in methanol (2 mL) was added to a solution of 2,3-monoepoxy per-*O*-methylated β-CD (1.0 g, 0.72 mmol) in aqueous 0.1M $NaHCO_3$ (25 mL). The mixture was refluxed under Ar for 24 h. After cooling the reaction mixture to room temperature, it was extracted with $CHCl_3$ (50 mL × 5) and the organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel with gradient elution from $CHCl_3$/acetone (5:2 (v/v)) to $CHCl_3$, and then to $CHCl_3$/MeOH 10:1 (v/v) to give **1** as a colorless solid (0.21 g, 20%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.50$ (s, 2H, pyH^2), 7.81 (s, 1H, pyH^4), 5.28–5.00 (m, 12H, H^{1B-G}), 4.62 (d, 2H, $J = 7.6$ Hz, H^{1A}), 4.35 (m, 2H, H^{5A}), 4.10–3.14 (m, 200H, $-OCH_3$ and other H$^{2-6}$ protons), 3.03 ppm (dd, 2H, $J = 10.8$, 2.8 Hz, H^{3A}). The assignments of the ^{1}H NMR spectrum were performed by measuring the H–H COSY spectrum and by comparison with literature values.[9a,18b] MS (FAB, *m*-nitrobenzyl alcohol): *m*/*z* 2938 (calcd for M^+: 2938.23). Elemental analysis (%) calcd for $C_{129}H_{221}NO_{68}S_2$: C 52.73, H 7.58, N 0.48, O 37.03; found: C 51.96, H 7.31, N 0.62, O 36.89. The ^{1}H NMR and H–H COSY spectra of **1** are shown in the Supporting Information.

UV/Vis absorption spectra were measured on a Shimadzu UV-2100 spectrophotometer with a thermostatic cell holder. ^{1}H NMR spectra were recorded on a JEOL JNM-A400 spectrometer (400 MHz) in D_2O (CEA, 99.9%) using sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TSP, Aldrich) as an external standard or in $CDCl_3$ (CEA, 99.8%) and tetramethylsilane as an internal standard. Mixed O_2–N_2 gases with various partial pressures of oxygen were prepared with a KOFLOC GM-4B gas-mixing apparatus (Kyoto, Japan). $P_{1/2}$ values were determined according to the procedure described in the literature.[6b,20] The average value of five repeated measurements was employed.

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