Supramolecular Medicine

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Supramolecular Iron Porphyrin/Cyclodextrin Dimer Complex that Mimics the Functions of Hemoglobin and Methemoglobin**

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Our previous studies on a porphinato iron/per-*O*-methylated β-cyclodextrin dimer supramolecular system revealed that 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinato iron (FeTPPS), encapsulated by two cyclodextrin units and ligated by a nitrogenous axial ligand at a linker position of the cyclodextrin dimer, captures molecular oxygen,[1] carbon monoxide,[1,2] nitrogen monoxide,[1d] or a cyanide anion[3] in aqueous solution. Because these functions of the supramolecular system are similar to those of hemoglobin (Hb), myoglobin (Mb), and their ferric derivatives, the FeTPPS/cyclodextrin dimer complexes have potential for use in basic research and practical applications. For example, the complex of Fe^{II}TPPS and Py3CD (hemoCD1, Scheme 1) is a powerful candidate to remove CO from the blood stream.[2] The Fe^{III}TPPS/Im3CD complex is expected to function as an effective and safe cyanide antidote.[3] However, synthesis of the per-*O*-methylated β-cyclodextrin dimers (Py3CD,[1a] Py2CD,[1d] and Im3CD[1c]) shown in Scheme 1 is time consuming, because of the multiple steps involved. An easy-to-synthesize per-*O*-methylated β-cyclodextrin dimer whose FeTPPS complex remains highly functional could serve a number of objectives. Herein, we tried to prepare a cyclodextrin dimer from the commercially available materials with a synthesis involving two steps.

Our target cyclodextrin dimer was Py3OCD (Scheme 1), the synthetic route to which is shown in Scheme 2. Py3OCD was obtained from heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DMe-β-CD) in 21% total yield (see Supporting Information).

Because a single crystal of an inclusion complex of Fe^{III}TPPS and Py3OCD could not be prepared, 5,10,15,20-tetrakis(4-hydroxyphenyl)porphinato iron(III) (Fe^{III}TPPOH) was used in place of Fe^{III}TPPS to determine the X-ray crystal structure of the supramolecule (see Supporting Information). The X-ray crystal structure of the 1:1 complex of Fe^{III}TPPOH and Py3OCD is shown in Figure 1.[4] Fe^{III}TPPOH is tightly encapsulated in a cavity formed by two cyclodextrin units and the pyridine ligand at the linker position coordinates to the ferric center with a slope (tilt angle = 29°). The plane of the axial pyridine is aligned with the line connecting the nitrogen atom of a pyrrole with that of the opposite pyrrole (eclipsed conformation). The latter orientation is the same as that in the case of Mb.[5] The effects of the axial ligand orientation have been discussed in relation to the O_2 affinity of hemoproteins.[6] A water molecule coordinates to iron(III) as the sixth axial ligand. The Fe–OH_2 distance was approximately 0.24 nm, though the accuracy seems to be poor due to a large amount of disorder in the crystal. The distance between iron(III) and the nitrogen of the pyridine ligand is 0.194 nm, which is shorter than the N(His)–Fe^{III} distance of metMb (0.2141 nm). Inclusion and ligation within a restricted area seem to cause the distorted structure of the supramolecule.

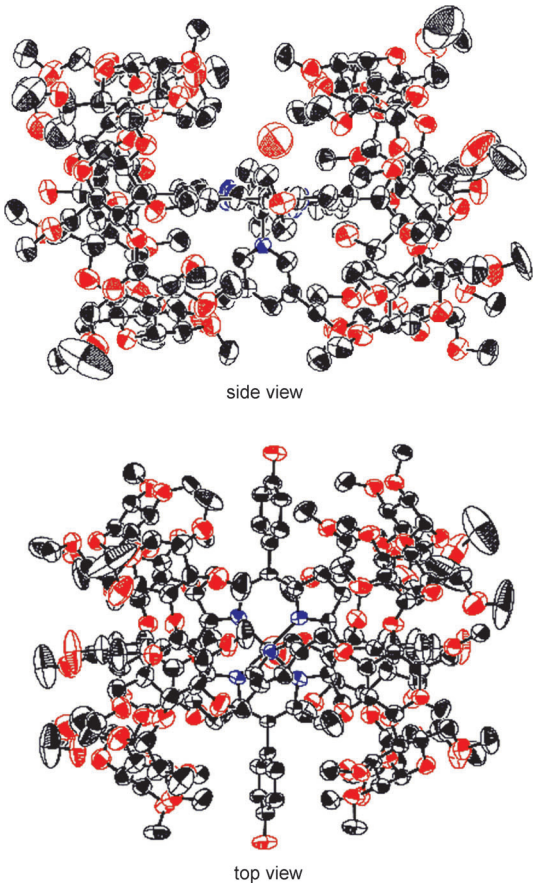

Figure 1. ORTEP diagram (50% probability) of the Fe^{III}TPPOH/Py3OCD inclusion complex.[4] Hydrogen atoms have been omitted for clarity. C black, O red, N blue.

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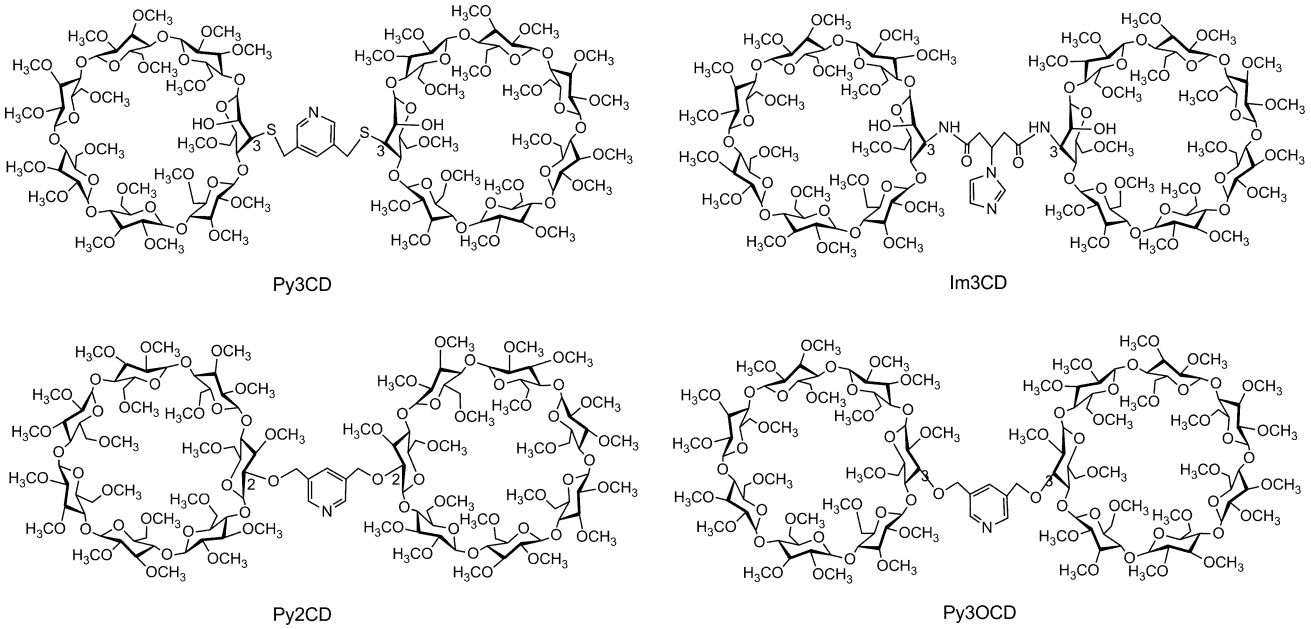
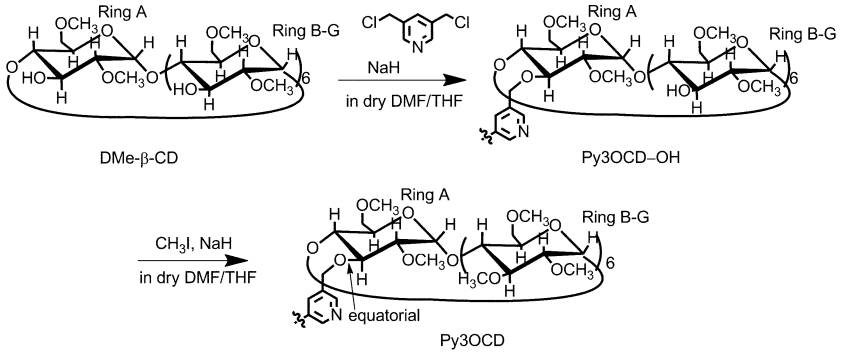


Scheme 1. Dimers of per-*O*-methylated β-cyclodextrin whose FeTPPS complexes show hemoprotein-like functions.



Scheme 2. Synthesis of Py3OCD. DMF = dimethylformamide, THF = tetrahydrofuran.

The Fe^{II}TPPS/Py3OCD complex (hemoCD3) bound to dioxygen ($p_{1/2}^{O_2}$ = 18 Torr) as well as carbon monoxide ($p_{1/2}^{CO} = 5.6 \times 10^{-4}$ Torr) in aqueous solution at pH 7.0 and 25 °C (Supporting Information). Because hemoCD3 shows functions similar to those of Hb and/or Mb, its ferric form (met-hemoCD3) is expected to show behavior similar to that of metHb and/or metMb. A traditional antidote for cyanide poisoning is amyl nitrate.[7] The amyl nitrate method is based on the excellent ability of metHb to bind CN^-, but a notable disadvantage is the undesired oxidation of Hb to metHb, which may be toxic for humans under anoxic conditions.[7] A recent treatment for cyanide poisoning is the capture of CN^- ions by hydroxocobalamin (HOCbl).[7] However, the greatest disadvantage of using HOCbl is its poor ability to bind CN^- in blood, because serum albumin strongly incorporates HOCbl in its pockets to cause marked reduction in the binding (K^{CN}) and rate constants (k_{on}^{CN}) of CN^-.[3,8] We previously reported that the 1:1 complex of Fe^{III}TPPS with Im3CD (Scheme 1) shows good capture of CN^- in the blood.[3] However, k_{on}^{CN} ($193\,M^{-1}s^{-1}$; Table 1) of the Fe^{III}TPPS/Im3CD complex is not high enough when compared with that of metHb ($320\,M^{-1}s^{-1}$) and metMb ($305\,M^{-1}s^{-1}$). In general, the faster a receptor captures the CN^- ion, the better it can act as a cyanide antidote. We have assumed that the k_{on}^{CN} of a receptor depends on the pK_a of the H_2O coordinated to the metal ion of the receptor. Such an assumption is based on the idea that an axial H_2O exchanges more easily with CN^- than OH^-. The spectroscopic pH titration of met-hemoCD3 provided a pK_a of 8.0, which is close to the pK_a values of metHb (8.33) and HOCbl (8.1) and is slightly larger than that of the H_2O-Fe^{III}TPPS/Im3CD complex (7.7).[3] Under physiological conditions, the axial ligand of the met-hemoCD3 is mostly H_2O, not OH^-.

Addition of NaCN to met-hemoCD3 (λ_{max} = 398 nm) in 0.05 M phosphate buffer at pH 7.0 and 37 °C caused systematic spectral changes and a new absorption band appeared at 419 nm. The ESI-TOF MS spectrum supported the formation of $[(CN^-)$-met-hemoCD3$]^{4-}$ (m/z = 985.6). The k_{on}^{CN} and dissociation rate constant of the cyanide adduct (k_{off}^{CN}) were determined by using stopped-flow and ligand-exchange methods (see Supporting Information), respectively, and the results are shown in Table 1. The k_{on}^{CN} of met-hemoCD3 was $736\,M^{-1}s^{-1}$ and the k_{off}^{CN} was $2.55 \times 10^{-4}\,s^{-1}$. As expected, the k_{on}^{CN} value of met-hemoCD3, which had a larger pK_a value for the axial H_2O, was much larger than that of the Fe^{III}TPPS/Im3CD complex with the smaller pK_a value (Figure 2a). Interestingly, the k_{on}^{CN} value of met-hemoCD3 was much larger than those of HOCbl ($81.5\,M^{-1}s^{-1}$) and metHb ($320\,M^{-1}s^{-1}$). The rapid binding of CN^- to a receptor is vital

Table 1: The equilibrium (K^{CN}, K^{N_3}) and rate constants (k_{on}^{CN}, k_{off}^{CN}, $k_{on}^{N_3}$, $k_{off}^{N_3}$) for binding of CN^- and N_3^- to receptors in 0.05 M phosphate buffer at pH 7.0 and 37 °C.

	k_{on}^{CN} [$M^{-1}s^{-1}$]	$k_{off}^{CN}\times10^4$ [s^{-1}]	$K^{CN}\times10^{-6}$ [M^{-1}]	$k_{on}^{N_3}$ [$M^{-1}s^{-1}$]	$k_{off}^{N_3}$ [s^{-1}]	K^{N_3} [M^{-1}]	pK_a	Reference
met-hemoCD3	736	2.55	2.89	3810	1.28	4890	8.0	this work
Fe^{III}TPPOH/Py3OCD	715	3.55	2.01	11200	5.86	1910	8.2	this work
Fe^{III}TPPS/Py3OCD-OH	78.9	24.0	0.0329	6460	17.9	360	7.2	this work
met-hemoCD1	14.9	0.071	2.11	nd[a]	nd[a]	nd[a]	5.5	[1e, 3]
met-hemoCD2[b]	39.3	0.179	2.20	nd[a]	nd[a]	nd[a]	6.9	[1e, 3]
Fe^{III}TPPS/Im3CD	193	0.739	2.61	nd[a]	nd[a]	nd[a]	7.7	[1e, 3]
HOCbl	81.5	0.102	7.96	nd[a]	nd[a]	nd[a]	8.1	[1e, 3]
metHb	320	3.93	0.814	nd[a]	nd[a]	nd[a]	8.3	[1e, 3]

[a] Not determined. [b] Fe^{III}TPPS/Py2CD complex.

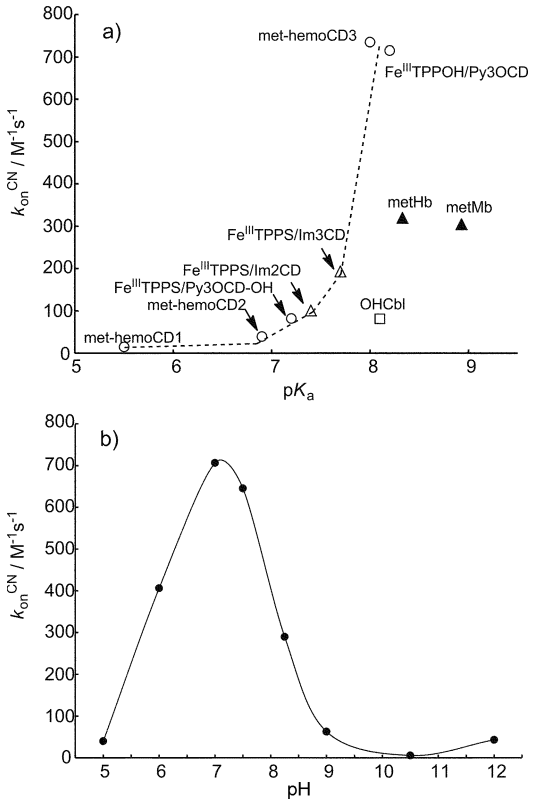
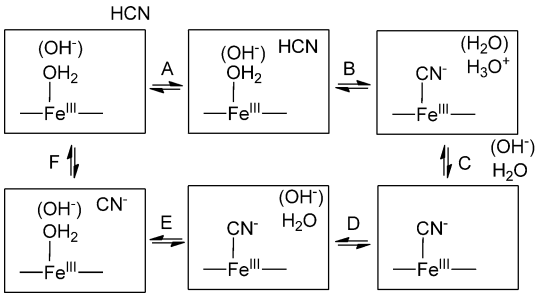


Figure 2. a) pK_a-rate profile for the coordination of CN^- to supramolecular ferric porphyrins and HOCbl and b) pH-rate profile for met-hemoCD3. The pK_a-rate profile was obtained from experiments carried out at pH 7.0 and 37 °C.



(1)

for acute medical care. The binding constant (K^{CN}) of met-hemoCD3 ($K^{CN}=k_{on}^{CN}/k_{off}^{CN}=2.89\times10^6\,M^{-1}$, Table 1) was smaller than that of HOCbl ($7.96\times10^6\,M^{-1}$) in phosphate buffer without serum proteins (see below).

As the X-ray crystal structure indicates, binding of CN^- to met-hemoCD3 occurs within a cyclodextrin capsule that is isolated from the aqueous bulk phase. Therefore, the binding process can be expressed by Equation (1):

A square in Equation (1) represents a cyclodextrin capsule. The k_{on}^{CN} clearly depends on the pK_a of the axial H_2O of the receptor (Figure 2a), indicating that ligand exchange of axial H_2O with CN^- occurs more easily than that of axial OH^- with CN^-. The preferred species of met-hemoCD3, with a pK_a of 8.0, at pH 7.0 is an aqua complex, in which the H_2O molecule is rapidly exchanged with CN^- (processes A and B in Equation (1)). A bell-shaped pH-k_{on}^{CN} profile was observed (Figure 2b). At pH values lower than five, the pyridine ligand may be protonated (see Supporting Information) and an unstable five-coordinate high-spin cyanide adduct is formed. The narrow space within the capsule of met-hemoCD3 seems to inhibit diaxial coordination of CN^-. When the pH increases from five to seven, however, the pyridine ligand coordinates to the ferric center to form a six-coordinate low-spin cyanide adduct that is more stable than the five-coordinate high-spin adduct. At pH values higher than seven, the formation of a hydroxo complex of met-hemoCD3 slows the CN^- coordination. A profile similar to Figure 2a was observed for the relationship between k_{off}^{CN} and pK_a of the axial H_2O of Fe^{III}porphyrin/per-*O*-methylated β-cyclodextrin dimer (see Supporting Information). The cyanide dissociation process of a ferric complex with a lower pK_a of the axial H_2O needs penetration of OH^- into a cyclodextrin capsule (process D), which is more unfavorable than that of H_2O.

The azide anion (pK_a of $HN_3=4.5$) also coordinated to met-hemoCD3 (Table 1). Although the k_{on}^{N3} for met-hemoCD3 was significantly larger than the k_{on}^{CN}, the binding constant (K^{N3}) was much smaller than K^{CN} because of the very large k_{off}^{N3}. The pH- k_{on}^{N3} profile (see Supporting Information) showed that k_{on}^{N3} at pH lower than 4 was almost zero and steeply increased at pH 4.5, which corresponds to pK_a of HN_3. Unlike cyanide binding, the azide adduct of met-hemoCD3 is formed only through penetration of the N_3^- anion into the cyclodextrin capsule. The k_{on}^{N3} remarkably decreased with further increase in pH. Because the pK_a values of HN_3 and axial H_2O of met-hemoCD3 are 4.5 and 8,

respectively, the k_{on}^{N3} is expected not to change remarkably in a pH range of 4.5 and 8. In spite of the expectation, the k_{on}^{N3} decreased in pH larger than 4.5. Such a pH-rate profile for the azide binding could not be explained clearly. The binding behavior of the azide anion remarkably differs from that of the cyanide anion.

Before using met-hemoCD3 as a cyanide antidote, its binding behavior to CN^- ions was studied in serum (Table 2). Serum contains various biological components such as proteins, lipids, sugars, vitamins, and inorganic ions. Therefore, it is a good medium for a preliminary survey of cyanide binding before a full in vivo study. k_{on}^{CN} as well as K^{CN} for HOCbl were markedly lower in the serum. Serum albumin is the main serum protein, and HOCbl is known to be incorporated in pockets of serum albumin, causing a reduction in its cyanide-binding ability.[3] However, the decrease in k_{on}^{CN} and K^{CN} was not remarkable in the case of met-hemoCD3, which scarcely interacts with serum proteins. This is a major advantage to using met-hemoCD3 as a cyanide antidote.

Table 2: Binding of CN^- to met-hemoCD3 and other receptors in serum at 37 °C.

	k_{on}^{CN} [$M^{-1}s^{-1}$]	$k_{off}^{CN}\times10^4$ [s^{-1}]	$K^{CN}\times10^{-6}$ [M^{-1}]	Reference
met-hemoCD3	514	2.99	1.72	this work
Fe^{III}TPPS/Im3CD	104	0.776	1.34	[3]
metHb	208	6.44	0.323	this work
HOCbl	6.5	5.0	0.013	[3]

The effectiveness of met-hemoCD3 as a cyanide antidote was examined by using male Wistar rats. NaCN (10 mM in PBS) was infused into the femoral vein of the rats (300–360 g, 3 individuals) under anesthesia at a rate of 0.15 mL min^{-1}. The respiration rate of the rat increased in the initial eight minutes and then decreased between eight and ten minutes after the infusion. The respiration completely stopped at 12 minutes, after which the infusion of NaCN was terminated. All untreated rats died after respiratory arrest. However, all rats (3 individuals) were revived upon injection of met-hemoCD3 (20 mM in PBS) into the same femoral vein at the same rate (0.15 mL min^{-1}). Injection of the met-hemoCD3 solution was initiated immediately after the respiratory arrest. The injection time was adjusted to be the same as that of the infusion of the NaCN solution. Therefore, the total injected-amount of met-hemoCD3 was two equivalents relative to NaCN. The urine in the bladder was collected every 30 minutes after the injection of met-hemoCD3 and spectroscopically analyzed. About 66% of the injected met-hemoCD3 was excreted in the urine in the forms of H_2O- and CN^--met-hemoCD3 complexes and ferrous hemoCD3 (CO- and/or O_2-hemoCD3) within 30 minutes after the initiation of injection. The urine collected at 30 minutes contained 45% H_2O-met-hemoCD3, 45% CN^--met-hemoCD3, and 10% ferrous hemoCD3. Ferrous hemoCD3 was formed by way of reduction of met-hemoCD3 in the blood and bound endogenous carbon monoxide and/or molecular oxygen. Most injected met-hemoCD3 molecules (95%) were eliminated through the urine after 360 minutes. Our analysis suggests that approximately 81% of the injected cyanide was eliminated through the urine in the form of CN^--met-hemoCD3. Additionally, the cytotoxicity of met-hemoCD3 was examined by an MTT assay using HeLa cells. No cytotoxicity was observed.

In summary, we have developed a versatile per-*O*-methylated β-cyclodextrin dimer (Py3OCD) that can be prepared using a simple procedure involving just two chemical reactions. The X-ray structure of the 1:1 inclusion complex of Fe^{III}TPPOH and Py3OCD (met-hemoCD3) indicated that the two cyclodextrin units provide a hydrophobic environment around the iron center, which demonstrated the biomimetic functions of this supramolecule. Ferric hemoCD3 rapidly and strongly binds to the cyanide anion. Lastly, met-hemoCD3 was confirmed to be a better cyanide antidote than either HOCbl, met-Hb, or met-Mb.

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