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A Diatomic Molecule Receptor That Removes CO in a Living Organism**

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A living body is an organized assembly composed of various supramolecules such as bilayer lipid membranes, DNA, RNA, enzymes, hemoglobin (Hb), myoglobin (Mb), and so on. Many chemists have tried to artificially mimic the functions of biological molecules.[1] Initial efforts were focused on the modeling of hydrolases such as chymotrypsin[2] and papain.[3] Numerous studies on hydrolase models yielded valuable scientific knowledge of hydrolysis reactions and led to new research fields such as micellar catalysis,[4] cyclodextrin catalysis,[5] and artificial bilayer membranes.[6] Accidental discovery of dibenzo[18]crown-6[7] caused explosive development in clathrate chemistry related to biological ionophores.[8] Modeling the functions of heme[9] and nonheme proteins[10] has activated coordination and bioinorganic chemistry. One seminal study in bioinorganic chemistry concerns the "picket-fence porphyrin" that reversibly binds dioxygen (O_2) in an organic solvent.[11] Although the picket-fence porphyrin has offered a possibility for developing an artificial dioxygen carrier in blood, few successful examples of O_2 binding under physiological conditions have been reported, because water molecules prevent stable formation of the O_2 complex of the ferrous porphyrin.[9] Recently, we synthesized a water-soluble supramolecular complex (hemoCD) composed of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(II) (Fe^{II}TPPS) and a per-O-methylated β-cyclodextrin dimer with a pyridine linker (Py3CD; Scheme 1) in which two cyclodextrin units are linked by a SCH_2PyCH_2S (Py = pyridin-3,5-diyl) bridge at the 3- and 3′-positions of the glucopyranoses.[12] As the encapsulation of Fe^{II}TPPS by Py3CD prevents the attack of a water molecule to the ferrous center of the porphyrin, the 1:1 inclusion complex "hemoCD" forms the stable O_2 adduct even in aqueous solution at room temperature. Similar to Hb and Mb, hemoCD reversibly binds O_2 and carbon monoxide; the O_2 ($P_{1/2}^{O_2}$) and CO affinities ($P_{1/2}^{CO}$) of hemoCD at pH 7.0 and 25 °C are 17 and 0.000015 Torr, respectively.[13,14] The CO affinity is approximately 100 times higher than that of Hb (R-state) under the same conditions.[9] This unique property of hemoCD suggested to us the potential of this material as a CO stripper in living organisms. Carbon monoxide is continuously produced in the mammalian body during the catabolism of heme by the heme oxygenase enzymes, and endogenous CO controls various physiological phenomena such as blood pressure, antibacterial action, and cell apoptosis.[15,16] For a detailed study on the physiological role of CO, a test organism from which all internal CO is removed is desired. In the present study, we demonstrate hemoCD as an ideal sequestering agent of CO in a living body.

The solution of hemoCD used for administration was prepared by the reduction of Fe^{III}TPPS (1.0 molar equiv) and Py3CD (1.2 molar equiv) with $Na_2S_2O_4$ in phosphate-buffered saline (PBS) at pH 7.4. The excess reducing agent was removed by passing the solution through a gel permeation column. During this treatment, hemoCD binds atmospheric O_2 to form the O_2 adduct (oxy-hemoCD). In all experiments, freshly prepared oxy-hemoCD solutions were used, because oxy-hemoCD is gradually autoxidized ($t_{1/2}$ = 30.1 h at pH 7.0 and 25 °C) to the ferric form (met-hemoCD). After the solution of oxy-hemoCD in PBS (0.20 mM, 1.0 mL) was infused into the femoral vein of a rat (Wistar male, 270–350 g), the administered hemoCD began to be excreted into the urine within 30 min. The UV/Vis absorption spectrum of the urine collected after the infusion shows the characteristic Soret band at 422 nm and Q band at 540 nm (Figure 1). The spectral features (λ_{max} and half bandwidth of the Soret band, Table S1 in the Supporting Information) of the urine are in agreement with those of CO-bound hemoCD (CO-hemoCD), thus indicating that the infused oxy-hemoCD is converted to CO-hemoCD during systemic circulation in the rat body.[17] Formation of CO-hemoCD in the rat was further confirmed by resonance Raman (rR) spectroscopy (Figure 1 inset). The characteristic rR band at 480 cm^{-1} is assigned to the Fe–C bond stretching of CO-hemoCD.[14] As we treated the rats under normal atmosphere, the excreted CO must be endogenous.

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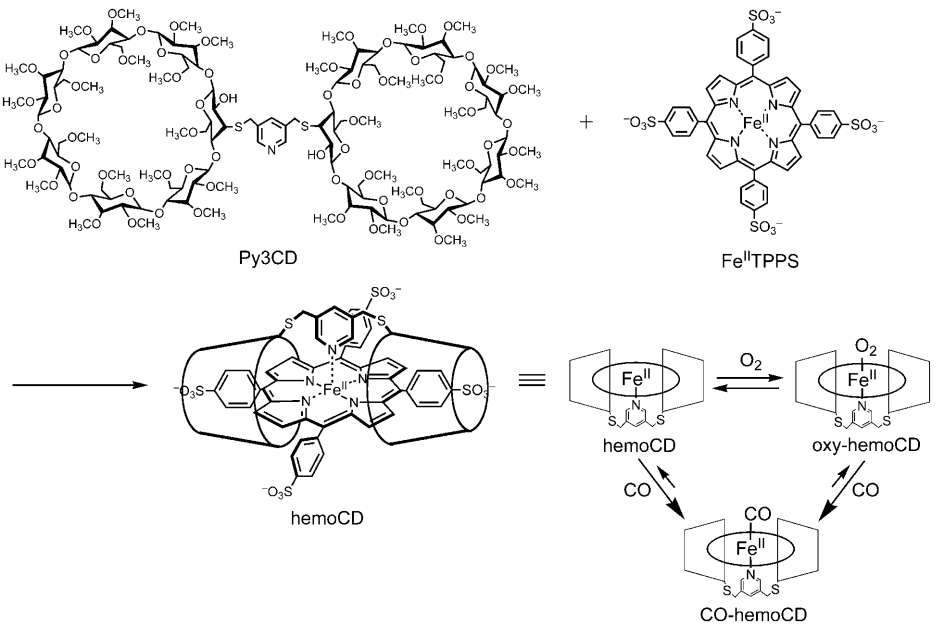

Scheme 1. Supramolecular interaction between Py3CD and Fe^{II}TPPS to form a 1:1 inclusion complex (hemoCD) and schematic representation of the binding of O_2 and CO to hemoCD.

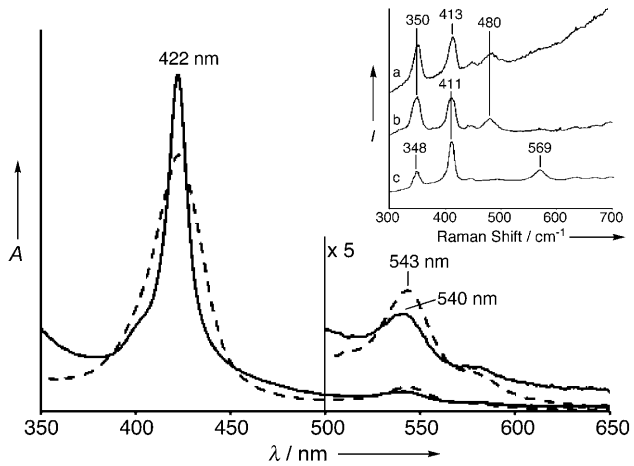

Figure 1. UV/Vis absorption spectra of a solution of oxy-hemoCD in PBS at pH 7.4 (– – –) and of rat urine after the infusion of oxy-hemoCD (——) at 25 °C. The urine sample was appropriately diluted by PBS. Inset shows the rR spectra a) of the rat urine after the infusion of oxy-hemoCD and of the reference samples b) CO-hemoCD (ν_{Fe-C} = 480 cm^{-1}) and c) oxy-hemoCD (ν_{Fe-O} = 569 cm^{-1}).[12,14] The band at 480 cm^{-1} has been assigned to the Fe–C bond stretching of CO-hemoCD, as confirmed by isotope substitution experiments.[14]

To confirm the effect of encapsulation of Fe^{II}TPPS by Py3CD, a solution of Fe^{III}TPPS (0.50 mM)[18] in PBS in the absence of Py3CD was continuously infused into the rat vein at a rate of 1.0 mL h^{-1} for 120 min. In this case, no excretion of the iron porphyrin complex into the urine occurred. After ceasing the Fe^{III}TPPS infusion, Py3CD (0.60 mM) in PBS was infused into the opposite femoral vein at the same rate of 1.0 mL h^{-1}. Just after the start of infusion of Py3CD, porphyrin excretion began (Figure 2). Quite interestingly, the UV/Vis absorption spectrum of the excreted porphyrin was identified as that of CO-hemoCD (Figure 2 inset). Such a result indicates the occurrence of a series of events in the rat body: the selective encapsulation of Fe^{III}TPPS by Py3CD, the reduction of Fe^{III}TPPS to Fe^{II}TPPS by some internal reductase(s), and binding to endogenous CO.[19] The fact that Fe^{III}TPPS is not excreted in the urine before the Py3CD infusion is interpreted in terms of the strong affinity of serum albumin to bind Fe^{III}TPPS.[20] The binding of Fe^{III}TPPS to rat serum albumin (RSA) and the absence of interaction of the Fe^{III}TPPS/Py3CD complex (met-hemoCD) with RSA were confirmed by titration experiments in vitro (Figure S3 in the Supporting Information). A large Fe^{III}TPPS/RSA complex (ca. 70 kDa) in blood cannot be eliminated through the kidney, while the smaller CO-hemoCD (4 kDa) flows from the kidney. A glomerular filtration model experiment using an ultrafiltration membrane (molecular weight cutoff 30 kDa; Figure S4 in the Supporting Information) clearly showed that Fe^{III}TPPS bound to RSA hardly passed through the membrane, whereas hemoCD derivatives flow easily even in the presence of RSA. The encapsulation of Fe^{II}TPPS by Py3CD is essential not only to capture endogenous CO in vivo but also to achieve the renal excretion of the hemoCD species from the body.

Figure 3 shows the time courses in the urinary excretion profiles during and after the infusion of oxy-hemoCD (0.63 mM in PBS, infused for 220 min at a rate of 1.0 mL h^{-1}). The urine samples were collected at 30 min intervals. The amount of hemoCD excreted during each interval (M_{hemoCD}) was determined by converting all hemoCD

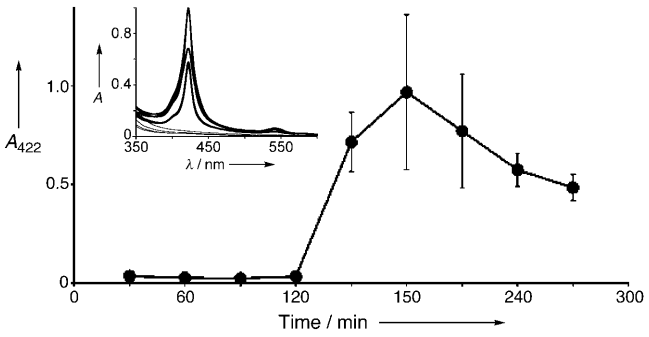

Figure 2. Plot of the absorbance at 422 nm of the urine sampled for each 30 min interval versus time during infusion of Fe^{III}TPPS (0.50 mM in PBS, 1.0 mL h^{-1}, initial 120 min) and subsequent additional infusion of Py3CD (0.6 mM in PBS, 1.0 mL h^{-1}, additional 120 min). Each point represents the mean ± standard deviation (SD) of the data obtained from three experiments using three independent rats. Inset shows the UV/Vis absorption spectra of the urine solutions before (thin lines) and after the infusion of Py3CD (thick lines).

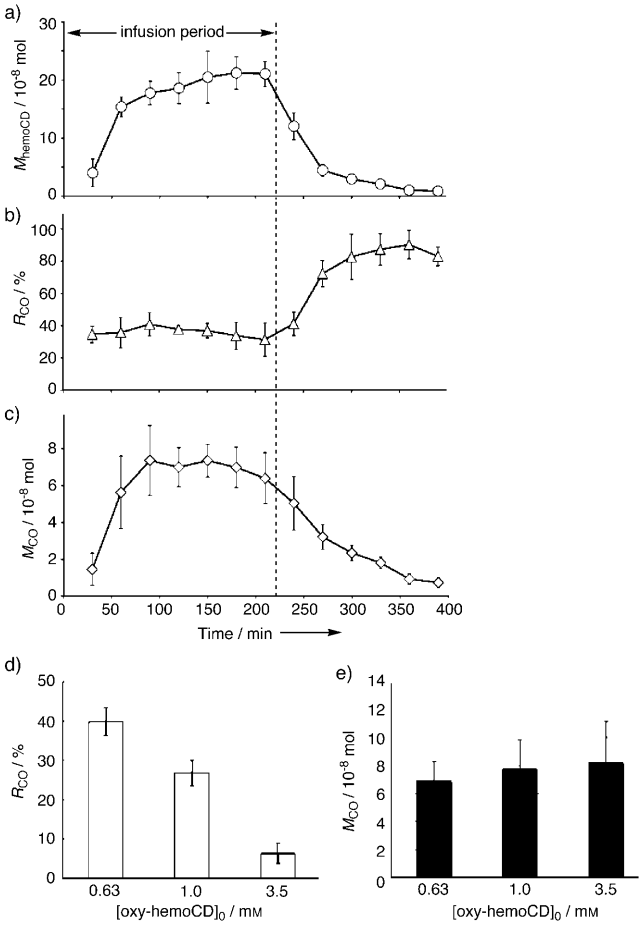

Figure 3. a–c) Excretion profiles for the rat urine collected during and after the infusion of oxy-hemoCD (0.63 mM in PBS, 1.0 mL h^{-1}, initial 220 min). a) Amount of hemoCD excreted during each 30 min interval (M_{hemoCD}). b) Molar fraction of CO-hemoCD in the total hemoCD derivatives in the urine sampled at each 30 min interval (R_{CO}). c) Amount of CO excreted in the urine during each 30 min interval (M_{CO}). Each point represents the mean ± SD of the data obtained from four experiments using four independent rats. d,e) Effects of the initial concentration of infused oxy-hemoCD ([oxy-hemoCD]$_0$) on the R_{CO} (d) and M_{CO} values (e) at the plateau regions during the infusions. Each column represents the mean ± SD of the data obtained from three (1.0 and 3.5 mM) or four (0.63 mM) independent experiments.

derivatives (mostly oxy- and CO-hemoCD) in the urine into CO-hemoCD by adding $Na_2S_2O_4$ and CO into the urine and measuring the absorbance at 422 nm (ε_{422} = 222 600 L mol^{-1} cm^{-1}).[12] As shown in Figure 3a, M_{hemoCD} values are almost constant during infusion and concomitantly decrease after completion of the infusion. Within 6.5 h after starting the infusion of oxy-hemoCD, 65–75% of the infused hemoCD molecules were recovered as the excretion product. From the absorbances at 422 nm for the urine before and after the addition of $Na_2S_2O_4$ and CO (Figure S5 in the Supporting Information), the molar fraction of CO-hemoCD in the total hemoCD (oxy-hemoCD + CO-hemoCD) in the urine sampled at each 30 min interval (R_{CO}) can be determined by Equation (1):[21]

$$R_{CO}\ (\%) = ([\text{CO-hemoCD}]/[\text{hemoCD}]_t)\,100 \qquad (1)$$

The time course of R_{CO} is shown in Figure 3b. The R_{CO} values were almost constant (35–45%) during the infusion of oxy-hemoCD, whereas these values increased to 70–90% after the infusion stopped. The results are explained as follows: removable CO in the rat body is totally excreted by forming CO-hemoCD when the system involves a sufficiently large amount of hemoCD, and excess hemoCD exists as the O_2 adduct in the rat body. After infusion stops, remaining hemoCD takes up endogenous CO that is continuously produced by the action of heme oxygenase. From the values of [CO-hemoCD] and the urine volume, the amount of CO excreted in the urine during each 30 min interval (M_{CO}) can be calculated (Figure 3c). Constant CO excretion (6–8 × 10^{-8} mol per 30 min) was observed during the infusion. At first, we assumed that the amount of CO in the urine rapidly decreases because CO in the rat body will be totally removed. However, the M_{CO} values were almost constant during the infusion, thus indicating that endogenous CO production proceeds rapidly. Figure 3d,e shows the dependency of the initial concentration of infused oxy-hemoCD ([oxy-hemoCD]$_0$) on the R_{CO} and M_{CO} values at the plateau regions during the infusions. As [oxy-hemoCD]$_0$ increases from 0.63 to 3.5 mM, R_{CO} decreases from 40 to 6%. Nevertheless, M_{CO} is independent of [oxy-hemoCD]$_0$, and the mean amount of excreted CO during 30 min is (7.6 ± 2.3) × 10^{-8} mol. Normalization of the mean M_{CO} value by weight (mean weight of the rats is 300 g) and time leads to a CO production rate in the rat body of (5.1 ± 1.6) × 10^{-7} mol kg^{-1} h^{-1}. The value is quite close to the reported value for the human body ((2.8 ± 0.5) × 10^{-7} mol kg^{-1} h^{-1}).[22] To our knowledge, this is the first evaluation of the endogenous CO production rate in a mammalian body other than human.

The present study demonstrates that carbon monoxide in the rat body is selectively captured and excreted by the action of a supramolecular system (hemoCD) composed of a water-soluble ferrous porphyrin (Fe^{II}TPPS) and a cyclodextrin dimer (Py3CD). Such a function of hemoCD in vivo is accomplished through the highly selective CO binding[23] and the unimpeded renal excretion of hemoCD. Furthermore, administration of hemoCD did not affect the rat vital functions at all.[24] These attractive properties of hemoCD would make it possible to 1) measure the amount of CO in bodies of any mammals, 2) provide a "pseudo-knockout" animal that lacks endogenous CO, and 3) remove excessive inhaled CO for detoxification.[25] These subjects are currently under investigation.

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[13] The O_2 and CO affinities are represented by the O_2 and CO partial pressures $P_{1/2}^{O_2}$ and $P_{1/2}^{CO}$, respectively, at which half of the hemoCD molecules are coordinated by O_2 and CO.
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[17] Since the spectral patterns (UV/Vis and rR) of the urine are in agreement with those of CO-hemoCD, the supramolecular structure of hemoCD should be maintained after circulation in the body. In addition, the unchanged Py3CD scaffold was detected in the urine by means of MALDI-TOF mass spectrometry (Figure S1 in the Supporting Information).
[18] Fe^{II}TPPS was very unstable and rapidly oxidized (in less than 1 s) to the ferric state (Fe^{III}TPPS) in the absence of Py3CD under aerobic conditions. Therefore, we used Fe^{III}TPPS as the control.
[19] In the case of the infusion of met-hemoCD, the excreted urine solution also contained CO-hemoCD (Figure S2 in the Supporting Information), thus indicating that the reduction of met-hemoCD to hemoCD occurred in the rat body.
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[21] The derivation of the equation for determining R_{CO} is given in the Supporting Information.
[22] This value was calculated from $(0.42 \pm 0.07)\,mL\,h^{-1}$ of CO production (see Reference [15] and R. F. Coburn, W. S. Blakemore, R. E. Forster, *J. Clin. Invest.* **1963**, *42*, 1172 – 1178) based on the assumptions of a human body weight of 60 kg and body temperature of 37 °C.
[23] Nitric oxide (NO), which is a potent ligand to the ferrous porphyrin, does not bind to hemoCD, though NO slightly decomposes hemoCD, see: K. Kano, Y. Itoh, H. Kitagishi, T. Hayashi, S. Hirota, *J. Am. Chem. Soc.* **2008**, *130*, 8006 – 8015.
[24] The biochemical tests of rat plasma were performed by an IDEXX VetTest chemistry analyzer before and after the infusion of oxy-hemoCD (Table S2 in the Supporting Information). The rat heart rate and the blood pressure were preliminarily monitored during the infusion.
[25] In the case of the infusion of oxy-hemoCD into the CO-exposed rat, the amount of excreted CO in the urine was greatly enhanced (Figure S6 in the Supporting Information), thus suggesting that hemoCD is also able to remove exogenously inhaled CO from the rat body.

