Supramolecular Chemistry

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Supramolecular Complex of Cytochrome *c* with a Polyanionic β-Cyclodextrin**

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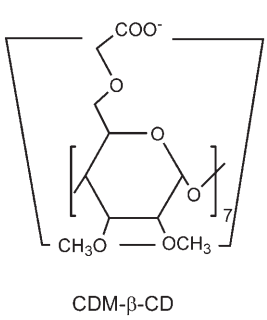
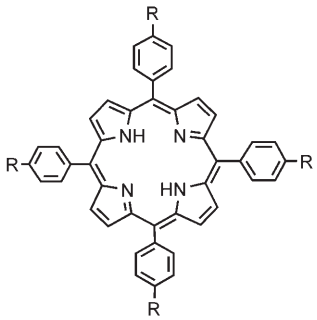
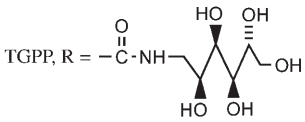
In biological systems, cytochrome *c* (cyt *c*) is bound to its partners (cytochrome *c* reductase and oxidase) through electrostatic interactions between positively charged lysine residues (Lys) of cyt *c* and acidic residues of the partners.[1] Cyt *c* ($M_r = 12400$) has 19 lysines and two arginines as the basic residues and 12 acidic residues (aspartic and glutamic acids). At pH 7.0, cyt *c* (isoelectric point (pI) = 10.4)[2] has nine positive charges in its ferric form. Therefore, many artificial, anionic receptors of cyt *c* have been devised as recognition elements of the protein surface that bind with cyt *c* through electrostatic interactions.[3] Clark-Ferris and Fisher found strong electrostatic interactions between cyt *c* and an anionic porphyrin, 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (TPPC), the binding constant (K) being $2 \times 10^5\,\text{M}^{-1}$.[4] Polyanionic heteropolytungstates were studied as modifiers of the structure of cyt *c*.[5] Uroporphyrin I with eight carboxylate groups caused formation of a ground-state complex with cyt *c* leading to static fluorescence quenching of uroporphyrin I by cyt *c*.[6] Groves and co-workers constructed a sophisticated multiporphyrin assembly by partly using electrostatic interactions between an anionic zinc porphyrin and cyt *c*.[7] In 1997, Hamilton and co-workers reported a well-designed protein receptor composed of a calix[4]arene building block and four peptide loops with eight carboxylate groups.[8] This calixarene receptor strongly binds with cyt *c* ($K = 3 \times 10^6\,\text{M}^{-1}$) and inhibits the reduction of ferricytochrome *c* (ferricyt *c*) with ascorbate, suggesting that a hemin pocket of cyt *c* is covered by the receptor. Systematic investigations by Hamilton's group have revealed that well-designed anionic, artificial receptors show their distinguished functions in inhibition of protein–protein interactions,[9] unfolding of cyt *c*,[10] and proteolysis of cyt *c* by trypsin.[11] Besides the studies mentioned above, many examples of utilization of electrostatic interactions in the chemical biology of cyt *c* have been demonstrated.[12] In the present study, we tried to introduce a supramolecular concept into protein chemistry. We are currently interested in the extremely strong ability of per-O-methylated β-cyclodextrin to include peripheral aryl groups of water-soluble *meso*-tetraarylporphyrins in its cavity.[13] We know that O-methylation of the secondary hydroxy groups of β-cyclodextrin is essential to form stable *trans*-type 1:2 inclusion complexes of water-soluble porphyrins, such as 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TPPS), and the cyclodextrin.[14] We then used heptakis(6-*O*-carboxymethyl-2,3-di-*O*-methyl)-β-cyclodextrin (CDM-β-CD) as an anionic supramolecular receptor of cyt *c* which can be used to prepare a ternary complex consisting of cyt *c*, CDM-β-CD, and an additional guest.



CDM-β-CD



TPPS, R = $-SO_3^-$



Complexation of horse heart ferricyt *c* with CDM-β-CD was studied by means of isothermal titration calorimetry (ITC). The determined thermodynamic parameters for complexation of cyt *c* with CDM-β-CD are listed in Table 1 as a function of NaCl concentration.

In 1×10^{-2} M NaCl solution at pH 7.0, the K value is $2.3 \times 10^4\,\text{M}^{-1}$, that is, much smaller than that for the complex of the calixarene receptor.[8] As CDM-β-CD ($pK_a \approx 4$, the NMR data indicated that all carboxy groups of CDM-β-CD

Table 1: Thermodynamic parameters for complexation of cyt *c* with CDM-β-CD in aqueous NaCl solutions at pH 7.0 and 25 °C.[a]

NaCl [M]	K [M^{-1}]	ΔH [kJ mol^{-1}]	ΔS [J mol^{-1} K^{-1}]
1×10^{-2}	$(2.3 \pm 0.2) \times 10^4$	9.3 ± 0.3	115 ± 2
2×10^{-2}	$(9.2 \pm 0.4) \times 10^3$	9.6 ± 0.3	108 ± 2
5×10^{-2}	$(1.9 \pm 0.1) \times 10^3$	8.8 ± 0.1	92 ± 1
1×10^{-1}	too small	–	–

[a] The titration curve was obtained by adding 25 aliquots (10 μL each) of CDM-β-CD (4×10^{-2} M) into a solution of cyt *c* (2×10^{-4} M).

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dissociate in water above pD 6) has seven carboxylate anion groups on the side of the primary hydroxy group, this complex has a bucket-type shape (the bottom consists of the side of secondary OCH_3 groups) because of electrostatic repulsion between the anionic groups. The MM2 calculation suggests a maximum surface area of CDM-β-CD of 3.5 nm^2, which may be relatively smaller than that of a protein–protein interaction domain of cyt *c*. It has been reported that cyt *c* reductase electrostatically interacts with cyt *c*, whose interaction sites are Lys 13, 72, 86, 27, and 87. On the basis of the NMR spectroscopic data archived in the Protein Data Bank, the electrostatic interaction domains of cyt *c* are estimated to be approximately 4–13 nm^2 by considering the location of the Lys residues on the front surface where the hemin pocket is located (see the Supporting Information). The surface area of the calixarene receptor[8] is wider than that of CDM-β-CD. Furthermore, both hydrophobic and hydrogen-bonding interactions between the calixarene receptor with four peptide loops and cyt *c* may also participate in complexation together with electrostatic interactions. Meanwhile, electrostatic interactions seem to be the preferential binding force in the cyt *c*–CDM-β-CD system. The thermodynamic parameters support this assumption. The complexation of cyt *c* with CDM-β-CD is endothermic and is dominated by the positive and large entropy change. Positive entropy changes are common in the complexation of polyanionic cyclodextrin hosts with oppositely charged guests.[15] Extended dehydration upon complexation promotes salt-bridge-type complexation leading to the positive entropy change. The marked effect of NaCl on complexation strongly supports the electrostatic association between cyt *c* and CDM-β-CD. Interestingly, the complexation of cyt *c* with cyt *c* peroxidase ($\Delta H = 9.6\ kJ\,mol^{-1}$, $\Delta S = 130\ J\,mol^{-1}\,K^{-1}$ in a solution with an ionic strength of 5×10^{-2} M)[16] and with cytochrome b_5 ($\Delta H = 4.2\ kJ\,mol^{-1}$, $\Delta S = 142\ J\,mol^{-1}\,K^{-1}$ in 2×10^{-3} M Tris-HCl buffer solution; Tris = tris(hydroxymethyl)aminomethane)[17] is also endothermic and the positive and large entropy changes are the origin of these protein–protein interactions. Dehydration associated with the electrostatic binding of cyt *c* with its partner seems to greatly contribute to the complexation to cause electron transfer from cyt *c* reductase to cyt *c* oxidase as a consequence.

Analysis of the calorimetric titration curves showed the 1:1 complex formation ($n = 1.07$–1.10). The ESI mass spectrum also indicated the 1:1 complex of cyt *c* and CDM-β-CD (see the Supporting Information). To verify the binding site of CDM-β-CD, we measured ^1H NMR spectroscopic changes of ferricyt *c* as a function of the CDM-β-CD concentration (see the Supporting Information). The signals arising from the methyl protons at the 3- and 8-positions of hemin in cyt *c* and of the methionine residue (Met 80) that coordinates to Fe^{III} of cyt *c* were observed at $\delta = 32.22$, 35.31, and -24.13 ppm, respectively, in D_2O.[18] The signals arising from the 3-CH_3 and 8-CH_3 protons shifted to lower and higher magnetic fields, respectively, and the signal of Met 80 shifted to a lower magnetic field upon the addition of CDM-β-CD. The alterations in the chemical shifts were saturated at two equivalents of CDM-β-CD, with $\Delta\delta = 0.11$ ppm for 3-CH_3, $\Delta\delta = -0.33$ ppm for 8-CH_3, and $\Delta\delta = -0.11$ ppm for Met 80. These results suggest that CDM-β-CD is bound to the protein surface in the vicinity of the hemin pocket (the crevice of cyt *c*).[8,12a]

If CDM-β-CD is bound to the surface near the hemin pocket of cyt *c*, CDM-β-CD should inhibit the reduction of cyt *c* with ascorbate ion. It has been reported that phosvitin, a phosphoglycoprotein, complexed with cyt *c* inhibits the reduction of cyt *c* with anionic reducing agents such as ascorbate, dithionite, and ferricyanide.[19] Figure 1 shows the

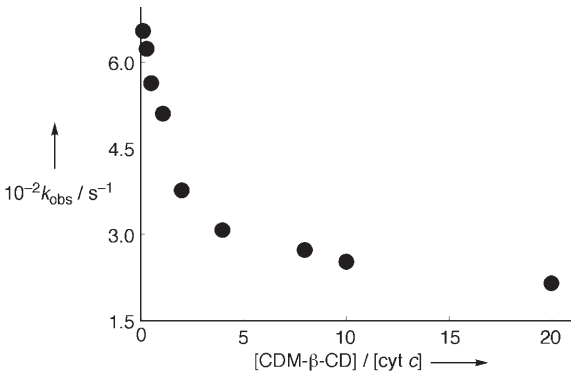

Figure 1. Pseudo-first-order rate constants for the reduction of ferricyt *c* (1×10^{-5} M) with ascorbate (5×10^{-4} M) in water containing various amounts of CDM-β-CD at pH 7.0 and 25 °C.

effect of CDM-β-CD on the reduction of ferricyt *c* with ascorbate. Although the reduction rate was depressed to one-third upon addition of 20 equivalents of CDM-β-CD, the reduction was not inhibited completely by this receptor even if an excess amount of CDM-β-CD was added. Such a result clearly indicates that CDM-β-CD is bound to cyt *c* at multiple places. A portion of the cyt *c* molecules cover the hemin pocket of cyt *c* completely, but another portion misaligns. If an additional CDM-β-CD molecule is bound to the 1:1 complex of CDM-β-CD and cyt *c*, further inhibition in the reduction of cyt *c* by ascorbate should occur. Electrostatic repulsion between a bound CDM-β-CD molecule and an additional one may prohibit further binding of the CDM-β-CD molecule in the vicinity of the crevice of cyt *c*. There is a possibility that an ascorbate anion penetrates the cavity of CDM-β-CD to reach the hemin center, leading to the reduction of hemin to heme. We then added an excess amount of cyclooctanol, which is included into the cyclodextrin cavity, into the system and followed the rate of the reduction of ferricyt *c*. Only slight inhibition by cyclooctanol was observed (see the Supporting Information), suggesting that penetration of ascorbate can be negligible.

CDM-β-CD inhibited the interaction between cyt *c* and cyt *c* reductase (porcine heart, Sigma, type 1, lyophilized powder, ≥ 1.0 units mg^{-1} protein) as shown in Figure 2. In the absence of CDM-β-CD, the initial rate of the reduction (from the tangent line at $t = 0$) was 4×10^{-7} M min^{-1}, whereas those for the reduction in the presence of 100 and 1000 equivalents of CDM-β-CD were 7×10^{-8} and 3×10^{-8} M min^{-1}, respectively. Although CDM-β-CD inhibited the protein–protein interaction, complete inhibition could not be realized even in the presence of a large excess of CDM-β-CD. Such a result is

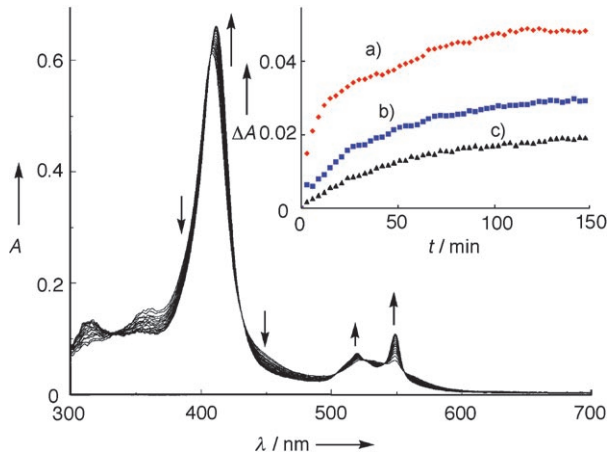

Figure 2. Reduction of ferricyt *c* (5×10^{-6} M) with cyt *c* reductase (porcine heart, 0.4 mg mL^{-1}) in 1×10^{-2} M Tris-HCl buffer solution at pH 7.0 and 25 °C in the absence (a) and the presence of 100 (b) and 1000 equivalents (c) of CDM-β-CD.

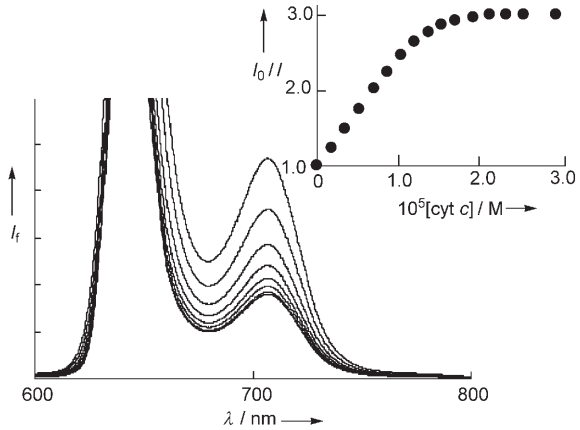

Figure 3. Fluorescence quenching of TGPP (1×10^{-5} M) complexed with CDM-β-CD (2×10^{-5} M) by cyt *c* in 1×10^{-2} M Tris-HCl buffer solution at pH 7.0 and 25 °C. TGPP was excited at 643 nm and the fluorescence was monitored at 707 nm. The inset shows the Stern–Volmer plot for fluorescence quenching in which the inner-filter effect was cancelled from the data obtained for the TGPP–TMe-β-CD complex. I_f = fluorescence intensity, I_0 = initial intensity.

consistent with that for the reduction of cyt *c* with ascorbate. The Michaelis–Menten constant (K_m) for the cyt *c*–cyt *c* reductase system has been reported to be 1×10^{-5} M.[1c] Therefore, competition binding of CDM-β-CD and cyt *c* reductase to cyt *c* occurs when a larger amount of CDM-β-CD coexists in the system. The CD spectrum of cyt *c* was not affected by CDM-β-CD at room temperature, suggesting that CDM-β-CD does not cause unfolding of cyt *c* (see the Supporting Information).

On the basis of these data, it can be concluded that CDM-β-CD is bound to the surface of cyt *c* to cover the hemin pocket. We then tried to form a supramolecule of cyt *c* by applying a novel character of the β-cyclodextrin, whose secondary hydroxy groups are O-methylated, in complexation with water-soluble *meso*-tetraarylporphyrins.[13,14] The supramolecular system was composed of cyt *c*, CDM-β-CD, and 5,10,15,20-tetrakis(4-glucaminocarbonylphenyl)porphyrin (TGPP). As it is possible that an anionic porphyrin binds with cyt *c*, TGPP (without charge) was used as an additional guest even though its solubility in water was not satisfactory. Although TGPP did not dissolve in water without cyclodextrin, it was solubilized in water by complexing with CDM-β-CD. Our previous studies[13,14] strongly suggest the formation of a *trans*-type 1:2 TGPP–CDM-β-CD complex. Indeed, the ^{1}H NMR spectrum of a mixture of TGPP and CDM-β-CD in D_2O supports the formation of the symmetrical 1:2 complex (see the Supporting Information). Figure 3 shows the fluorescence spectral changes of TGPP complexed with CDM-β-CD upon addition of cyt *c*. Fluorescence from the TGPP–CDM-β-CD complex was effectively quenched by cyt *c* and the Stern–Volmer plot was saturated upon addition of 1.5 equivalents of cyt *c*. The Stern–Volmer constant (K_{SV}) obtained from the straight line in Figure 3 is 2.9×10^5 M^{-1}, and the quenching rate constant (k_q) is roughly estimated to be 3×10^{13} M^{-1} s^{-1} by assuming a fluorescence lifetime of TGPP of 10 ns.[20] Such a k_q value is much larger than the diffusion-controlled rate constant, clearly indicating that a static fluorescence quenching takes place in the TGPP–CDM-β-CD–cyt *c* ternary system. Saturation in the Stern–Volmer plot also supports the theory that fluorescence quenching occurs through a ground-state complex. A convincing structure of the ternary complex is shown in Figure 4, in which a TGPP–CDM-β-CD complex electrostatically binds with two cyt *c*

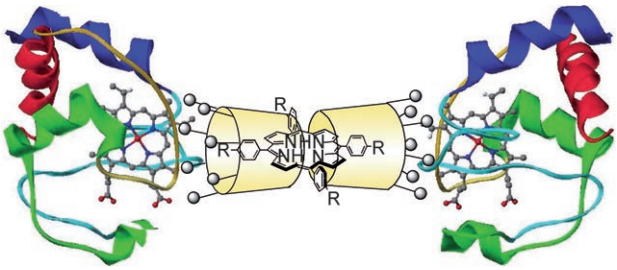

Figure 4. A plausible structure of the ternary complex of cyt *c*, CDM-β-CD, and TGPP.

molecules. The results of the ITC measurements strongly support such a structure (see the Supporting Information). To confirm the role of electrostatic interactions, heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMe-β-CD) was used in place of CDM-β-CD. Although TMe-β-CD forms a stable 1:2 TGPP–TMe-β-CD complex, it cannot bind with cyt *c* through electrostatic interactions. In the TGPP–TMe-β-CD–cyt *c* system, no fluorescence quenching occurred at all, indicating the essential role of electrostatic interactions between cyt *c* and the TGPP–CDM-β-CD complex.

As shown in Figure 3, one-third of the TGPP molecules were not quenched by cyt *c*, indicating that the remaining two-thirds of the cyt *c* molecules bound to the TGPP–CDM-β-CD complex to put their hemin centers in the vicinity of TGPP. The hemin centers of the other third of the cyt *c* molecules are

located relatively far away from TGPP. Such a result is consistent with that of the ascorbate reduction.

So far, a lot of artificial anionic receptors of cyt *c* have been originated.[3] Most of the previous systems are composed of only two components, a protein and a receptor. In the present study, we tried to prepare a ternary complex of cyt *c*, a receptor with supramolecular character, and an additional guest that is bound to the receptor. Such a system could be a tailor-made system in which protein function is intentionally controlled by choosing a third guest appropriately.

Experimental Section

CDM-β-CD was synthesized according to the procedures described in the literature.[21] Cyt *c* (horse heart, Sigma, C2506) and cyt *c* reductase (porcine heart, Sigma, C3381) were purchased and used without further treatment.

TGPP was prepared by a condensation reaction of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (200 mg) with D-glucamine (186 mg) in dimethylformamide (10 mL) containing 1-hydroxy-1*H*-benzotriazole monohydrate (HOBT, 144 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC, 200 mg) at room temperature for 20 h. After the solvent was evaporated from the reaction mixture, the residue was dissolved in 0.1M aqueous HCl, and the acidic solution was neutralized with aqueous NaOH. The precipitates were filtered, washed with water, and dried under vacuum to yield TGPP·4.5 H_2O (73% yield). MS (ESI^+, 0.1% aqueous formic acid) *m*/*z* calcd for $[M+H]^+$: 1443.54; found: 1443.99. Elemental analysis (%) calcd for $C_{72}H_{83}N_8O_{24}$·4.5 H_2O: C 56.72, H 6.02, N 7.35, O 29.91; found: C 56.45, H 6.16. N 7.03, O 29.38.

UV/Vis absorption and fluorescence spectra were recorded with a Shimadzu UV-2100 spectrophotometer and an RF-5300PC spectrofluorometer, respectively, with thermostatic cell holders. CD spectra were recorded on a Jasco J600 spectropolarimeter. ^1H NMR spectra were measured on a JEOL JNM-ECA-500 spectrometer (500 MHz) in D_2O (CEA, 99.9%) by using sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]-propionate (TSP, Aldrich) as external standard.

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