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Intramolecular Oxidative *O*-Demethylation in a Per-*O*-methylated β-Cyclodextrin–Iron Porphyrin Inclusion Complex in Aqueous Solution

Takunori Ueda, Saki Kumeda, Hiroaki Kitagishi, and Koji Kano*

Department of Molecular Chemistry and Biochemistry, Faculty of Science and Engineering, Doshisha University, Kyotanabe, Kyoto 610-0321

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The reaction of a supramolecular heme enzyme model (met-hemoCD2), a 1:1 inclusion complex of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron(III) (Fe^{III}TPPS) and a per-*O*-methylated β-cyclodextrin dimer having a OCH_2PyCH_2O (Py: pyridin-3,5-diyl) linker (Py2CD), with peroxy acid (*m*-chloroperbenzoic acid or peracetic acid) caused intramolecular mono- and di-*O*-demethylations of the OCH_3 group(s) of Py2CD. The *O*-demethylation seemed to proceed through the hydroxylation of a C–H bond of OCH_3 by an oxoiron(IV) porphyrin π-cation radical ($O{=}Fe^{IV}P^{\bullet+}$).

The hydroxylation of C–H bonds catalyzed by cytochrome P450 (Cyt P450) is an important chemical reaction among metabolic processes.[1] An oxoiron(IV) porphyrin π-cation radical ($O{=}Fe^{IV}P^{\bullet+}$, Cpd I), known to be the active species in Cyt P450-catalyzed oxidation,[1,2] is formed via the heterolytic cleavage of the O–O bond of a hydroperoxoiron(III) porphyrin ($HOO{-}Fe^{III}P$, Cpd 0) produced by reductive activation of an oxyiron(II) complex ($O_2{-}Fe^{II}P$).[2–4] Strong electron donation from an axial thiolate ligand to the iron is considered to be essential for the O–O bond heterolysis in Cpd 0. A number of model reactions have been studied. In the model systems, *m*-chloroperbenzoic acid (*m*-CPBA) has been widely used as an oxidant to produce the Cpd I-like active species, because the electron-attracting nature of the RCO-group of an acylperoxoiron(III) porphyrin ($RC(O)OO{-}Fe^{III}P$) causes heterolytic cleavage of the O–O bond even in the absence of a thiolate ligand.[3–8] Previous model studies revealed that the oxoiron(IV) porphyrin π-cation radicals are the active reaction intermediates in the hydroxylation of alkanes[8] and oxidative *N*- and *O*-demethylations of amines and ethers, respectively.[6,9]

We have been studying new types of supramolecular heme protein models composed of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron (FeTPPS) and per-*O*-methylated β-cyclodextrin dimers having nitrogenous axial ligands.[10–14] We designed two types of supramolecular complexes, namely, Fe^{II}TPPS and Fe^{III}TPPS called hemoCD*n* ($n = 1$ and 2) and met-hemoCD*n*, respectively (Figure 1). The FeTPPS in these models is surrounded by hydrophobic cyclodextrin cavities, similar to the manner in which heme or hemin is surrounded by proteins. Such supramolecules behave similar to myoglobin[11–13] and met-myoglobin[10,14] in aqueous solution. We recently found that the reaction of an inclusion complex of Fe^{III}TPPS and Py3CD (met-hemoCD1) with hydrogen peroxide yielded a very stable oxoiron(IV) porphyrin ($O{=}Fe^{IV}P$) through the homolytic cleavage of the O–O bond of $HOO{-}Fe^{III}P$ in the cyclodextrin (CD) cage.[15,16] In the absence of Py3CD, the porphyrin ring of Fe^{III}TPPS was rapidly decomposed upon the addition of hydrogen peroxide due to the attack of the hydroxyl radical (•OH) generated by the homolysis of the O–O bond of $HOO{-}Fe^{III}P$.[15,17] Protection of the porphyrin ring by the CD dimer enabled us to observe the formation of the high-valent oxoiron porphyrin complex in aqueous solution. In the present study, we investigated the reaction of met-hemoCD2 with *m*-CPBA and peracetic acid (PAA) to explore whether $O{=}Fe^{IV}P^{\bullet+}$ is formed in the CD cage.

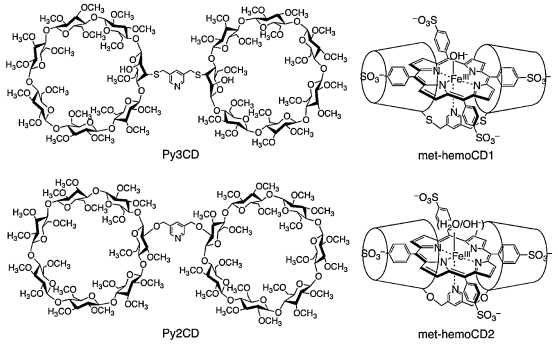


Figure 1. Structures of Py3CD, Py2CD, met-hemoCD1, and met-hemoCD2.

The addition of *m*-CPBA (20 equiv) to met-hemoCD1 ($[Fe^{III}TPPS] = [Py3CD] = 1 \times 10^{-5}$ M) in 0.05 M phosphate buffer at pH 7 and 25 °C caused significant bleaching due to decomposition of the porphyrin ring (Figure 2A). In addition, because met-hemoCD1 has two thioether bonds at the linker position of the CD dimer, Py3CD may be directly oxidized to the sulfoxide and/or sulfone by *m*-CPBA.[18] Previously, we observed intramolecular oxygen transfer from $O{=}Fe^{IV}P$ to a sulfide bond of Py3CD.[16] It was concluded, therefore, that met-hemoCD1 was not suitable for studying the formation of $O{=}Fe^{IV}P^{\bullet+}$ in the reaction with *m*-CPBA. In the present study, we used met-hemoCD2 (a 1:1 complex of Fe^{III}TPPS and Py2CD, Figure 1), in which the two cyclodextrin units were bound together by a OCH_2PyCH_2O (Py: pyridin-3,5-diyl) linker. Unlike in the case of met-hemoCD1, no bleaching occurred and biphasic spectral changes were observed by UV–vis spectroscopy for the reaction of met-hemoCD2 with *m*-CPBA (Figure 2B). An intermediate species with λ_{max} values of 400, 531, and 700 nm (Cpd X) appeared immediately after the addition of *m*-CPBA to met-hemoCD2 ($\lambda_{max} = 409$ and 569 nm) in 0.05 M phosphate buffer at pH 7 and 25 °C. The spectrum of Cpd X gradually changed to show λ_{max} at 422 and 558 nm, indicating the formation of the oxoiron(IV) complex ($O{=}Fe^{IV}P$).[15] The resulting $O{=}Fe^{IV}P$ gradually returned to Cpd X upon standing for 30 h (Figure S1).[19] The results suggested that Cpd X was not $O{=}Fe^{IV}P^{\bullet+}$.

The UV–vis spectrum of Cpd X at pH 7.0 was almost identical to that of a monoaqua form of met-hemoCD2 at pH 5

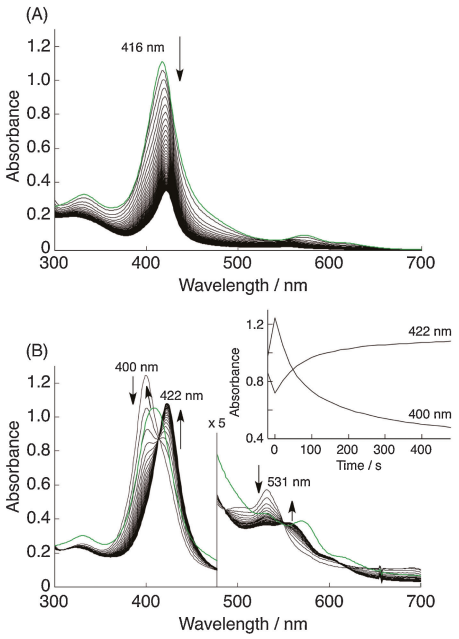


Figure 2. UV–vis spectra of met-hemoCD1 (1×10^{-5} M) (A) and met-hemoCD2 (1×10^{-5} M) (B) before (green) and after addition of *m*-CPBA (2×10^{-4} M, black) in 0.05 M phosphate buffer at pH 7 and 25 °C. The spectra after the addition of *m*-CPBA were recorded at time intervals of 20 s. Inset of (B) shows time course of the absorbances at 400 and 422 nm.

(Figure S2).[19] Because the pK_a value of the axial H_2O of met-hemoCD2 is 6.9,[12] both H_2O and OH^- can act as the sixth axial ligand of met-hemoCD2 before the reaction at pH 7. Therefore, it was predicted that Cpd X would have a structure similar to met-hemoCD2, but the pK_a of the axial H_2O of Cpd X would be higher than that of met-hemoCD2. The EPR spectrum of met-hemoCD2 at 77 K (Figure S3)[19] showed a pattern typical for a high-spin iron(III) porphyrin,[15] with a pH-dependent $g_\perp$ signal. At pH 7, split peaks at $g_\perp = 6.09$ and 6.34 were observed, whereas a single peak at $g_\perp = 6.09$ was measured at pH 5. These results suggested that the signals at $g_\perp = 6.09$ and 6.34 could be assigned to the H_2O- and OH^--coordinated met-hemoCD2s, respectively. The EPR spectrum of Cpd X, showing a single peak at $g_\perp = 6.09$ at pH 7, was quite similar to that of met-hemoCD2 at pH 5, indicating that the iron center of Cpd X was in the iron(III) state with an axial H_2O ligand. The pH titration for Cpd X (Figure S4)[19] showed spectral changes ascribable to an acid–base equilibrium of the axial H_2O[15] at around pH 7–10 (pK_a = ca. 8.2, vide infra). The increase in the pK_a value of the axial H_2O of Cpd X suggested a dramatic change in the coordination environment at the distal site of the iron porphyrin.

To identify the structure of Cpd X, the CD dimer component was extracted from the aqueous solution of Cpd X with $CHCl_3$. In order to exclude the oxidant and its derivative from the $CHCl_3$ extract, water-soluble peracetic acid (PAA), which showed the same behavior as *m*-CPBA (Figure S5),[19] was used. The reaction mixture obtained by mixing met-hemoCD2 ($[Fe^{III}TPPS] = [Py2CD] = 1.0 \times 10^{-4}$ M) with PAA (1.5×10^{-4} M) at room temperature for 5 min was extracted with $CHCl_3$. The organic layer was concentrated, and the residue was

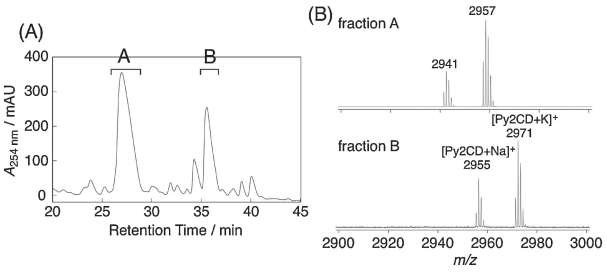
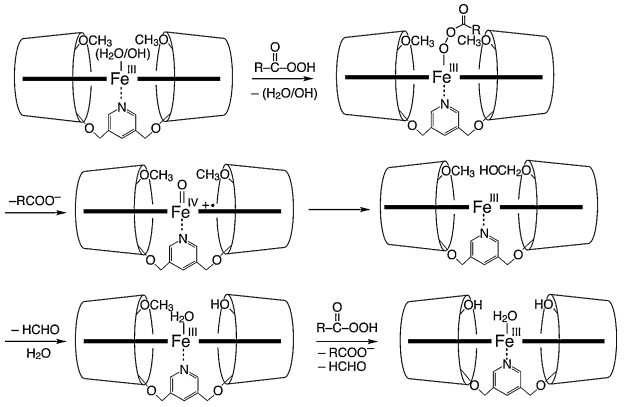


Figure 3. (A) HPLC traces for the CD component extracted from the aqueous solution containing Cpd X. (B) MALDI-TOF mass spectra for the fractionated solutions of (A). The calculated mass number of Py2CD ($C_{131}H_{225}NO_{70}$) is 2932.

analyzed by reverse-phase HPLC (Figure 3A). The MALDI-TOF mass spectra of peaks A and B in the HPLC trace (Figure 3B) corresponded to demethylated Py2CD and Py2CD itself, respectively. The mass peaks of fraction A were observed at m/z 2957 and 2941, which corresponded to $[Py2CD + K - 14]^+$ and $[Py2CD + Na - 14]^+$, respectively. The results strongly suggested that the compound corresponding to peak A was a monohydroxylated Py2CD ($Py2CD^{OH}$), wherein an OCH_3 group of Py2CD was converted to an OH group. The reactions of met-hemoCD2 with excess PAA (2–5 equiv) yielded dihydroxylated Py2CD ($Py2CD^{2OH}$), along with the monohydroxylated product ($Py2CD^{OH}$) (Figure S6).[19] Interestingly, more hydroxylated Py2CD products were not observed despite the presence of 14 primary (6-OCH_3) and 26 secondary OCH_3 groups (2- and 3-OCH_3) in Py2CD. The ^1H NMR spectrum of $Py2CD^{OH}$ isolated by HPLC was quite complex because of its dissymmetric structure, but showed marked changes in the signals of the secondary OCH_3 groups while slight changes were observed in the signals of the primary OCH_3 groups (Figure S7).[19] The H–H COSY spectrum indicated that the signal at 5.07 ppm could be ascribed to an OH group at the 3-position, formed by the demethylation reaction (Figure S7). It was found that the reaction of met-hemoCD2 with peroxy acids such as *m*-CPBA and peracetic acid caused the conversion of one and/or two OCH_3 groups at the 3-position(s) of Py2CD to OH group(s) through oxidative *O*-demethylations.

Oxidative *N*- and *O*-demethylations catalyzed by Cpd I[20,21] and its mimics[6,9] are known. The plausible mechanism for the oxidative *O*-demethylation reaction of met-hemoCD2 is shown in Scheme 1. The *O*-demethylation reaction proceeds through the hydroxylation of the OCH_3 group by $O{=}Fe^{IV}P^{\bullet+}$, followed by the elimination of formaldehyde from the resultant acetal. In the present system, formaldehyde was detected in the reaction mixture of met-hemoCD2 with PAA (Figure S8).[19] Furthermore, *O*-demethylation did not occur when Py2CD or a Zn^{II}TPPS/Py2CD complex was reacted with *m*-CPBA (data not shown). These results indicate that the iron center of met-hemoCD2 plays an essential role in the *O*-demethylation reactions. The fact that an oxoiron(IV) derivative of hemoCD1 ($O{=}Fe^{IV}P$) did not induce demethylation of Py3CD[15,16] strongly suggests that $O{=}Fe^{IV}P^{\bullet+}$ is the reactive intermediate. A stopped-flow analysis did not reveal the formation of the $O{=}Fe^{IV}P^{\bullet+}$ intermediate, but provided a pseudo-first-order rate constant (k_{obs}) for the formation of Cpd X (Figure S9).[19] A linear relationship between k_{obs} and [*m*-CPBA] indicated that the formation of an acylperoxo



Scheme 1. Schematic representation for the reaction of met-hemoCD2 with peroxy acids.

intermediate (RC(O)OO–Fe^{III}P) was the rate-determining step. Very rapid heterolysis of the O–O bond of RC(O)OO–Fe^{III}P afforded O=$Fe^{IV}P^{\bullet+}$, which then oxidized the OCH_3 group to yield the acetal (OCH_2OH) and Fe^{III}P. The acetal decomposed to the alcohol (Cpd X) and formaldehyde. In the presence of excess oxidant, two OCH_3 groups at the 3-positions of Py2CD were converted to the OH groups. Because further demethylation was impossible due to the inadequate positional relationships between other OCH_3 groups in Py2CD and the oxygen of O=$Fe^{IV}P^{\bullet+}$, subsequently formed O=$Fe^{IV}P^{\bullet+}$ seemed to be reduced by its surroundings to form O=Fe^{IV}P. The one-electron reduction of O=$Fe^{IV}P^{\bullet+}$ in Cpd X to O=Fe^{IV}P seemed to proceed very rapidly because O=$Fe^{IV}P^{\bullet+}$ could not be detected during the conversion of Cpd X to O=Fe^{IV}P.

The mono-*O*-demethylated product ($Py2CD^{OH}$) isolated by HPLC formed an inclusion complex with Fe^{III}TPPS. The pK_a of the axial H_2O of the Fe^{III}TPPS/$Py2CD^{OH}$ complex was determined to be 8.2 (Figure S10).[19] Reduction of the Fe^{III}TPPS/$Py2CD^{OH}$ complex with $Na_2S_2O_4$, followed by purification using gel filtration chromatography (Sephadex G-25) under aerobic conditions gave a dioxygen adduct of the Fe^{III}TPPS/$Py2CD^{OH}$ complex (O_2–Fe^{II}TPPS/$Py2CD^{OH}$) (Figure S11).[19] This O_2–Fe^{II}TPPS/$Py2CD^{OH}$ complex could be converted to the Fe^{II}TPPS/$Py2CD^{OH}$ and CO–Fe^{II}TPPS/$Py2CD^{OH}$ complexes under N_2 and CO atmospheres, respectively. The oxygen binding affinity ($P_{1/2}$) of the Fe^{II}TPPS/$Py2CD^{OH}$ complex and the autoxidation rate of the O_2–Fe^{II}TPPS/$Py2CD^{OH}$ complex were quite different from those of hemoCD2 (Table 1). $P_{1/2}$ is the partial O_2 pressure, at which half of the O_2-receptor molecules are oxygenated. The $P_{1/2}$ of the Fe^{II}TPPS/$Py2CD^{OH}$ complex was determined to be 17 Torr in phosphate buffer at pH 7.0 and 25 °C, whereas that of hemoCD2 was 176 Torr. It was noteworthy that the O_2 affinity dramatically increased upon the modification of only one OCH_3 group in Py2CD to an OH group. Although we need to carry out a more detailed investigation, a possible mechanism for the higher O_2 affinity of the Fe^{II}TPPS/$Py2CD^{OH}$ complex is hydrogen bonding between the OH group at the 3-position of $Py2CD^{OH}$ and the O_2 bound to Fe^{II}TPPS, leading to a slower dissociation rate of the O_2–Fe^{II}TPPS/$Py2CD^{OH}$ complex.[22] The replacement of a single OCH_3 group by an OH group in Py2CD may allow the surrounding water molecules to penetrate into the cyclodextrin cavity and effect the water-catalyzed autoxidation of the O_2–Fe^{II}TPPS/$Py2CD^{OH}$ complex.[23]

Table 1. O_2 binding affinities ($P_{1/2}$) and half-lifetimes for autoxidation ($t_{1/2}$) of hemoCD2 and Fe^{II}TPPS/$Py2CD^{OH}$ in 0.05 M phosphate buffer at pH 7 and 25 °C

	$P_{1/2}$/Torr	$t_{1/2}$/h	Ref
Fe^{II}TPPS/Py2CD (hemoCD2)	176	∞	12
Fe^{II}TPPS/$Py2CD^{OH}$	17	4	this work

In conclusion, we found that the reaction of a 1:1 inclusion complex of Fe^{III}TPPS and Py2CD (met-hemoCD2) with *m*-CPBA or PAA generated a very active oxoiron(IV) porphyrin π-cation radical (O=$Fe^{IV}P^{\bullet+}$), which oxidized a neighboring OCH_3 group in Py2CD, leading to demethylation and affording the Fe^{III}TPPS/$Py2CD^{OH}$ complex. The only structural difference between $Py2CD^{OH}$ and Py2CD was the OH group introduced into the 3-position of the per-*O*-methylated cyclodextrin dimer. The single hydroxylation of Py2CD drastically altered the properties of its FeTPPS complexes, such as the pK_a of the axial H_2O, the dioxygen affinity, and the autoxidation rate of the O_2 adduct.

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