A water-soluble supramolecular complex that mimics the heme/copper hetero-binuclear site of cytochrome c oxidase†‡

Hiroaki Kitagishi, a∗a Daiki Shimoji,a Takehiro Ohta, b Ryo Kamiya,a Yasuhiro Kudo,a Akira Onoda, c Takashi Hayashi, c Jean Weiss, d Jennifer A. Wytkod and Koji Kanoa

In mitochondria, cytochrome c oxidase (CcO) catalyses the reduction of oxygen (O2) to water by using a heme/copper hetero-binuclear active site. Here we report a highly efficient supramolecular approach for the construction of a water-soluble biomimetic model for the active site of CcO. A tridentate copper(ii) complex was fixed onto 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron(III) (FeIII TPPS) through supramolecular complexation between FeIII TPPS and a per-O-methylated β-cyclodextrin dimer linked by a (2,2′:6′,2″-terpyridyl)copper(ii) complex (CuII Terpy CD2). The reduced FeIII TPPS/CuII Terpy CD2 complex reacted with O2 in an aqueous solution at pH 7 and 25 °C to form a superoxo-type FeIII- O2-/CuI complex in a manner similar to CcO. The pH-dependent antioxidation of the O2 complex suggests that water molecules gathered at the distal Cu site are possibly involved in the FeIII- O2-/CuI superoxo complex in an aqueous solution. Electrochemical analysis using a rotating disk electrode demonstrated the role of the FeTPPS/Cu Terpy CD2 hetero-binuclear structure in the catalytic O2 reduction reaction.

Introduction

Cytochrome c oxidase (CcO) is the terminal enzyme in the mitochondrial respiratory chain. CcO consumes most of the molecular oxygen (O2) processed by living organisms by reducing it to water (H2O).1 The four-electron/four-proton reduction process (O2 + 4e− + 4H+ → 2H2O) takes place at the heme a3/CuA hetero-binuclear active centre of CcO (Fig. 1a).1−5 For the catalytic O2 reduction reaction, the reaction mechanism schematically depicted in Fig. 1b has been proposed.1,2 In the catalytic cycle, the fully reduced heme a3/CuA site (FeII/CuI, compound R) reacts with O2 to form an oxymyoglobin-like superoxo complex of heme a3 (FeIII-O2-/CuI, compound A).1,6 Compound A is rapidly (~0.5 ms) converted to an oxoferryl intermediate (FeIV═O/CuII-OH, compound P) via O−O bond cleavage assisted by H atom injection from a vicinal tyrosine residue.1−6 Mechanistic investigations have suggested that one or more water molecules near the bound O2 can facilitate the conversion of compound A to compound P.1−6

To understand the reaction mechanism, synthetic heme/copper models have been constructed using tetraarylporphinoiron(II) (PFeII) combined with CuI complexes (CuILn, where L is a nitrogen donor ligand; n (coordination number) = 3 or 4).4,5 However, upon oxygenation of the PFeII/CuILn model systems in anhydrous organic solvents, μ-peroxo-type bridged structures, i.e., PFeIII – O2−–CuII Ln complexes, tend to form instead of compound A-like superoxo species.4,5−12 In native CcO, the μ-peroxo-type bridged structure has not been

Fig. 1 (a) Heme a3/CuA hetero-binuclear active site of CcO (PDB ID: 1OCO) and (b) the simplified mechanism for the O2 reduction reaction catalysed by CcO.
experimentally identified, although it has been proposed as a transitional precursor of compound P.19,23,24 The structural differences between the native and model systems (superoxo vs. \( \mu \)-peroxo)14 might be attributed to the influence of water.7,8,11 A model study by Naruta and co-workers demonstrated that the \( \mu \)-peroxo complex (PFe\(^{III}\).O\(_2\).Cu\(^{II}\)O\(_{\alpha}\)) formed at \(-70\) °C was converted to the superoxo complex (PFe\(^{III}\).O\(_2\)/Cu\(^{II}\)O\(_{\alpha}\)) at \(-30\) °C by the action of water molecules.15 In native CcO, highly ordered water molecules have been detected in the vicinity of heme \( \alpha / \gamma \)CuB.7,15 A quantum chemical calculation suggested that a water molecule in the vicinity of CuB decreases the energy barrier of the transformation of compound A to compound P.8 In this context, a water-soluble PFe\(^{III}\)/Cu\(^{II}\)A model compound would be useful to investigate the role of water on the reactivity of the Fe/Cu hetero-binuclear complex with O\(_2\). However, very few heme/copper mimics functioning under aqueous conditions have been prepared so far, except for the system constructed in the engineered heme pocket of myoglobin.

In this study, we describe an aqueous synthetic PFe/CuL\(_3\) hetero-binuclear model system built on a porphyrin/ cycloextrin supramolecular complex (Scheme 1). This system takes advantage of the very stable formation of a self-assembling 1 : 2 complex of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron (FeTPPS) with per-O-methylated \( \beta \)-cyclodextrins (CDs).19 We have previously studied the porphyrin/cyclodextrin complexes as simple biomimetic models of heme proteins that function under aqueous conditions,20–21 where the molecular cage of per-O-methylated \( \beta \)-CDs provided a microscopic hydrophobic environment for FeTPPS similar to the heme pocket of heme proteins.24 Here, we have synthesised a per-O-methylated \( \beta \)-CD dimer linked by a Cu\(^{II}\)-terpyridine complex (Cu\(^{II}\)TerpyCD\(_2\), Scheme 1) to replicate the distal tridentate CuB site of CcO. The structural characterisation of the supramolecular FeTPPS/CuTerpyCD\(_2\) complex and its reactivity towards O\(_2\) are described.

Results and discussion
Synthesis of a water-soluble Fe\(^{III}\)/Cu\(^{II}\) hetero-binuclear complex

The synthetic route of a supramolecular Fe\(^{III}\)TPPS/Cu\(^{II}\)TerpyCD\(_2\) complex is shown in Scheme 1 and experimental details are described in (ESI†). Briefly, the terpyridyl ligand was inserted as a linker of the CD dimer (TerpyCD\(_2\)) by the reaction of 5,5′′-bis(mercaptomethyl)-2,2′,6′,2″-terpyridine with 2,3-monoepoxy- per-O-methylated \( \beta \)-CD (Epo-OMe-\( \beta \)-CD).20 The addition of CuSO\(_4\).5H\(_2\)O to TerpyCD\(_2\) in an aqueous solution generated two absorption bands at 336 and 350 nm (Fig. 2a), which corresponded to the ligand to metal charge transfer bands of the terpyridyl-Cu\(^{II}\) 1 : 1 complex.25 In the UV-vis titration, a biphasic spectral change was observed (Fig. 2a inset), indicating that the 1 : 2 complex of Cu\(^{II}\) with TerpyCD\(_2\) (2\(_{\max }\) = 333 nm) was first formed and then it was converted to the thermodynamically stable 1 : 1 complex upon further addition of Cu\(^{II}\). The spectral

Scheme 1 Preparation of the supramolecular Fe\(^{III}\)TPPS/Cu\(^{II}\)TerpyCD\(_2\) complex.
Changes were completed at one equivalent of Cu$^{2+}$. The complexation between TerpyCD$_2$ and Cu$^{2+}$ was also monitored by electrospray mass spectrometry. In the 1 : 1 mixture of CuSO$_4$ and TerpyCD$_2$ in H$_2$O, the 1 : 1 complex (Cu$^{II}$TerpyCD$_2$) was observed at $m/z$ 1577 and 1059 (Fig. 2b), which corresponds to [Cu$^{II}$TerpyCD$_2$]$^{2+}$ and [(H$_2$O)Cu$^{II}$TerpyCD$_2$ + H]$^+$, respectively. The 1 : 2 complex was also detected as a small ion peak when the 1 : 2 mixture of CuSO$_4$ and TerpyCD$_2$ in H$_2$O was analysed by electrospray mass spectrometry (data not shown).

The Cu$^{II}$TerpyCD$_2$ complex was then titrated with Fe$^{III}$TPPS (Fig. 3a). The Soret band of Fe$^{III}$TPPS shifted from 408 nm to 418 nm, indicating that a μ-oxo-dimer of Fe$^{III}$TPPS dissociated to the monomeric monohydroxo complex (Fe$^{III}$OH$^{-}$TPPS)$^+$ through interaction with Cu$^{II}$TerpyCD$_2$. The spectral changes were completed upon addition of one equivalent of Cu$^{II}$TerpyCD$_2$ to Fe$^{III}$TPPS, indicating a quantitative 1 : 1 complexation. The obtained complex was then analysed by electrospray mass spectroscopy. The two main ion peaks were detected at $m/z$ 1385 and 2078 as tri- and di-anionic species, respectively (Fig. 3b). Considering total charges of the complexes, the peaks at $m/z$ 1385 and 2078 were assigned to the μ-oxo and μ-hydroxo Fe$^{III}$TPPS/Cu$^{II}$TerpyCD$_2$ complexes, i.e., [PFe$^{III}$-O-Cu$^{II}$CD$_2$]$^+$ and [PFe$^{III}$-(OH)-Cu$^{II}$CD$_2$]$^-$, respectively. The assignments were confirmed by isotopic pattern simulations (Fig. 3b inset). Evidence of the μ-oxo (Fe$^{III}$-O-Cu$^{II}$)

Characterisation of an O$_2$ adduct of the Fe$^{III}$/Cu$^{I}$ complex

The Fe$^{III}$TPPS/Cu$^{II}$TerpyCD$_2$ complex was reduced with excess sodium dithionite (Na$_2$S$_2$O$_4$) to obtain the fully reduced [PFe$^{II}$/Cu$^{I}$CD$_2$]$^0$ complex in the deoxy state in an O$_2$-free solution ($I_{max}$ at 430, 554, and 601 nm, Fig. 5, black line). The dissolved O$_2$ in the solution was completely consumed by excess dithionite, and the redox potential of dithionite is negative

![Fig. 3](image-url) Characterisation of the supramolecular Fe$^{III}$TPPS/Cu$^{II}$TerpyCD$_2$ complex. (a) UV-vis spectral changes of Fe$^{III}$TPPS (3 μM) upon stepwise addition of Cu$^{II}$TerpyCD$_2$ in 0.05 M phosphate buffer at pH 7.0 and 25 °C. The inset shows the changes in absorbance at 418 nm as a function of the molar ratio ([Cu$^{II}$TerpyCD$_2$]/[Fe$^{III}$TPPS]). (b) Electrospray mass spectrum (negative mode) of the 1 : 1 mixture of Fe$^{III}$TPPS and Cu$^{II}$TerpyCD$_2$ in H$_2$O. The inset shows the simulated isotope distribution patterns for the μ-oxo- and μ-hydroxo-bridged Fe$^{III}$TPPS/Cu$^{II}$TerpyCD$_2$ complexes.

![Fig. 4](image-url) Optimized molecular structures of the FeTPPS/CuTerpyCD$_2$ inclusion complexes in the Fe/Cu non-bridged and Fe/Cu oxo-bridged forms. The models are shown from both side and top views. Hydrogen atoms are omitted for clarity. Molecular mechanics calculations were carried out using CONFLEX/MM3 (extensive search) parameters in Scigress version 2.2.1 software program (Fujitsu).
enough to reduce both FeIII and CuII to FeII and CuI.29,30 After the reduction, the solution was passed through a short gel-filtration column (Sephadex G-25) under aerobic conditions to remove excess S2O42− and its oxidised products. The UV-vis spectrum of the resulting solution showed absorption maxima at 411 nm and 542 nm (Fig. 5, blue line); the Q-band was very different from that of the oxidised state (FeIIITPPS/CuIITerpyCD2, λmax (Q-bond) = 570 nm, green line) and similar to that of the O2 complex of the previously reported FeIIITPPS/CD dimer system.31 Introduction of CO gas into the solution caused further spectral changes with absorption maxima at 418 nm and 535 nm (Fig. 5, red line). The sharp Soret band is characteristic of the CO–FeIIITPPS complex,32 indicating that a ligand exchange from O2 to CO occurs in this system.

The O2 complex was further characterized by EPR and resonance Raman (rR) spectroscopic analyses. The EPR spectrum of the O2 adduct of FeIIITPPS/CuIITerpyCD2 measured at 77 K was completely silent (Fig. S4†), which was consistent with the spectra of other O2 complexes of the PFeIII/CuIILa heterobinuclear systems.31-33 The rR analysis at 77 K (frozen solution of the O2 adduct) using 405 nm excitation revealed a characteristic band at 578 cm−1, which shifted to 551 cm−1 under an O2 atmosphere (Fig. 5 inset). The isotope shift (Δν = 27 cm−1) corresponds to the expected value for the νFe=O stretching mode.15 The wavenumber is quite similar to those of the PFeIII-O2/CuIILa superoxo complexes in the previously reported native34 and synthetic model systems as listed in Table 1.14,15,35 Furthermore, the O–O bond stretching mode (νO=O) was not enhanced in this system. This is a relevant observation as the νO=O band is often observed in the range of 750–900 cm−1 in the FeIII–O2–CuIIμ-peroxo complexes, but not in the case of the FeII–O2/CuIILa superoxo complexes (Table 1).14,15,35,37 Based on the rR data, the configuration of the present O2-adduct of FeIIITPPS/CuIITerpyCD2 is assigned as the superoxo-type PFeIII–O2–/CuIILa complex (Fig. 6), which is the same coordination mode as in compound A of native CrO.1,14,38

The superoxo PFeIII–O2–/CuIICD2 complex was gradually converted to another state when the solution was allowed to stand at pH 7 and 25 °C under aerobic conditions (Fig. 7). The absorption spectra showed several isosbestic points and the final spectrum (shown as a green line in Fig. 7) was coincident to that of the oxidised FeIIITPPS/CuIITerpyCD2 complex (Fig. S4†). EPR spectral changes also support oxidation of the superoxo PFeIII–O2–/CuIICD2 species to the FeIIITPPS/CuIIterpyCD2 complex (Fig. S4†). The first-order rate constants (kobs) for the conversion were determined from the absorbance change at various pH conditions. Interestingly, the superoxo complex was more rapidly converted at lower pH (Fig. 7 inset). The linear pH/log kobs dependency at pH 7–10 (slope = −0.11) suggests that the conversion is partially accelerated by a proton-coupled process.39 Collman et al. have reported that the rate of the O2 reduction catalysed by their PFe/CuIILa model complex is pH-dependent and increases at lower pH.40 We have previously reported that the autoxidation rate of the O2 complex in the PFeII/CD dimer system without any distal functions is independent of pH in the neutral pH region (7–10), whereas it is accelerated at

### Table 1

<table>
<thead>
<tr>
<th>Medium</th>
<th>νFe–O/cm−1 (16O2)</th>
<th>νO–O/cm−1 (18O2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O, pH 7.4</td>
<td>572 (548)</td>
<td>—</td>
</tr>
<tr>
<td>H2O, pH 7.2</td>
<td>571 (545)</td>
<td>—</td>
</tr>
<tr>
<td>CH2Cl2</td>
<td>570 (544)</td>
<td>—</td>
</tr>
<tr>
<td>DMF</td>
<td>575 (549)</td>
<td>—</td>
</tr>
<tr>
<td>CH3CN/THF</td>
<td>574 (548)</td>
<td>—</td>
</tr>
<tr>
<td>H2O, pH 7.0</td>
<td>578 (551)</td>
<td>—</td>
</tr>
</tbody>
</table>

### Figure 6

Oxygenation of the FeIIITPPS/CuIITerpyCD2 complex to form a superoxo PFeIII–O2–/CuIICD2 complex.
pH below 6 and above 10.\textsuperscript{24} Therefore, the pH-rate dependency at the neutral pH region suggests that the water molecules gathered at the distal Cu site promote the conversion of the Fe\textsuperscript{III}–O\textsubscript{2}/Cu\textsuperscript{I}CD\textsubscript{2} complex to the oxidised Fe\textsuperscript{III}–(OH)–Cu\textsuperscript{II}CD\textsubscript{2} complex.

The quantum chemical study on native CcO\textsuperscript{5} proposes that a water molecule coordinating to the distal copper ion facilitates the conversion of compound A to compound P through the formation of the hydroperoxo Fe\textsuperscript{III}–O\textsubscript{2} intermediate that has not been experimentally detected. Thus, the involvement of a water molecule in the present Fe\textsuperscript{III}–O\textsubscript{2}/Cu\textsuperscript{I}CD\textsubscript{2} complex is likely to occur. In addition, molecular modelling suggests that a water molecule bound to the distal copper ion can induce protonation of the superoxo complex (Fig. 8a), where the proton-donation to the superoxo Fe\textsuperscript{III}–O\textsubscript{2}– moiety is likely to occur at low pH.

Electrochemical analysis for the O\textsubscript{2} reduction

To evaluate the CcO-like function of this system, we monitored the electrocatalytic O\textsubscript{2} reduction reaction.\textsuperscript{45–47} The cyclic voltamogram (CV) of the Fe\textsuperscript{III}TPPS/Cu\textsuperscript{II}TerpyCD\textsubscript{2} complex immobilized on a glassy carbon electrode showed a reversible redox couple at $E_{1/2} = -0.21$ V (vs. Ag/AgCl) in a deoxygenated buffer solution (under Ar, Fig. 9a, black line). The result is similar to those of the previously reported PFe/CuL\textsubscript{aq} heterobinuclear systems; the Fe\textsuperscript{III}/Fe\textsuperscript{II} and Cu\textsuperscript{II}/Cu\textsuperscript{I} redox waves appear at the same potentials.\textsuperscript{31,46} In an air-saturated buffer, the CV of the Fe\textsuperscript{III}TPPS/Cu\textsuperscript{II}TerpyCD\textsubscript{2} complex showed a large catalytic current below $-0.25$ V because of O\textsubscript{2} reduction (Fig. 9a, blue line). A comparison of the CVs of the Fe\textsuperscript{III}TPPS/Cu\textsuperscript{II}TerpyCD\textsubscript{2} complex with those of the reference samples, i.e., Fe\textsuperscript{III}TPPS and Fe\textsuperscript{III}TPPS/TerpyCD\textsubscript{2} (Fig. 9b), clearly indicates the effect of the Fe/Cu hetero-binuclear structure in the O\textsubscript{2} reduction; the Fe\textsuperscript{III}TPPS/Cu\textsuperscript{II}TerpyCD\textsubscript{2} complex showed a very large catalytic current starting from a lower onset potential ($\Delta E_{\text{onset}} = -40$ mV). The O\textsubscript{2} reduction process was then studied by linear sweep voltammetry (LSV) using a rotating disk electrode (RDE, Fig. 9c). The LSVs of the Fe\textsuperscript{III}TPPS/Cu\textsuperscript{II}TerpyCD\textsubscript{2} and Fe\textsuperscript{III}TPPS/TerpyCD\textsubscript{2} complexes showed diffusion limited catalytic O\textsubscript{2}– reduction currents below $-1.0$ V vs. Ag/AgCl. In the case of FeTPPS without the CD dimer, the current was never saturated in LSV due to a slow reaction rate of the iron porphyrin with O\textsubscript{2}.
involvement of water molecules in the formation of the super-oxy complex in aqueous solution. We believe that our aqueous model system will help to clarify the long-standing arguments with regard to the native and synthetic model systems in CoO chemistry.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We thank (late) Prof. Takashi Ogura (University of Hyogo) for the use of rP instruments and helpful discussion. This work was financially supported by MEXT/JSPS KAKENHI (Grant No. 15H02569, 16K13092, 17H02208), the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (2015–2019), the Naito Foundation, Iketani Science and Technology Foundation, and Suntory Foundation for Life Sciences. JW and TH thank financial support from the bilateral France–Japan ANR-JST program TMOL”Molecular Technology” project “MECANO” AT R.1 4.JTIC.OOO2.O 1.

Notes and references

This article is licensed under a Creative Commons Attribution 3.0 Unported Licence.


38 The νFe–O stretching frequency of 578 cm⁻¹ is higher than the reported values for the κ-coordinated PFeIII–O₂⁻ complexes, indicating that the Lewis acidity of the distal Cu complex significantly alters the Fe–O bonding. For the νFe–O data of PFeIII–O₂⁻ complexes, see: K. M. Vogel, P. M. Kozlowski, M. Z. Zgierski and T. G. Spiro, J. Am. Chem. Soc., 1999, 121, 9915–9921.

39 If this conversion would have been completely conjugated with the proton transfer, the slope of the pH/log kobs plot would be –1.0.


42 In the present system, any intermediate species ascribed to ferrylxo (FeIV=O) complex could not be observed during conversion from the superoxo to the oxidised complexes in aqueous solution at room temperature.

43 The O₂ complex was also formed in the FeIII–TPPS/TerpyCD₂ complex without copper. The UV-vis spectrum of this complex (λmax = 419 and 542 nm) was found to be consistent with that of the PFeIII–O₂⁻/CuICD₂ superoxo complex, which further supports the absence of a direct interaction between the distal Cu¹ and the bound O₂ in the PFeIII–O₂⁻/CuCD₂ complex.


49 The selectivity of the electrocatalytic O₂ reduction by the FeTPPS/CuTerpyCD₂ and its copper free complexes could not be determined using a rotating ring disk electrode system because of very low currents detected at the ring electrode.