



Cite this: *Chem. Sci.*, 2018, 9, 1989

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

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

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) (Fe^{III}TPPS) through supramolecular complexation between Fe^{III}TPPS and a per-*O*-methylated β-cyclodextrin dimer linked by a (2,2':6',2''-terpyridyl)copper(II) complex (Cu^{II}TerpyCD₂). The reduced Fe^{III}TPPS/Cu^ITerpyCD₂ complex reacted with O₂ in an aqueous solution at pH 7 and 25 °C to form a superoxo-type Fe^{III}-O₂⁻/Cu^I complex in a manner similar to CcO. The pH-dependent autoxidation of the O₂ complex suggests that water molecules gathered at the distal Cu site are possibly involved in the Fe^{III}-O₂⁻/Cu^I superoxo complex in an aqueous solution. Electrochemical analysis using a rotating disk electrode demonstrated the role of the FeTPPS/CuTerpyCD₂ hetero-binuclear structure in the catalytic O₂ reduction reaction.

Received 2nd November 2017
Accepted 12th January 2018

DOI: 10.1039/c7sc04732k

rsc.li/chemical-science

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/Cu_B 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.¹⁻³ In the catalytic cycle, the fully reduced heme a₃/Cu_B site (Fe^{II}/Cu^I, compound R) reacts with O₂ to form an oxymyoglobin-like superoxo complex of heme a₃ (Fe^{III}-O₂⁻/Cu^I, compound A).^{3,6} Compound A is rapidly (~0.5 ms) converted to an oxoferryl intermediate (Fe^{IV}=O/Cu^{II}-OH, compound P) via O-O bond cleavage assisted by H atom injection from a vicinal tyrosine

residue.³⁻⁶ Mechanistic investigations have suggested that one or more water molecules near the bound O₂ can facilitate the conversion of compound A to compound P.^{7,8}

To understand the reaction mechanism, synthetic heme/copper models have been constructed using tetraarylporphinatoiron(II) (PFe^{II}) combined with Cu^I complexes (Cu^IL_{*n*}, where L is a nitrogen donor ligand; *n* (coordination number) = 3 or 4).^{4,5} However, upon oxygenation of the PFe^{II}/Cu^IL_{*n*} model systems in anhydrous organic solvents, μ-peroxo-type bridged structures, *i.e.*, PFe^{III}-O₂-Cu^{II}L_{*n*} complexes, tend to form instead of compound A-like superoxo species.⁹⁻¹² In native CcO, the μ-peroxo-type bridged structure has not been

^aDepartment of Molecular Chemistry and Biochemistry, Faculty of Science and Engineering, Doshisha University, Kyotanabe, Kyoto 610-0321, Japan. E-mail: hkitagis@mail.doshisha.ac.jp

^bPicobiology Institute, Graduate School of Life Science, University of Hyogo, RSC-UH LP Center, Hyogo 679-5148, Japan

^cDepartment of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita 565-0871, Japan

^dInstitut de Chimie de Strasbourg, UMR 7177, CNRS, Université de Strasbourg, 4 Rue Blaise Pascal, 67000 Strasbourg, France

† This paper is dedicated to (late) Professor Takashi Ogura.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c7sc04732k

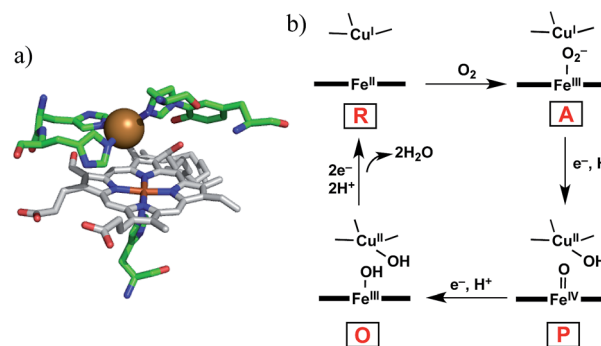


Fig. 1 (a) Heme a₃/Cu_B hetero-binuclear active site of CcO (PDB ID: 1OCO) and (b) the simplified mechanism for the O₂ reduction reaction catalysed by CcO.



experimentally identified, although it has been proposed as a transitional precursor of compound P.^{3,12,13} The structural differences between the native and model systems (superoxo *vs.* μ -peroxo)¹⁴ might be attributed to the influence of water.^{7,8,13} A model study by Naruta and co-workers demonstrated that the μ -peroxo complex ($\text{PFe}^{\text{III}}\text{-O}_2\text{-Cu}^{\text{II}}\text{L}_3$) formed at -70°C was converted to the superoxo complex ($\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{II}}\text{L}_3$) 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 a_3/Cu_B .^{7,16} A quantum chemical calculation suggested that a water molecule in the vicinity of Cu_B decreases the energy barrier of the transformation of compound A to compound P.⁸ In this context, a water-soluble $\text{PFe}^{\text{II}}/\text{Cu}^{\text{II}}\text{L}_n$ 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.^{17,18}

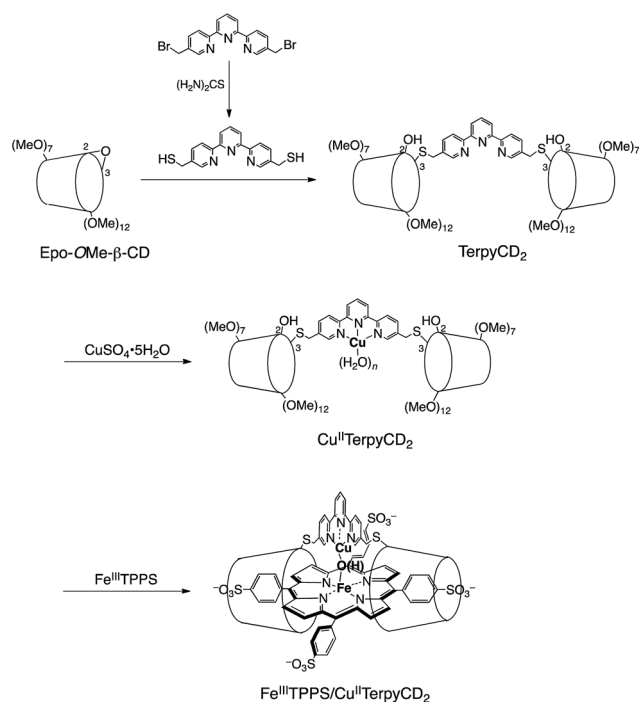
In this study, we describe an aqueous synthetic PFe/CuL_3 hetero-binuclear model system built on a porphyrin/cyclodextrin supramolecular complex (Scheme 1). This system takes advantage of the very stable formation of a self-assembling 1 : 2 complex of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinatoiron (FeTPPS) with per-*O*-methylated β -cyclodextrins (CDs).¹⁹ We have previously studied the porphyrin/cyclodextrin complexes as simple biomimetic models of heme proteins that function under aqueous conditions,^{20–23} where the molecular cage of per-*O*-methylated β -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 β -CD dimer linked by a Cu^{II} -

terpyridine complex ($\text{Cu}^{\text{II}}\text{TerpyCD}_2$, Scheme 1) to replicate the distal tridentate Cu_B site of CcO. The structural characterisation of the supramolecular $\text{FeTPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex and its reactivity towards O_2 are described.

Results and discussion

Synthesis of a water-soluble $\text{Fe}^{\text{III}}/\text{Cu}^{\text{II}}$ hetero-binuclear complex

The synthetic route of a supramolecular $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ 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-monoepoxyper-*O*-methylated β -CD (Epo-*OMe*- β -CD).²⁰ The addition of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 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^{2+} with TerpyCD_2 ($\lambda_{\text{max}} = 333$ nm) was first formed and then it was converted to the thermodynamically stable 1 : 1 complex upon further addition of Cu^{2+} . The spectral



Scheme 1 Preparation of the supramolecular $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex.

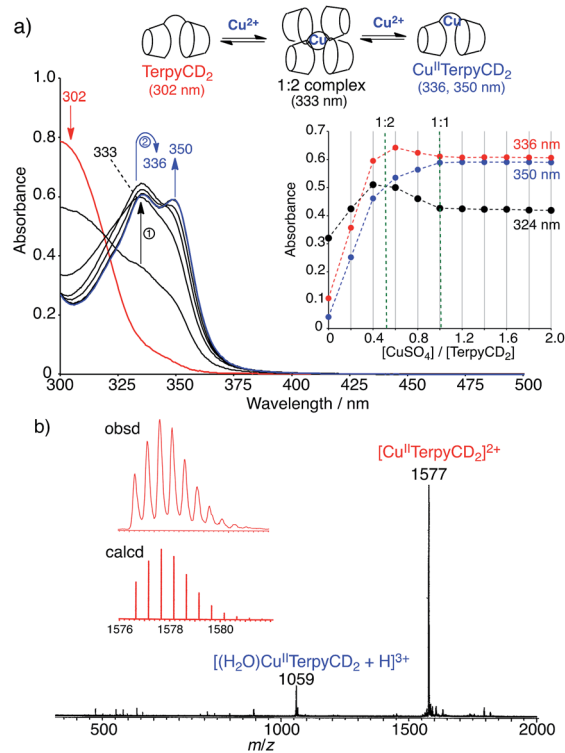


Fig. 2 Complexation of TerpyCD_2 with Cu^{2+} in aqueous solution. (a) UV-vis spectral change of TerpyCD_2 ($33\ \mu\text{M}$) upon stepwise addition of CuSO_4 in water at 25°C . The inset shows changes in absorbances as a function of $[\text{CuSO}_4]/[\text{TerpyCD}_2]$. The biphasic titration curve indicates transient formation of the 1 : 2 complex before forming the thermodynamically stable 1 : 1 complex ($\text{Cu}^{\text{II}}\text{TerpyCD}_2$) during the titration. (b) Electrospray mass spectrum (positive mode) of the 1 : 1 mixture of TerpyCD_2 and CuSO_4 in H_2O . The inset shows the simulated isotope distribution patterns for the $[\text{Cu}^{\text{II}}\text{TerpyCD}_2]^{2+}$ complex.



changes were completed at one equivalent of Cu^{2+} . The complexation between TerpyCD₂ and Cu^{2+} was also monitored by electrospray mass spectroscopy. In the 1 : 1 mixture of CuSO_4 and TerpyCD₂ in H_2O , the 1 : 1 complex ($\text{Cu}^{\text{II}}\text{TerpyCD}_2$) was observed at m/z 1577 and 1059 (Fig. 2b), which corresponds to $[\text{Cu}^{\text{II}}\text{TerpyCD}_2]^{2+}$ and $[(\text{H}_2\text{O})\text{Cu}^{\text{II}}\text{TerpyCD}_2 + \text{H}]^{3+}$, respectively. The 1 : 2 complex was also detected as a small ion peak when the 1 : 2 mixture of CuSO_4 and TerpyCD₂ in H_2O was analysed by electrospray mass spectroscopy (data not shown).

The $\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex was then titrated with $\text{Fe}^{\text{III}}\text{TPPS}$ (Fig. 3a). The Soret band of $\text{Fe}^{\text{III}}\text{TPPS}$ shifted from 408 nm to 418 nm, indicating that a μ -oxo-dimer of $\text{Fe}^{\text{III}}\text{TPPS}$ dissociated to the monomeric monohydroxo complex ($\text{Fe}^{\text{III}}(\text{OH}^-)\text{TPPS}$)¹⁹ through interaction with $\text{Cu}^{\text{II}}\text{TerpyCD}_2$. The spectral changes were completed upon addition of one equivalent of $\text{Cu}^{\text{II}}\text{TerpyCD}_2$ to $\text{Fe}^{\text{III}}\text{TPPS}$, 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 $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complexes, *i.e.*, $[\text{PFe}^{\text{III}}\text{-O-Cu}^{\text{II}}\text{CD}_2]^{3-}$ and $[\text{PFe}^{\text{III}}\text{-(OH)-Cu}^{\text{II}}\text{CD}_2]^{2-}$, respectively. The assignments were confirmed by isotope pattern simulations (Fig. 3b inset). Evidence of the μ -oxo ($\text{Fe}^{\text{III}}\text{-O-Cu}^{\text{II}}$)

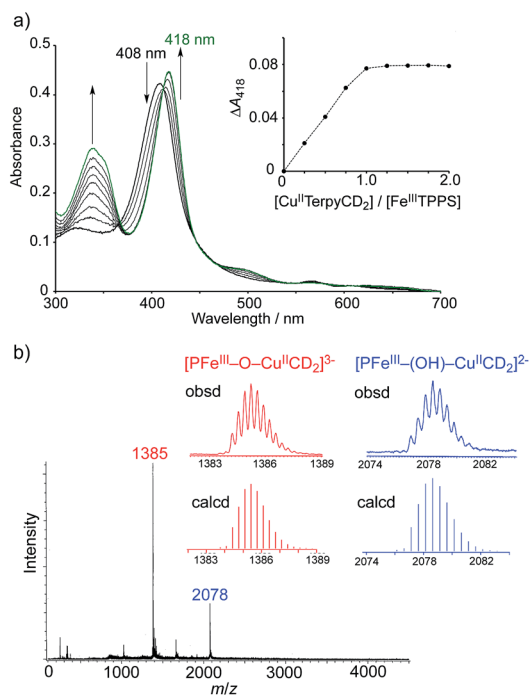


Fig. 3 Characterisation of the supramolecular $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex. (a) UV-vis spectral changes of $\text{Fe}^{\text{III}}\text{TPPS}$ ($3 \mu\text{M}$) upon stepwise addition of $\text{Cu}^{\text{II}}\text{TerpyCD}_2$ in 0.05 M phosphate buffer at pH 7.0 and $25 \text{ }^\circ\text{C}$. The inset shows the changes in absorbance at 418 nm as a function of the molar ratio ($[\text{Cu}^{\text{II}}\text{TerpyCD}_2]/[\text{Fe}^{\text{III}}\text{TPPS}]$). (b) Electrospray mass spectrum (negative mode) of the 1 : 1 mixture of $\text{Fe}^{\text{III}}\text{TPPS}$ and $\text{Cu}^{\text{II}}\text{TerpyCD}_2$ in H_2O . The inset shows the simulated isotope distribution patterns for the μ -oxo- and μ -hydroxo-bridged $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complexes.

structure was also provided by its characteristic absorption bands at 453 and 567 nm, which appeared when the pH of the solution was increased (Fig. S3†). The red-shifted Soret band at alkaline conditions indicates formation of the $\text{PFe}^{\text{III}}\text{-O-Cu}^{\text{II}}$ μ -oxo complex.^{26–28} The pH titration revealed the acid-base equilibrium of $[\text{PFe}^{\text{III}}\text{-O-Cu}^{\text{II}}\text{CD}_2]^{3-}$ and $[\text{PFe}^{\text{III}}\text{-(OH)-Cu}^{\text{II}}\text{CD}_2]^{2-}$ with $\text{pK}_a = 8.8$. This pK_a value is consistent with that previously predicted by Karlin and Blackburn ($\text{pK}_a = 8 \pm 2.5$).²⁸ The electron paramagnetic resonance (EPR) spectra showed significantly weak signals at $g = 6.09$ and 2.08 in the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex (Fig. S4†) because of the antiferromagnetic coupling between the two metal ions as a result of their close proximity. The optimized molecular structure (Fig. 4) also illustrates the proximity of Fe and Cu ions in the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex; the Fe/Cu distances for the non-bridged and oxo-bridged forms are 5.23 and 3.52 \AA , respectively. The distances are similar to those in native CcO , in which the oxidised heme a_3/Cu_B distance were found in the range of $4.4\text{--}4.9 \text{ \AA}$.⁴

Characterisation of an O_2 adduct of the $\text{Fe}^{\text{II}}/\text{Cu}^{\text{I}}$ complex

The $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex was reduced with excess sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) to obtain the fully reduced $[\text{PFe}^{\text{II}}/\text{Cu}^{\text{I}}\text{CD}_2]^{3-}$ complex in the deoxy state in an O_2 -free solution (λ_{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

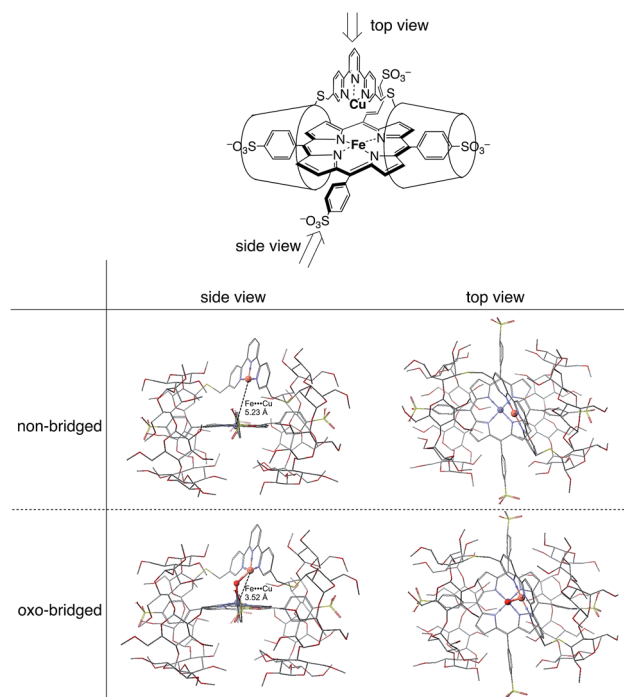


Fig. 4 Optimized molecular structures of the $\text{Fe}/\text{TPPS}/\text{CuTerpyCD}_2$ inclusion complexes in the Fe/Cu non-bridged and Fe/Cu oxo-bridged forms. The models are shown from both side and top views. Hydrogen atoms are omitted for clarity. Molecular mechanics calculations were carried out using CONFLEX/MM3 (extensive search) parameters in Scigress version 2.2.1 software program (Fujitsu).



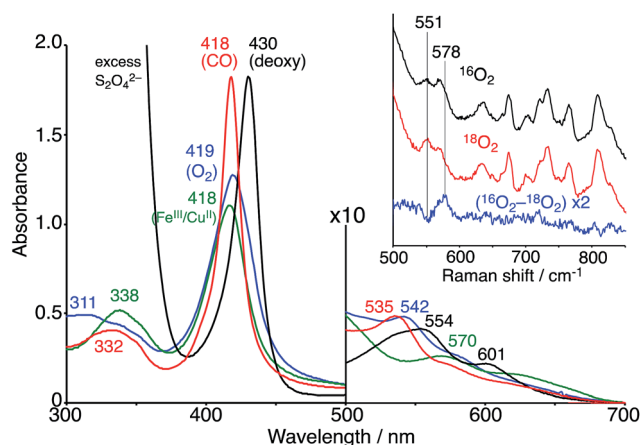


Fig. 5 UV-vis spectra of the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ (green) and its reduced $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ complexes in the deoxy (black), oxy (blue) and CO (red) forms in 0.05 M phosphate buffer at pH 7.0 and 25 °C. The inset shows the resonance Raman spectra of the $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ complexes obtained by excitation at 405 nm under $^{16}\text{O}_2$ atmosphere (black), $^{18}\text{O}_2$ atmosphere (red), and the difference $^{16}\text{O}_2$ - $^{18}\text{O}_2$ (blue). Conditions: 0.05 M phosphate buffer at pH 7.0, 77 K (frozen solution).

enough to reduce both Fe^{III} and Cu^{II} to Fe^{II} and Cu^{I} .^{29,30} After the reduction, the solution was passed through a short gel-filtration column (Sephadex G-25) under aerobic conditions to remove excess $\text{S}_2\text{O}_4^{2-}$ and its oxidised products. The UV-vis spectrum of the resulting solution showed absorption maxima at 419 nm and 542 nm (Fig. 5, blue line); the Q-band was very different from that of the oxidised state ($\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$, λ_{max} (Q-band) = 570 nm, green line) and similar to that of the O_2 complex of the previously reported $\text{Fe}^{\text{II}}\text{TPPS}/\text{CD}$ dimer system.²⁰ 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- $\text{Fe}^{\text{II}}\text{TPPS}$ complex,²⁰ indicating that a ligand exchange from O_2 to CO occurs in this system.

The O_2 complex was further characterized by EPR and resonance Raman (rR) spectroscopic analyses. The EPR spectrum of the O_2 adduct of $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ measured at 77 K was completely silent (Fig. S4†), which was consistent with the spectra of other O_2 complexes of the $\text{PFe}^{\text{II}}/\text{Cu}^{\text{I}}\text{L}_n$ heterobinuclear systems.^{31–33} The rR analysis at 77 K (frozen solution of the O_2 adduct) using 405 nm excitation revealed a characteristic band at 578 cm^{-1} , which shifted to 551 cm^{-1} under an $^{18}\text{O}_2$ atmosphere (Fig. 5 inset). The isotope shift ($\Delta\nu = 27 \text{ cm}^{-1}$) corresponds to the expected value for the $\nu_{\text{Fe-O}}$ stretching mode.¹⁵ The wavenumber is quite similar to those of the $\text{PFe}^{\text{III}}-\text{O}_2^-/\text{Cu}^{\text{I}}\text{L}_n$ 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 ($\nu_{\text{O-O}}$) was not enhanced in this system. This is a relevant observation as the $\nu_{\text{O-O}}$ band is often observed in the range of 750–900 cm^{-1} in the $\text{PFe}^{\text{III}}-\text{O}_2-\text{Cu}^{\text{I}}\text{L}_n$ μ -peroxo complexes, but not in the case of the $\text{Fe}^{\text{III}}-\text{O}_2^-/\text{Cu}^{\text{I}}\text{L}_n$ superoxo complexes (Table 1).^{14,15,35–37} Based on the rR data, the configuration of the present O_2 -adduct of $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ is assigned as the superoxo-type $\text{PFe}^{\text{III}}-$

Table 1 The Fe-O and O-O stretching frequencies ($\nu_{\text{Fe-O}}/\text{cm}^{-1}$, $\nu_{\text{O-O}}/\text{cm}^{-1}$) in the O_2 complexes of native CcO and synthetic PFe/CuL_n compounds

	$\nu_{\text{Fe-O}}/\text{cm}^{-1}$ ($^{18}\text{O}_2$)	$\nu_{\text{O-O}}/\text{cm}^{-1}$ ($^{18}\text{O}_2$)	Medium
Superoxo group			
CcO (beef heart) ^a	572 (548)	—	H_2O , pH 7.4
CcO (bovine heart) ^b	571 (545)	—	H_2O , pH 7.2
$\text{Fe}/\text{Cu}[\text{NMePr}]^c$	570 (544)	—	CH_2Cl_2
FeCuArOH^d	575 (549)	—	DMF
$[(\text{L}^{\text{N}4\text{-OH}})\text{Cu}/\text{Fe}(\text{TMPIm})]^e$	574 (548)	—	$\text{CH}_3\text{CN}/\text{THF}$
$\text{FeTPPS}/\text{CuTerpyCD}_2^f$	578 (551)	—	H_2O , pH 7.0
μ-Peroxo group			
LS-4DCHIm ^g	585, 591 (564)	876, 863 (820)	MeTHF
$[\text{L}^{\text{OH}}\text{Fe}/\text{Cu}]^h$	—	799 (752)	$\text{CH}_3\text{CN}/\text{toluene}$
$[(\text{L}^{\text{N}4\text{-OH}})\text{Cu}/\text{Fe}(\text{TMPIm})]^e$	611 (584)	787, 803 (751)	$\text{CH}_3\text{CN}/\text{THF}$

^a Ref. 34. ^b Ref. 6. ^c Ref. 14. ^d Ref. 35. ^e Ref. 15. ^f This work. ^g Ref. 37. ^h Ref. 36.

$\text{O}_2^-/\text{Cu}^{\text{I}}\text{L}_3$ complex (Fig. 6), which is the same coordination mode as in compound A of native CcO.^{3,14,38}

The superoxo $\text{PFe}^{\text{III}}-\text{O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ 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 $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex (Fig. 5). EPR spectral changes also support oxidation of the superoxo $\text{PFe}^{\text{III}}-\text{O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ species to the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex (Fig. S4†). The first-order rate constants (k_{obs}) 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/ $\log k_{\text{obs}}$ 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 O_2 reduction catalysed by their PFe/CuL_n model complex is pH-dependent and increases at lower pH.⁴⁰ We have previously reported that the autoxidation rate of the O_2 complex in the $\text{PFe}^{\text{II}}/\text{CD}$ dimer system without any distal functions is independent of pH in the neutral pH region (7–10), whereas it is accelerated at

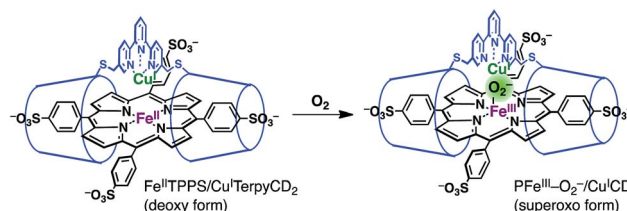


Fig. 6 Oxygenation of the $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ complex to form a superoxo $\text{PFe}^{\text{III}}-\text{O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ complex.



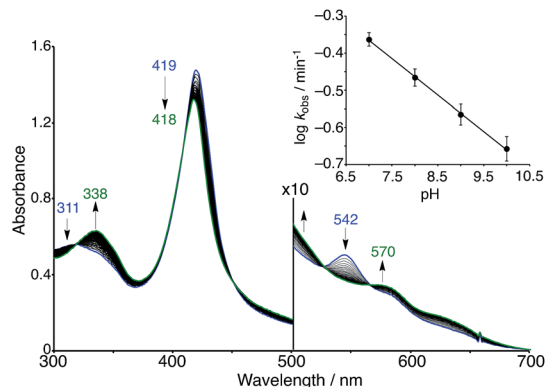


Fig. 7 Spontaneous conversion of the superoxo ($\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$) complex in 0.05 M phosphate buffer at pH 7.0 and 25 °C. The spectrum was recorded at 15 s intervals. The inset shows the logarithmic first-order rate constants (k_{obs}) for the conversion as a function of the pH of the solution.

pH below 6 and above 10.²⁴ 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 $\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ complex to the oxidised $\text{PFe}^{\text{III}}\text{-(OH)-Cu}^{\text{II}}\text{CD}_2$ complex.

The quantum chemical study on native Cco^8 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 $\text{Fe}^{\text{III}}\text{-OOH}$ intermediate that has not been experimentally detected. Thus, the involvement of a water molecule in the present $\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ 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 methoxy groups of the CD dimer are suitable to provide two hydrogen bonding sites to the water. The pH-dependent decomposition of the superoxo complex, as shown in Fig. 7, might be explained by the acid–base equilibrium of the water molecule (Fig. 8b), where the proton-donation to the superoxo complex is likely to induce the O–O bond cleavage as proposed in Cco^8 and/or the proton-assisted autoxidation reaction similar to myoglobin.^{41,42}

The O_2 binding in the present complex was practically irreversible; the O_2 complex of $\text{Fe}^{\text{II}}\text{TPPS}/\text{Cu}^{\text{I}}\text{TerpyCD}_2$ was never converted to its $\text{Fe}^{\text{II}}/\text{Cu}^{\text{I}}$ deoxy complex, even when the O_2 complex once formed was dissolved in a deoxygenated buffer (Fig. S5†). In contrast, the deoxy complex was observed in the $\text{Fe}^{\text{II}}\text{TPPS}/\text{TerpyCD}_2$ complex without copper under the same experimental conditions.⁴³ This result indicates that the O_2 bound to PFe^{II} is tightly held by the distal $\text{Cu}^{\text{I}}\text{L}_3$ complex, as previously demonstrated by the Fe/Cu superoxo complex.¹⁴ The tight O_2 binding was also confirmed by observing ligand exchange with CO. The ligand exchange occurred slowly over ~30 min when the Fe/Cu superoxo complex was dissolved in a CO saturated buffer (Fig. S5†), whereas it occurred instantaneously in the absence of distal Cu complex or in the absence of O_2 (Fig. S5†). The ligand exchange of O_2 with CO also rapidly occurs in the previous $\text{Fe}^{\text{II}}\text{TPPS}/\text{CD}$ dimer systems.^{20,24} The

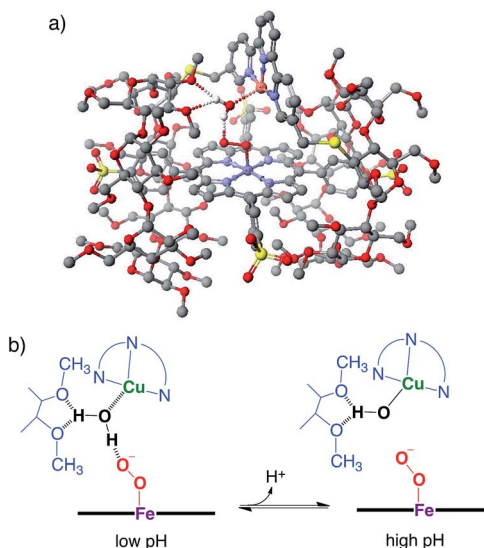


Fig. 8 The superoxo $\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ complex with a water molecule. (a) The molecular model constructed using CONFLEX/MM3 (extensive search) parameters in Scigress version 2.2.1 software program (Fujitsu). Hydrogen atoms, except for water, are omitted for clarity. (b) The possible acid–base equilibrium of the water, where the proton-donation to the superoxo $\text{PFe}^{\text{III}}\text{-O}_2^-$ moiety is likely to occur at low pH.

significantly slow ligand exchange of $\text{PFe}^{\text{II}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{L}_3$ with CO caused by distal Cu complex might be related to the lower CO/ O_2 affinity ratio of native Cco (0.1) in comparison to that of myoglobin (20–50) or haemoglobin (200–250).⁴⁴

Electrochemical analysis for the O_2 reduction

To evaluate the Cco -like function of this system, we monitored the electrocatalytic O_2 reduction reaction.^{45–47} The cyclic voltammogram (CV) of the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ 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_n hetero-binuclear systems; the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ and $\text{Cu}^{\text{II}}/\text{Cu}^{\text{I}}$ redox waves appear at the same potentials.^{31,46} In an air-saturated buffer, the CV of the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex showed a large catalytic current below -0.25 V because of O_2 reduction (Fig. 9a, blue line). A comparison of the CVs of the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex with those of the reference samples, *i.e.*, $\text{Fe}^{\text{III}}\text{TPPS}$ and $\text{Fe}^{\text{III}}\text{TPPS}/\text{TerpyCD}_2$ (Fig. 9b), clearly indicates the effect of the Fe/Cu hetero-binuclear structure in the O_2 reduction; the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ complex showed a very large catalytic current starting from a lower onset potential ($\Delta E_{\text{onset}} = -40$ mV). The O_2 reduction process was then studied by linear sweep voltammetry (LSV) using a rotating disk electrode (RDE, Fig. 9c). The LSVs of the $\text{Fe}^{\text{III}}\text{TPPS}/\text{Cu}^{\text{II}}\text{TerpyCD}_2$ and $\text{Fe}^{\text{III}}\text{TPPS}/\text{TerpyCD}_2$ complexes showed diffusion limited catalytic O_2 -reduction currents below -1.0 V vs. Ag/AgCl . In the case of FeTTPS without the CD dimer, the current was never saturated in LSV due to a slow reaction rate of the iron porphyrin with O_2



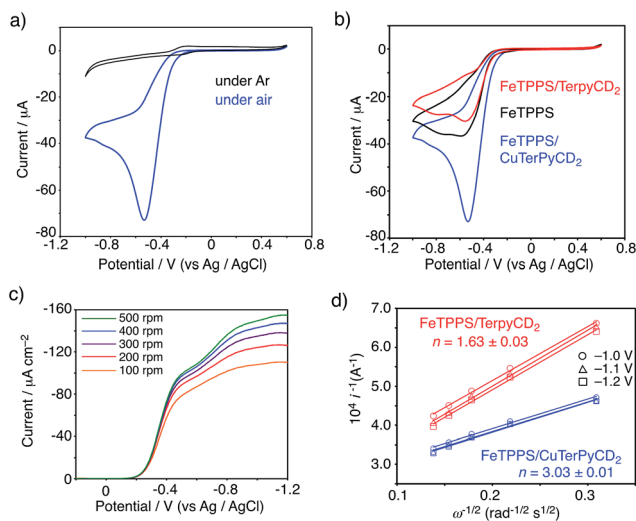


Fig. 9 (a, b) CV of the FeTPPS/CuTerpyCD₂ complex and its reference samples absorbed on the glassy carbon electrode with Nafion (5 wt% dispersion, 10 μL) in pH 7 phosphate buffer at a scan rate of 0.1 V s⁻¹ using Ag/AgCl and Pt wire as the reference counter electrodes, respectively. (c) LSV data for the FeTPPS/CuTerpyCD₂ complex (10 nmol) coated with Nafion (5 wt% dispersion, 10 μL) on a glassy carbon electrode in air saturated pH 7.0 phosphate buffer at a scan rate of 10 mV s⁻¹ at multiple rotations using Ag/AgCl and a Pt wire as the reference and counter electrodes, respectively. (d) Koutecky–Levich plots for the FeTPPS/CuTerpyCD₂ and FeTPPS/TerpyCD₂ complexes at the potentials of -1.0, -1.1 and -1.2 V to determine the average number of electrons (*n*) used for the O₂ reduction reaction.

on the disk electrode (Fig. S6†). The saturated currents observed in the Fe^{III}TPPS/Cu^{II}TerpyCD₂ and Fe^{III}TPPS/TerpyCD₂ complexes at various rotation rates were analysed using the Koutecky–Levich equation to determine the average number of electrons (*n*) used in the O₂ reduction (Fig. 9d).⁴⁸ A significant increase in the *n* value was observed for the Fe/Cu hetero-binuclear complex (*n* = 3.03 ± 0.01) compared to the control sample without copper (*n* = 1.63 ± 0.03).⁴⁹ Therefore, we conclude that the terpyridyl Cu complex associated with FeTPPS in our model system facilitates the catalytic O₂ reduction as an electron source, as proposed in the mechanism of native CcO³ and as proven using the synthetic model systems.^{5,45,48}

Conclusions

In conclusion, we have synthesized a water-soluble biomimetic model complex for the heme a₃/Cu_B hetero-binuclear active centre of CcO by utilizing a supramolecular complexation, and characterised its reactivity with O₂. To the best of our knowledge, this is the first example of a totally synthetic CcO model that works in a completely aqueous solution. In common with compound A of native CcO, we have identified the PFe^{III}-O₂⁻/Cu^ICD₂ superoxo complex as the O₂ adduct in our model system in aqueous solution, whereas the PFe^{III}-O₂-Cu^{II}L_n μ-peroxo complexes tend to form in the other synthetic model systems in anhydrous organic solvents. The pH-dependent conversion of the PFe^{III}-O₂⁻/Cu^ICD₂ superoxo complex to its oxidised μ-hydroxo PFe^{III}-(OH)-Cu^{II}CD₂ complex suggested the

involvement of water molecules in the formation of the superoxo 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 CcO 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 rR 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” AI\1.1 4.JTIC.OOO2.O 1.

Notes and references

- G. T. Babcock, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 12971–12973.
- S. Ferguson-Miller and G. T. Babcock, *Chem. Rev.*, 1996, **96**, 2889–2907.
- S. Yoshizawa and A. Shimada, *Chem. Rev.*, 2015, **115**, 1936–1989.
- E. Kim, E. E. Chufán, K. Kamaraj and K. D. Karlin, *Chem. Rev.*, 2004, **104**, 1077–1133.
- J. P. Collman, R. Boulatov, C. J. Sunderland and L. Fu, *Chem. Rev.*, 2004, **104**, 561–588.
- T. Ogura, S. Hirota, D. A. Proshlyakov, K. Shinzawa-Itoh, S. Yoshikawa and T. Kitagawa, *J. Am. Chem. Soc.*, 1996, **118**, 5443–5449.
- K. Muramoto, K. Ohta, K. Shinzawa-Itoh, K. Kanda, M. Taniguchi, H. Nabekura, E. Yamashita, T. Tsukihara and S. Yoshikawa, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 7740–7745.
- M. R. A. Blomberg, P. E. M. Siegbahn, G. T. Babcock and M. Wilkström, *J. Am. Chem. Soc.*, 2000, **122**, 12848–12858.
- E. Kim, J. Shearer, S. Lu, P. Moënne-Loccoz, M. E. Helton, S. Kaderli, A. D. Zuberbühler and K. D. Karlin, *J. Am. Chem. Soc.*, 2004, **126**, 12716–12717.
- T. Chishiro, Y. Shimazaki, F. Tani, Y. Tachi, Y. Naruta, S. Karasawa, S. Hayami and Y. Maeda, *Angew. Chem., Int. Ed.*, 2003, **42**, 2788–2791.
- E. E. Chufán, S. C. Puiu and K. D. Karlin, *Acc. Chem. Res.*, 2007, **40**, 563–572.
- S. Hematian, I. Garcia-Bosch and K. D. Karlin, *Acc. Chem. Res.*, 2015, **48**, 2462–2474.
- A. W. Schaefer, M. T. Kieber-Emmons, S. M. Adam, K. D. Karlin and E. I. Solomon, *J. Am. Chem. Soc.*, 2017, **139**, 7958–7973.



- 14 J. P. Collman, C. J. Sunderland, K. E. Berg, M. A. Vance and E. I. Solomon, *J. Am. Chem. Soc.*, 2003, **125**, 6648–6649.
- 15 J.-G. Liu, Y. Naruta and F. Tani, *Angew. Chem., Int. Ed.*, 2005, **44**, 1836–1840.
- 16 M. Ralle, M. L. Verkhovskaya, J. E. Morgan, M. I. Verkhovsky, M. Wilkström and N. J. Blackburn, *Biochemistry*, 1999, **38**, 7185–7194.
- 17 J. A. Sigman, B. C. Kwok and Y. Lu, *J. Am. Chem. Soc.*, 2000, **122**, 8192–8196.
- 18 A. Bhagi-Damodaran, M. A. Michael, Q. Zhu, J. Reed, B. A. Sandoval, E. N. Mirts, S. Chakraborty, P. Moënnelocoz, Y. Zhang and Y. Lu, *Nat. Chem.*, 2017, **9**, 257–263.
- 19 K. Kano, H. Kitagishi, S. Tamura and A. Yamada, *J. Am. Chem. Soc.*, 2004, **126**, 15202–15210.
- 20 K. Kano, H. Kitagishi, M. Kodera and S. Hirota, *Angew. Chem., Int. Ed.*, 2005, **44**, 435–438.
- 21 H. Kitagishi, M. Tamaki, T. Ueda, S. Hirota, T. Ohta, Y. Naruta and K. Kano, *J. Am. Chem. Soc.*, 2010, **132**, 16730–16732.
- 22 K. Watanabe, H. Kitagishi and K. Kano, *Angew. Chem., Int. Ed.*, 2013, **52**, 6894–6897.
- 23 H. Kitagishi, S. Kurosawa and K. Kano, *Chem.-Asian J.*, 2016, **11**, 3213–3219.
- 24 K. Kano, H. Kitagishi, C. Dagallier, M. Kodera, T. Matsuo, T. Hayashi, Y. Hisaeda and S. Hirota, *Inorg. Chem.*, 2006, **45**, 4448–4460.
- 25 V. M. Manikandamathavan, V. Rajapandian, A. J. Freddy, T. Weyhermüller, V. Subramanian and B. U. Nair, *Eur. J. Med. Chem.*, 2012, **57**, 449–458.
- 26 M.-A. Kopf, Y.-M. Neuhold, A. D. Zuberbühler and K. D. Karlin, *Inorg. Chem.*, 1999, **38**, 3093–3102.
- 27 E. Kim, M. E. Helton, I. M. Wasser, K. D. Karlin, S. Lu, H.-w. Huang, P. Moënnelocoz, C. D. Incarvito, A. L. Rheingold, M. Honecker, S. Kaderli and A. D. Zuberbühler, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 3623–3628.
- 28 S. Fox, A. Nanthakumar, M. Wilkström, K. D. Karlin and N. J. Blackburn, *J. Am. Chem. Soc.*, 1996, **118**, 24–34.
- 29 S. G. Mayhew, *Eur. J. Biochem.*, 1978, **85**, 535–547.
- 30 B. Liu, Y. Chen, T. Doukov, S. M. Soltis, C. D. Stout and J. A. Fee, *Biochemistry*, 2009, **48**, 820–826.
- 31 Z. Halime, H. Kotani, Y. Li, S. Fukuzumi and K. D. Karlin, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13990–13994.
- 32 P. Vorburger, M. Lo, S. Choua, M. Bernard, F. Melin, N. Oueslati, C. Boudon, M. Elhabiri, J. A. Wytko, P. Hellwig and J. Weiss, *Inorg. Chim. Acta*, 2017, **468**, 232–238.
- 33 C. Kahlfuss, J. A. Wytko and J. Weiss, *ChemPlusChem*, 2017, **4**, 584–594.
- 34 C. Varotsis, W. H. Woodruff and G. T. Babcock, *J. Biol. Chem.*, 1990, **265**, 11131–11136.
- 35 J. P. Collman, R. A. Decréau, Y. Yan, J. Yoon and E. I. Solomon, *J. Am. Chem. Soc.*, 2007, **129**, 5794–5795.
- 36 J.-G. Liu, Y. Naruta, F. Tani, T. Chishiro and Y. Tachi, *Chem. Commun.*, 2004, 120–121.
- 37 S. M. Adam, I. Garcia-Bosch, A. W. Schaefer, S. K. Sharma, M. A. Siegler, E. I. Solomon and K. D. Karlin, *J. Am. Chem. Soc.*, 2017, **139**, 472–481.
- 38 The $\nu_{\text{Fe-O}}$ stretching frequency of 578 cm^{-1} is higher than the reported values for the penta-coordinated $\text{PFe}^{\text{III}}\text{-O}_2^-$ complexes, indicating that the Lewis acidity of the distal Cu complex significantly alters the Fe–O bonding. For the $\nu_{\text{Fe-O}}$ data of $\text{PFe}^{\text{III}}\text{-O}_2^-$ complexes, see: K. M. Vogel, P. M. Kozłowski, 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 $\text{pH}/\log k_{\text{obs}}$ plot would be -1.0 .
- 40 J. P. Collman, S. Ghosh, A. Dey, R. A. Decréau and Y. Yang, *J. Am. Chem. Soc.*, 2009, **131**, 5034–5035.
- 41 K. Shikama, *Chem. Rev.*, 1998, **98**, 1357–1373.
- 42 In the present system, any intermediate species ascribed to ferrylxo ($\text{Fe}^{\text{IV}}=\text{O}$) complex could not be observed during conversion from the superoxo to the oxidised complexes in aqueous solution at room temperature.
- 43 The O_2 complex was also formed in the $\text{Fe}^{\text{II}}\text{TTPPS}/\text{TerpyCD}_2$ complex without copper. The UV-vis spectrum of this complex ($\lambda_{\text{max}} = 419$ and 542 nm) was found to be consistent with that of the $\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ superoxo complex, which further supports the absence of a direct interaction between the distal Cu^{I} and the bound O_2 in the $\text{PFe}^{\text{III}}\text{-O}_2^-/\text{Cu}^{\text{I}}\text{CD}_2$ complex.
- 44 R. Motterlini and R. Foresti, *Am. J. Physiol.: Cell Physiol.*, 2017, **312**, C302–C313.
- 45 J. P. Collman, N. K. Devaraj, R. A. Decréau, Y. Yang, Y.-L. Yan, W. Ebina, T. A. Eberspacher and C. E. D. Chidsey, *Science*, 2007, **315**, 1565–1568.
- 46 R. Boulatov, J. P. Collman, I. M. Shiryayeva and C. J. Sunderland, *J. Am. Chem. Soc.*, 2002, **124**, 11923–11935.
- 47 F. Melin, A. Trivella, M. Lo, C. Ruzié, I. Hijazi, N. Oueslati, J. A. Wytko, B. Boitrel, C. Boudon, P. Hellwig and J. Weiss, *J. Inorg. Biochem.*, 2012, **108**, 196–202.
- 48 S. Chatterjee, K. Sengupta, S. Hematian, K. D. Karlin and A. Dey, *J. Am. Chem. Soc.*, 2015, **137**, 12897–12905.
- 49 The selectivity of the electrocatalytic O_2 reduction by the $\text{FeTTPPS}/\text{CuTerpyCD}_2$ 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.

