Single-turnover intermolecular reaction between a Fe^{III}–superoxide–Cu^{I} cytochrome *c* oxidase model and exogeneous Tyr244 mimics†

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An Fe^{III}–superoxide–Cu^{I} cytochrome *c* oxidase model reacts intermolecularly with hindered phenols leading to phenoxyl radicals, as was observed in the enzyme and evidence for the formation of an Fe^{IV}–oxo is presented.

Cytochrome *c* oxidase (C*c*O) is a respiratory enzyme that catalyzes the reduction of O$_2$ to H$_2$O without releasing highly-toxic, partially reduced oxygen species (PROS).[1] To carry out the 4e$^-$ reduction, a fully reduced enzyme has 6e$^-$ available stored in five redox active centers: heme *a*, Cu$_A$, heme a$_3$, Cu$_B$ and a tyrosine Tyr244.[1] The catalytic cycle begins when dioxygen binds to the reduced C*c*O Fe$_{a3}^{II}$–Cu^{I} active site to form a heme–superoxide complex with no oxidation of Cu^{I} (Fe$_{a3}^{III}$O$_2^{•-}$–Cu^{I}).[2] Regardless of the sequence, two more electrons are provided, one from Cu$_B^{I}$, leading to Cu$_B^{II}$, and another one from heme a$_3$, leading to iron^{IV}=O, respectively. However there is still controversy about the role of the tyrosine residue, illustrated by two different mechanisms proposed in the literature.[2] In the fully reduced mechanism, the 4th electron and a proton come from heme *a*–Cu$_A$ and proton transduction occurs across the membrane, leaving the tyrosine unreacted. In contrast, in the mixed-valence mechanism, the tyrosine provides both the proton and the 4th electron, leading to the resonance Raman and EPR signatures of the so-called **P$_M$** intermediate: a ferryloxo, a copper(II) and a tyrosyl radical.[2] The present study is designed to demonstrate spectroscopically the validity of this scenario by showing that soluble hindered phenols such as **2–H/D** can act as an efficient source of a proton and an electron when reacting intermolecularly with a dioxygen complex of C*c*O model **1** (Fig. 1).
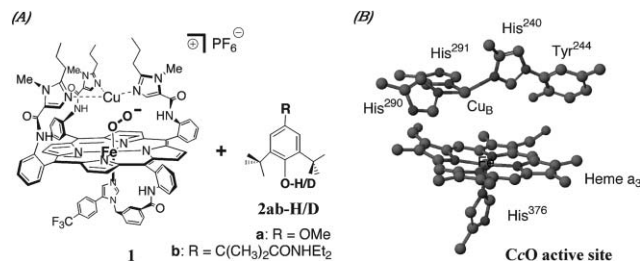
We previously reported **1** to be a true biomimetic C*c*O model: (a) not only it does reproduce the structural heme a$_3$–Cu$_B$ motif (Fig. 1),[3a] (b) but also it carries out reduction of O$_2$ to H$_2$O,[3b] and (c) it binds O$_2$ in an Fe^{III}–superoxide–Cu^{I} fashion[3c,d] exactly as the enzyme,[2] with Cu^{I} not binding to O$_2$, and not being oxidized.[3e]

The reaction of EPR-silent **1**[3c] with 3–12 equiv. of the phenols **2–H** in dichloromethane under careful exclusion of O$_2$ leads to two equiv. of phenoxyl radical **2**$^{•}$ which displays an intense EPR signal at *g* = 2.0082 (Fig. 2).[4a] The yield of **2**$^{•}$ was determined by comparing the integration of the EPR signal to a standard solution of this phenoxyl radical generated by the reaction of the phenol with K$_3$Fe^{III}CN$_6$ and quantified by iodometric titration.[4b,c]† Both phenols **2a/b–H** were chosen based on the stability of their phenoxyl radicals **2**$^{•}$ resulting in a slow decay of the EPR signals.[4a–c]

The kinetics of this reaction studied by EPR, best fit in a pseudo first-order rate law (Fig. 3A–C); the pseudo-first-order constant (k_{obs}) is proportional to the concentration of **2** (Fig. 3C). The second-order rate constant k_2 obtained from the slope was found to be 0.012 M^{-1} min^{-1} for the reaction with **2a–H**. This kinetic behavior demonstrates that the reaction between **1** and **2** is a bimolecular process. The rate law is: d[**2**$^{•}$]/d*t* = k_2[**2**][**1**] with k_{obs} = k_2[**2**].

A small kinetic isotope effect of k_H/k_D = 2 was observed from the reaction between **1** and **2–D**.† This result implies proton

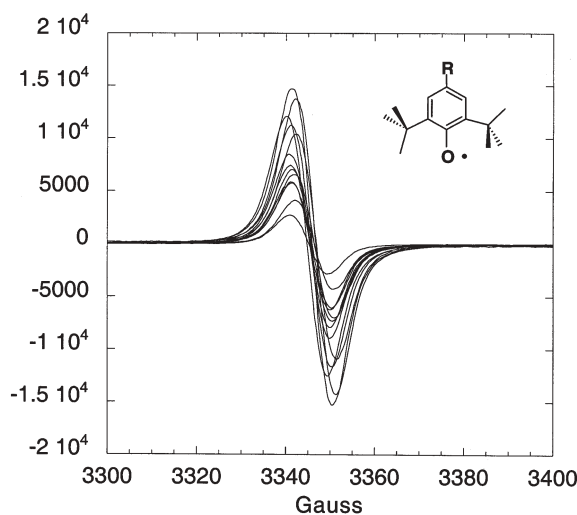**Fig. 1** (A) Proposed intermolecular reaction between a Fe^{III}–superoxide–Cu^{I} C*c*O complex model **1** and phenols (**2–H/D**). Details of this reaction are shown in Scheme 1. (B) Structural comparison of **1** with the active site of C*c*O.

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† Electronic supplementary information (ESI) available: Experimental procedures, MS and UV spectra. See DOI: 10.1039/b607277a

Fig. 2 Spectroscopic evidence for a biomimetic reaction between an Fe^{III}–superoxide–Cu^{I} C*c*O model (**1**) and phenols **2–H** (3–12 equiv.) leading to the phenoxyl radicals **2**$^{•}$: X-band EPR spectrum of phenoxyl radical **2a**$^{•}$ (*g* = 2.0082).†
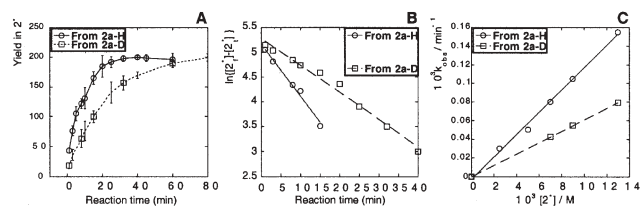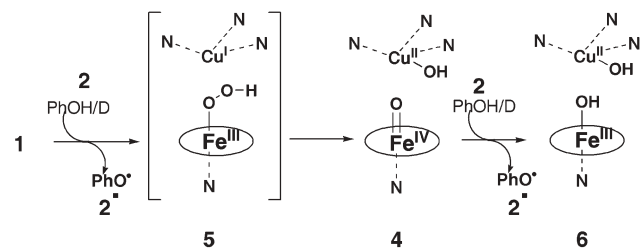
Fig. 3 Intermolecular reaction between **1** and an excess of **2–H/D** in CH$_2$Cl$_2$ at room temperature. A. Concentration of **2˙** at different times. Each dot is the mean of two sets of duplicates. B. Pseudo-first-order plots based on the change of concentration of **2˙**: [**2˙**]$_f$ final concentration of **2˙**, [**2˙**]$_t$ concentration of **2˙** at time t. C. Plots of k_{obs} against [**2˙**].

Fig. 4 Ferryloxo models **7a** and **7b**.

transfer occurs in the rate-determining step from the phenol **2–H** to the O$_2$-bound heme **1**, consistent with formation of a hydroperoxo species **5**. Compound **5** has never been observed spectroscopically in the enzyme, but was previously postulated based on DFT calculations (Scheme 1).[5] The rate of the reaction measured with **2b–H** is three times slower than for **2a–H**. This result indicates that the weaker O–H bond strength of the *p*-methoxy phenol **2a–H** (bond dissociation energy (BDE) *ca.* 3 kcal mol^{-1} smaller) is more reactive than *p*-alkylamide phenol **2b–H** which is more acidic (by *ca.* 1 pK_a unit). The latter is closer to the values found for the pK_a of the cross-linked histidine–tyrosine models (6.6–8.4).[4d–f]

The rate of this reaction is also influenced by the polarity of the solvent: the reaction is 4 times slower in a non-polar dichloromethane–toluene mixture (1 : 2 vol.) than in a polar dichloromethane–acetonitrile mixture (1 : 2 vol.) that is more relevant to physiological conditions (dielectric constants ($\varepsilon_{25^\circ C}$) for: water: 78.5; acetonitrile: 36.6; dichloromethane: 9.1; toluene: 2.3).[6] The expected intramolecular reaction of a hydroperoxo–Cu^I species **5** leads to rupture of the O–O bond and subsequent formation of **4**. This intramolecular reaction is expected to be faster than another intermolecular reaction between **5** and **2–H**, especially when the concentration of **2–H** is low. The reduction of the ferryloxo species **4** by one molecule of phenol **2–H** is reminiscent of the reactivity of the peroxidase non radical compound-II species with phenols.[7] Such a reaction would lead to a ferric-hydroxo species **6** and is favored thermodynamically. Evidence of such a reaction is given by the formation of **2˙** from the reaction of **2–H** with *in situ* generated ferryloxo porphyrin species such as model **7a** and ferryloxo tetrakismesitylporphyrin (TMP) **7b**[8a–c] (Fig. 4). The ferryloxo **7a** was synthesized at *ca.* −60 °C (acetonitrile–acetone–dry ice bath) by adaptation of a method described for simple porphyrins,[8d] oxygen-atom transfer from a soluble iodosylbenzene (1-iodosyl-2-(2-methyl-propane-2-sulfonyl)-benzene)[8e] to a deoxy-Fe-only precursor form of **1**. It was
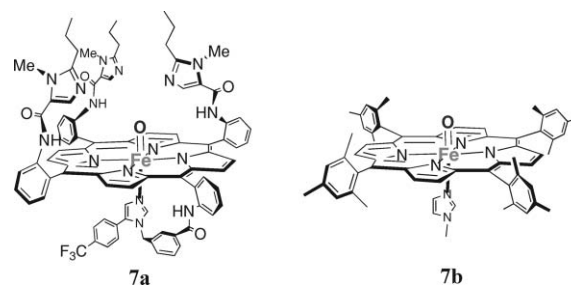
prepared with a slight deficit of iodosylbenzene to ensure that **2** would primarily react with **7a**. The ferryloxo nature of **7a** was established using methods previously reported for other ferryloxo porphyrins lacking superstructures:[8a–c,f] (a) spectroscopic investigations carried out at low temperature showed dramatic ^{1}H-NMR paramagnetic upfield shifts, and 4–10 nm shifts for the Q bands in the absorption spectrum, (b) the reaction of **7a/b** with triphenylphosphine at low temperature led to triphenylphosphine oxide, whereas reaction with ammonium hydroxide led to a species (presumably an Fe^{III}-only version of **6**) that no longer oxidizes triphenylphosphine.

The yield of the phenoxyl radical implies that 2 electrons are abstracted from the phenol pool. It is expected that the Cu would be oxidized to Cu^{II} under these conditions accounting for the additional electron. The reaction product **6** is presumably magnetically coupled, as no Cu^{II} signal was observed in the EPR spectrum of the mixture at the end of the reaction.

Primary evidence for the formation of an oxoferryl species **4** comes from the formation of triphenylphosphine oxide (tppo) following treatment of the mixture containing the putative species **4** with triphenylphosphine (tpp, after *ca.* 1 equiv. of **2˙** was formed). The mixture was analyzed by TLC and GC against authentic standards.† High yields of tppo were obtained at −40 °C or even at room temperature, although greater stability of the ferryloxo species is expected at low temperature.[8a–d,f,g] This result is unambiguous evidence of an oxygen-atom transfer from **4** to triphenylphosphine.[8f] A series of careful control reactions conducted at room temperature between **1** and triphenylphosphine showed that no tppo is formed. Overall the yield in tppo is satisfactory, given the possible competition between this intermolecular oxidation reaction and another intermolecular reduction of **4** by **2–H** as well as the low stability expected for **4** at room temperature.

Further evidence for the formation of an oxoferryl species **4** comes from mass spectrometry.† A nanospray infusion technique using a low ionization potential at room temperature was employed to analyze a cold mixture resulting from the reaction between **1** and **2–H** leading to **4** (−40 to −78 °C, by injection from a pre-cooled syringe). The spectrum was carefully examined taking into account the charges of the species resulting from the loss of the PF$_6$ counterion or the hydroxyl Cu^{II} species. The major peak had an isotope distribution of peaks centered at m/z = 1661.43. This pattern is consistent with the theoretical spectrum of a singly charged potassium chloride adduct of a Fe^{IV}=O/Cu form of **4**, that may result from the loss of HO from Cu followed by Cl insertion during ionization.

Scheme 1 Intermolecular reaction between Fe^{III}–superoxide–Cu^I (**1**) and phenols (**2–H/D**). Proposed mechanism and intermediates formed.
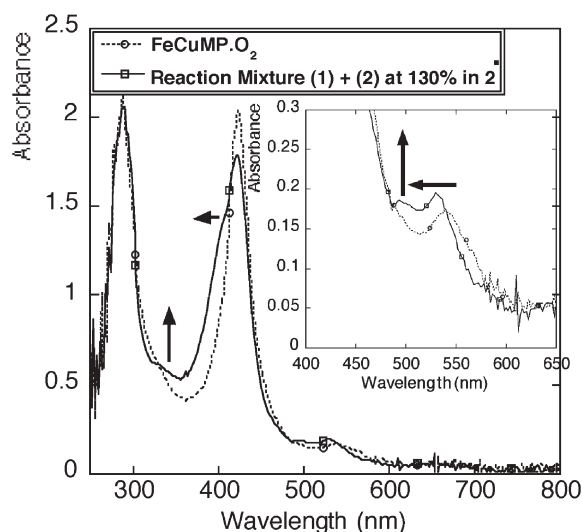
Fig. 5 UV-Vis absorption of Fe^{III}–superoxide–Cu^I (**1**) before reaction with phenol **2a** (dotted line) and after (dark line). Inset: magnification of the Q bands.

The intermolecular reaction between **1** and **2–H** was also monitored by UV-vis spectroscopy (Fig. 5) and analyzed in light of the absorption spectra of **1**[3c] and of an authentic phenoxyl radical **2·** generated *in situ*.† Of the four bands characteristic of phenoxyl radical **2·**(beside the absorption of the parent molecule **2–H** at 300 nm), only the most intense absorption band that gradually appears at 339 nm ($\varepsilon = 3.8 \times 10^3$ M^{-1} cm^{-1}) could be used to correlate the UV-vis data with the kinetic data from EPR. The absorptions at 388 nm and 405 nm were barely visible at the end of the reaction as shoulders on the Soret band. The broad band centered at 540 nm in **2·** and typical of phenoxyl radicals was too weak ($\varepsilon = 120$ M^{-1} cm^{-1}) to be distinguished from the porphyrin Q bands. Moreover, significant shifts in the Q bands were observed during the reaction.

This study demonstrates that **1**, an iron^{III}–superoxo–copper^I biomimetic model of the **oxy** intermediate observed in CcO,[2] reacts with an exogenous soluble Tyr244 mimic **2** to generate a phenoxyl radical **2·** as occurs in the enzyme.[2] However unlike the tyrosyl radical in the enzyme, the phenoxyl radical formed (**2·**) is well resolved and does not couple nor interfere with the neighboring Cu^{II} species. Strong evidence is provided regarding the formation of an hydroperoxo-type intermediate **5** and of a heme ferryloxo product **4**. Thorough mechanistic studies of this intermolecular reaction and complete characterization of the ferryloxo species **4** and **7a** (resonance Raman) are in progress and should pave the way to future studies of a similar intramolecular reaction in more advanced CcO models having a covalently attached phenol.[9]

Notes and references

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