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Intramolecular Single-Turnover Reaction in a Cytochrome *c* Oxidase Model bearing a Tyr244 Mimic

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In the terminal step of respiration, cytochrome c oxidase (C*c*O) carries out the 4e- reduction of dioxygen to water.[1] This reaction is coupled to the ATP synthesis, the main energy storage source in the body. In healthy organisms C*c*O performs without releasing toxic partially reduced oxygen species.[1] Three electrons involved in the reduction originate from the $Fe^{II}a_3$/Cu^{I} active site. The fourth electron and a proton come either from a tyrosine-244 (mixed valence enzyme) or from FeA/CuA (fully reduced enzyme, with proton translocation across the membrane) leading to an oxoferryl-cupric-tyrosyl radical intermediate (P_M) or oxoferryl-cupric intermediate (P_R), respectively.[2a–f] We previously reported a stable Fe^{III}-superoxide-Cu^{I} C*c*O model[3a] that reacts intermolecularly with exogeneous Tyr244 mimics leading to phenoxyl radicals and an oxoferryl-cupric species, mimicking the P_M intermediate.[3b] Based on the crystal structure of the enzyme,[4ab] we have constructed an $Fe^{II}Cu^{I}$ CcO model **1** (Figure 1) that faithfully reproduces the structural heme a_3-Cu_B motif with a built-in histidine-tyrosine cross link.[5a–c] The present study is designed to explore the validity of the mixed-valence scenario by showing that **1** having all the three redox centers present in the enzyme active site, can first react with O_2 to form ***oxy*-1** that subsequently reacts intramolecularly to give spectroscopic features that are associated with the $\mathbf{P_M}$ intermediate (species **2**, Scheme 1).

Oxygenation of **1** at −60C° leads to *oxy*-**1**, a stable species that has the features of a Fe^{III}-superoxide-Cu^{I}.[3b,7a–c] This intermediate is EPR silent, and resonance Raman spectroscopy showed an oxygen isotope sensitive band at 575/549 cm^{-1} ($^{16}O_2/^{18}O_2$) characteristic of a heme-superoxide (*Oxy*) species (Figure 2A).[3b,7a–c] Moreover slight modification of the UV/Vis spectrum is noticed upon formation of *oxy*-**1**.

Upon warming to −40C°, the Fe-O_2 stretching mode decays while intermediate species *oxy*-**1** undergoes a subsequent intramolecular redox process similar to that which is thought to take place in CcO. In this process leading to species **2** (scheme 1), the distal Cu^{I} group becomes oxidized to an aquo or hydroxo Cu^{II} complex as the O-O bond is heterolytically ruptured; the Fe^{III} is further oxidized to an Fe^{IV} oxoferryl. In the same reaction sequence the phenol is oxidized to a phenoxyl radical. During the process, proton transfer is thought to occur leading to an hydroperoxo intermediate postulated from DFT calculations.[8]

First indication of the oxoferryl-cupric-phenoxyl radical nature of **2** is given by spectrophotometric studies[6] with growing absorptions at 580–620 nm as was shown in C*c*O for the P_M state (610 nm) and the $F^{\bullet}$ state (575 nm).[2ef] Nanospray and electrospray mass spectrometry analyses[3b] indicate the formation of **2** with a peak at m/z = 1613.2871 matching the simulated spectrum of a potassium chloride adduct of compound **2**.[6] An increase of 2 amu is observed when **1** is reacted with isotopic $^{18}O_2$. Evidence for the formation of the oxoferryl nature of **2** was also established by an oxygen-atom transfer reaction with

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triphenylphosphine[9] leading in high yield to triphenylphosphine oxide.[6,9] Previous studies have shown that such a reaction does not occur with ***oxy*-1**-like species.[3b]

The radical nature of **2** is evidenced by EPR spectroscopy, which we examined in light of the controversy about the EPR spectrum of the P_M intermediate.[1] Early studies performed on the enzyme did not show any EPR-signal for the Cu(II) in a P_M-type oxidized enzyme.[2a] The unpaired electrons of the tyrosyl radical (S=½) and of Cu_B (II) (S=½) are expected to be spin-coupled (with possible delocalization of spin density onto the imidazole) resulting in an overall silent EPR spectrum for the P_M intermediate. But subsequent studies have reported an EPR-active intermediate with a Cu EPR signal that is distorted by the neighboring oxoferryl paramagnet (S=1).[2b–e,j] Another paper invoked a three-electron oxidized enzyme in a oxoferryl/cupric P_R intermediate where the phenol is not oxidized,[2j] although another study using iodide labeling and protein peptide analysis suggested that a tyrosine radical was formed.[2i] Also, a P_M intermediate generated artificially by treating the enzyme with hydrogen peroxide revealed partial uncoupling for the CuB/Tyr244 system and the presence of a tyrosine radical but the Cu(II) signal was not assigned to Cu_B.[2f–h] In addition, upon photolysis of the oxidized enzyme, a radical signal presumably from Tyr244, and a Cu(II) signal were detected.[2k]

The EPR spectrum of our complex **2** has features reminiscent of a free-base porphyrin cross-linked imidazole-phenoxyl radical, such as a broad signal with shoulders at 3366G and 3445G. It is significantly different than that of a tyrosyl radical[2f–h,j] or that of an analogous CcO model bearing zinc in the porphyrin and Cu(II) in the distal site.[5b] Broad features at 2800–3000G in our spectrum are reminiscent of the one observed by Karlsson or Blair in the enzyme.[2b,c,e] The signal of **2** was observed upon warming ***oxy*-1** to −40°C and was recorded at an early stage because of the high reactivity of **2** as reported earlier on similar species.[3b] Low temperature, high power experiments did not reveal a signal underlying the observed one at g~2.[6] Our spectroscopic data suggest a paramagnetic Cu(II)/cross-linked imidazole-phenoxyl radical/ oxoferryl species as depicted in **2**, that might represent a model of the P_M intermediate. But because of **2**'s complex spin system, possible contributions from several species, and disagreements in the literature, we regard this interpretation of our EPR spectrum to be very tentative; empirical comparisons with reports of the enzyme are dangerous. In future work we plan to clarify this by studying models that contain diverse pairs of the paramagnetic species.

This single-turnover model study shows that phenol behaves as a H^+/e^- donor involved in the O-O bond cleavage. It validates a scenario in which the enzyme operates in the mixed valence state, and supports the existence of a Tyr244 radical in the enzyme.[10] Model **1** is a good mimic of the CcO active site to lead to a P_M intermediate. Model **1** is also a better structural mimic of the enzyme active site than any other models reported to date[5d–h] because it contains all three redox centers with the right Fe/Cu distance and a proximal imidazole. When the redox state of **1** is changed to a mixed valence Fe^{II}/Cu^{II} species, reaction with O_2 does not lead to **2** although Resonance Raman shows that O_2 binding still occurs. Moreover other studies with an analogous version of **1** immobilized on SAM electrode, have shown that the tyrosine mimic is crucial to severely limit the release of PROS during steady state turnover under a rate limiting electron flux.[11]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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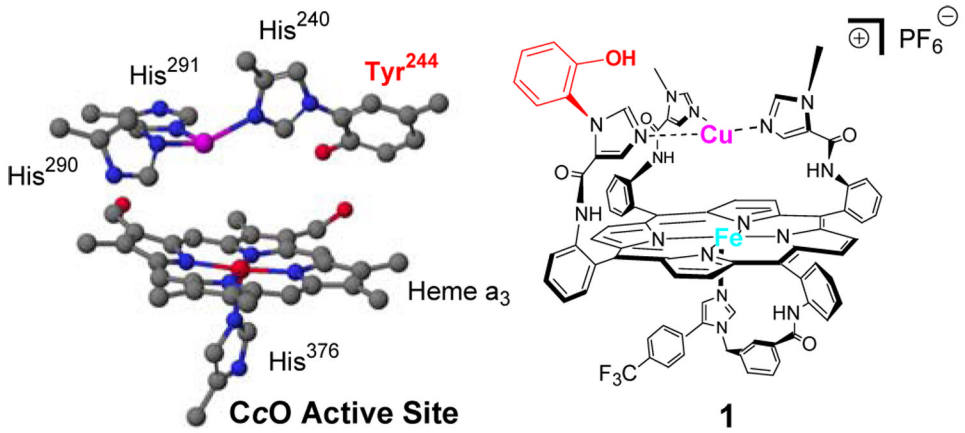


Figure 1.
(Left) Heme a_3/Cu_B of bovine cytochrome c oxidase. (Right) Chemical Structure of **1**.

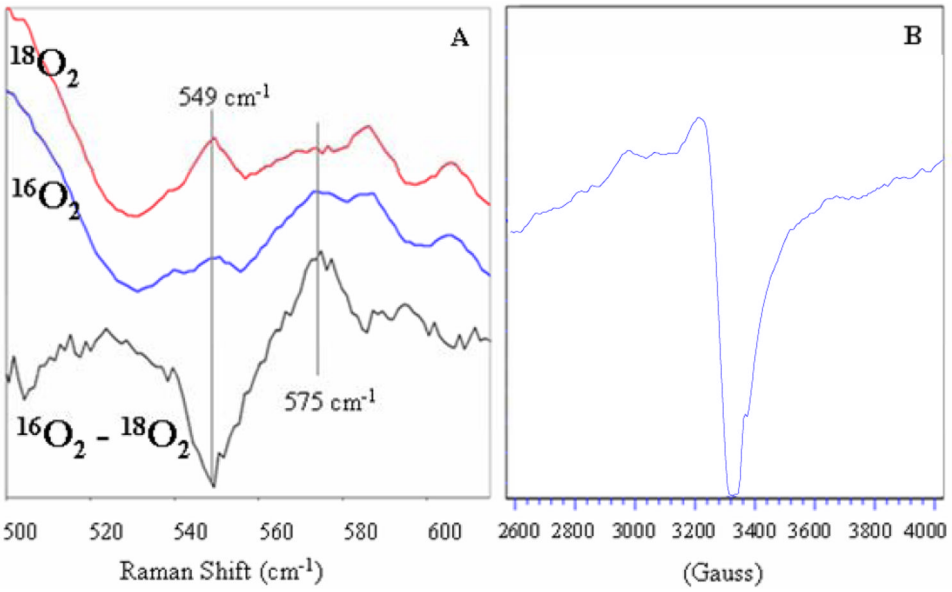
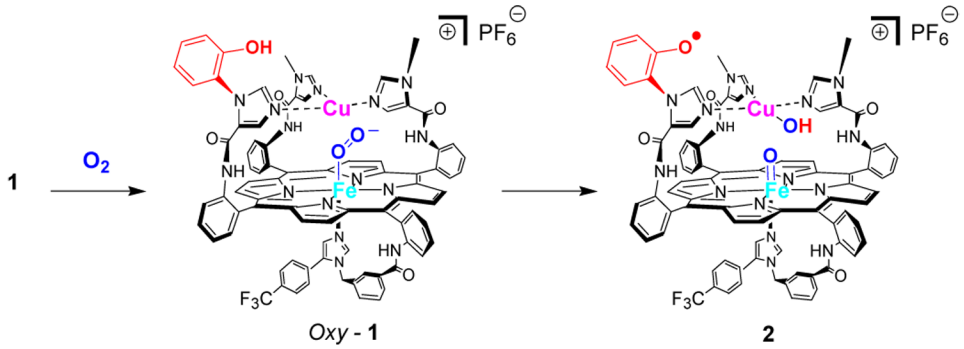


Figure 2.

(A) Evidence of an Fe(III)-superoxo-Cu(I) species *oxy*-**1** formed by reaction of **1** with dioxygen: Resonance Raman (77K, DMF) of *oxy*-**1**-$^{18}\mathbf{O_2}$, *oxy*-**1**-$^{16}\mathbf{O_2}$, and the difference spectrum. (B) X-band EPR spectrum (77K in DMF) obtained upon warming up *oxy*-**1** at −40° C.



SCHEME 1.

Single turnover intramolecular reaction of 1 with dioxygen leading to *oxy*-1 at −60°C, and oxoferryl-cupric-tyrosyl radical mimic species 2 upon warming at −40°C.