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

James P. Collman,* Richard A. Decréau, Yilong Yan, Jungjoo Yoon, and Edward I. Solomon

Department of Chemistry, Stanford University, Stanford, California 94305-5080

Received December 19, 2006; E-mail: jpc@stanford.edu

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] On the basis of the crystal structure of the enzyme,[4ab] we have constructed an Fe^{II}Cu^I C*c*O 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 P_M intermediate (species **2**, Scheme 1).

Oxygenation of **1** at −60 C° 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 −40 °C, 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 C*c*O. 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$^•$ 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 triphenylphosphine[9] leading in high yield

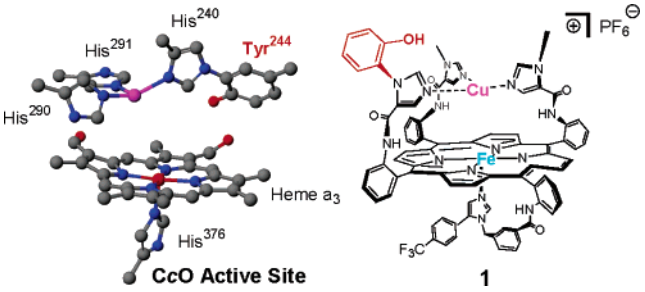


Figure 1. (left) Heme a_3/Cu$_B$ of bovine cytochrome *c* oxidase; (right) chemical structure of **1**.

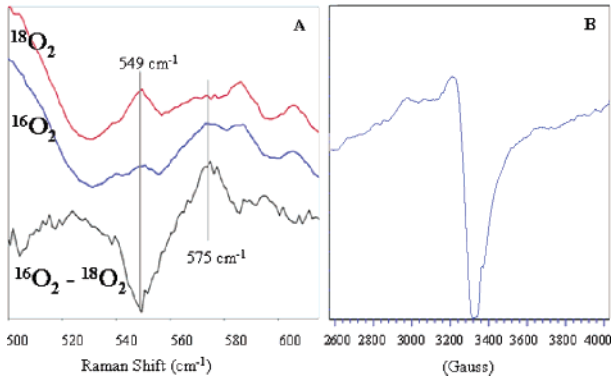
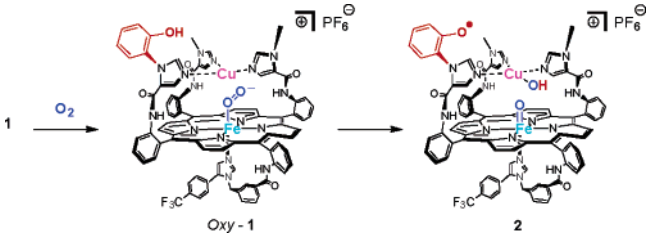


Figure 2. (A) Evidence of an Fe(III)-superoxo-Cu(I) species *oxy*-**1** formed by reaction of **1** with dioxygen: resonance Raman (77 K, DMF) of *oxy*-**1**-$^{18}O_2$, *oxy*-**1**-$^{16}O_2$, and the difference spectrum. (B) X-band EPR spectrum (77 K in DMF) obtained upon warming up *oxy*-**1** at −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.



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 = $^1/_2$) and of Cu$_B$ (II) (S = $^1/_2$) 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

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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 3366 and 3445 G. It is significantly different than that of a tyrosyl radical[2f−h,l] or that of an analogous C*c*O model bearing zinc in the porphyrin and Cu(II) in the distal site.[5b] Broad features at 2800−3000 G 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 $g \approx 2$.[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 the complex spin system of **2**, 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 C*c*O 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]

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Supporting Information Available: Further experimental data including procedures for the preparation of **1**, *oxy*-**1**, and **2**, and also NMR, MS, resonance Raman, and UV−vis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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