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Catalytic Reduction of O_2 by Cytochrome *c* Using a Synthetic Model of Cytochrome *c* Oxidase

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Cytochrome *c* oxidase (C*c*O) performs a four-electron reduction of oxygen to water in the last step of respiration.[1] The active site in C*c*O consists of a heme/Cu site having a post-translationally modified tyrosine residue covalently bound to one of the histidine ligands of the distal Cu (Figure 1A).[2] Cytochrome *c* (Cyt*c*), a small electron-transfer protein, is the source of electrons for C*c*O. Cyt*c* has a coordinatively saturated low-spin heme active site (Figure 1B).[1] Mimicking of the structure and function of C*c*O using synthetic model complexes has attracted significant attention over the last two decades.[3−5] Recently, a model of C*c*O that incorporates a heme with a covalently attached proximal imidazole, a trisimidazole distal binding pocket for Cu, and a covalently modified tyrosine (Y244) analogue has been reported (FeCuPhOH, Figure 1C).[6] This functional model catalyzes the selective four-electron reduction of oxygen at physiological pH using an electrode as the source of electrons and generates negligible (<4%) partially reduced oxygen species (PROS) during catalytic turnover.[7] Herein we report the selective catalytic four-electron reduction of oxygen by the biological one-electron reductant Cyt*c* (from horse heart) using this functional C*c*O model in a homogeneous mixed solvent system.

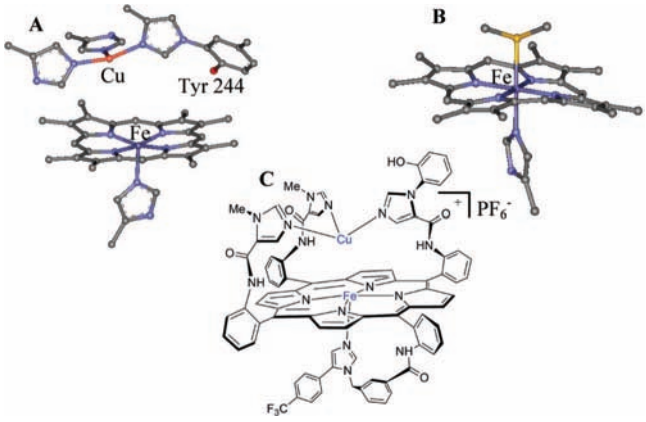

Figure 1. Active-site structures of (A) C*c*O, (B) Cyt*c*, and (C) FeCuPhOH, a synthetic model of C*c*O.

This homogeneous reaction was monitored by following the oxidation of reduced Cyt*c* by O_2 in the presence of 2% FeCuPhOH catalyst. Figure 2 shows the kinetic traces. These data show a decrease in the percentage of reduced Cyt*c* at a pseudo-first-order rate of 1.3×10^{-3} s^{-1}, which is much greater than that for the slow auto-oxidation of reduced Cyt*c* ($\sim 1 \times 10^{-5}$ s^{-1}).[8] Monitoring of the O_2 concentration of the solution before and after the reaction showed that 3.9 ± 0.1 equiv of reduced Cyt*c* is oxidized per equivalent of O_2 consumed, indicating that this oxidation process is *stoichiometric* within experimental error. The rate of catalysis (k_{obs}/[catalyst]) was pH-dependent, decreasing from $(3.9 \pm 0.2) \times 10^3$ M^{-1} s^{-1} at pH 6−7 to $(1.8 \pm 0.1) \times 10^3$ M^{-1} s^{-1} at pH 8 [Figure S1A in the Supporting Information (SI)], indicating that protonation may be rate-determining at high pH.

Because of limitations in the solubility of Cyt*c* under these experimental conditions, only 25 turnovers (i.e., 1 equiv of catalyst oxidized 100 equiv of Cyt*c*) could be obtained.[9] The turnover number for this catalyst was determined by studying its electrocatalytic O_2 reduction. The catalyst modified with an alkyne linker was "clicked" onto a $C_{16}SH$ thiol in a self-assembled monolayer (SAM; see the SI for details). The SAM limits the rate of electron transfer from the electrode to the catalyst to 4 s^{-1}.[7] Electrolysis of this covalently bound catalyst at physiological potentials gave a turnover number of $(9 \pm 1) \times 10^3$.

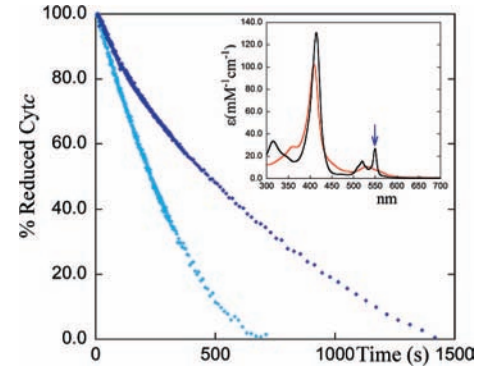

Figure 2. Kinetic traces showing the decrease of reduced Cyt*c* (following the 550 nm band intensity) in the presence of 2% FeCuPhOH by aerated (dark-blue) and O_2-saturated (light-blue) 1:1 aqueous buffer/acetonitrile solvent at pH 7 and 25 °C. Inset: absorption spectra of reduced (black) and oxidized (red) Cyt*c*. The blue arrow indicates the 550 nm band.

Reduction of O_2 using reduced Cyt*c* with the catalyst was studied under various conditions with the aim of elucidating the mechanism and identifying the rate-determining step (rds) during catalytic turnover. The proposed mechanism for the four-electron reduction of O_2 by Cyt*c* is presented in Scheme 1. In the first step, O_2 binds to the reduced catalyst, forming an Fe^{III}−superoxo species (Scheme 1, step A). Since the catalyst contains four electrons (two from Fe^{II}, one from Cu^{I}, and one from phenol), the next step leads to the formation of a "P_M" intermediate comprising of an oxidized Cu^{II}, an Fe^{IV}═O ferryl radical, and a phenoxide radical (Scheme 1, step B).[6b] This oxidized intermediate is then reduced by 4 equiv of Cyt*c*, regenerating the fully reduced active catalyst (Scheme 1, steps C and D). The rds could involve (a) O_2 binding, (b) O−O bond cleavage, or (c) electron transfer from reduced Cyt*c* to the oxidized catalyst.

The turnover rate increased by more than a factor of 2 when an O_2-saturated solution was used instead of an air-saturated solution (Figure 2). This indicates that O_2 binding may be the rds. The catalytic reaction showed a modest inverse deuterium isotope effect (k_H/k_D) of 0.82. Also, there was no change in the rate of O_2 reduction when the concentration of reduced Cyt*c* was varied at constant catalyst concentration (Figure S1B in the SI). This indicates that electron transfer from reduced Cyt*c* is rapid and is not involved in the rds during

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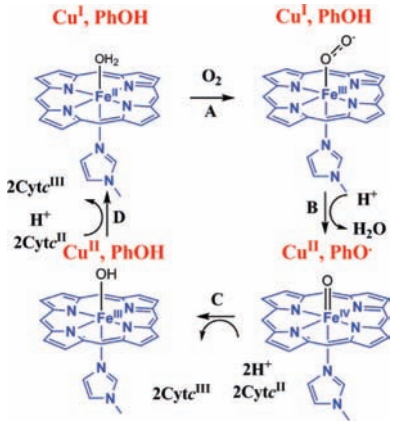
catalytic turnover. Single turnover kinetics experiments were performed with the FeCuPhOH catalyst and the "Fe-only" complex (without Cu and phenol) to obtain the corresponding rates of O_2 binding and electron transfer.

The Fe-only complex acts as a control to measure the rate of O_2 binding to the catalyst (Scheme 1, step A), as this complex lacks the necessary reducing equivalents for the O−O bond cleavage (Scheme 1, step B). The reaction of O_2 with the reduced Fe^{II} complex was monitored by following the characteristic Fe^{II} absorption at 434 nm (Figure 3); this showed an O_2 binding rate of 0.5 s^{-1} to form a ferric superoxide species, followed by its slow hydrolysis with a rate of 0.05 s^{-1} (Figure S3). Parallel monitoring of the Fe^{II} state of the FeCuPhOH catalyst at 434 nm showed monophasic O_2 binding with a rate of 0.1 s^{-1} (Figure 3). EPR and UV−vis spectra of the reaction product at 40 s indicated that it is identical to the chemically produced $Fe^{III}Cu^{II}$ species (Figures S3 and S4 in the SI). Since the FeCuPhOH catalyst reduces O_2 stoichiometrically (see above) and the amount of the hydrolysis side reaction is negligible (<4% PROS),[7] the monophasic kinetics of the O_2 reaction (i.e., no intermediates observed) implies that rates of O−O bond cleavage (Scheme 1, step B) and decay of the high-valent intermediate (Scheme 1, step C) must be much greater than 0.1 s^{-1}. The rate of O_2 binding to the fully reduced FeCuPhOH catalyst is less than that to the Fe-only complex, possibly because of greater steric hindrance in the former due to the phenol substituent. It should be noted that the rates of O_2 binding (Scheme 1, step A) for these complexes are much less that those reported for C*c*O and other O_2-binding heme proteins and model complexes.[10−12] We have recently shown that this slow O_2 binding is due to the presence of an axial water ligand that H-bonds to additional H_2O molecules in the distal pocket, making the ferrous catalyst low-spin in nature (Figure S5 in the SI), in contrast to the high-spin five-coordinate ferrous active site of the enzyme.[1,13] The small inverse kinetic isotope effect observed during catalytic turnover is consistent with displacement of a bound water in the rds.[14]

Scheme 1. Possible Mechanism of O_2 Reduction by Cyt*c* in the Presence of the FeCuPhOH C*c*O Model Complex



The second-order rate constants (k^{second}) for electron transfer between reduced Cyt*c* and the oxidized $Fe^{III}Cu^{II}$PhOH catalyst and Fe^{III}-only complex (Scheme 1, step D) under anaerobic conditions were independently estimated to be $(4 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (Figure S6). This translates to a pseudo-first-order rate constant ($=k^{second}[Cytc^{2+}]$) of ~1.2 s^{-1} under catalytic turnover. The rate of O_2 binding (0.1 s^{-1}) is at least 10 times less than the rate of electron transfer from reduced Cyt*c* to the $Fe^{III}Cu^{II}$PhOH catalyst. The rates of reduction of the ferryl and tyrosyl radical species (Scheme 1, step C) are arguably greater than the rate of reduction of $Fe^{III}Cu^{II}$ (Scheme 1, step D), as they have higher driving forces for electron transfer. Comparison of these rates indicates that O_2 binding is the rds during catalytic turnover at physiological pH, whereas both the O−O bond cleavage and electron transfer steps are relatively fast.

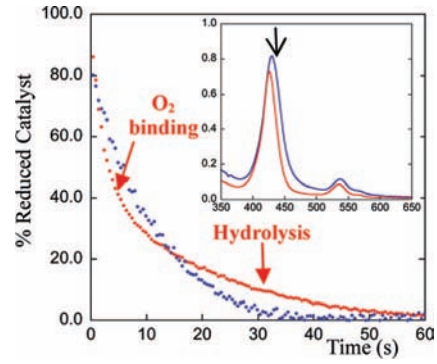


Figure 3. Kinetic traces showing the decrease of 434 nm absorption intensity of the Fe-only complex (red) and the FeCuPhOH catalyst (blue) in the presence of O_2. Inset: absorption spectra of the reduced Fe-only (red) and FeCuPhOH (blue) complexes. The black arrow indicates the 434 nm band.

In summary, we have demonstrated that our functional model of C*c*O can catalyze the selective four-electron reduction of O_2 using the biological reductant Cyt*c*. The rate-determining step in the catalytic cycle is O_2 binding to the catalyst. The rate of O−O bond cleavage is $\gg 0.1$ s^{-1}. The oxidized products are rapidly reduced back to the active form by reduced Cyt*c*. We believe this is the first report of kinetically inert O_2 being reduced to H_2O by Cyt*c* using a synthetic functional model as a catalyst.

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Supporting Information Available: Experimental details; plots showing pH dependence, Cyt*c* concentration dependence, and turnover number; UV−vis, EPR, and resonance Raman spectra; and an electron transfer rate plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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