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Catalytic Reduction of O₂ by Cytochrome c Using a Synthetic Model of Cytochrome c Oxidase

James P. Collman^{*}, Somdatta Ghosh, Abhishek Dey, Richard A. Decréau, and Ying Yang

Department of Chemistry, Stanford University, Stanford, CA 94305, USA.

Abstract

Cytochrome *c* oxidase (CcO) catalyzes the four-electron reduction of oxygen to water, the one-electron reductant Cytochrome *c* (Cyt_c) being the source of electrons. Recently we reported a functional model of CcO that electrochemically catalyzes the four-electron reduction of O₂ to H₂O (Collman *et al. Science*, **2007**, 315, 1565). The current paper shows that the same functional CcO model will catalyze the four-electron reduction of O₂ using the actual biological reductant Cyt_c in a homogeneous solution. Both single and steady-state turnover kinetic studies indicate that O₂ binding is rate determining and O-O bond cleavage and electron transfer from reduced Cyt_c to the oxidized model complex are relatively fast.

Cytochrome *c* oxidase (CcO) performs a four electron reduction of oxygen to water, in the last step of respiration.¹ The active site in CcO is comprised of a heme/Cu site having a post translationally modified tyrosine residue covalently bound to one of the histidine ligands of the distal Cu (Fig. 1A).² Cytochrome *c* (Cyt_c), a small electron transfer protein, is the source of electrons for CcO. Cyt_c has a co-ordinatively saturated low-spin heme active site (Fig. 1B).¹ Mimicking the structure and function of CcO using synthetic model complexes has attracted significant attention over the last two decades.^{3,4,5} Recently a model of CcO that incorporates a heme with a covalently attached proximal imidazole, a tris-imidazole distal binding pocket for Cu, and a covalently modified tyrosine (Y244) analogue has been reported (FeCuPhOH, Fig. 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 CcO model in a homogeneous mixed solvent system.

This homogeneous reaction was monitored by following the oxidation of reduced Cyt_c by O₂ in the presence of 2% FeCuPhOH catalyst. Fig. 2 shows the kinetic traces. These data show a decrease in the percentage of reduced Cyt_c at a pseudo 1st order rate of $1.3 \times 10^{-3} \text{ s}^{-1}$, which is much faster than the slow auto-oxidation of reduced Cyt_c ($\sim 1 \times 10^{-5} \text{ s}^{-1}$).⁸ Monitoring the O₂ concentration of the solution before and after the reaction shows that 3.9 ± 0.1 equivalents of reduced Cyt_c are oxidized per equivalent of O₂ consumed, indicating that this oxidation process is *stoichiometric* within error. The rate of catalysis ($k_{\text{obs}}/[\text{Catalyst}]$) is pH dependent decreasing from $3.9 \pm 0.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ between pH 6–7 to $1.8 \pm 0.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ at pH 8 (Fig. S1) indicating that protonation may be rate limiting at high pH.

Due to limitations in the solubility of Cyt_c under these experimental conditions only 25 turnovers (i.e. 1 equivalent catalyst:100 equivalents Cyt_c) could be obtained.⁹ The turnover

number for this catalyst has been determined by studying its electrocatalytic O₂ reduction. The catalyst modified with an alkyne linker was “clicked” on to a C₁₆SH thiol in a self assembled monolayer (SAM, details in SI). 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 gives a turnover number of 9 ± 1 × 10³.

Reduction of O₂ using reduced Cyt_c with the catalyst was studied at 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 O₂ by Cyt_c is presented in Scheme 1. In the first step, O₂ binds to the reduced catalyst forming an Fe^{III}-superoxo species (step A, Scheme 1). 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 “P_M” intermediate (step B, Scheme 1) which is comprised of an oxidized Cu^{II}, an Fe^{IV}=O ferryl and a phenoxide radical.^{6b} This oxidized intermediate is then reduced by four equivalents of Cyt_c regenerating the fully reduced active catalyst (steps C and D). The rds could involve (a) O₂ binding, (b) O-O bond cleavage, or (c) electron transfer from reduced Cyt_c to the oxidized catalyst.

The turnover rate increases by more than two fold (Fig. 2) upon using an O₂ saturated solution instead of an air saturated solution. This indicates that O₂ binding may be the rds. The catalytic reaction shows a modest inverse deuterium isotope effect (k_H/k_D) of 0.82. Also there is no change in the rate of O₂ reduction by varying the concentration of reduced Cyt_c with a constant catalyst concentration (Fig. S1). This indicates that electron transfer from reduced Cyt_c is rapid and is not involved in the rds during catalytic turnover. Single turnover kinetic experiments were performed with the FeCuPhOH and the “Fe-only” complex (without Cu and phenol) to obtain the rates of O₂ binding and electron transfer.

The Fe-only complex acts as a control to measure the rate of O₂ binding to the catalyst (step A, Scheme 1), as this complex lacks the necessary reducing equivalents for the O-O bond cleavage (step B, Scheme 1). The reaction of O₂ with the reduced Fe^{II} complex was monitored following the characteristic Fe^{II} absorption at 434 nm, which shows an O₂ binding rate of 0.5 s⁻¹ (Fig. 3) forming a ferric superoxide species, followed by its slow hydrolysis with a rate of 0.05 s⁻¹ (Fig. S3). Parallel monitoring of the Fe^{II} state of the FeCuPhOH catalyst at 434 nm shows a monophasic O₂ binding with a rate constant of 0.1 s⁻¹ (Fig. 3). EPR and UV-Vis spectra of the reaction product at 40s indicate it is identical to the chemically produced Fe^{III}Cu^{II} species (Fig. S3, S4). Since the FeCuPhOH catalyst reduces O₂ stoichiometrically (vide supra) and the amount of hydrolysis side reaction is negligible (PROS <4%)⁷, the monophasic kinetics of the O₂ reaction (i.e. no intermediates observed) implies that the O-O bond cleavage (step B, Scheme 1) and decay of the high valent intermediate (step C, Scheme 1) must be much greater than 0.1 s⁻¹. The rate of O₂ binding to the fully reduced FeCuPhOH catalyst is slower than the Fe-only complex possibly because of greater steric hindrance in the former due to the phenol substituent. Note that the O₂ binding rates (step A, Scheme 1) of these complexes are much slower than those reported for CcO and other O₂ binding heme proteins and model complexes.^{10,11,12} We have recently shown that this slow O₂ binding is due to the presence of an axial water ligand, which H-bonds to additional H₂O molecules in the distal pocket, making the ferrous catalyst low-spin in nature (Fig. S5), 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

The second order rate constants (k^{2nd}) for electron transfer between reduced Cyt_c and oxidized Fe^{III}Cu^{II}PhOH catalyst or the Fe^{III}-only complex (step D, Scheme 1), under anaerobic conditions, were independently estimated to be ~4 ± 0.1 × 10⁴ M⁻¹s⁻¹ (Fig. S6). This translates

to a pseudo first-order rate of $\sim 1.2 \text{ s}^{-1}$ ($k^{2\text{nd}} \times [\text{Cyt}c^{2+}]$) under catalytic turnover. The rate of O_2 binding (0.1 s^{-1}) is at least 10 times slower than the rate of electron transfer from reduced Cyt c to the $\text{Fe}^{\text{III}}\text{Cu}^{\text{II}}\text{PhOH}$ catalyst. The rates of reduction of the ferryl and tyrosyl radical species (step C, Scheme 1) are arguably greater than the rate of reduction of $\text{Fe}^{\text{III}}\text{Cu}^{\text{II}}$ (step D, Scheme 1) 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.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Active site structures of A) CcO B) Cytc and C) synthetic model of CcO (Fe Cu and phenol analogue).

Figure 2.

Kinetic traces of oxidation of the 550 nm band of reduced Cyt c in the presence of 2% FeCuPhOH by aerated (dark blue) and O₂ saturated (light blue) 50:50 aqueous buffer acetonitrile solvent at pH 7 and 25°C. Inset: absorption spectra of reduced (black) and oxidized (red) Cyt c. Blue arrow indicates the 550 nm band.

Figure 3. Kinetic traces showing the decrease of 434 nm absorption intensity of the Fe-only (red) complex and FeCuPhOH (blue) catalyst in the presence of O₂. Inset: absorption spectra of reduced Fe-only (red), and FeCuPhOH (blue) complexes. The black arrow indicates the 434 nm band.

Scheme 1.
Possible mechanism of O_2 reduction by $Cytc$ in the presence of the $FeCuPhOH$ CcO model complex.