Published in final edited form as:

Methods Enzymol. 2009 ; 456: 507–520. doi:10.1016/S0076-6879(08)04428-5.

Use of Ruthenium Photoreduction Techniques to Study Electron Transfer in Cytochrome Oxidase

Lois Geren, Bill Durham, and Francis Millett

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, lgeren@uark.edu, Phone: 479-575-6203, FAX: 479-575-4049

Abstract

Ruthenium photoreduction methods are described to study electron transfer from cytochrome c to cytochrome c oxidase, and within cytochrome oxidase. Methods are described to prepare a ruthenium cytochrome c derivative, Ru-39-Cc, by labeling the single sulfhydryl group on horse K39C with (4-bromomethyl-4'-methylbipyridine) (bis-bipyridine)ruthenium(II). The ruthenium complex attached to Cys-39 on the opposite side of the heme crevice does not interfere with the interaction with cytochrome oxidase. Laser flash photolysis of a 1:1 complex between Ru-39-Cc and bovine cytochrome oxidase results in photoreduction of heme c within 1 μ s, followed by electron transfer from heme c to Cu_A in cytochrome oxidase with a rate constant of 60,000 s⁻¹ and from Cu_A to heme a with a rate constant of 20,000 s⁻¹. A new ruthenium dimer, Ru₂Z, has been developed to reduce Cu_A within 1 μ s with a yield of 60%, followed by electron transfer from Cu_A to heme a and then to the heme a₃/Cu_B binuclear center. Methods are described to measure the single-electron reduction of each of the intermediates involved in reduction of oxygen to water by cytochrome oxidase, including P_m, F, O_H, and E.

1. Introduction

Cytochrome c oxidase (CcO) is the terminal electron transfer complex in the respiratory chains of mitochondria and many prokaryotes (Babcock and Wikstrom, 1992). Electron transfer of four electrons from cytochrome c (Cc) to molecular oxygen is coupled to the uptake of four “chemical” protons to form 2 H₂O, and the translocation of four “pumped” protons across the membrane. Cc initially transfers an electron to Cu_A located in subunit II, followed by electron transfer to heme a and then to the heme a₃/Cu_B binuclear center located in subunit I, where oxygen is reduced to water. The D-channel containing D-132 is thought to be involved in the uptake of both chemical and pumped protons from the matrix side of the membrane, while the K-channel containing K-362 is involved in proton uptake during reduction of the binuclear center (Branden et al., 2006). X-ray crystal structures of bovine (Yoshikawa *et al.*, 1998), *Paracoccus denitrificans* (Ostermeier *et al.*, 1997) and *Rhodocacter sphaeroides* (Qin *et al.*, 2006) CcO have revealed the locations of the redox centers and proton channels in great detail. However, determination of the kinetics of the electron and proton transfer steps and the mechanism of coupling between them remains a challenging problem.

One of the most widely used methods to study rapid electron transfer in CcO involves flash photolysis of CO-bound heme a₃ in reduced CcO, followed by reaction with oxygen (Hill, 1991; Faxen et al., 2005). In another approach, our laboratory has developed a new ruthenium photoreduction technique to inject single electrons into Cc or CcO and measure the subsequent electron transfer reactions on a rapid time scale (Millett and Durham, 2002). To study electron

transfer involving cytochrome c, a polypyridyl ruthenium complex [Ru(II)] is covalently attached to Cys-39 on Cc to form Ru-39-Cc (Geren et al, 1995). Photoexcitation of Ru(II) to the metal-to-ligand charge-transfer state, Ru(II*), a strong reductant, leads to rapid reduction of the ferric heme group in Cc. This new technique has been used to determine that Cc initially transfers an electron into Cu_A of CcO with a rate constant of 60,000 s⁻¹ (Geren et al., 1995). In another approach, several new ruthenium dimers have been developed which bind with high affinity to cytochrome oxidase and can photoreduce Cu_A within 1 μs (Zaslavsky et al., 1998). This new technique has been used to measure the kinetics for electron transfer from Cu_A to heme a and then to the heme a₃/Cu_B binuclear center, as well as coupled proton pumping.

2. Preparation of Ruthenium-labeled Cytochrome c Derivatives

We have developed four different methods for the covalent attachment of a ruthenium complex to a protein (Millett and Durham, 2002). In the method to be described here, a ruthenium bromomethyl reagent is used to label a cysteine sulfhydryl group on the surface of the protein (Geren *et al.*, 1991, 1995). A single free cysteine can be engineered onto the surface of the protein by site-directed mutagenesis to specifically address the goals of the experiment. It is generally desirable to place the ruthenium complex on a surface remote from the binding site with other proteins, so the complex does not interfere with interprotein interactions. It is also important to have a good pathway for electron transfer from the ruthenium complex to the redox center to maximize the rate of electron transfer. Another advantage of this labeling method is that all three chelating ligands can be altered to change the redox potentials of the ruthenium complex, allowing measurement of the reorganization energy, and optimization of the rate and yield of photoinduced electron transfer (Figure 1, Table 1).

2.1 Materials

The yeast cytochrome c mutant H39C,C102T is made as described by Geren *et al.* (1995), and the preparation of horse cytochrome c mutant K39C is described in Patel *et al.* (2001). The synthesis of (4-bromomethyl-4'-methylbipyridine) (bis-bipyridine)ruthenium(II) reagent is described by Geren *et al.*, (1995), with care being taken to maintain dryness and eliminate metal contamination. The bromination method described in that paper has been modified slightly. After reflux in 48 % HBr for 3 hours, the PF₆ precipitation was eliminated due to the product's poor solubility in water. Instead, the excess HBr was allowed to boil off carefully without allowing any overheating of the dried product, with the final drying done under vacuum and low heat. The dry powder was immediately dissolved in very dry DMF and stored frozen under dry N₂. Extreme care is required to maintain dryness and prevent hydrolysis of the bromine group.

2.2. Preparation of Ruthenium-labeled Cytochrome c

The protein labeling technique is similar for all the bromo-methyl ruthenium reagents and is similar to that described in Geren *et al.*, (1995), but has been altered somewhat with experience. The protein to be labeled is first treated with a ten fold excess of dithiothreitol for ten minutes to reduce any cross-linked disulfide bonds. The sample is then washed two times as quickly as possible using concentrators or a P-2 column into 50 mM sodium borate, pH 9.0, to remove any remaining dithiothreitol. Three to five mg of cytochrome c in approximately 300 μl of 50 mM sodium borate, pH 9.0, with a concentration of about 1 mM is best. Raising the pH increases the reactivity but decreases the selectivity for cysteines. A pH of 10 to 10.5 will produce labeled lysines as well as cysteines. After degassing under N₂, a 0.5 equivalent of dithiothreitol is added and stirred 5 minutes. Using a very dry syringe to protect the ruthenium stock solution and continuing to stir, an approximate 3 fold excess (sometimes more, depending upon the reactivity of the complex) of the ruthenium reagent is slowly added. The ruthenium reagent is very quickly hydrolyzed by water and care must be taken to keep the stock dry and

use only syringes which have been rinsed with dry ether or dry DMF. After 5 minutes of stirring the sample is allowed to react overnight (about 18 hours) at room temperature, or 3 hours at 37°C, protected from light. All of the unreacted ruthenium reagent will have hydrolyzed by this time and can then be removed by buffer exchange via dialysis, Amicon concentrators, or Bio-Gel P-2 column. The labeled protein is then purified by ion exchange or HPLC chromatography. The amount of ruthenium reagent used can be adjusted to minimize the amount of unlabeled protein without producing too much multi-labeled material. The reaction may be scaled up or down. If the labeling does not cause any disturbance of the protein structure, the absorbance spectra of the ruthenium complex and the protein should be additive. The spectrum of Ru-39-Cc is the same as that of equimolar Ru(bpy)₂(dmb) and horse Cc. The specific residue on the protein labeled with ruthenium should be confirmed with HPLC separation of a trypsin digest, followed by peptide sequencing as described by Geren *et al.* (1991).

3.0. Measurement of Intraprotein Electron Transfer in Horse Ru-39-Cc

Internal electron transfer between the ruthenium complex and the heme in a ruthenium-labeled protein is measured using laser flash photolysis (Durham et al., 1989). The excitation pulse is the third harmonic of a Nd:YAG laser, with a pulse width of 20 ns and wavelength of 356 nm. The probe source is a pulsed 75 W xenon arc lamp, and the photomultiplier detector has a response time of 10 ns. Photoexcitation of Ru(II) in 10 μM oxidized horse Ru-39-Cc results in electron transfer from Ru(II*) to Fe(III) to form Ru(III) and Fe(II), followed by reverse electron transfer to form Ru(II) and Fe(III) according to Scheme 1 (Figure 2). The reduction of Fe(II) is detected at 550 nm after subtracting a small contribution from Ru(II*) detected at 556 nm, an isobestic for heme c. The photoexcitation and recovery of Ru(II) is detected at 434 nm, a heme c isobestic. Both transients are fit using equations (1) and (2) based on Scheme 1 (Durham et al., 1989).

$$i(\text{Fe}^{\text{II}}) = \text{Absorbance}(550\text{nm} - 556\text{nm}) = A [e^{-(k_1+k_d)t} - e^{-k_2t}] \quad \text{Eq. (1)}$$

$$i(\text{Ru}^{\text{II}}) = \text{Absorbance}(434\text{ nm}) = \Delta\epsilon_{434} C_0 [1 - Be^{-(k_1+k_d)t} + Ce^{-k_2t}] \quad \text{Eq. (2)}$$

where $A = k_1\Delta\epsilon_{550}C_0/(k_2-k_1-k_d)$, $B = (k_2-k_d)/k_2-k_1-k_d$, $C = k_1/(k_2-k_1-k_d)$, $\Delta\epsilon_{550}=18.6\text{ mM}^{-1}$, and $\Delta\epsilon_{434}=12\text{ mM}^{-1}$. The best fit for horse Ru-39-Cc is obtained with $k_1 = (6 \pm 1) \times 10^5\text{ sec}^{-1}$, $k_2 = (7 \pm 1) \times 10^5\text{ sec}^{-1}$, and $k_d = (5.5 \pm 1.0) \times 10^6\text{ sec}^{-1}$ (Figure 2). These rate constants are independent of protein concentration and are similar to the rates obtained for the yeast Ru-39-Cc (Geren *et al.*, 1995). Residue 39 was chosen for attachment of the ruthenium complex because it is on the back side of Cc remote from the binding site with CcO. There is an efficient electron transfer pathway between the ruthenium complex and the heme consisting of 13 covalent bonds and one hydrogen bond with a total distance of 12.6 Å. The metal-to-ligand charge-transfer excited state Ru(II*) is a strong reducing agent, and can rapidly transfer an electron to heme c Fe(III) according the scheme 1. Since the driving forces for k_1 and k_2 of 1.1 and 1.0 eV, respectively, are near the expected reorganizational energy of 0.8 eV, the rate constants k_1 and k_2 should be near the maximum, activationless rate constant (Durham *et al.* 1989).

The sacrificial electron donor aniline can be used to reduce Ru(III) and lead to the photoreduction of the heme in Ru-39-Cc through k_1 and k_6 steps in scheme 1 with a yield of 5%. Aniline does not have a low enough redox potential to directly reduce Ru(II*). Higher

photoreduction yields can be obtained using the Ru(bpd)₂(dmb) complex, which has a redox potential of 0.98 for the Ru(II^{*})/Ru(I) transition, allowing the sacrificial donor DMAB to directly reduce Ru(II^{*}) to Ru(I) (Table 1, Scheme 1). The yeast Ru_D-39-Cc is photoreduced through the k₅ and k₄ steps in Scheme 1 with a yield of 35%. Reduction potentials for a number of useful ruthenium complexes are listed in Table 1.

4.0. Measurement of Electron Transfer from Ru_D-39-Cc to Cytochrome c Oxidase

Intracomplex electron transfer from Ru_D-39-Cc to CcO is studied using laser flash photolysis. A Phase R model DL1400 flash lamp-pumped dye laser emitting a 480 nm light flash of < 0.5 μs duration is used as the excitation source (Geren et al. 1995). The sample contains 5 μM yeast Ru_D-39-Cc and 5 μM bovine CcO in 5 mM sodium phosphate, pH 7.0, 5 mM DMAB, and 0.1% dodecyl maltoside at 25 °C. Under these low ionic strength conditions, Ru_D-39-Cc is tightly bound to CcO, allowing measurement of intracomplex electron transfer according to scheme 2. Excitation of Ru(II) to Ru(II^{*}) with a single laser flash results in rapid photoreduction of heme c, followed by electron transfer to Cu_A and then to heme a. The reaction of heme c is followed at 550 nm, while Cu_A and heme a are followed at 830 nm and 605 nm, respectively (Figure 3). The decrease in 830 nm absorbance indicates electron transfer from heme c to Cu_A with a rate constant of 60,000 s⁻¹. The recovery of the 830 nm signal resulting from the reoxidation of Cu_A is matched by the reduction of heme a seen in the 605 nm transient with a rate of k_b = 2 × 10⁴ sec⁻¹. Both k_a and k_b are independent of protein concentration and ionic strength up to 100 mM, indicating intracomplex electron transfer. Site-directed mutagenesis studies have shown that positively charged lysines surrounding the heme crevice of Cc interact electrostatically with negatively charged carboxylates near Cu_A on subunit II of CcO (Wang et al., 1999). A highly conserved tryptophan residue on subunit II mediates electron transfer from the heme group of Cc to Cu_A. Theoretical calculations of the rate of electron transfer based on a computational model of the Cc:CcO complex are in good agreement with the experimental value of 2 × 10⁴ s⁻¹ (Roberts and Pique, 1999; Wang et al., 1999).

4.1. Measurement of Formation and Dissociation Rate Constants for the Complex between Ru-Cc and CcO

When the ionic strength is increased above 50 mM, the amplitude of the intracomplex phase of electron transfer between Ru-39-Cc and CcO decreases, and a new phase appears due to bimolecular electron transfer between the two proteins according to Scheme 2. At intermediate ionic strengths between 50 and 100 mM, both intracomplex and bimolecular phases are seen in the kinetics, allowing the fraction f of Ru-39-Cc:CcO complex to be determined. Using the total concentrations of CcO (O_T), and Ru-39-cyt c (Cc_T), K_D may be calculated from equation 3.

$$K_D = (1 - f)[(O_T) - f(Cc_T)]/f \quad \text{Eq. (3)}$$

The dissociation constant K_d is given by:

$$K_D = k_{\text{diss}}/k_{\text{form}} \quad \text{Eq. (4)}$$

Since the rate constant k_a is independent of ionic strength up to 100 mM, the presence of two phases indicates the absence of rapid equilibrium, and k_{diss} must be much smaller than k_a. Using steady state assumptions, the bimolecular reaction is given by equation 5:

$$k_{2nd} = k_{form} k_a / (k_{diss} + k_a) \quad \text{Eq. (5)}$$

Measurement of k_{2nd} and k_a , along with the calculation of K_D thus also allows the calculation of k_{diss} and k_{form} (Geren *et al.*, 1995). For beef oxidase and yeast Ru-39-C c at 100 mM ionic strength these were found to be $k_a = 6 \times 10^4 \text{ s}^{-1}$, $k_{form} = 1.8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{diss} = 8 \times 10^3 \text{ s}^{-1}$ (Scheme 2).

5.0 Measurement of Electron Transfer and Oxygen Reduction in Cytochrome c Oxidase using Electrostatically Bound Ruthenium Complexes

A number of positively charged ruthenium complexes have been developed that bind to CcO and directly photoreduce Cu_A (Figure 1). The ruthenium dimer Ru_2C has a charge of +4 that allows it to bind to the acidic patch on subunit II of CcO (Zaslavsky et al, 1998). The same laser flash photolysis instrumentation described in section 4.0 above is used to photoreduce CcO by Ru_2C . Laser flash photolysis of 5 μM oxidized bovine CcO and 25 μM Ru_2C in 5 mM TrisHCl, pH 8.0 with 10 mM aniline and 1 mM 3CP results in electron transfer from the excited state $\text{Ru}(\text{II}^*)$ of Ru_2C to Cu_A in CcO within 1 μs , followed by electron transfer from Cu_A to heme a with a rate constant of $20,000 \text{ s}^{-1}$. The mechanism of photoreduction of Cu_A follows the k_1 and k_6 steps of Scheme 1, and the total yield of photoreduced heme a is 6.2%. Another ruthenium dimer, Ru_2Z , has recently been developed which can photoreduce CcO with a yield of 60% (Brand et al., 2007). Replacement of the bipyridine ligands in Ru_2C with the bipyrazine ligands in Ru_2Z changed all four redox potentials (Table 1). The redox potential of the $\text{Ru}(\text{II}^*)/\text{Ru}(\text{I})$ transition was increased from 0.83 V to 1.16 V, allowing the sacrificial donor aniline to reduce the excited state $\text{Ru}(\text{II}^*)$ to $\text{Ru}(\text{I})$, followed by electron transfer from $\text{Ru}(\text{I})$ to $\text{Cu}_A(\text{II})$ along the k_5 and k_4 steps in scheme 1. Cu_A is 16% reduced and heme a is 52% reduced after electron transfer equilibrium is established between them. The equilibrium constant K for electron transfer between Cu_A and heme a is thus 3.2, and the redox potential of heme a is 30 mV more positive than that of Cu_A (Brand et al., 2007).

5.1. Measurement of Single Electron Reduction of CcO state P_m to F

A mechanism for the reduction of O_2 by CcO is shown in Figure 4 (Branden et al, 2006). State **O** containing a fully oxidized heme a_3/Cu_B binuclear center is reduced by one electron to state **E** and then by a second electron to state **R**₂, which rapidly binds oxygen to form state **A**. State **A** is reduced to state **P**_m in a rapid 4-electron reaction in which electrons come from heme a_3 , Cu_B , and a neighboring tyrosine. The tyrosine radical in state **P**_m is reduced to form state **F**, and then oxyferryl heme a_3 is reduced to form state **O**. There is growing agreement that each of the one-electron transfers to the heme a_3/Cu_B binuclear center is coupled to pumping one proton across the membrane, as indicated in Figure 4 (Verkhovsky et al., 2006; Bloch et al., 2004). Methods have been developed to study each of the one-electron reduction steps in the mechanism using the ruthenium photoreduction technique.

The **P**_m state is formed by treating resting state CcO with CO under aerobic conditions (Siletsky et al, 2006). A solution containing 10 μM oxidized bovine CcO and 30 μM Ru_2C in 5 mM Tris-HCl, pH 8.0 is gently bubbled with CO under aerobic conditions for 60 seconds and incubated until the difference spectrum indicates formation of **P**_m. The yield of the **P**_m state is calculated from $\Delta\epsilon_{607-630} = 11 \text{ mM}^{-1} \text{ cm}^{-1}$, and is typically about 75%. After the **P**_m state is completely formed, 10 mM aniline and 1 mM 3CP are added as sacrificial donors, followed by laser flash photolysis. Photoreduced Cu_A transfers an electron to heme a, followed by electron transfer from heme a to the tyrosine radical near the binuclear center with a rate constant of $4,000 \text{ s}^{-1}$ (Siletsky et al., 2006). The reduction and reoxidation of heme a is followed at 650 nm, while the reduction of the binuclear center is followed at 580 nm. Measurement of

transmembrane voltage generation under the same conditions indicated two phases of electrogenic proton transfer with rate constants of 3300 s^{-1} and 770 s^{-1} (Siletsky et al., 2006). The fast phase of proton transfer is coincident with electron transfer to the tyrosine radical, and is associated with proton delivery to Cu_B bound hydroxide to form water. The slow phase of proton transfer could be due to proton translocation across the membrane.

5.2 Measurement of Single Electron Reduction of F state to O state

The **F** state is generated by treating resting CcO with 4 mM H_2O_2 (Fabian and Palmer, 1995). A solution containing 8 μM bovine CcO and 22 μM Ru_2C in 5 mM Tris-HCl, pH 8.0 with 10 mM aniline and 1 mM 3CP is treated with 4 mM H_2O_2 and incubated for 5 minutes to form state **F**. The yield of **F** is determined from the peak in the difference spectrum (**F** – **O**) at 583 nm using extinction coefficients of $\Delta\epsilon_{583-630} = 5.3\text{ mM}^{-1}\text{ cm}^{-1}$ and $\Delta\epsilon_{436-414} = 67\text{ mM}^{-1}\text{ cm}^{-1}$ (Fabian and Palmer, 1995). Laser flash photolysis results in photoreduction of Cu_A , followed by electron transfer to heme a with a rate constant of $20,000\text{ s}^{-1}$, and electron transfer from heme a to oxyferryl heme a_3 to form state **O** with a rate constant of 470 s^{-1} . The reduction and reoxidation of the heme a is followed at 605 nm, while the reduction of oxyferryl heme a_3 is followed at 580 nm (Zaslavsky et al., 1998). A deuterium isotope effect of 4.3 indicates a proton-transfer rate-limiting step. Measurement of transmembrane voltage generation indicated two phases of electrogenic proton transfer with rate constants of 830 s^{-1} and 220 s^{-1} in a 1:3 ratio (Konstantinov, 1998). The fast phase is coincident with reduction of oxyferryl heme, and is associated with proton transfer to the oxygen on the oxyferryl heme to form water. The slow phase of proton transfer is associated with proton translocation (Konstantinov, 1998). A fast phase of proton release is also observed coincident with reduction of oxyferryl heme (Zaslavsky et al., 2004).

5.3. Measurement of Single Electron Reduction of State O_H to state E

There are several forms of the fully oxidized state of CcO, including the resting state **O** (Moody, 1996), and the state O_H which is formed immediately after fully reduced state **R₄** reacts with oxygen (Bloch et al., 2004). If an additional electron is not injected into the O_H state within approximately 30 seconds, then it relaxes back to the resting state **O**. It has been reported that reduction of state O_H is coupled to proton pumping, while reduction of state **O** is not (Bloch et al., 2004; Belevich et al., 2007; Verkhovsky et al., 2006). A stopped-flow-flash technique is used to study 1-electron reduction of state O_H (Brand et al., 2007). 5 μM bovine CcO is anaerobically reduced with 2 mM ascorbate and 1 μM PMS in 5 mM HEPES buffer, pH 7.9 in one syringe of the stopped-flow, and then rapidly mixed with oxygenated buffer containing 20 μM Ru_2Z , 10 mM aniline, and 1 mM 3CP. State **R₄** reacts with oxygen within 5 msec to form state O_H , and then the laser is fired to excite Ru_2Z to the $\text{Ru}(\text{I})$ state, which injects a single electron into Cu_A . An electron is transferred from Cu_A to heme a with a rate constant of $20,000\text{ s}^{-1}$, followed by biphasic electron transfer from heme a to Cu_B with rate constants of 750 s^{-1} and 110 s^{-1} and relative amplitudes of 25% and 75% (Brand et al., 2007). There is no change in absorbance at 436 nm, indicating that the electron acceptor in the binuclear site is Cu_B rather than heme a_3 . One electron reduction of *P. denitrificans* CcO state O_H has been studied by Belevich et al. (2007) using $\text{Ru}(\text{bpy})_3$ as photoreductant. Correlation of the kinetics of electron transfer and transmembrane voltage generation led to a mechanism for coupling between electron transfer and proton translocation.

5.4. Measurement of Single Electron Reduction of State E to States R_2 and P_m

The one-electron reduction of state **E** is studied by a stopped-flow-flash technique. 5 μM bovine CcO and 5 μM horse heart cytochrome c mutant K13E is anaerobically reduced with 2 mM ascorbate and 1 μM PMS in 5 mM HEPES, pH 8.0, 0.1% LM in one syringe of the stopped-flow. This syringe is then mixed rapidly with the other syringe of the stopped-flow containing

20 μM Ru₂Z, 10 mM aniline, and 1 mM 3CP in buffer. After mixing, the reduced CcO which is fully oxidized to the **O_H** state within 5 ms, is then reduced by the ferrous K13E Cc within 10 ms. The laser is flashed 20 ms after mixing to excite Ru₂Z and inject a single electron into Cu_A, which transfers an electron the heme a. Biphasic electron transfer from heme a to heme a₃ to form state **R₂** then occurs with rate constants of 1100 s⁻¹ and 90 s⁻¹, and relative amplitudes of 11% and 89%. The 436 nm transient indicates that O₂ rapidly binds to state **R₂** to form state **P**. Additional laser flashes result in transients with a rate constant of 3000 s⁻¹, consistent with the sequential **P_M** to **F** transition (Siletsky *et al.*, 2006), demonstrating the advantage of using higher yield photoreductants.

Acknowledgments

Supported by NIH grants GM20488 and NCRR COBRE RR15569

Abbreviations

Cc	cytochrome c
CcO	cytochrome c oxidase
DMAB	dimethylaminobenzoate
3CP	3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical
PMS	phenazine methosulfate
bpy	2,2'-bipyridine
dmb	4,4'-dimethyl-2,2'-bipyridine
bpz	2,2'-bipyrazine
bphb	1,4-bis(2,2'-bipyridyl-4'-yl)-benzene
bpd	3,3'-bipyridazine
Ru₂C	[Ru(bpy) ₂] ₂ (bphb)
Ru₂Z	[Ru(bpz) ₂] ₂ (qpy)
Ru-39-Cc	cytochrome c labeled at Cys-39 with Ru(bpy) ₂ (dmb)
Ru_D-39-Cc	cytochrome c labeled at Cys-39 with Ru(bpd) ₂ (dmb)

References

- Babcock GT, Wikstrom M. Oxygen Activation and the Conservation of Energy in Cell Respiration. *Nature* 1992;356:301–309. [PubMed: 1312679]
- Belevich, Ilya; Bloch, Dmitry A.; Belevich, Nikolai; Wikstrom, Marten; Verkhovsky, Michael I. Exploring the proton pump mechanism of cytochrome *c* oxidase in real time. *Proc Natl Acad Sci U S A* 2007;104:2685–2690. [PubMed: 17293458]
- Bloch D, Belevich I, Jasaitis A, Ribacka C, Puustinen A, Verkhovsky MI, Wikstrom M. The catalytic cycle of cytochrome *c* oxidase is not the sum of its two halves. *Proc Natl Acad Sci U S A* 2004;101:529–533. [PubMed: 14699047]
- Brand SE, Rajagukguk S, Ganesas K, Geren L, Fabian M, Han D, Gennis R, Durham B, Millett F. A New Ruthenium Complex to Study Single-Electron Reduction of the Pulsed OH State of Detergent-Solubilized Cytochrome Oxidase. *Biochemistry* 2007;46:14610–14618. [PubMed: 18027981]
- Branden G, Gennis RB, Brzezinski P. Transmembrane proton translocation by cytochrome *c* oxidase. *Biochim Biophys Acta* 2006;175(7):1052–1063.
- Durham B, Pan LP, Long J, Millett F. Photoinduced Electron-Transfer Kinetics of Singly Labeled Ruthenium Bis-(bipyridine) Dicarboxybipyridine Cytochrome *c* Derivatives. *Biochemistry* 1989;28:8659–8665. [PubMed: 2557906]
- Fabian M, Palmer G. The Interaction of Cytochrome Oxidase with Hydrogen Peroxide: The Relationship of Compounds P and F. *Biochemistry* 1995;34:13802–13810. [PubMed: 7577973]
- Faxen K, Gilderson G, Adelroth P, Brzezinski P. A mechanistic principle for proton pumping by cytochrome *c* oxidase. *Nature* 2005;437:286–289. [PubMed: 16148937]
- Geren LM, Beasley JR, Fines BR, Saunders AJ, Hibdon S, Pielak GJ, Durham B, Millett F. Design of a Ruthenium-Cytochrome *c* Derivative to Measure the Electron Transfer to the Initial Acceptor in Cytochrome *c* Oxidase. *J Biol Chem* 1995;270:2466. [PubMed: 7852307]
- Geren L, Hahm S, Durham B, Millett F. Photoinduced Electron Transfer Between Cytochrome *c* Peroxidase and Yeast Cytochrome *c* Labeled at Cys 102 with (4-Bromomethyl-4'-methylbipyridine) [bis(bipyridine)]ruthenium²⁺. *Biochemistry* 1991;30:9450. [PubMed: 1654098]
- Hill B. The Reaction of the Electrostatic Cytochrome *c*-Cytochrome Oxidase Complex with. 1991
- Konstantinov AA. Cytochrome *c* oxidase as a proton-pumping peroxidase: reaction cycle and electrogenic mechanism. *J Bioenerg Biomembranes* 1998;30:121–130.
- Millett F, Durham B. Design of Photoactive Ruthenium Complexes to Study Interprotein Electron Transfer. *Biochemistry* 2002;41:11315–11324. [PubMed: 12234172]
- Moody AJ. As-prepared forms of fully oxidised haem/Cu terminal oxidases. *Biochimica Biophysica Acta* 1996 Aug 7;1276(1):6–20.
- Ostermeier C, Harrenga A, Ermler U, Michel H. Structure at 2.7 Å Resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody Fv fragment. *Proc Nat Acad Sci USA* 1997;94:10547–10553. [PubMed: 9380672]
- Patel CN, Lind MC, Pielak GJ. Characterization of horse cytochrome *c* expressed in *Escherichia coli*. *Protein Expr Purif* 2001;22:220–224. [PubMed: 11437597]
- Qin L, Hiser C, Mulichak A, Garavito R, Ferguson-Miller S. Identification of conserved lipid/detergent-binding sites in a high-resolution structure of the membrane protein cytochrome *c* oxidase. *Proc Natl Acad Sci USA* 2006;103:16117–1622. [PubMed: 17050688]
- Roberts VA, Pique ME. Definition of the Interaction Domain for Cytochrome *c* on Cytochrome *c* Oxidase. III Prediction of the docked complex by a complete systematic search. *J Biol Chem* 1999;274:38051–38060. [PubMed: 10608874]
- Siletsky SA, Han D, Brand S, Morgan JE, Fabian M, Geren L, Millett F, Durham B, Konstantinov AA, Gennis RB. Single-electron photoreduction of the PM intermediate of cytochrome *c* oxidase. *Biochimica et Biophysica Acta* 2006;1757:1122–1132. [PubMed: 16938268]
- Verkhovsky MI, Belevich I, Bloch DA, Wikstrom M. Elementary steps of proton translocation in the catalytic cycle of cytochrome oxidase. *Biochimica Biophysica Acta* 2006 May-June;1757(5–6):401–407.
- Wang K, Zhen Y, Sadoski R, Grinnell S, Geren L, Ferguson-Miller S, Durham B, Millett F. Definition of the interaction domain for cytochrome *c* on cytochrome *c* oxidase: II. Rapid kinetic analysis of

electron transfer from cytochrome c to *Rhodobacter sphaeroides* cytochrome oxidase surface mutants. *J Biol Chem* 1999;274:38042–38050. [PubMed: 10608873]

Yoshikawa S, Shinzawa-Iroh K, Nakashima R, Yaono R, Yamashita E, Inoue N, Yao M, Fei MJ, Libeu CP, Mizushima T, Yamagichi H, Tomizaki T, Tsukihara T. Redox-Coupled Structural Changes in Bovine Heart Cytochrome c Oxidase. *Science* 1998;280:1723–1729. [PubMed: 9624044]

Zaslavsky D, Sadoski RC, Wang K, Durham B, Gennis RB, Millett F. Single Electron Reduction of Cytochrome c Oxidase Compound F: Resolution of Partial Steps by Transient Spectroscopy. *Biochemistry* 1998;37:14910–14916. [PubMed: 9778367]

Zaslavsky D, Sadoski RS, Rajagukguk S, Geren L, Millett F, Durham B, Gennis RB. Direct measurement of proton release by cytochrome c oxidase in solution during the F to O transition. *Proc Natl Acad Sci USA* 2004;101:10544–10547. [PubMed: 15247424]

Figure 1.
Chemical structures of selected ruthenium complexes

Figure 2.

Photoinduced electron transfer within horse Ru-39-Cc. A solution containing 10 μM Ru-39-horse Cc in 100 mM phosphate buffer pH 7.0 was excited with a 10 ns 356 nm laser flash, and the transients detected at 550 – 556 nm and 434 nm. The smooth lines are fitted to equations 1 and 2 of Scheme 1 with $k_1 = 0.6 \times 10^6$, $k_2 = 0.7 \times 10^6$, $k_d = 5.5 \times 10^6$.

Figure 3.

Photoinduced electron transfer in $\text{Ru}_D\text{-39-Cc:CcO}$ complex. A sample containing 5 μM yeast $\text{Ru}_D\text{-39-Cc}$ and 5 μM bovine CcO in 5 mM sodium phosphate, pH 7.0, 5 mM DMAB, and 0.1% dodecyl maltoside at 25 $^\circ\text{C}$ was excited with a 480 nm laser flash. Cu_A was detected at 830 nm, while heme a was detected at 605 nm.

Figure 4. Cytochrome c oxidase catalytic cycle showing states of the heme a_3 /Cu $_B$ binuclear center and conserved tyrosine. Reactions steps indicated by solid arrows are coupled to proton pumping.

Scheme 2.

Table I
Standard Reduction Potentials of Ruthenium Complexes

Complex	(III)/(II)	(III)/(II*)	(II)/(I)	(II*)/(I)
Ru(bpy) ₃	1.27	−0.87	−1.31	0.83
Ru(bpy) ₂ (dmb)	1.27	−0.83	−1.36	0.79
Ru(bpz) ₂ (dmb)	1.76	−0.25	−0.79	1.22
Ru(bpd) ₂ (dmb)	1.49	−0.49	−1.00	0.98
Ru(bpm) ₂ (dmb)	1.55	−0.30	−0.95	0.90
Ru ₂ C	1.27	−0.87	−1.31	0.83
Ru ₂ Z	1.70	−0.28	−0.82	1.16