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Review

Design of photoactive ruthenium complexes to study electron transfer and proton pumping in cytochrome oxidase☆

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ABSTRACT

This review describes the development and application of photoactive ruthenium complexes to study electron transfer and proton pumping reactions in cytochrome c oxidase (CcO). CcO uses four electrons from Cc to reduce O_2 to two waters, and pumps four protons across the membrane. The electron transfer reactions in cytochrome oxidase are very rapid, and cannot be resolved by stopped-flow mixing techniques. Methods have been developed to covalently attach a photoactive tris(bipyridine)ruthenium group [Ru(II)] to Cc to form Ru-39-Cc. Photoexcitation of Ru(II) to the excited state Ru(II*), a strong reductant, leads to rapid electron transfer to the ferric heme group in Cc, followed by electron transfer to Cu_A in CcO with a rate constant of 60,000 s^{-1}. Ruthenium kinetics and mutagenesis studies have been used to define the domain for the interaction between Cc and CcO. New ruthenium dimers have also been developed to rapidly inject electrons into Cu_A of CcO with yields as high as 60%, allowing measurement of the kinetics of electron transfer and proton release at each step in the oxygen reduction mechanism. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Energy conversion and utilization are critical processes for all living organisms. In aerobic organisms, electrons derived from the oxidation of metabolites are transferred down a respiratory chain to cytochrome oxidase, which reduces oxygen to water. Electron transfer through the complexes of the respiratory chain is coupled to proton pumping, establishing a membrane potential which drives the synthesis of ATP. The topic of respiratory oxidases is extremely broad, as indicated by this current special issue. This review is focused on the development and application of new photoinduced ruthenium rapid kinetics methods to study the electron transfer and proton pumping reactions of cytochrome oxidase, as illustrated in Scheme 1.

Cytochrome c oxidase (CcO) is the terminal member of the respiratory chains of mitochondria and many prokaryotes. It is a redox-linked proton pump which uses four electrons from cytochrome c to reduce molecular oxygen to water [1,2]. Electron transfer is coupled to the uptake of 4 "chemical" protons from the matrix to combine with O_2 to form 2 H_2O, and the translocation of 4 additional "pumped" protons from the matrix to the cytoplasmic side of the membrane [3,4]. X-ray crystal structures of CcO from bovine mitochondria [5–7], *Paracoccus denitrificans* [8,9] and *Rsp.* [10] have provided detailed structural information on the four redox active metal centers: Cu_A, located in subunit II, and heme a, heme a_3, and Cu_B, located in subunit I. Cu_A consists of two copper atoms bridged by the sulfur atoms of two cysteine residues [5–10]. Fig. 1 shows the relative locations of the metal centers in bovine CcO revealed by X-ray diffraction studies.

The electron-transfer reactions involved in the reduction of O_2 are sufficiently established to warrant inclusion in most current biochemistry textbooks. The basic scheme is illustrated in Scheme 2. The CcO reaction begins with reduction of Cu_A by Cc, followed by electron transfer from Cu_A to heme a, and then to the binuclear heme a_3-Cu_B center (Scheme 1, Fig. 1) [1–4,11,12]. The fully oxidized binuclear center, state O, is reduced in successive one-electron transfer steps to form state E and state R, in which heme a_3 and Cu_B are reduced. Molecular oxygen rapidly binds to heme a_3 in state R and is reduced in a concerted, 4-electron reaction to form state P_M, which contains an oxyferryl heme a_3, oxidized Cu_B, and a radical on tyrosine 244 [13,14]. In successive one-electron transfer reactions, the tyrosine radical in state P_M is reduced to form state F and then the oxyferryl heme a_3 is reduced to form the ferric heme a_3 in state O. There is growing experimental support for models in which each of the 1-electron transfers to the binuclear catalytic site in CcO is coupled to pumping one proton across the membrane [15–21]. Konstantinov and coworkers have used an electrometric method to determine that protons were translocated across the membrane in both the P→F and F→O electron-transfer steps [22]. Mutations in the "D

Abbreviations: CcO, cytochrome c oxidase; Cc, cytochrome c; bpy, 2,2′-bipyridine; dmb, 4,4′-dimethyl-2,2′-bipyridine; bpz, bipyrazine; bpd, bipyridazine; bpyCOOH, 4,4′-dicarboxy-2,2′-bipyridine; qpy, 2,2′:4′,4″:2″,2‴-quaterpyridine; Ru_2D, $[Ru(bpy)_2]_2qpy^{4+}$; Ru_2Z, $[Ru(bpz)_2]_2qpy^{4+}$; *Rps.*, *Rhodobacter sphaeroides*

☆ This article is part of a Special Issue entitled: Respiratory Oxidases.

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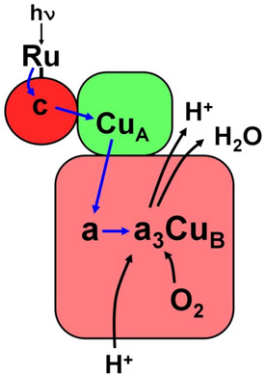


Scheme 1. Photoinduced electron transfer in Ru-39-Cc:CcO complex.

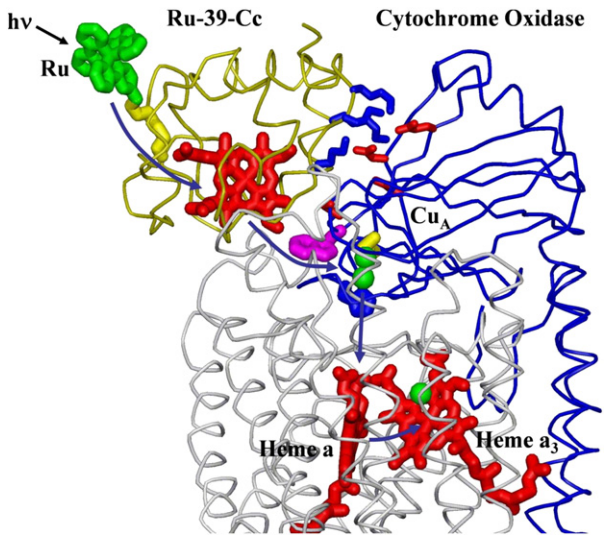
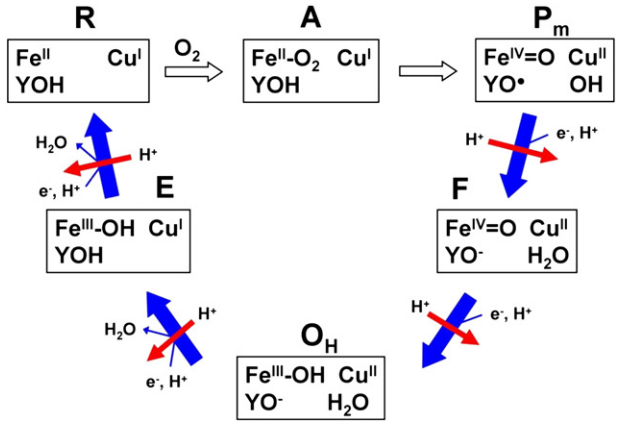


Fig. 1. Model of the Cc:CcO complex created from X-ray crystal structures of bovine CcO and cytochrome c [44]. A ruthenium polypyridine complex has been added to position 39 of cytochrome c (Ru-39-Cc) to illustrate the electron transfer pathways (PDB ID: 2OCC).

channel" (D132N), shown in Fig. 2, strongly inhibited electrogenic proton transfer in the F→O step, suggesting that the D channel is involved in uptake of both "chemical" and "pumped" protons [23]. Glu-286 is a possible branching point where protons are transferred either to the catalytic binuclear site to form water (chemical protons) or to the outside of the membrane (pumped protons) [23,24]. The "K channel" containing Lys-362 is not involved in proton pumping, but may be used for proton uptake during reduction of the binuclear center [23,24].



Scheme 2. Oxygen reduction mechanism of CcO.

2. Laser flash photolysis and kinetic measurements

Many of the electron-transfer reactions of CcO are extremely fast and far beyond the capability of rapid mixing kinetic techniques. Laser flash photolysis is capable of probing reactions that cover the range of reaction times from seconds to nanoseconds and faster. The technique relies on a photochemical event, triggered by a very short duration pulse of light, to initiate the reactions of interest. The subsequent reactions are typically monitored spectrophotometrically. CcO has a number of well-defined absorption bands which reflect the oxidation states of the metallic centers and so it is very well suited to investigation by laser flash photolysis. Unfortunately, CcO is not photochemically active, so another reaction must be used to initiate the electron-transfer reactions.

Derivatives of the parent compound $Ru(bpy)_3^{2+}$ have been extensively investigated and offer a number of properties that make them exceptional candidates for the role of photoredox initiator [25]. The complexes are photoredox active, i.e., the excited state is both a strong oxidant and a strong reductant. This allows for a large number of photoinitiation schemes. The complexes have long-lived excited states that allow ample opportunity for reaction. They are extremely stable in the ground state and do not readily degrade in the excited state. The compounds are typically orange or red which allows the use of lasers that emit in the visible region of the spectrum and thus minimizes UV damage to proteins. In addition, the redox properties and structure can be modified to suit a particular application. An extensive literature that describes this chemistry has been developed.

The use of these complexes to study redox reactions in metalloproteins spans more than three decades starting with the pioneering work of Gray and coworkers [26]. Some of the very first successful investigations relied on solution phase $Ru(bpy)_3^{2+}$ to photochemically reduce $Ru(NH_3)_5$ that was covalently linked to histidine in cytochrome c [26]. These investigations were aimed at developing a better

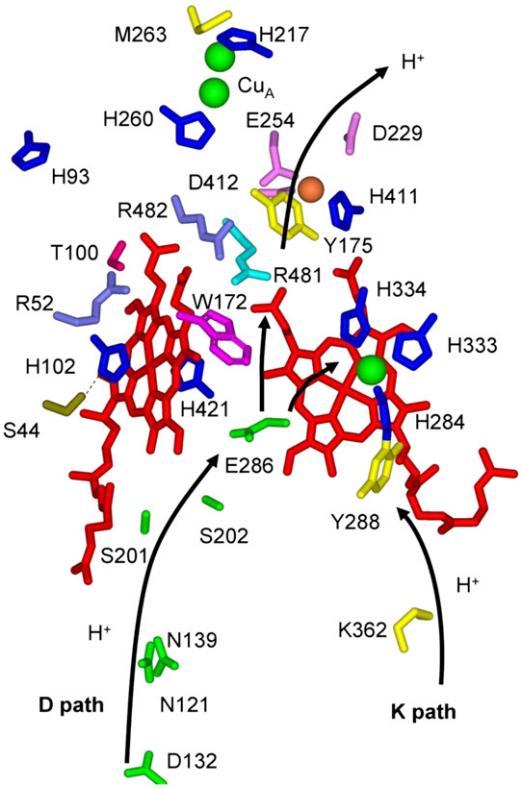


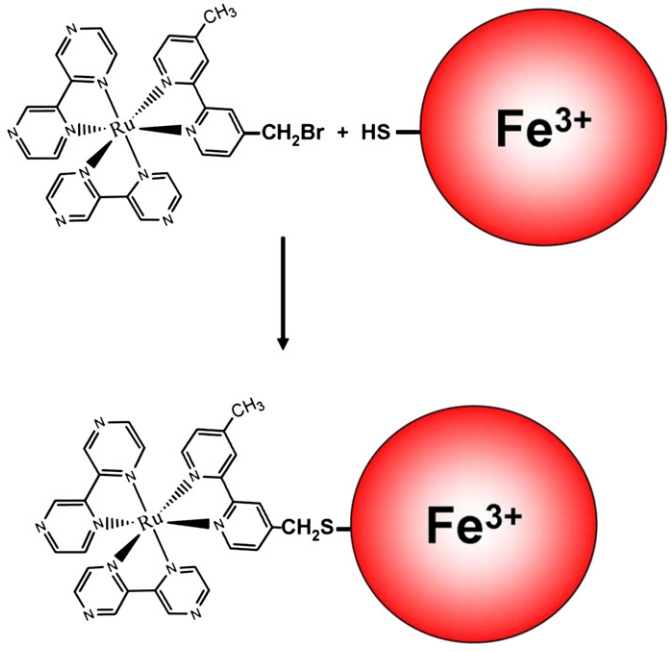
Fig. 2. Structure of components of CcO, including Cu_A, heme a, heme a_3, Cu_B, Y288, D path residues, and K path residues [10]. *R. sphaeroides* sequence numbering is used (PDB ID: 2GSM).

understanding of electron transfer within a metalloprotein. Subsequent work in our laboratory led to the preparation of a series of cytochrome c derivatives that contained photoactive $Ru(bpy)_2$(bpyCOOH) covalently linked to surface lysines [27,28,29]. Derivatives based on coordinating $Ru(bpy)_2$(imidazole) to surface histidines were also developed [30,31].

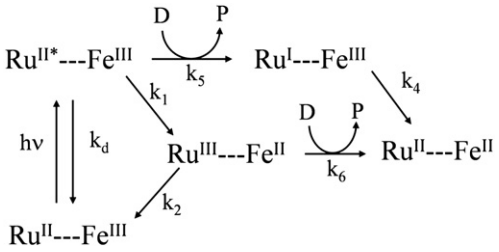
The earliest application to CcO took place in the early 90s [32]. Several reaction schemes have been devised to use the photochemical redox properties of the ruthenium complexes to initiate electron transfer. In one, cytochrome c is covalently linked to a derivative of $Ru(bpy)_3^{2+}$ [32]. Excitation of the ruthenium label with a short duration laser pulse results in very rapid reduction of the Fe(III) of cytochrome c. Under appropriate solution conditions, a significant population of the cytochrome c is electrostatically bound to CcO and poised for electron transfer following the reduction of Fe(III) to Fe(II) (Scheme 1).

A number of ways to covalently link the ruthenium complexes to cytochrome c have been developed since the early work. One of the most efficient relies on use of mutants with a strategically placed cysteine on the surface of cytochrome c which reacts with a derivative of the parent ruthenium complex that contains one α-bromodimethylbipyridine as illustrated in Scheme 3 [33–36]. This reagent reacts specifically with the cysteine under appropriate solution conditions to form a thioether bond and HBr. Yields of up to 80% have been obtained with this method.

The photoinitiation process is illustrated in Scheme 4 in which Ru(II)–Fe(III) represents the ruthenium and heme iron states in a Ru–Cc derivative. The asterisk indicates the excited state. Irradiation with a short duration light pulse results in formation of the Ru(II)* metal-to-ligand charge transfer excited state. The excited state can undergo an electron transfer reaction (k_1) to yield Ru(III)–Fe(II). The intermediate Ru(III) can reoxidize the Fe(II), as indicated by k_2, and return the system to the starting point with no net reaction. This unproductive back reaction is prevented by a solution phase donor, D, which reacts irreversibly to reduce Ru(III) and yield Ru(II)–Fe(II). Aniline has been used extensively in this role. At this point the photoinitiator is restored to the original oxidation state and is ready for the next light pulse. Decay of the excited state back to the ground state by processes that do not involve electron transfer is represented by k_d. If the Ru–Cc derivative is bound to CcO, then photoexcitation of the ruthenium complex results in rapid reduction of heme c, followed by electron transfer to Cu_A, heme a, and finally the heme a_3/Cu_B binuclear center in CcO as shown in Scheme 1 [35].



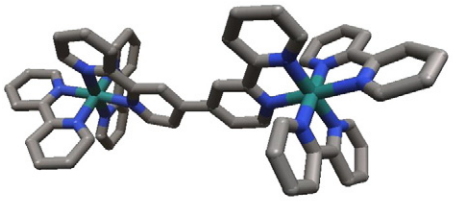
Scheme 3. Synthetic scheme to covalently attach ruthenium complexes to protein cysteines.



Scheme 4. Reaction pathways for photochemical reduction of the heme in Cc.

A second approach, first described by Nilsson [37], simply involves adding relatively high concentrations of $Ru(bpy)_3^{2+}$ to a solution of CcO with no cytochrome c. This approaches relies on the fortuitous weak binding of $Ru(bpy)_3^{2+}$ to a location on CcO that allows the oxidation quenching by the Cu_A. The reaction scheme is essentially the same as that shown in Scheme 4 with Cu_A in place of Fe(III). Laser flash photolysis results in the very rapid reduction of Cu_A in CcO followed by electron transfer to heme a then to heme a_3. This second strategy has the advantage that it removes the spectroscopic complications contributed by cytochrome c but it also eliminates the opportunity to study the initial electron transfer event.

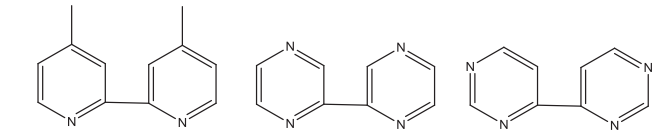
Subsequent work in our laboratory has demonstrated that improved yields can be obtained by using much lower concentrations of ruthenium complexes with +4 overall charge [38–40]. This discovery allowed us to apply a substantial body of ruthenium chemistry to the kinetic investigation of CcO. Initially, experiments were performed with dimeric complexes prepared with only bipyridine as the coordinating ligands. For example, the dimer $[Ru(bpy)_2]_2qpy^{4+}$ (Ru_2D) has redox and excited-state properties that are nearly identical with $Ru(bpy)_3^{2+}$ [38]. The 4+ charge increases the binding constant between the photoinitiator and CcO.



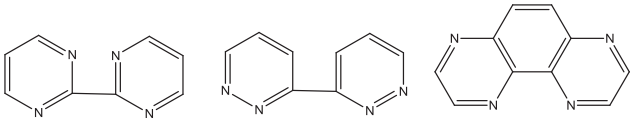
Ru_2D

The strategies described above rely on using CcO as a redox quencher. Addition of a redox quencher that reacts with the ruthenium complex can provide a means of forming Ru(I), a powerful reducing agent, as shown in Scheme 5 as k_5. In Scheme 5, Ru(II)–Cu_A(II) represents the ruthenium based photoinitiator bound near Cu_A on CcO.

A variety of heterocyclic ligands can be used in place of bipyridine. Some representative examples are shown below. Substitution of one or more of the bipyridine ligands provides a means of "tuning" the redox properties of the resultant complexes.



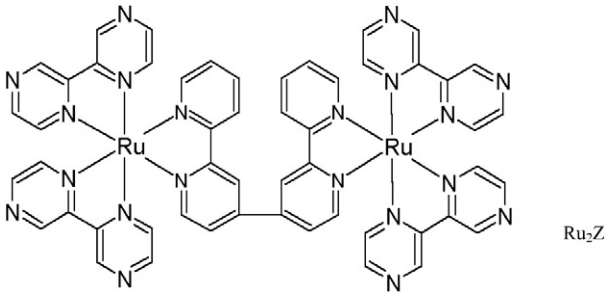
4,4'-dimethyl-2,2'-bipyridine, dmb 2,2'-bipyrazine, bpz 4,4'-bipyrimidine, bpm



2,2'-bipyrimidine, bpm 3,3'-bipyridazine, bpdz tetraazaphenanthroline, TAP

Table 1 summarizes the redox properties of several of the complexes made by substituting other heterocycles for bipyridine. These complexes

have been investigated as potential photoinitiators in the schemes described above and others in our laboratory. Dimeric complexes prepared with bipyrazine, Ru_2Z, have proven to be very effective when used in Scheme 5 with CcO [41].



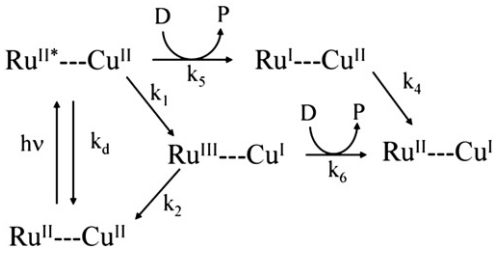
Comparison of the excited state potentials of the bipyridine and bipyrazine dimers shows that the bipyrazine dimer is a significantly stronger oxidant in the excited state and capable of undergoing a reductive quenching reaction with aniline. The excited state of corresponding bipyridine complex is not quenched by aniline. The Ru(I) is a strong reductant and reduces Cu_A of CcO rapidly in the absence of cytochrome c and in very high yield, approximately 60% [41].

It should be noted that Schemes 4 and 5 contain two pathways for photoreduction. In most instances the choice of reaction paths can be dictated by choice of solution conditions. For example, a high concentration of solution phase quencher and unfavorable binding conditions will make k_5 the dominant reaction.

3. Electron transfer results

3.1. Overview

Early kinetic studies of the reaction of CcO [35] with the physiological donor, cytochrome c, were performed using a derivative of cytochrome c specifically designed for the application. The derivative, abbreviated Ru-39-Cc, was prepared from a yeast cytochrome c mutant containing a single cysteine at position 39 that was labeled with a ruthenium complex as shown in Scheme 3. Ru-39-Cc satisfies two important design criteria. Position 39 is located outside of the binding domain and the ruthenium complex in this position is not expected to interfere with the interaction of cytochrome c with CcO. The binding constant and second-order rate constant for the reaction of Ru-39-Cc with CcO were measured and found to be the same as for native yCc [35], indicating that the ruthenium group on Ru-39-Cc does not affect the interaction with CcO. The second design criteria is rapid reduction of the iron center in Ru-39-C with respect to the reduction of Cu_A by the iron center, i.e., the photoinitiation step is fast with respect to the reactions of interest. In this particular derivative, good electronic coupling between the ruthenium complex and the heme group is expected because there are 13 covalent bonds and one hydrogen bond separating the two metal centers as shown in Fig. 1. Experimental measurements revealed a rate constant for electron transfer from Ru(II*) to the heme group of $5\times10^5\ s^{-1}$ (k_1 in Scheme 4). More recently, Ru-39-Cc was found to give a higher yield (35%) of reduced cytochrome c when the complex $Ru(bpd)_2(dmb)^{2+}$ was used in place of $Ru(bpy)_2(dmb)^{2+}$. Beyond the differences in photochemical yields, the substitution has no effect on the reactions of interest. Laser excitation of the 1:1 complex between Ru-39-Cc and beef CcO at low ionic strength leads to the electron-transfer sequence: Ru(II*) → heme c^{3+} → Cu_A^{2+} → heme a^{3+} with rate constants of $k_1=5\times10^5\ s^{-1}$, $k_a=6\times10^4\ s^{-1}$, and $k_b=2\times10^4\ s^{-1}$, summarized in Scheme 6 and illustrated in (Fig. 3) [35]. The redox changes of Cu_A, and heme a were followed at 830 nm and 605 nm, respectively (Fig. 3). The redox changes associated with the heme of Cc were followed at 550 nm but are not shown.



Scheme 5. Reaction pathways for photochemical reduction of copper A in CcO.

Observations over a wide range of ionic strengths reveal kinetic behavior that reflects the dissociation of Cc and CcO with increasing ionic strength [35]. At low ionic strength, the equilibrium constant for dissociation is low enough that only a small percentage of cytochrome c is not bound to CcO. Under these conditions only a simple exponential transient is observed as indicated in Fig. 3. As the ionic strength was increased from 40 mM to 100 mM, the transients became biphasic and the amplitude of the fast phase, also referred to as the bound phase and corresponding to the electron transfer in the associated complex, decreased as the fraction of 1:1 complex decreased. At the same time, a slow phase appeared due to intermolecular reaction between free Ru-39-Cc and CcO. The rate constant for the bound phase, k_a, does not change over the range of ionic strength of very low to over 100 mM, indicating that the orientation of the complex does not change as a function of ionic strength. Simultaneous observation of both bound and intermolecular phases in the ionic strength range 55–105 mM, provided a means of determining the complex formation and dissociation rate constants. At physiological ionic strength (100 mM) the complete reaction involves substrate complex formation between Cc^{2+} and CcO with rate constant $k_f=1.8\times10^8\ M^{-1}\ s^{-1}$, bound-phase electron transfer from Cc^{2+} to Cu_A^{2+} with rate constant $k_a=6\times10^4\ s^{-1}$, and product complex dissociation with rate constant $k_d=8\times10^3\ s^{-1}$ (Scheme 6) [35].

3.2. What is the interaction domain of CcO with cytochrome c?

Analysis of the X-ray crystal structures of bovine, *Rsp* and *P. nitrificans* CcO reveals a prominent cluster of acidic residues on subunit II near the binuclear Cu_A center [5–10]. Trp-143 is located at the center of this acidic cluster and this aromatic residue is highly conserved. It is in van der Waals contact with the Cu_A center and is potentially part of the electron-transfer pathway between the surface of subunit II and Cu_A. The location of the binding domain between Cc and CcO has been investigated by using a double mutant cycle involving *Rsp*. CcO mutants in which acidic residues were replaced with neutral Asn or Gln residues, and Ru-39-Cc mutants in which lysine residues were replaced with Ala. The sites of the mutations are illustrated in Fig. 4 and the rate constants are summarized in Tables 2 and 3 [42]. The mutations D214N, E157Q, E148Q, and D195N in CcO significantly reduced the bound-phase rate constant k_a and the complex formation rate constant k_f, and increased the dissociation constant K_d, indicating that these mutations affect both binding strength and orientation [42] (Table 2). The mutations K13A, K27A, K72A, K86A, and K87A in Ru-39-Cc also decreased the rate constant k_f for binding Cc to CcO (Table 3) indicating that these lysines which surround the heme crevice

Table 1
Standard ground and excited-state reduction potentials (vs NHE) of selected ruthenium polypyridine complexes.

Complex	(II)/(III)	(II*)/(III)	(II)/(I)	(II*)/(I)
$Ru(bpy)_3^{2+}$	1.27	−0.87	−1.31	0.83
$Ru(bpy)_2(dmb)^{2+}$	1.27	−0.83	−1.36	0.79
$Ru(bpz)_2(dmb)^{2+}$	1.76	−0.25	−0.79	1.22
$Ru(bpd)_2(dmb)^{2+}$	1.49	−0.49	−1.00	0.98
$Ru(TAP)_2(phen)^{2+}$	1.73	−0.16	−0.83	1.06

of Cc are involved in binding CcO [43]. Mutations at CcO Trp-143 decreased the electron-transfer rate constant k_a for the bound phase by more than 700-fold, without significantly affecting the binding strength, indicating that removing the aromatic side chain causes a substantial reduction in the electron coupling for electron transfer [42].

3.3. Electron transfer pathway between Cc and CcO

Roberts and Pique have used a computational program to propose a structure for the complex between Cc and CcO, illustrated in Fig. 4, that is consistent with the kinetic studies [44]. The interaction consists of a central hydrophobic domain, surrounded by complementary electrostatic interactions between Cc lysines 8, 13, 86/87, 72, and CcO Asp-195, Glu-157, Glu-148, and Asp-214, respectively. In this model, the indole ring of Trp-143 is in van der Waals contact with the heme CBC methyl group at the center of the hydrophobic domain. The closest edge-to-edge distance between the porphyrin ring of cytochrome c and the Cu_A ligand, Met-263, is 13 Å.

Moser et al. [45] have shown that the rate constants for electron transfer in a wide variety of proteins can be described by Eq. (1).

$$k_{et} = k_o \exp[-\beta(r-r_o)\exp\left(-(\Delta G^{o'}+\lambda)^2/4\lambda RT\right)] \quad (1)$$

This equation combines the basic free energy dependence and general electronic coupling originally described by Marcus [46] with the empirically derived distance dependence demonstrated by Dutton and coworkers. In this equation β describes how rapidly the rate constant for electron transfer declines with the distance between the redox centers. The distance of separation is described by r, and r_o is the distance at which the electronic coupling is expected to be near unity, 3.6 Å. Moser et al. [45] have shown that a value of $\beta = 1.4\ Å^{-1}$ best described the data obtained with a large set of proteins. The term k_o corresponds to the maximum rate constant obtained when the electronic coupling is unity, i.e., close contact, and a value of $1\times10^{13}\ s^{-1}$ was obtained by Moser et al. [45]. The term λ is the reorganizational energy and corresponds to the energy required to rearrange the reactants and the solvent prior to electron transfer. The reorganization energy for electron transfer from Cc to Cu_A in CcO can be calculated from the intrinsic reorganizational energies of Cc and Cu_A using the Marcus cross relation $\lambda = (\lambda_{Cc}+\lambda_{Cu})/2 = (1.1+0.2)/2 = 0.65$ eV [35]. Eq. (1) yields an electron-transfer rate constant of $6\times10^4\ s^{-1}$ when $r = 13$ Å, $\beta = 1.4\ Å^{-1}$, $\lambda = 0.65$ eV and $\Delta G^{o'} = -0.03$ eV. This is in good agreement with the experimental value for k_a of $6\times10^4\ s^{-1}$ and $9\times10^4\ s^{-1}$ for the reaction between Ru-39-Cc and Cu_A in beef CcO [35], and *Rhodobacter sphaeroides* CcO, respectively [42]. The indole ring of Trp-143 in direct van der Waals contact with both the Cc heme and the Cu_A ligands Cys-256 and Met-263 (Fig. 4) appears to contribute significantly to the electronic coupling between the iron and copper centers.

The Cu_A center has a novel binuclear structure in which the two copper atoms are bridged by the sulfur atoms of Cys 252 and Cys 256 (5–10). His 217 and Met 263 are terminal ligands to one copper atom and His 260 and the backbone carbonyl of Glu 254 are terminal ligands to the other copper atom (Fig. 2). The mutations H260N and R482P decrease the rate constant for electron transfer from Cu_A to heme a from $90{,}000\ s^{-1}$ to $45\ s^{-1}$ and $50\ s^{-1}$, respectively [47,48], supporting a proposal that the pathway for electron transfer involves a hydrogen-bond network through the Cu_A ligand His-260, the peptide backbone and the highly conserved subunit I residue Arg-482, to the heme a propionates [6,7,49].

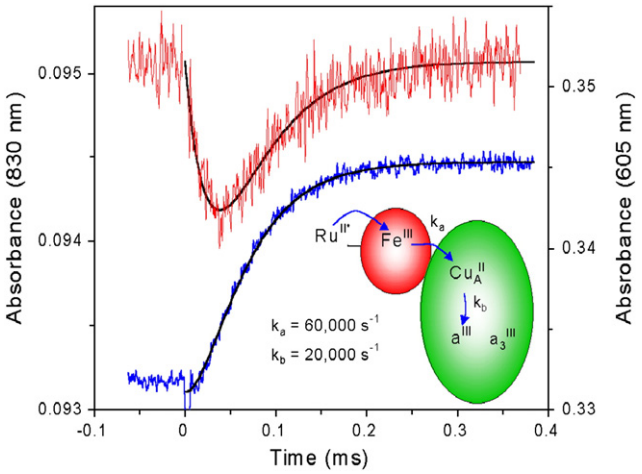
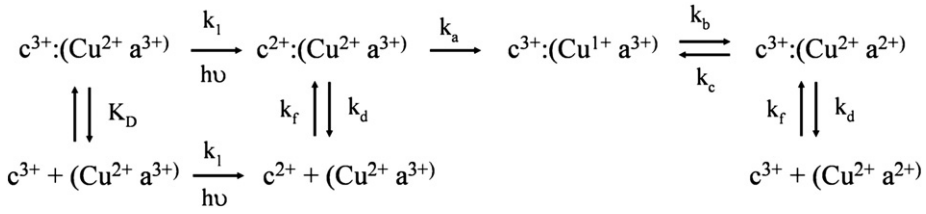


Fig. 3. Photoinduced electron transfer in Ru-39-Cc:CcO complex after a single laser flash. The 830 nm transient (red) shows the reduction of Cu_A by Ru-39-Cc with rate constant $k_a = 60{,}000\ s^{-1}$ followed by oxidation of Cu_A by heme a with rate constant $k_b = 20{,}000\ s^{-1}$. The 605 nm transient (blue) shows the reduction of heme a by Cu_A with rate constant $k_b = 20{,}000\ s^{-1}$. The solid black lines are best fits to the kinetic equations describing the reaction in Scheme 6 [35]. The sample contained 5 μM yeast Ru-39-Cc and 5 μM bovine CcO in 5 mM sodium phosphate, pH 7.0, 5 mM dimethylaminobenzoate, and 0.1% dodecyl maltoside at 25 °C.

3.4. Reactions at the heme a_3-Cu_B site

Electron transfer to heme a is followed by electron transfer to heme a_3 which is strongly coupled to the Cu_B site and in close proximity to Y244 (bovine), that participates in the overall catalytic cycle through transient formation of a radical. Kinetic measurements of the steps that follow electron transfer to heme a are complicated by the numerous forms in which CcO can be prepared experimentally. For example, the oxidized enzyme can be prepared in a "resting", "slow", "fast", "pulsed", "O" and "O_H" state [50–56]. Electron transfer from Cu_A to heme a is similar in these forms but transfer to heme a_3/Cu_B varies widely. The "slow" and "resting" states appear to be similar and are characterized by slow rates of catalysis and slow electron transfer between heme a and heme a_3/Cu_B, $k = 90\ s^{-1}$ [50–56]. Likewise the "pulsed" and "fast" states are similar and are characterized by much faster heme a to heme a_3/Cu_B electron transfer. The "pulsed" state is prepared by exposing the fully reduced enzyme to oxygen. The "pulsed" state reverts to the "slow" state slowly in solution. The "O_H" state has been described by Wikstrom and colleagues, and is a transient state formed at the early stages of the "pulsing" process [4,52–55]. Wikstrom and colleagues have shown that injection of one electron from photoexcited $Ru(bpy)_3^{2+}$ into the metastable O_H form of *P. denitrificans* CcO resulted in electron transfer from heme a to the heme a_3-Cu_B center and coupled proton pumping [55]. In the O_H state, the midpoint potential



Scheme 6. Reaction pathways for electron transfer between Cc and CcO.

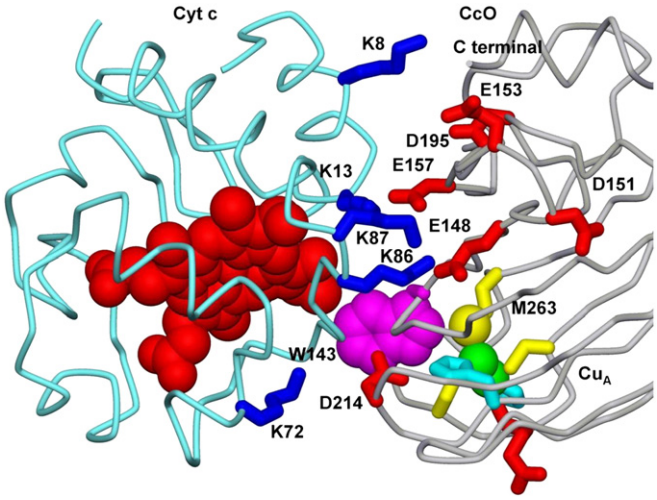


Fig. 4. Model of the Cc:CcO complex showing charged residues in the protein–protein interaction domain [44].

of Cu_B is 100 mV higher than the potential of heme a and the electron transfer from heme a to heme a_3/Cu_B is complete within 2 ms.

In order to further study the reaction $O_H \rightarrow E$, a stopped-flow-flash technique was used to form the O_H state from several sources (bovine heart mitochondria, *R. sphaeroides* and *P. denitrificans*) [41]. Upon mixing the fully reduced anaerobic enzyme with oxygenated buffer containing Ru_2Z, the oxidized O_H state was formed within 5 ms. Ru_2Z was then excited with a laser flash to inject 1 electron into Cu_A (Scheme 7, Fig. 5). Electron transfer from Cu_A to heme a occurred with a rate constant of 20,000 s^{-1} in bovine CcO. Electron transfer from heme a to the heme a_3/Cu_B center in the O_H state was 63% complete and occurred with biphasic kinetics with rate constants of 750 s^{-1} and 110 s^{-1} and relative amplitudes of 25% and 75%. In contrast, 1-electron injection into the "fast" O form of the bovine oxidase was only 30% complete and occurred with monophasic kinetics with a rate constant of 90 s^{-1}. This was the first evidence for a difference between the "fast" O form of the bovine oxidase and the "O_H" form. No reduction of heme a_3 was observed indicating that Cu_B accepts the electron from heme a in the transition from the O_H state to the E state.

A protocol has also been demonstrated to generate the 1-electron reduced E form of the enzyme with K13E Cc, and then to photoreduce the E form with Ru_2Z to study the kinetics of the $E \rightarrow R$ transition [41]. The resulting 2-electron reduced R form of the enzyme reacted very rapidly with O_2 to form the P_M state.

3.5. Kinetics of proton pumping

Electron transfer that results in reduction of oxygen to water in cytochrome c oxidase is coupled to pumping protons across the inner mitochondrial membrane. Electrons enter on the cytoplasmic side of the membrane at the Cu_A site. From Cu_A, they move to heme a near the middle of the bilayer, and finally to heme a_3/Cu_B and oxygen. Thermodynamically, the overall process results in a free energy change that is the difference between the negative free energy of electron transfer and the positive free energy required to create a proton gradient across the membrane. Exactly how these are coupled on a molecular level is still not clear. It is, however, reasonable to postulate that movement of the protons is associated with neutralization of the charge developed by the hemes as they undergo reduction. The propionate groups of heme *a* and/or heme a_3 (Fig. 2) and the conserved carboxylate E286 could be sites of transient protonation that accompany electron transfer. Arginines R481 and R482 are of particular interest since these amino acids can interact with the propionates through ionic and hydrogen bonds.

Table 2
Rate constants k_a for electron transfer from Ru-55-Cc to CcO Cu_A for various mutants of CcO in 5 mM TrisCl, pH 8.0. The dissociation constant K_D for the Cc:CcO complex was measured in 5 mM TrisCl, pH 8.0 with 45 mM NaCl [42].

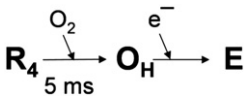
Mutant	k_a, s^{-1}	K_D, μM
Wild type	38,000	1.0
E148Q	15,000	2.3
D151N	45,000	1.6
E157Q	12,000	3.9
D188N	45,000	1.3
D195N	25,000	2.3
D214N	700	3.2
W143A	32	0.9
W143F	85	1.5

Table 3
Second-order rate constant for the reaction of Ru-39-Cc with CcO in 5 mM TrisCl, pH 8.0 and 100 mM NaCl [43].

Mutant	k_f, $\mu M^{-1}\ s^{-1}$
None	528
K86A	214
K72A	295
K87A	192
K27A	260
K13A	18

Ruthenium-based kinetics studies revealed that the difference in midpoint potentials between Cu_A and heme a in the mutant R481K was significantly reduced relative to the wild type [57]. It was also found that this mutant was slower to react with dithionite and it had lower activity at high pH [57]. The mutant D132A was also implicated in proton transfer. It has only 4% of the activity of wild type and uptake of protons from the internal side of the membrane is seriously compromised. The activity of this mutant also appears to be dependent on the uptake of protons from the outside. When the mutations are combined, the double mutant D132A/ R481K has only 1% of the activity of the wild type [57]. The observation that the double mutant has a higher impact than expected from the two single mutants suggests that R481K not only lowers the midpoint potential of heme *a* but also impairs uptake of protons from the outside of the membrane that occurs in the D132A single mutant. The results support an important role of R481 and heme a/a_3 propionates in proton movement in a reversible exit path [57].

Laser flash photolysis with a ruthenium complex has also been applied to the measurement of the kinetics of proton release from CcO [40]. It is generally assumed that proton uptake "charges" an internal proton reservoir in CcO with protons, which are released to the outer aqueous layer in a later stage of the mechanism. However, due to the rapid uptake of protons from water, proton release had not been previously observed in CcO in the absence of coupling membranes. In the laser flash photolysis experiments, compound F was formed at pH 9.5 by treatment of monomeric CcO with 5 mM H_2O_2, and the ruthenium dimer Ru_2D was used to photoreduce Cu_A, which rapidly transfers an electron to heme a as indicated by the initial increase in 605 nm absorbance (Fig. 6) [40]. Heme a then transfers an electron to the oxyferryl heme a_3 in compound F with a rate constant of 1500 s^{-1}. Proton release which accompanied the reduction of compound F was monitored with a solution phase pH indicator. The rate constant for proton release was



Scheme 7. Redox states of CcO accessed in stopped-flow-flash experiments.

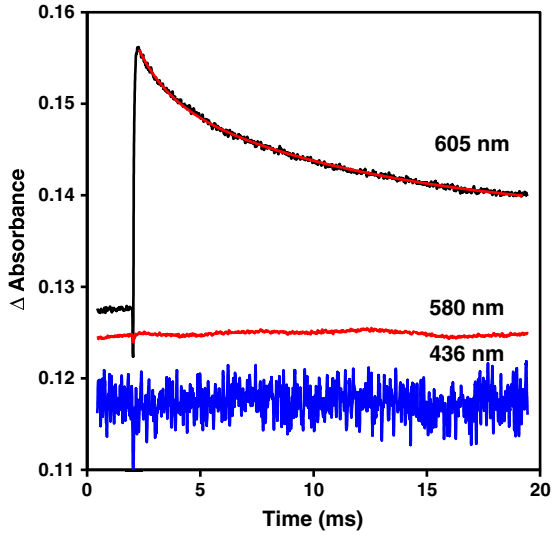


Fig. 5. Transient absorbance changes following injection of one electron from Ru_2Z into the O_H state after rapid oxidation of the fully reduced bovine CcO by O_2 [41]. The 605 nm transient for heme a shows very rapid formation of reduced heme a followed by slow biphasic decay. The solid red line corresponds to the best-fit line to a biexponential decay with rate constants of 750 and 100 s^{-1}. No absorbance changes were observed at 436 nm (blue) indicating no reduction of heme a_3, nor at 580 nm (red) indicating compound F was not involved. The sample contained 5 μM CcO, 20 μM Ru_2Z, 10 mM aniline, and 1 mM 3CP in 5 mM HEPES buffer, pH 7.9 with 0.1% lauryl maltoside.

1500 s^{-1}. (Fig. 6). The amplitude of the transient corresponded to the release of 0.5–0.8 protons per compound F reduced [40].

A conclusion drawn from this study is the existence of an internal "proton pool" in CcO of at least two protons. Proton transfer studies of CcO oriented in small unilamellar vesicles have also provided evidence of an internal proton pool [58]. One of the protons is involved in the reaction at the active site during the reduction of compound F and a second proton is released to the aqueous phase. The second proton appears to be located at a site with a pK_a greater than 9.5 and must be present when the enzyme is in the peroxide-generated F state. The proton is ejected after an electron is transferred from heme a to oxyferryl heme a_3, suggesting that the effective pK_a of the site is lowered below 9.5. E286 has been shown to be protonated in the F state as well as the oxidized and fully reduced states of the protein, and it has a $pK_a > 9.5$ in these states [59–61]. E286 was found to have a pK_a of 9.4 from studies of the transition from the P_r to F states [62]. It is reasonable to assume that E286 is one of the internal transient sites of protonation. The second site is not currently well defined and may be one of the heme propionate groups or possibly another residue in the exit channel (Fig. 2).

4. Conclusions

A variety of ruthenium complexes have been applied to the laser flash photolysis kinetic investigations of the reactions of CcO. The schemes provide considerable flexibility in how they are applied. The very fast intracomplex CcO/Cc electron transfer has been investigated by using ruthenium complexes covalently attached to Cc. Internal electron transfer between Cu_A and heme a and between heme a and heme a_3/Cu_B has been investigated most effectively by direct electron transfer from a ruthenium complex to Cu_A. Details of the reactions have been revealed by combining this kinetic methodology with genetic modifications of both Cc and CcO. In this way details of the electron pathway and the docking domain have been revealed. A stopped-flow flash photolysis method has been utilized to study 1-electron reduction of the transient O_H state formed by reaction of the fully reduced CcO by O_2. In addition, pH sensitive dyes have been used with the ruthenium photoactivation method to provide insight into the proton pumping process.

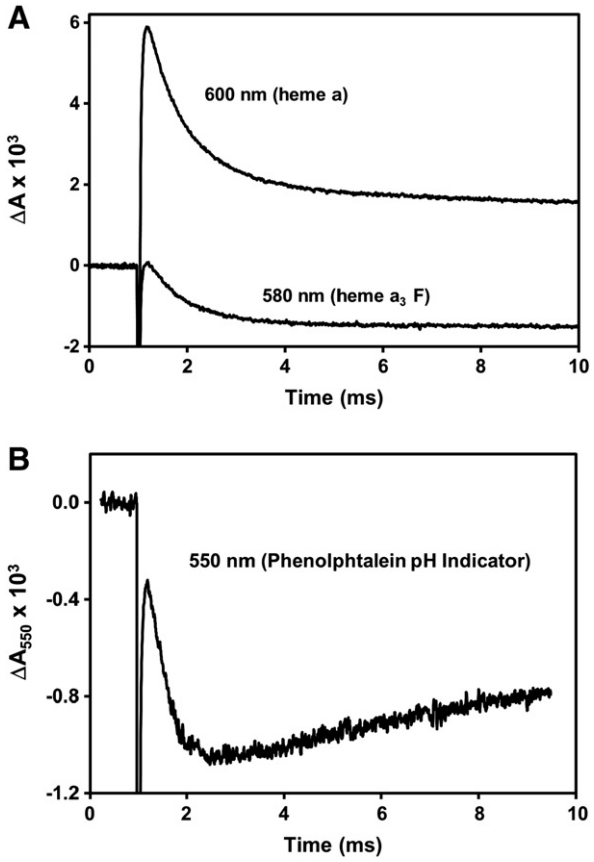


Fig. 6. Transient absorbance changes showing proton release from monomeric CcO during the F to O transition at pH 9.5 [40]. The 600 nm transient shows the rapid reduction of heme a by photoactivated Ru_2D, followed by reoxidation. The 580 nm transient shows the reduction of compound F. The 550 nm transient shows the release of protons from CcO and their uptake by the phenolphthalein pH indicator. CcO was treated with H_2O_2 to form compound F, and then one electron was photochemically injected from Ru_2D [40].

Acknowledgments

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