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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*

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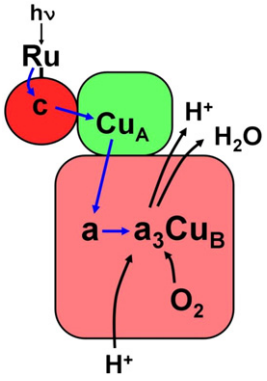


Scheme 1. Photoinduced electron transfer in Ru-39-Cc:CcO complex.

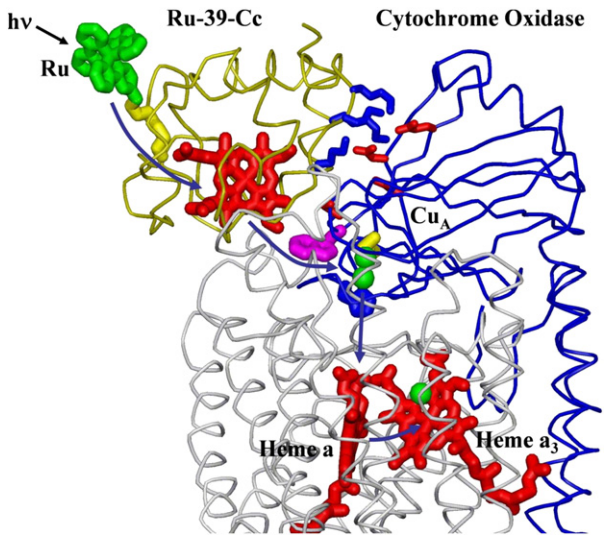
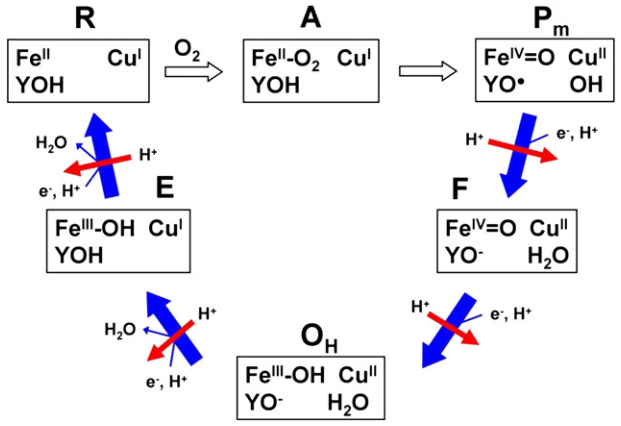


Fig. 1. Model of the Cc:CcO complex created from X-ray crystal structures of bovine CcO and cytochrome c [44]. A ruthenium polypyridine complex has been added to position 39 of cytochrome c (Ru-39-Cc) to illustrate the electron transfer pathways (PDB ID: 2OCC).

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

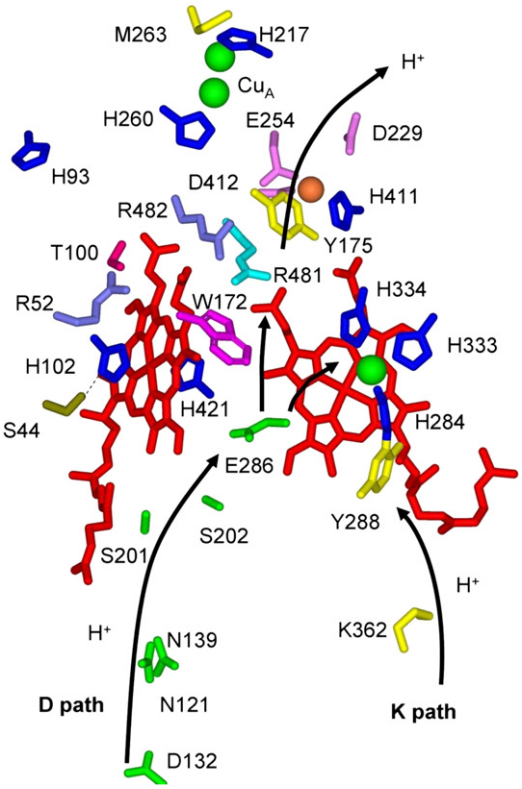


Fig. 2. Structure of components of CcO, including Cu_A, heme a, heme a_3, Cu_B, Y288, D path residues, and K path residues [10]. *R. sphaeroides* sequence numbering is used (PDB ID: 2GSM).