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Review

# Gating and regulation of the cytochrome c oxidase proton pump ☆

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ABSTRACT

As a consumer of 95% of the oxygen we breathe, cytochrome c oxidase plays a major role in the energy balance of the cell. Regulation of its oxygen reduction and proton pumping activity is therefore critical to physiological function in health and disease. The location and structure of pathways for protons that are required to support cytochrome c oxidase activity are still under debate, with respect to their requirements for key residues and fixed waters, and how they are gated to prevent (or allow) proton backflow. Recent high resolution structures of bacterial and mammalian forms reveal conserved lipid and steroid binding sites as well as redox-linked conformational changes that provide new insights into potential regulatory ligands and gating modes. Mechanistic interpretation of these findings and their significance for understanding energy regulation is discussed. This article is part of a Special Issue entitled: Respiratory Oxidases.



## 1. Introduction

The proton pumping function of cytochrome c oxidase (CcO) has been the subject of intense study and controversy for many years, beginning with the question of whether CcO pumps protons at all [1]. While the pumping question is no longer in dispute and much progress has been made in defining the underlying mechanism of oxygen reduction, it remains unclear how this exergonic process drives proton translocation across a membrane. Part of the difficulty in addressing the issue relates to the apparently critical role of water in facilitating and gating the movement of protons [2–5]. Mutagenesis and crystallography are powerful tools for determining the structural features important for protein function, but they are less incisive when it comes to detecting the positions and roles of water molecules. Residues can be mutated but water cannot, and very high resolution crystal structures are necessary to reliably track the whereabouts of water. Obtaining the necessary resolution (usually better than 2.5 Å) with a membrane protein is a challenge. In this regard, some success has been achieved with CcO isolated from bovine and bacterial sources, clarifying some aspects of the pumping process but leading to conflicting conclusions regarding others.

A major physiological question that drives the quest to understand the pumping mechanism is: how is the efficiency of the process controlled? As the consumer of 95% of the oxygen we breathe and the terminal member of the mitochondrial electron transfer chain, cytochrome c oxidase is a major player in the energy equilibrium of organisms, contributing to the balance between ATP and heat production, exerting control over the level of aerobic metabolism, and affecting upstream production of reactive oxygen species [6,7]. The intrinsic efficiency of the CcO proton pump has been proposed to be regulated by the rate of proton backflow through the protein [8–12] possibly via reversal of the normal exit pathway for pumped protons. However, neither the exit nor the backflow pathway has been located, although various routes are postulated [5,13,14]. It is clear that defining the proton pathways and understanding what structural features determine the rates of flow, during uptake, exit and backflow, are of fundamental importance to understanding energy balance and metabolic control in health and disease.

This review will summarize the insights gained from studies on the *Rhodobacter* enzyme (RsCcO) focusing on evidence for the importance of conserved lipid and steroid binding sites in structure and regulation, and for a role of conformational change and water positioning in the gating of proton pathways.

## 2. Conserved lipid binding sites

The original RsCcO crystal structure ([15]; PDB ID: 1M56) revealed six phospholipids embedded in the structure, two buried in a cleft in subunit III and four surrounding the single transmembrane helix of subunit IV, in an arrangement that almost completely separated the latter subunit from interaction with the rest of the protein (Fig. 1). The structure was unprecedented in its demonstration of major involvement of lipid in a membrane protein's structural integrity.

Also striking were the images of lipids associated with the bovine enzyme: when structures were obtained at 1.8 Å resolution, a total of 13 different lipids per 13-subunit monomer were resolved and each binding site was specific for a particular lipid [16] (PDB ID: 2DYR) (Fig. 2).

The importance of specific lipid associations was further emphasized when a second structure of the *Rhodobacter* enzyme [17] (PDB

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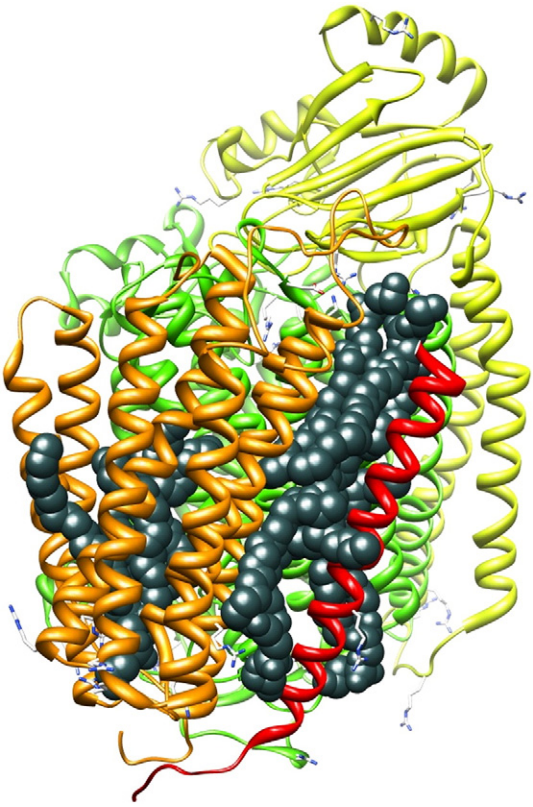

**Fig. 1.** *Rhodobacter sphaeroides* cytochrome c oxidase (RsCcO) showing associated lipid. Four subunits: I, green; II, yellow; III, orange; IV, red. Lipid, phosphatidylethanolamine (PE), indicated as dark gray spheres. Drawing created in Chimera (UCSF) from PDB ID: 1M56.

ID: 2GSM) was obtained at higher resolution (2.0 Å). This structure contained the two core subunits I and II, but was missing subunits III and IV that were initially seen as having the major lipid interactions. In the new structure, alkyl chains are seen embedded in the membrane domain of the protein surface, some with defined head groups of the sugar-containing detergents used in the purification and others without a resolved head group. Interestingly, the sites occupied by lipids and detergents in the RsCcO structure were found to overlay precisely with positions occupied by lipid or detergent in both *Paracoccus* and bovine CcO. Analysis of the residues that created the grooves holding the alkyl chains also showed a high level of conservation in all three structures [18], emphasizing the significance of the lipid binding sites. Such conserved sites have also been noted in other membrane proteins [19–22]. What these specifically-bound lipids contribute to the structural and functional properties of intrinsic membrane proteins remains to be clarified. A possibility is that they could act as flexible caulking between subunits and helices, shielding internal water channels while allowing conformational change. In fact, both proton uptake pathways, D and K, when inhibited by removal of a carboxyl at their entrance, can be chemically rescued by addition of μmolar levels of lipidic molecules with a carboxyl group, indicating a lipid binding site in close proximity [8,23,24].

Another interesting facet of crystallographically-defined, specific lipid binding sites is the observation that certain detergents, particularly sugar-based detergents, are capable of substituting for lipid molecules in these sites. The positioning of the sugar head groups indicates that they are particularly good substitutes because they stack and hydrogen-bond effectively with the aromatic residues that are concentrated in the region of the protein at the membrane interface [17]. This stable interaction may account for their unusual success in purifying [25,26] and crystallizing membrane proteins [27].

## 3. A conserved steroid binding site

Of even greater significance with respect to the function and regulation of CcO is the discovery that one of the steroid/bile salt binding sites first recognized in the bovine crystal structure [28] and attributed to possible nucleotide binding, is also conserved in the bacterial enzyme [24]. In both cases, bile salts bound in this site appear to influence the activity of CcO by interacting with a carboxyl residue ($E101_{II}$ in Rs, $E62_{II}$ in bovine) at the entrance to a key proton uptake route, the K pathway (Fig. 3).

Initial clues about the existence of this site in RsCcO came from studies of a mutant in which the conserved carboxyl, $E101_{II}$, is replaced with an

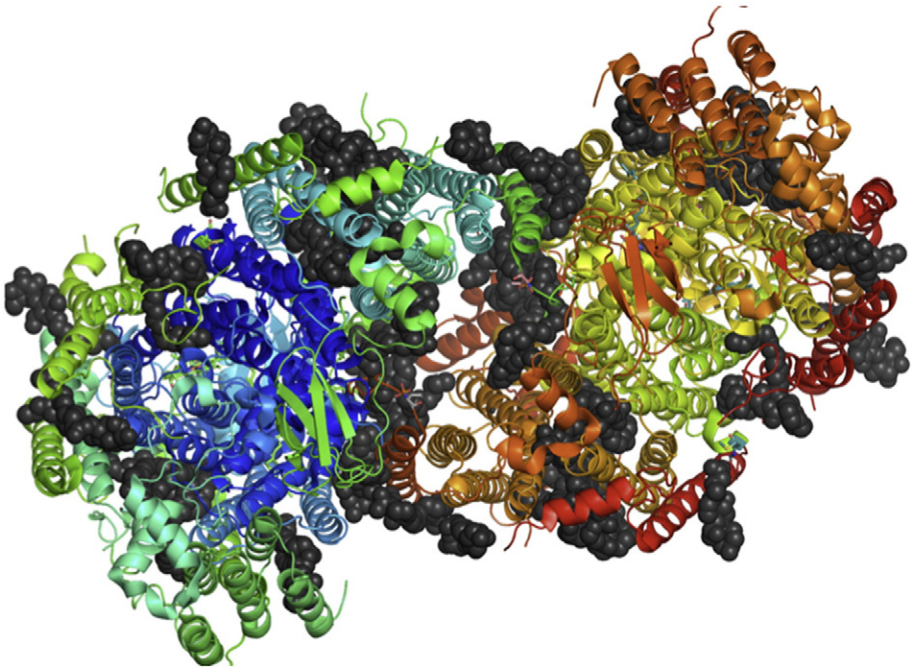

**Fig. 2.** Bovine cytochrome c oxidase dimer at 1.8 Å showing associated lipids (dark gray spheres). Drawing created in Pymol from PDB ID: 2DYR.

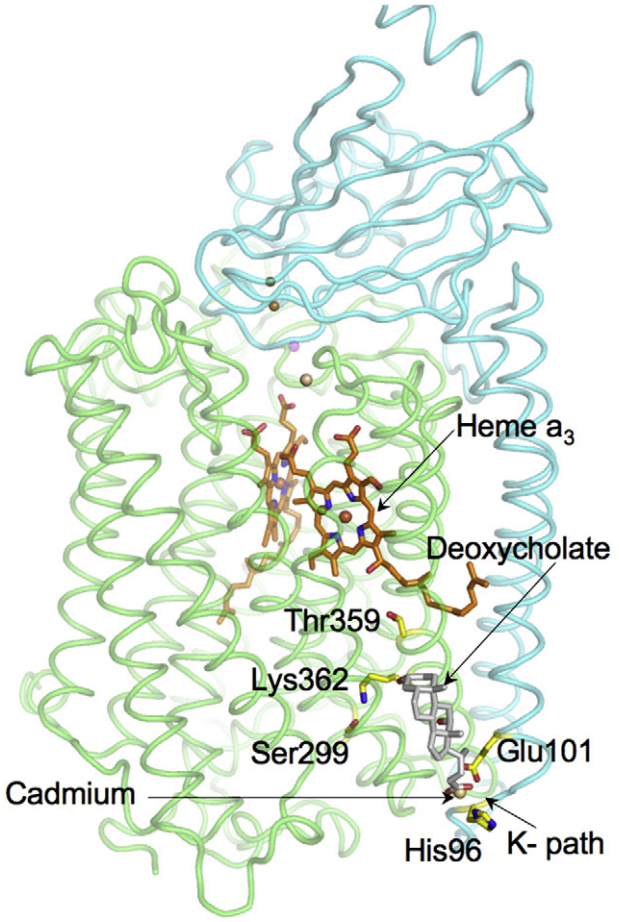


**Fig. 3.** RsCcO two-subunit form (PDB ID: 3DTU) showing the position of deoxycholate (light gray) in proximity to the K proton pathway. Subunit I, green; subunit II, cyan. Labeled residues are those with a role in K path. Cadmium is located at the entrance of the path, bound to E101, His96, deoxycholate and water (not shown). Drawing was created in Pymol by Dr. Ling Qin.

alanine, resulting in a strongly inhibited form (5% WT activity). Certain bile salts at μM levels were found to rescue the mutant, increasing its activity by more than 10 fold, to 50% of WT [23]. The most powerful chemical rescue was provided by deoxycholate, and a crystal structure of RsCcO determined in its presence resolved a single deoxycholate with its carboxyl group in close proximity to that of $E101_{II}$ (PDB ID: 3DTU), as predicted from its activating ability.

An unexpected finding, not predicted from the activation effects on the K-path, was that deoxycholate in RsCcO occupied a site that closely aligned with that of a cholate molecule in the bovine CcO structure [24]. This cholate site was previously noted to be associated with subunit 6a of bovine CcO at the interface of the dimer, rather than with subunits I and II of the alternate monomer, but in fact both steroids show a strong interaction with the homologous carboxyls at the mouth of the K channel (Fig. 4). While it has long been known that certain detergents, including bile salts and Triton X100, inhibit bovine CcO [29], this new finding suggested that the inhibitory effect of cholate on the bovine enzyme is due to its ability to compromise the K path; a similar conclusion was reached by other investigators studying inhibition of bovine CcO by Triton X100 [30]. In contrast, the stimulatory effect on the $E101_{II}$ mutant is apparently due to the bile acid's ability to bind and replace the needed carboxyl thus restoring some of the proton uptake capacity of the K-path. The $E101_{II}$A mutant therefore provides as a sensitive assay for ligand binding at the site, allowing the measurement of a compound's ability to activate or inhibit the mutant form, or compete with a known activator or inhibitor.

In the course of studies utilizing this system, it was found that there is a complex competition between ligands at the site. Notably, the level of activity of the $E101_{II}$A mutant is influenced not only by carboxyl containing compounds but also by certain detergents used for routine assay, even at the low concentrations that support maximal activity of wildtype (e.g. 0.06% dodecylmaltoside). The results indicate that the binding pocket in the $E101_{II}$A mutant has high affinity for several detergents that, when bound, increase the blockage of the K path beyond the mutation itself; when these non-ionic detergents are displaced by bile salts or other molecules that provide a carboxyl, activity is partially restored. This detergent/ligand competition does not appear to affect so markedly the wildtype RsCcO, presumably because the pocket is less hydrophobic in the wildtype due to the strategic placement of the conserved carboxyl. The findings raise questions about which properties of the K path entrance region are most important in controlling proton uptake, the carboxyl itself or the ability of the carboxyl to create a more hydrophilic environment that allows water to be organized in the entrance region and prevents hydrophobic ligands from occupying it. The specificity of the K path entrance pocket for binding certain lipidic ligands may also explain conflicting experimental findings regarding the effects of mutations in this region [19,31], likely due to different experimental or purification conditions, or different structural properties of various CcO forms.

A number of compounds appear to compete at the steroid binding site, as evidenced by their effects on the $E101_{II}$A mutant. Affinities in the micromolar concentration range have been determined for several bile salts, cholesterol-hemisuccinate, protoporphyrin IX, bilirubin, phytanic acid, arachidonic acid and some detergents [32]. It is important to note that the cholate binding sites in the bovine CcO have been postulated to represent regulatory nucleotide binding sites [33]; however, we were unable to demonstrate experimentally an effect of nucleotides in our system under a variety of conditions including long preincubation, even though computational modeling of the site supports that possibility.

In general, stimulation of the $E101_{II}$A mutant occurs when the effector molecule has hydrophobic character and certain structural features that allow it to bind, as well as a carboxyl group or some ability

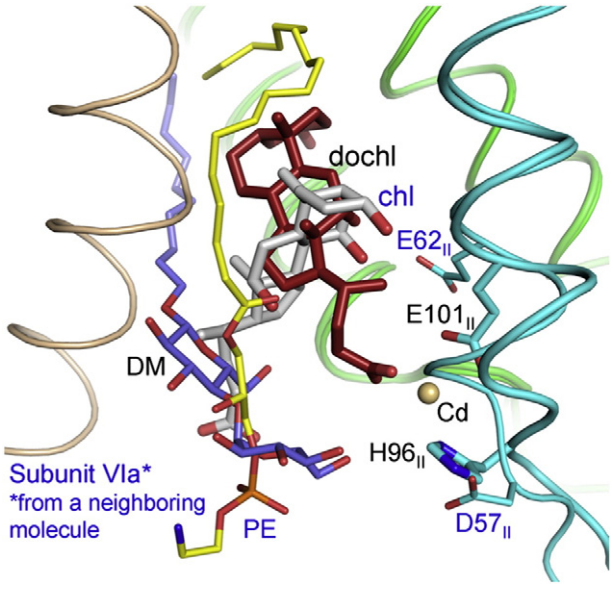


**Fig. 4.** Overlay of bovine CcO (PDB ID: 2DYR) and RsCcO (PDB ID: 3DTU) showing proximity of binding sites for cholate chl, gray sticks) and deoxycholate (dochl, red sticks). Associated lipids are shown: phosphatidyl ethanolamine (PE) in bovine (yellow) and decylmaltoside (DM) in *Rhodobacter* (purple). Subunit VIa from the other monomer in the bovine dimer is shown (wheat). Subunit I (green). Subunit II (cyan). Drawing created in Pymol by Dr. Ling Qin.

to facilitate proton uptake into the K path, such as organizing water. Inhibition occurs when the compound can bind but interferes with proton uptake. Striking in this regard is the fact that, in all the crystal structures obtained of the native CcO from bacteria or bovine sources, neither detergent nor lipid is resolved in the steroid site, only water or cholate or deoxycholate when present. This may be an indication that the site is designed to be open to allow proton access via water chains. Competition between activators and inhibitors of $E101_{II}A$ suggest that they are binding at the same site as the bile salts [32], but the physiological significance of any of these molecules and the precise nature of their influence on protein conformation or water organization in or near the K-path remains unclear. One clue to possible clinical importance comes from a report [34] that bilirubin, at levels found in neonatal jaundice, has a direct inhibitory effect on neuronal CcO, an effect that is counteracted by bile salts. Since these are both competing ligands at the K-path steroid site, it is tempting to think that this might be the site of interaction responsible for the observed cellular response.

Computational analysis and crystallographic studies are currently aimed at understanding the molecular details of the interactions involved and identifying other ligands, including nucleotides, that may be able to bind at the site and exert physiological regulatory effects.

## 4. Conformational change and conserved water

A prevalent view of the mechanism of CcO is that proton pumping does not depend on any significant conformational changes beyond rearrangements of side chain positions and water molecules [35–37]. In contrast, in the case of the bovine CcO, structural changes in the vicinity of heme a have been observed in reduced crystals and interpreted to be involved in a pumping mechanism associated with a proton pathway designated the H path [38], but neither the pathway [39] nor the conformational changes were apparent in the bacterial enzyme [22,40]. However, recent crystal structures of the *Rhodobacter* CcO in dithionite-reduced compared to oxidized states, have revealed conformational changes that differ in some respects from those seen in bovine CcO, involving a shift in position of heme $a_3$ and of closely associated regions of the structure [2,41].

These new structures provide evidence for involvement of conformational change in a gating mechanism that allows the opening of the K path for substrate protons to be correlated with closing of the D path that conveys pumped protons. In the oxidized structure, entrance of protons from the K path into the heme $a_3$-$Cu_B$ active site appears to be blocked by a tight hydrogen bond (2.6 Å) between the OH of the $Cu_B$ His-Tyr-ligand and the OH of the farnesyl group of heme $a_3$ (Fig. 5). In the reduced RsCcO structure, movement of the heme $a_3$ porphyrin ring away from $Cu_B$ breaks the bond and creates a larger opening (4.1 Å) at the bottom of the active site. Formation of a chain of waters leading into the heme $a_3$–$Cu_B$ crevice is seen in the reduced crystal, suggesting a path for proton uptake from the K pathway below. A shift in helix VIII and the K-path residue T359 appears to be associated with the water rearrangement. In addition, the reduced crystal shows the loss of a water from a position close to $Cu_B$ ([2]; W301) that has been invoked as part of a connection between the top of the D path and the active site [4]. This change suggests a mechanism to allow alternating access of protons from K and D paths, a necessary feature of the CcO pumping mechanism.

This finding is particularly interesting because it fits with a previously proposed mechanism for proton pumping [13] that predicts that reduction of $Cu_B$ will trigger the loss of the tight hydrogen bond at the bottom of the heme $a_3$ crevice, as actually observed in the reduced crystal structure. In the model, reduction of $Cu_B$ followed by movement of a proton from the D-path to the vicinity of its His334 ligand, weakened the bonding of $Cu_B$ to the His-Tyr ligand, resulting in internal electronic rearrangements within this unique cross-linked pair. The internal rearrangement

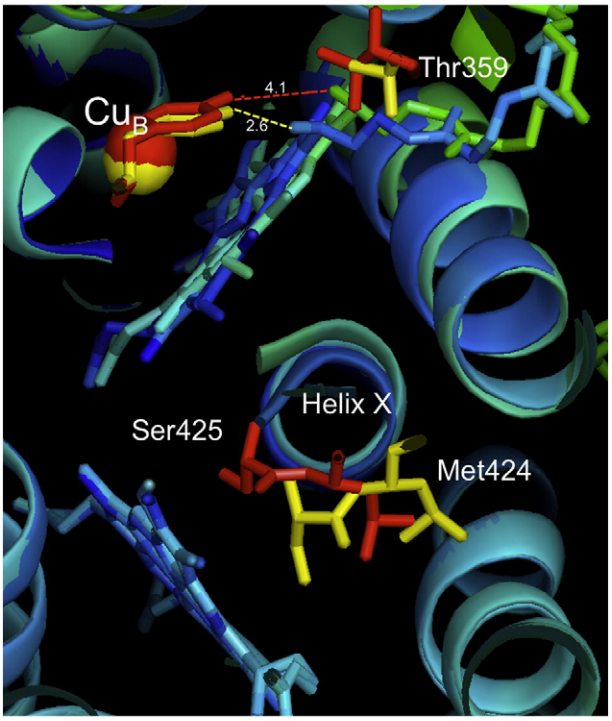


**Fig. 5.** Overlay of crystal structures of oxidized (PDB ID: 2GMS) and reduced (PDB ID: 3FYE) forms of two-subunit RsCcO. Red indicates reduced and yellow indicates oxidized, for $Cu_B$, Tyr288, Thr359, Met424 and Ser425 where significant movement is observed. Hydrogen bond lengths between the Tyr288-OH and the heme $a_3$ farnesyl-OH are indicated by dotted lines, 2.6 Å (yellow, oxidized) and 4.1 Å (red, reduced). Heme $a_3$ is cyan and green (reduced), blue (oxidized). The central helix X is rendered in turquoise (oxidized) dark blue (reduced). The drawing is a cross-section viewed from the inside of the membrane, created in Pymol.

in turn caused the His-Tyr-OH hydrogen bond with the farnesyl-OH to be broken, opening access to water molecules and K-path protons [13]. Aside from giving a new perspective on the role of this unusual $Cu_B$ ligand as an important gate for the K path, the model and the reduced structure emphasize the critical function of water positioning in forming and breaking proton paths.

It is important to note that these conformational changes are also observed in crystals of the four-subunit RsCcO, and so are not dependent on the crystal type [2]. In addition, the effects of reduction are reversible: when a crystal is reduced and then allowed to reoxidize, it returns to the oxidize conformation. However there are other concerns regarding the significance of the structural changes observed in Rs and bovine CcO, since they differ in several respects.

A concern regarding all crystal structures of redox active metalloproteins is the known ability of high intensity X-rays to release electrons from the protein and solvent, so that the metal centers in an oxidized crystal may become reduced during data collection [2,22,41–45]. To address this question we have examined the spectra of frozen RsCcO crystals during X-ray exposure [41] and found the noteworthy result that, although heme a appears to be rapidly reduced even at 70 K, the observed spectrum is not that of the native reduced CcO, but shows a split peak at 589 and 610 nm in the alpha-band region. When the crystal is briefly annealed (warmed for a few seconds and refrozen) the spectrum then becomes that of a normal reduced heme a at 606 nm (Fig. 6). This finding suggests that the frozen crystal retains the protein structure of the oxidized form even when the metal center is reduced, leading to a “strained” configuration at the metal center giving rise to the altered spectrum. This finding explains why conformational differences can be seen between reduced and oxidized forms, but could imply that we are not seeing the full extent of change. An even more rigorous test was applied by Yoshikawa and colleagues working with the bovine CcO [46]. Using 400 crystals

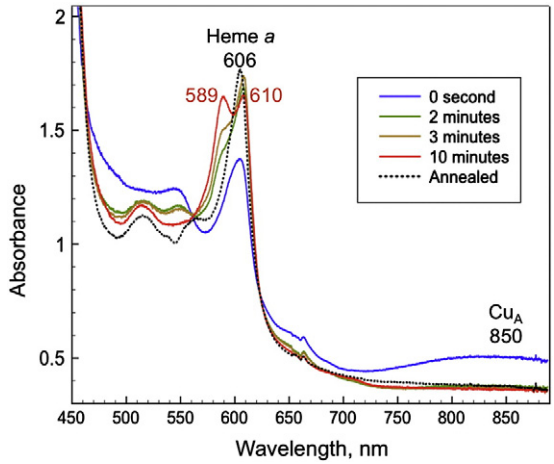


**Fig. 6.** Spectra of crystals of wildtype RsCcO during X-ray irradiation at 100 K. Time of exposure and peak wavelengths are indicated on graph. A double peak at 589 and 610 nm is shown to develop during 10 minute radiation. A peak at 606 nm is observed after annealing. Similar results were obtained with wildtype four-subunit crystals. Drawing created in PLOT.

and a few seconds of exposure each, they showed that the same oxidized structure was obtained as when they collected data for much longer periods on one crystal.

It is important to note that there are some commonalities between the Rs and bovine reduced structures: the bovine CcO shows the same significant movement of residues in helix X between the two hemes as RsCcO (Fig. 5) and a similar, though smaller, shift in the heme $a_3$ porphyrin ring in the CN/reduced form [47]. But the structures differ in the degree of heme $a_3$ movement and the resultant opening of the bottom of the active site crevice, as well as the movement of helix VIII. The RsCcO also shows no changes in the vicinity of the equivalent of the H pathway (close to the position of Asp 51, bovine numbering). These inconsistencies and the lack of change found in *Paracoccus* CcO [40] and the thermophilic $ba_3$ oxidase [22] remain to be resolved, but could well be the result of differences in completeness of reduction and deoxygenation, differences in water content, and/or restrictions imposed by dissimilar crystal contacts.

## 5. Conclusions

The mechanism and regulation of coupling between electron transfer and proton pumping in cytochrome c oxidase remains a complex unsolved problem of major physiological importance, given the central role of this enzyme in aerobic energy metabolism. The ability to obtain high-resolution structures in which lipid and water positions can be observed and conformational changes dependent on redox state detected, is providing new insight into possible proton gating modes and regulatory ligands. The crystallographic capture of new catalytic intermediates and the discovery of new crystal forms, with better resolved water and lipidic ligands, will be key to further illuminating this subtle and intricate process.

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