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Role of conformational change and K-path ligands in controlling cytochrome *c* oxidase activity

Jian Liu, Carrie Hiser, and Shelagh Ferguson-Miller

Department of Biochemistry and Molecular Biology, Michigan State University, 603 Wilson Rd, East Lansing, MI 48824, U.S.A

Abstract

Given the central role of cytochrome *c* oxidase (CcO) in health and disease, it is an increasingly important question as to how the activity and efficiency of this key enzyme are regulated to respond to a variety of metabolic states. The present paper summarizes evidence for two modes of regulation of activity: first, by redox-induced conformational changes involving the K-proton uptake path; and secondly, by ligand binding to a conserved site immediately adjacent to the entrance of the K-path that leads to the active site. Both these phenomena highlight the importance of the K-path in control of CcO. The redox-induced structural changes are seen in both the two-subunit and a new four-subunit crystal structure of bacterial CcO and suggest a gating mechanism to control access of protons to the active site. A conserved ligand-binding site, first discovered as a bile salt/steroid site in bacterial and mammalian oxidases, is observed to bind an array of ligands, including nucleotides, detergents, and other amphipathic molecules. Highly variable effects on activity, seen for these ligands and mutations at the K-path entrance, can be explained by differing abilities to inhibit or stimulate K-path proton uptake by preventing or allowing water organization. A new mutant form in which the K-path is blocked by substituting the conserved carboxyl with a tryptophan clarifies the singularity of the K-path entrance site. Further study in eukaryotic systems will determine the physiological significance and pharmacological potential of ligand binding and conformational change in CcO.

Introduction

The mechanism by which CcO (cytochrome *c* oxidase) reduces oxygen to water and conserves the free energy released by this reaction in the form of a proton gradient has been the subject of intensive investigation for many years, resulting in an increasingly nuanced understanding of the chemical and structural elements involved. There is general agreement on the sequence and nature of the steps in the oxygen reduction reaction, but how these steps are precisely linked with proton transfer events is by no means clear. Also lacking is an understanding of how the activity of this essential enzyme is regulated in response to a variety of metabolic challenges. But new insight is provided by the observation of redox-linked conformational change and the discovery of a conserved ligand-binding domain.

Correspondence: Shelagh Ferguson-Miller (fergus20@msu.edu).

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Kadenbach and colleagues were among the first to address the issue of how CcO might contribute to, and be regulated by, metabolic conditions [1–3]. They recognized that the well-known phenomenon of ‘respiratory control’, mediated by changes in membrane potential and/or the pH gradient, could not adequately account for the critical role of mitochondria and particularly cytochrome oxidase in the fine-tuning of energy supply to support complex cellular functions. In seminal studies on mammalian CcO, Kadenbach and co-workers demonstrated the regulation of activity and efficiency by many physiological ligands, including ATP, ADP, and thyroxine [1–4]. They further recognized that the enzyme was subject to phosphorylation, an early observation that is now supported by a great deal of data indicating numerous phosphorylation [5] and acetylation sites in mammalian CcO [6,7]. Equally significant was the discovery by the Kadenbach group that many isoforms of the nuclear encoded subunits existed and were differentially expressed in different tissues [8,9]. The physiological roles of these subunits and their isoforms remain to be fully defined. It is evident, however, that there are complicated regulatory processes at work, whose mechanism and significance we have only begun to comprehend.

Recently, we have added to this array of regulatory mechanisms by the fortuitous discovery of a ligand-binding site close to the interior entrance to the most conserved and essential proton uptake pathway, the K-path (Figure 1). The site was initially identified as a position where bile salts could bind and rescue a K-path mutant that was strongly inhibited by the deletion of a highly conserved carboxyl, E101 (*Rhodobacter sphaeroides* numbering throughout). The position of the site at the entrance of the K-path was confirmed in crystal structures of the bacterial enzyme [10]. Surprisingly, the same location was noted to be occupied by a bile salt in the crystallized bovine CcO (bovCcO), an enzyme that is normally purified and crystallized in the presence of the bile salt cholate. It is well established that cholate and deoxycholate are strong, but reversible, inhibitors of the bovine enzyme [11–13], yet the site of inhibition was not recognized. When the specificity of the site was studied in more detail [14], a variety of amphipathic compounds were found to bind and alter the activities of mutant and wild-type bacterial CcO in a manner consistent with a direct effect on the K-path. But a puzzling observation by us and others [15,16] was that the inhibitory/activating effects of various mutants and ligands were only partial, suggesting that there was more than one way for protons to access the K-path. However, a recently characterized mutant at the K-path entrance, substituting the conserved carboxylate residue with a tryptophan, results in essentially complete inhibition of the oxidase, providing more conclusive evidence of the structural requirements for proton entry into the K-path.

Evidence that the well-conserved K-path is a central player in the activity and regulation of CcO also comes from crystallographically observed conformational change. When oxidized crystals of *R. sphaeroides* CcO (*RsCcO*) are reduced by dithionite and rapidly frozen, a significant change in conformation is seen, involving heme a₃, helix VIII, and residues in helix X between the two hemes [17]. Some of the same alterations are seen in the bovCcO crystals [18,19] but not all are identical with those seen in the bacterial enzyme.

In the present study, we summarize the data that point to the importance of the K-path in the regulation of CcO activity, emphasizing the potential mechanistic implications of ligand effects and redox-linked structural changes. Our current hypothesis is that conformational

change, particularly as it affects water organization, is a major player in determining proton conduction pathways and gating thereof, and further that the ligand-binding site at the entrance of the K-path represents a physiologically important regulatory domain.

Crystallographically determined conformational change

Technical issues

It is difficult to evaluate the meaning of changes in structure that occur within the confines of a crystal where packing can be a restraint. However, membrane proteins may have an advantage in their being encased in detergent and lipid that can potentially allow more flexibility — but with the negative effect of making it more difficult to obtain high-resolution structures. Membrane protein crystals also have a relatively high solvent to protein ratio, often up to 70% solvent. In the case of CcO, there are further issues to consider since it is a metalloprotein whose metal centers can be reduced by electrons released in the sample during exposure to X-rays. This concern initially called into question whether any of the crystal structures showed a fully oxidized enzyme. This concern was well addressed by Yoshikawa and colleagues who combined diffraction data from many crystals, each with only minimal (seconds) exposure to X-rays, and obtained the same structure as when longer exposure was employed [20]. It was also addressed by spectral studies during X-ray irradiation of the *RsCcO* crystals [21], which indicated that although heme a and Cu_A became reduced during several minutes of X-ray exposure, the frozen protein was not converted into a native reduced form until the crystal was briefly thawed, implying that the protein structure was not altered in spite of the redox change in the metal.

Equally critical issues pertain to capturing the fully reduced structure. Because CcO has such a high affinity for oxygen and because of the high optical density of the Soret band (400 nm region) where heme a₃ reduction can be quantified, it is difficult to determine whether the whole crystal is reduced. Nevertheless, many crystal forms of CcO show some or all [21] of the conformational alterations that are observed in the wild-type *RsCcO*.

Observed changes and mechanistic implications

In the oxidized forms of both the two-subunit (PDB 2GSM) and four-subunit (PDB 1M56) *RsCcO* crystals, the entry of the K-path to the active site is closed by a strong hydrogen bond (2.6/2.7 Å) between the hydroxyl of the His-Tyr ligand of Cu_B and the hydroxyl of the farnesyl-heme a₃ side chain. In contrast, in the reduced forms of both the two-subunit (PDB 3FYE) and four-subunit (PDB 5WEH) *RsCcO*, the K-path connection to the heme a₃–Cu_B active center is open, as evidenced by increased length of the hydrogen bond (lengthened from 2.7 to 4.7 Å). The altered bonds are indicated by shorter and longer yellow dashed lines in Figure 2. The formation of a water chain into the site is not shown in Figure 2, but is clearly seen in the reduced *RsCcO* structure (PDB 3FYE) [17]. The loss of this strong bond in the reduced form appears to be the basis for the observed movement of heme a₃ and helix VIII (Figure 3), along with major shifts of side chains in helix X, affected by heme a₃ and also seen in the boVCcO [18]. A model was previously described [22], suggesting an explanation for what is now seen crystallographically. It was proposed in this model that the first two-electron transfer events into the fully oxidized protein (each preceded by a D-path

proton uptake to a proton-loading site) caused altered electron distribution in the ligands of Cu_B. In particular, electron rearrangements in the His-Tyr ligand led to temporary loss of the strong hydrogen bond to the farnesyl hydroxyl. A K-path proton could then access the active site via a newly formed water chain, as seen in the crystal structure [17]. Another model has recently been put forward based on computational analysis that also emphasizes the importance of the His-Tyr cross-linked species K-path gating [23].

It is interesting to note that deuterium exchange studies on *RsCcO* show an increased accessibility of a portion of helix VIII (colored yellow in Figure 1 and boxed in Figure 3) upon two-electron reduction [24], a finding that is consistent with the movement in this region seen in the reduced, compared with the oxidized, structure (Figure 3). And indeed, computational analysis of flexibility (PROFLEX*; [25]) shows helix VIII to be one of the regions to exhibit the most flexibility.

In the reduced *bovCcO* [18,26], changes in the vicinity of heme a are more pronounced than in the bacterial enzyme and have been interpreted to indicate an alternative proton pathway in that area (the H-path). The most recent, even higher resolution (1.6 Å), crystals of *bovCcO* (e.g. PDB 5B1B) show most of the redox-induced structural differences in the regions above, below, and beside heme a, but little change in the heme a₃ position itself, unlike in the bacterial oxidase. Although the authors took thorough precautions, it is still the case that it is almost impossible to judge the degree of reduction of any oxidase crystals, especially large ones, because the strong absorption in the ~400 nm region, where the redox state of heme a₃ can be interrogated, exceeds the ability to measure. The difference between the findings with bovine and bacterial oxidases may simply relate to the size of the crystals (much smaller in the case of bacteria) and hence the ability to approach full reduction. It is interesting that the reduced cyanide-inhibited form of *bovCcO*, in which oxygen binding is prevented and may allow the reduced state to be maintained, as originally reported by Yoshikawa and colleagues (PDB 3AG4), did show some movement of the heme a₃ plane. It is important to note that all the *bovCcO* structures are prepared from a cholate-containing enzyme, where electron transfer from heme a to a₃ is inhibited by blockage of the K-path.

To be an effective gating mechanism, opening and closing of the K-path needs to be coordinated with proton uptake in the D-path. When the K-path opens (immediately after reduction of O to E, or E to R), the D-path should temporarily close. In fact, the crystal structure of the reduced *RsCcO* shows loss of a critical water in the region where proton transfer to the active site from the D-path is expected to occur [17].

Whether any of the crystallographically observed conformations relate to physiological states remains to be determined. But importantly, conformational changes captured in ordered crystal states are expected to underestimate actual changes, which might be better observed by cryoEM studies on membrane proteins (as seen with bacteriorhodopsin [27]) or by free electron laser studies at room temperature [28].

But does control of oxidase activity by regulating the K-path make physiological sense? If the aim is to control oxidase by mechanisms other than regulation by the membrane potential, then the K-path is arguably a good option. Blockage of the K-path not only

inhibits activity but does so by inhibiting reduction of the heme a_3 /Cu_B active site, since K-path protons are required to support the fully oxidized (O) to the two-electron reduced (R) conversion, which in turn is required for oxygen binding and oxygen chemistry to proceed. Catalytic turnover is necessary for suicide inactivation, by a yet-to-be-established mechanism [29–31]. But importantly, it is well documented that limiting proton uptake by the D-path, but *not* the K-path, strongly increases the probability of suicide inactivation [31]. Thus, slowing the K-path to control CcO activity would be advantageous since it favors an oxidized active site less prone to damage.

Ligand binding at K-path entrance

Evidence of chemical rescue

The discovery that certain ligands could rescue, or further inhibit, the activity of an *RsCcO* mutant close to the interior entrance to the K-path was unexpected. Both the residue and the pathway are highly conserved and essential to activity. Previous studies by ourselves and others [15,16] had shown that many mutants that eliminated the carboxyl at this position (*RsCcO* E101_{II}) caused significant but highly variable degrees of inhibition. Surprisingly, we found that the variability related to the nature and concentration of detergent used in the assay [14]. In addition, in spite of the known inhibitory effects of the bile salt detergents on the *bovCcO* [13], these strong anionic carboxylic detergents were found to be powerful *activators* of E101A, as were other detergents and compounds with carboxylic groups. But many nonionic detergents such as Triton X-100 and dodecyl maltoside were inhibitory even at low levels of 0.06%. The simplest interpretation of these findings was that the activating ligands were rescuing the mutation by supplying the missing carboxyl in such a position as to permit the K-path to function in proton uptake, while the inhibitory compounds were binding in a way to further block the K-path entrance.

Crystallographic evidence

A crystal structure of *RsCcO*, determined in the presence of deoxycholate, revealed a deoxycholate molecule bound very close to the K-path entrance (Figures 1 and 4) with its carboxyl precisely located where it would be expected to be able to rescue the loss of E101 [10]. In this structure, a cadmium ion is interacting at the same site, where it is known to inhibit [32].

The effects of the other ligands that rescued or further inhibited the mutant were found to be competitive with the bile salts and to have different effects on the mutant compared with the wild-type enzyme. The steroid-based compounds did not affect the activity of a D-path mutant, D132A, indicating that the effects were specific for the K-path. On the basis of these observations, it was concluded that many of the ligands observed to alter the mutant activity were exerting their effects by binding to the same region as the bile salts. As previously mentioned, in all the *bovCcO* crystal structures so far published, this same region is occupied by a cholate molecule. That molecule interacts with the same conserved carboxyl (E62_{II}) at the entrance of the K-path. These findings strongly suggest that the observed inhibitory effects of bile salt detergents on the *bovCcO* are also due to blockage of the K-path. Other studies on the detergent inhibition of *bovCcO* support this conclusion [33].

A new mutant, E101W

Different amino acid substitutions at E101, even though they all eliminate the carboxyl, have been found to have greater or lesser degrees of inhibition, raising the question of how important the carboxyl itself really is, and whether there are other routes into the K-path that do not require the carboxyl [15]. It had been suggested that an additional role for the carboxyl of E101 is to help organize an effective water chain to support proton transfer [34,35]. Thus, not only removal of the carboxyl, but also disruption of a water chain would be required to fully inhibit the K-path. With this in mind, we created the E101W mutant. The bulky hydrophobic side chain, when modeled in the 101 position, occupies the entrance to the K-path and displaces many water molecules. And indeed, the mutant form has <2% the activity of the wild type (Figure 5) and, unlike E101A, its activity is neither affected by detergent concentration nor rescued by ligands such as one of the most potent activators of E101A, docosahexaenoic acid. These preliminary data are convincing that the entry to the K-path is probably unique under normal conditions, and the variety of effects of mutations and ligands is due to their differing ability to disrupt or help organize a water chain that will support proton uptake.

A wide variety of amphipathic compounds have been identified already as ligands of this conserved binding domain [14], but the most important questions still remain: what is its physiological significance? And, what ligands are biologically important? With the aim of obtaining further clues to answer these questions, we applied various computational methods [36] to screening for compounds that could potentially occupy this site — taking the *Rhodobacter* crystal structure as a starting point for analysis, and using a three-pronged computational approach involving comparison of ligand shape and electrostatics, comparison of binding-site features, and screening by docking of potential ligands. The results of these studies showed interesting agreement, indicating commonalities of steroid, nucleotide, and porphyrin-binding specificity, consistent with previous docking studies [14]. Although the questions are not yet answered, the scope of the search for physiological and pharmacological ligands is broadened, and a relationship to earlier studies of regulation by nucleotides is suggested [1–4].

Conclusions

Conformational changes, suggesting a gating mechanism for the K-proton pathway and ligand binding to a conserved site adjacent to its entrance, argue for the K-path as an important site of regulation of CcO, with the advantage that its inhibition is not associated with suicide inactivation. Different effects of mutation of the conserved carboxyl at the mouth of the K-path and of an array of ligands that bind to that region can be explained by their differing abilities to allow the formation of a water chain into the K-path. This conclusion is supported by a new mutant form in which the carboxyl is substituted with a tryptophan and the K-path is fully blocked and not responsive to chemical rescue. The large rigid tryptophan side chain apparently can displace water and create an environment not conducive to proton transfer or to carboxylate ligand binding.

The significance of the observed conformational changes and ligand interactions remains to be fully explored, but it is clear that the K-pathway should be a focus as we proceed with

efforts to understand how CcO and the respiratory chain are controlled to meet metabolic demands. Current crystallographic methods of capturing conformational states have limitations, but the advent of higher-resolution protein tomography by cryoEM, as well as free electron laser capabilities and higher-intensity synchrotron beams, will provide new more powerful approaches. Similarly, the metabolic role of small-molecule ligands of CcO and how they synergize with posttranslational modifications such as phosphorylation and acetylation may be further elucidated by the mutational technology of CRISPR-CAS9, which has the potential to facilitate modification of mitochondrially encoded genes in eukaryotic systems.

Abbreviations

bovCcO	bovine CcO
CcO	cytochrome <i>c</i> oxidase
RsCcO	<i>Rhodobacter sphaeroides</i> CcO

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Figure 1. Ribbon representation of *RsCcO* (PDB 3DTU) showing proton uptake pathways, D and K, entrance residues (D132, E101, green), and position of the ligand-binding site occupied in this crystal structure by deoxycholate (DXC, black). Yellow indicates regions observed to undergo some conformational change when the protein is reduced (see Figures 2 and 3).

Figure 2. Overlay of reduced (PDB 5WEH) and oxidized (PDB 1M56) crystal structures of the four-subunit forms of *RsCcO*

Yellow dashed lines show the position of the hydrogen bond between the His-Tyr hydroxyl and the farnesyl hydroxyl in the oxidized heme a_3 (red sticks; 2.7 Å length) and reduced heme a_3 (white sticks; 4.7 Å length). Helices of the reduced *RsCcO* are green, and the oxidized are cyan. The His-Tyr cross-linked ligand to Cu_B (orange) is shown in cyan in the oxidized form and in white in the reduced form.

Figure 3. Overlay of reduced (3FYE; hemes pink, helices green) and oxidized (2GSM; hemes/helices yellow) two-subunit *RsCcO* showing overall structure and regions of conformational change (white boxes)

White oval above heme a is the area found to be altered in the *bovCcO* but not in *RsCcO*; the region between heme a and heme a₃ also showed significant movement of side chains on helix X in both *Rs* and *bovCcO*, but is not visible in this diagram.

Figure 4. *RsCcO* deoxycholate-binding site with conserved residues and bound water molecules (PDB 3DTU)

The steroid-binding residues are colored by degree of conservation in CcO sequences as calculated in Hiser et al. [14], where residues with >75, 50–75, or <50% conservation are colored dark blue, medium blue, and gray, respectively. The structure includes the bound deoxycholate (green sticks), resolved water molecules (red spheres), and a cadmium ion (orange sphere). Reproduced from Hiser et al. [14].

Figure 5. Oxygen consumption activities of K-path E101 mutants, E101A and E101W, with varying levels of the ligand, docosahexaenoic acid (DHA)

Assays used a Clark-type electrode at 25°C in 100 mM HEPES buffer (pH 7.4) containing 0.06% lauryl maltoside detergent and a saturating level of cytochrome *c* substrate (30 μM) as in ref. [14] and DHA at 0–10 μM. Activity was calculated as electrons per second per CcO molecule as described in ref. [14]. While E101A is strongly stimulated by DHA at μM levels, E101W is little affected and shows activity <2% of the wild type (which remains constant at 1200 s⁻¹ over this range of DHA).