Published in final edited form as:

Biochim Biophys Acta. 2018 September ; 1859(9): 725–733. doi:10.1016/j.bbabo.2018.03.017.

The K-path entrance in cytochrome *c* oxidase is defined by mutation of E101 and controlled by an adjacent ligand binding domain[★]

Carrie Hiser, Jian Liu, and Shelagh Ferguson-Miller^{*}

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, United States

Abstract

Three mutant forms of *Rhodobacter sphaeroides* cytochrome *c* oxidase (RsCcO) were created to test for multiple K-path entry sites (E101W), the existence of an “upper ligand site” (M350 W), and the nature and binding specificity of the “lower ligand site” (P315W/E101A) in the region of a crystallographically-defined deoxyholate at the K-path entrance. The effects of inhibitory and stimulatory detergents (dodecyl maltoside and Tween20) on these mutants are presented, as well as competition with other ligands, including the potentially physiologically relevant ligands cholesterol and retinoic acid. Ligands are shown to be able to compete with natural lipids to affect the activity of membrane-bound RsCcO. Results point to a single K-path entrance site at E101, with a single ligand binding pocket proximal to the entrance. The affinity of this pocket for amphipathic ligands is enhanced by removal of the E101 carboxyl and blocked by substituting a tryptophan in this area. A new crystal structure of the E101A mutant of RsCcO is presented that illustrates the structural basis of these results, showing that the loss of the E101 carboxyl creates a more hydrophobic groove consistent with altered ligand affinities.

Keywords

Cytochrome *c* oxidase; K-path; Steroids; Detergents; Retinoic acid; Lipids

1. Introduction

Cytochrome *c* oxidase (CcO)¹ is the terminal electron acceptor in the electron transport chain in the mitochondrial membrane. As such, it plays a critical role in metabolism, but in spite of much effort (reviewed in [1–3]), little is established regarding regulation of CcO activity. This is in part due to the genetic and structural complexity of the eukaryotic enzyme. Mammalian cytochrome *c* oxidase (CcO) has 13 unique subunits, 3 of which are

[★]This article is part of a Special Issue entitled 20th European Bioenergetics Conference, edited by László Zimányi and László Tretter

^{*}Corresponding author. hiser@msu.edu (C. Hiser), liujian1@msu.edu (J. Liu), fergus20@msu.edu (S. Ferguson-Miller).

Conflict of interests

There are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in online version.

encoded in the mitochondrial genome which is not readily accessible for genetic modification [4]. Fortunately, there are several bacterial model systems amenable to mutational analysis of this complex protein. In particular, the simpler CcO from the bacterium *Rhodobacter sphaeroides* (RsCcO) shows high homology with the three largest mitochondrially-encoded core subunits of the mammalian CcO [5].

Protons used in the oxygen reduction chemistry and pumped across the membrane to create an electrochemical gradient are taken up via two well-defined proton pathways, the K- and D-paths (Fig. 1), which are common to both mammalian and bacterial A-type CcOs. Considerable evidence from bacterial studies supports the model that the D-path supplies all of the protons to be pumped and some of the substrate protons to be used for oxygen reduction chemistry, while the K-path is critical for delivery of two substrate protons during initial reduction of the active site before oxygen can bind and react [6–14]. But there has been an ongoing debate as to where the K-path entry point is located. Many mutants of a highly-conserved glutamate in subunit II, E101 (RsCcO numbering is used throughout this paper.), indicated that this residue is a key player in proton entry to the K-path [15]. However, results from RsCcO and the related bacterium *Paracoccus denitrificans* (PdCcO) [16] suggested that E101 may not be the only entrance for K-path proton uptake, based on incomplete inhibition when the glutamate was mutated to different amino acids. To investigate this question further, a mutation of E101 to a tryptophan was created. Preliminary data on its properties [17] indicated essentially complete blockage of the K-path and provided strong evidence that this location is in fact the unique K-path entry. Further studies of this definitive mutant are provided in this paper, leading to a clearer picture of the entrance characteristics.

The importance of this pathway entrance was emphasized by the discovery that a variety of carboxylate compounds (at μM levels) could “chemically rescue” the E101A mutation in RsCcO, suggesting that a ligand binding site existed at the K-path entrance. These ligands were shown to be specific for the K-path and not the D-path [18]. One of these activating molecules, deoxycholate (DOC), was co-crystallized with RsCcO [Protein Data Bank (PDB): 3DTU] [19] with its carboxyl adjacent to E101 where it could replace the missing carboxyl. The significance of these findings was emphasized by the observation that essentially all the crystals of bovine CcO (bovCcO) [e.g., PDB:1OCC [4]] show cholate, a known inhibitor of bovCcO, bound in the same region of the K-path entry point and interacting with the bovine equivalent of E101 (E62_H). These findings showed conservation of this site from bacteria through to mammals, making a strong case for its physiological significance and the need to further define the ligand interactions at this site. Indeed, docking of cholesterol, porphyrin, retinoic acid and nucleotides into this site was computationally predicted [20].

Aside from important physiological implications, this phenomenon of chemical rescue of the E101 mutants provided a sensitive test for ligand binding in the region [18, 19]. Although most of the compounds that stimulated E101A were amphipathic carboxylates, several non-ionic detergents also interacted competitively with the site and modified the effects of other ligands [21]. In particular, the detergent dodecyl maltoside (DDM) strongly inhibited E101A activity, in contrast to its support of maximum activity of WT RsCcO [5] and bovCcO [22,

23]. Different activity levels observed for K-path mutants of RsCcO [15, 18, 19, 21] and PdCcO [16] may be explained by use of different DDM levels in assays. In contrast, Tween20 is a strong activator of RsCcO [21] and bovCcO [24–26].

To account for observed ligand interactions, a two-site model [21] was postulated involving “lower” and “upper” sites. The lower site was defined as the crystallographically-observed binding site near E101 for DOC in RsCcO and cholate in bovCcO. The location of the upper site, which would accommodate the tails of detergents and straight-chain ligands, was proposed to be a groove above the K-path entrance between subunits I and II. This groove is occupied by a partial alkyl chain in RsCcO structures [e.g., PDB ID: 3DTU [19] and 2GSM [27]] and by one fatty acyl chain of cardiolipin in bovCcO [PDB ID: 2DYR] [28]. In one of the crystal structures of PdCcO [PDB: 3HB3] this site is occupied by the alkyl tail of an unusual, upside-down DDM molecule whose head group overlaps with the steroid site [29]. It was hypothesized that if the tails of long-chain ligands bind in this upper site, their head groups could compete with or displace steroid-like ligands that bind in the lower site, consistent with observed ligand effects and with modeling of the positioning of the DDM head group [21].

In this work we report the behavior of three mutant forms designed to test the location of the K-path entrance (E101W), the existence of an “upper ligand site” (M350 W), and the nature and binding specificity of the “lower ligand site” (P315W/E101A) in the region of the crystallographically-defined deoxycholate site (Fig. 1). We investigated the properties of inhibitory (DDM) and stimulatory (Tween20) detergents and competition with other ligands as well as characterizing ligands with potential to be physiologically relevant in the mammalian system: cholesterol and retinoic acid (RA). These results, plus a new RsCcO E101A crystal structure, are consistent with a single K-path entrance site at E101 and with a single ligand binding pocket proximal to the entrance whose affinity for amphipathic ligands is enhanced by removal of the E101 carboxyl and strongly blocked by substituting a tryptophan at this position.

2. Materials and methods

2.1. Chemicals

Anapoe20 (Tween20) and dodecyl maltoside (lauryl maltoside; DDM) were purchased from Anapoe (Maumee, OH). Docosahexaenoic acid (DHA) was purchased from Cayman Chemicals (Ann Arbor, MI). Cholesterol, deoxycholate (DOC), and retinoic acid (RA) were purchased from Sigma. Asolectin (soybean polar lipids) was reported by the supplier (Avanti Polar Lipids, Alabaster, AL) to contain 45.7% phosphatidylcholine, 22.1% phosphatidylethanolamine, 18.4% phosphatidylinositol, 6.9% phosphatidic acid, and 6.9% unspecified phospholipids and pigments as determined by HPLC/ELSD.

2.2. Procedures

Mutants were created by using the QuikChange (Stratagene) mutagenesis procedure and the final plasmids were transferred by conjugation into *Rhodobacter sphaeroides* strain 123 [18] (for the E101W mutation in subunit II and the double mutant of P315W in subunit I

plus E101A in subunit II), 1–4 [27] (for the M350 W mutation in subunit I), or a new strain 2–4 (see Supplemental Materials and Methods) (for E101A for crystallography) as described [30]. *R. sphaeroides* strains overexpressing 37-2 WT RsCcO [30], E101A mutant RsCcO [18], and all mutant RsCcOs created herein were grown and cell membranes were prepared as described [30] and the RsCcO was isolated by metal affinity chromatography as described for crystallography [18]. Incorporation of RsCcO into lipid vesicles (COVs) followed the protocol of Hosler et al. [5].

Oxygen uptake rates were measured with a Clark-type electrode and enzyme activity was calculated as turnover number (TN, electrons per second per CcO molecule) [5]. Assay mixtures contained 100 mM HEPES pH 7.4, 24 mM KCl, 2.8 mM ascorbate, 5.6 μ M EDTA, 1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) with 30 μ M bovine heart cytochrome *c* as a substrate, and with varying levels of dodecyl maltoside (DDM) and other compounds as noted.

The steady-state reduction levels of hemes were determined by using a variation of the method of Konstantinov et al. [7]. Assays were performed in 100 mM HEPES-KOH pH 7.4 with 24 mM KCl, 0.01% DDM, 1 mM ascorbate and 200 μ M TMPD in an unstirred cuvette in a Perkin-Elmer Lambda 40 spectrophotometer. Curves from fully-oxidized, fully-reduced, and steady-state-turnover RsCcOs were aligned at 585 nm (the apparent isosbestic point) so that these conditions could be compared.

Fluorescence quenching assays were performed on a Cary Eclipse spectrophotometer at an excitation wavelength of 280 nm. Quenching of 1 μ M RsCcO was followed at the emissions peak (326–328 nm) upon sequential 0.5 μ L additions of 20 mM cholesterol or 0.13 mM RA in a buffer containing 100 mM HEPES-KOH pH 7.4, 24 mM KCL, and either 0.01% DDM or 0.1% DDM.

Details of the crystallization of the E101A RsCcO are listed in Supplemental Material and Methods. Models of the E101W and P315W/E101A oxidases were made with the program Chimera [31]. The protein coordinates of E101A were used as the template for modeling the tryptophan mutations. The coordinates for the new E101A structure have been deposited in the Protein Data Bank as PDB ID:6CI0.

3. Results

3.1. A tryptophan in the E101 position blocks the K-path entrance and associated steroid ligand binding site

While several previous mutants of E101 [15] including E101A [18, 19, 21] indicated that this residue was critical for proton entrance into the K-path, incomplete inhibition suggested that an alternative entrance site might exist or that water reorganization could compensate for the carboxyl loss [16]. To determine if only one entrance is involved, the glutamic acid was replaced by a bulky tryptophan in order to remove the carboxyl and prevent water reorganization.

Mutation of E101 to a tryptophan (Fig. 1, Introduction) resulted in essentially complete blockage ($< 2\%$ of WT activity) of the K-path with a single amino acid substitution [17]. Although the alanine mutant at E101 could be stimulated to near-WT activity with very low levels of the long-chain acid docosahexaenoic acid (DHA) [17, 21], the E101W mutant was barely stimulated by DHA in a concentration-dependent manner [17]. Since retinoic acid (RA) has similar ability stimulate E101A to near-WT activity at low micromolar levels, its effect on E101W was also tested. As with DHA, RA only stimulated to a maximum of $\sim 55 \text{ s}^{-1}$ ($< 5\%$ of WT activity) (Fig. 2). This suggests that RA and DHA can still bind to the E101W mutant but, since considerably more ligand was required to achieve even a low level of stimulation, they bind with much lower affinity. The bulky tryptophan side chain of the E101W mutant appears to block the K-path entrance much more effectively than the smaller alanine, making it harder for the ligands to bind and for the carboxyl groups to facilitate an effective water bypass. When the E101 carboxyl is removed and the entrance path is blocked by the strong steric hindrance of tryptophan at this position, the K-path is essentially eliminated. Spectral analysis supports this conclusion.

Under steady-state conditions the activity of the E101W mutant was $< 2\%$ of WT and spectral assays (Fig. 3) showed a fully reduced α -peak (605 nm; $\sim 80\%$ contributed by heme a) and partially reduced Soret peak (445 nm; $\sim 50\%$ contributed by heme a , plus $\sim 50\%$ contributed by heme a_3), indicating a reduced heme a and an oxidized heme a_3 , due to electron retention on heme a . The result was similar to, yet even more dramatic than, the effects of the inhibitory detergent CHOBIMALT on the E101A mutant, for which the effect of the ligand was attributed to inhibition of proton uptake into the K-path and thereby altered electron equilibrium between the hemes [21]. The same conclusion had been reached for the inhibitory effects of cholate [24, 32, 33] and TritonX100 [25] [26] on bovCcO. Although WT RsCcO activity at pH 7.4 was too fast to capture a steady-state spectrum, when its activity was slowed at pH 9.5 (Fig. 5 in [21]) or pH 8.0 (Fig. 1 in [7]), WT showed α and Soret peaks both to be oxidized, as expected in the presence of oxygen and rapid electron equilibrium between the hemes. It is important to note that the α and Soret peaks in the E101W mutant were at the native WT positions, demonstrating that its dramatically reduced activity is not the result of a denatured enzyme structure, which is sensitively monitored by the heme spectra.

3.2. The M350W mutant tests the upper ligand binding site above the K-path entrance

A ligand binding model involving “upper” and “lower” sites [21] was suggested by crystal structure data and computational analysis [20]. To test for ligand binding in the upper site groove, a mutant was designed to sterically modify the proposed alkyl chain interaction. M350 in subunit I, located close to the upper site groove between subunits I and II (see Fig. 1, Introduction), was mutated to a tryptophan. It was postulated that this bulky residue would hamper binding of long-chain ligands to the upper site and interfere with proposed competition of their head groups with steroid site ligands.

Although this mutant showed unaltered spectra (Supplemental Fig. 1A), its unliganded activity decreased to $\sim 800\text{--}900 \text{ s}^{-1}$ as compared to $\sim 1200\text{--}1400 \text{ s}^{-1}$ for the WT RsCcO. Despite lower overall activity, in all other respects its responses to ligands and detergents

were the same as WT. RA still stimulated the M350 W mutant in a manner nearly identical to its effect on WT (Fig. 4). In titrations with DDM (Supplemental Fig. 2), cholesterol, and DHA (data not shown), the bulky tryptophan group did not alter the way these ligands affect activity. These activity assays suggest that DDM, cholesterol, RA, and DHA do not bind in the proposed upper ligand site.

The added tryptophan also provided a good probe to test directly for binding of ligands by fluorescence quenching spectroscopy. M350 W increased the overall fluorescence of unliganded RsCcO by ~40% over that of WT. The M350 W and WT fluorescence quenching curves determined with varying levels of cholesterol (Fig. 5A) changed in parallel. The flat M350 W-minus-WT difference curve shows that the fluorescence of the W350 tryptophan itself was not quenched by cholesterol, demonstrating that this ligand does not bind in the upper site, as expected. Fluorescence quenching curves with RA also changed in parallel and the W350_I tryptophan itself was also not quenched by RA (Fig. 5B), indicating that RA does not bind close to the upper site groove.

Taken together, the binding and activity data argue strongly against ligand binding in the postulated upper site of the two-site model.

3.3. The P315W/E101A mutant further probes the ligand binding site associated with the K-path entrance

The P315 residue of subunit I lies beneath the crystallographically-identified DOC in the binding pocket (Fig. 1, Introduction). To better define the properties of this steroid binding site, we created the P315W/E101A double mutant. This double mutant was as severely inhibited as the E101W mutant. By adding the P315W mutation to the E101A mutant the activity was diminished to only ~15 s⁻¹, 1% of typical WT activity. As in the case of the E101W mutant, spectra of the double mutant were normal (Supplemental Fig. 1B), indicating the dramatic effect on activity was not due to a denatured enzyme structure.

Similar to its effects on the E101W mutant, RA could only stimulate the activity of the double mutant P315W/E101A to ~50 s⁻¹ (Fig. 6). RA stimulation was decreased in the higher DDM concentration, implying that both ligands could compete to bind, albeit with lower affinities than to E101A, but RA could not overcome the tryptophan block. In the case of DOC (Supplemental Fig. 3), the P315W mutation inhibited both binding and “rescue” of activity, implying that the bulky tryptophan interfered more with the binding of DOC than of RA.

The P315W/E101A mutant was also used to further test for RA binding by fluorescence quenching (Supplemental Fig. 4). The P315W tryptophan provided a readily-accessible probe to test this area, giving a starting fluorescence intensity greater than that of the two other mutants with added tryptophans (E101W and M350 W) and double that of WT and E101A. RA still quenched the fluorescence of the double mutant but the P315W/E101A-minus-E101A curve was not flat, unlike the difference curves with RA and cholesterol for M350 W. This indicates that a significant portion of the quenching by RA in the double mutant was due to quenching of the P315W tryptophan itself. These data suggest that the

tryptophan does not prevent RA binding and that it binds near P315W. This is consistent with the activity measurements showing that RA still activates (Fig. 6).

3.4. Activity assays support cholesterol binding in the steroid binding site

Unlike cholesteryl hemisuccinate, a stimulatory carboxylate ligand [20,21], cholesterol inhibited the E101A mutant in 0.01% DDM (Fig. 7). Cholesterol did not further inhibit the already strongly-inhibited E101A in 0.06% DDM, suggesting that both the detergent and the steroid are equally inhibitory and bind in the same or overlapping sites, since their effects are not additive. Cholesterol titration of E101A in the presence of 300 μM DOC showed that cholesterol inhibition was mostly prevented. This demonstrates directly that cholesterol competes with DOC to bind in the steroid site. As previously shown for DOC [20], kinetic analysis of WT RsCcO shows that cholesterol also exhibits inhibitory behavior that is non-competitive with respect to cytochrome *c* (Supplemental Fig. 5). This is expected from the location of the steroid binding site at the entrance to the K-path, distant from the cytochrome *c* binding site [34, 35].

3.5. Tween20 shares the common ligand binding site

Activity assays of E101A imply competition between DDM and ligands for binding near the K-path entrance. Unlike DDM, the detergent Tween20 was shown to stimulate the activity of E101A approximately 10-fold [21]. The E101W mutant could also be stimulated by Tween20 but only to a maximum of $\sim 100 \text{ s}^{-1}$ in 0.3% Tween20, while the P315W/E101A double mutant could only be stimulated to a maximum of $\sim 55 \text{ s}^{-1}$ (data not shown). This suggests that even in 0.01% DDM, E101W and the double mutant, like E101A, still retain bound DDM that can be removed by Tween20, indicating a common binding site. In both mutants with tryptophan substitutions, neither Tween20 nor DDM are prevented from affecting activity, but their effects are minimal due to the barrier imposed by the bulky tryptophans.

Higher concentrations of Tween20 were required to stimulate E101A in 0.06% DDM (black line) versus 0.01% DDM (red line) (Fig. 8), supporting the idea that these detergents compete to bind in the same location. However, steroid ligands, which might also be expected to compete with Tween20, showed an unexpected synergistic behavior. Deoxycholate, a stimulatory steroid ligand, accentuated the stimulation of E101A by Tween20 (blue line) as compared to the titration without DOC (red line). Similarly, cholesterol accentuated the stimulatory effect of Tween20, even though it, by itself, is inhibitory to E101A (Fig. 7, Section 3.4). The presence of cholesterol (100 μM) in a titration of E101A with Tween20 (Fig. 8, green line) showed higher maximal activity compared to the same titration without cholesterol (black line). The potential for synergistic effects is suggested by the crystal structure (Fig. 1, Introduction) where DOC and a detergent molecule (decyl maltoside, DM) are seen to be closely interacting.

3.6. Can ligands compete with membrane lipids to bind at the K-path?

In vivo, CcO exists within a membrane. This raises the question as to whether ligands can compete to bind at the K-path entry site with the natural lipids of the membrane, some of which are specifically bound [27, 36, 37].

A titration of E101A with asolectin (soybean polar lipids) showed that this lipid mixture can itself stimulate activity to a maximum of $\sim 700\text{--}750\text{ s}^{-1}$ in either 0.01% DDM or 0.06% DDM (Supplemental Fig. 6; [21]). This suggests that the mixture of lipids can displace the DDM detergent. This would be expected from the RsCcO structures [19,36] in which molecules of detergents like DM or DDM are seen to occupy the same positions as natural lipids in the bovCcO structure [28], and a lipid or detergent chain is observed to be closely associated with the bile salt bound at the entrance of the K-path in structures of both bovCcO ([PDB ID:2DYR] [28]) and RsCcO (Fig. 1, Introduction; [PDB ID:3DTU] [19]).

To further investigate the interaction of K-path ligands with membrane lipids, E101A was reconstituted into lipid vesicles (COVs [5]). As expected for controlled conditions, its activity ($\sim 42\text{ s}^{-1}$) was much lower than the activity of the free enzyme in 0.01% or 0.06% DDM when the equivalent amount of asolectin lipids was added ($\sim 258\text{ }\mu\text{M}$; see Supplemental Fig. 6), due to the presence of a membrane potential in coupled COVs. Uncoupling the COVs with FCCP to remove the transmembrane potential increased the activity to $\sim 170\text{ s}^{-1}$. Addition of RA stimulated E101A activity in COVs (Fig. 9) with the greatest stimulation seen when the COVs were uncoupled with FCCP, while RA itself had no uncoupling activity (respiratory control ratio without RA ~ 3.5 , with RA ~ 6). However, the stimulation by RA was less on COVs than on the free enzyme in detergent solution. This indicates that RA can still access its binding location in the presence of a lipid membrane, but with somewhat lower efficiency.

4. Discussion

We report here the behavior of three mutant RsCcOs designed to test the location of the K-path entrance (E101W), the existence of an “upper ligand site” (M350 W), and the nature and binding specificity of the “lower ligand site” (P315W/E101A) (Fig. 1, Introduction). In the case of the upper ligand site, the tryptophan substitution at M350_l had no significant effect on ligand binding, ruling out a role for this proposed site. In contrast, mutations that modified the binding pocket at the K-path entrance, E101W and P315W/E101A, severely inhibited the enzyme to $\sim 5\%$ of WT activity and none of the ligands that chemically rescue the E101A mutant [18–21] could overcome the limitations imposed by the tryptophans. This leads to the conclusion that there is a unique K-path entrance and a single associated ligand binding site. However, further studies were needed to clarify the competitive behavior of activating and inhibiting ligands.

The introduction of tryptophans at key locations provided an additional approach to testing for ligand binding behavior by enabling fluorescence quenching studies. Although fluorescence intensity was significantly increased in the mutants and quenching of the added tryptophans could be measured, quantitative correlation with activity assays was not observed in all cases. The problems of substrate depletion and non-specific binding introduce complexities that are difficult to correct for in the fluorescence quenching assays. Nevertheless, each added tryptophan provided a probe to determine if a ligand could bind close to that specific location. In the case of M350 W, the added fluorescence was not quenched by ligand (Fig. 5, Section 3.2), while that of P315W was (Supplemental Fig. 4).

Thus the fluorescence quenching analyses support the conclusion reached by activity studies: that the lower binding site, but not the upper site, is involved in ligand binding.

Although computational analysis [20] and activity titrations (Fig. 7, Section 3.4) suggest that most steroids bind similarly in the K-path entrance of the E101A mutant, detergents seem to bind in the same pocket but in slightly different positions or orientations. In the crystal structure of RsCcO with DOC [PDB ID:3DTU] a DM detergent molecule is closely associated with the DOC (Fig. 1, Introduction), and in bovCcO [PDB ID:2EIL] a phospholipid is also closely associated with the equivalent cholate. These structures suggest that a detergent molecule, DM or DDM, could wrap around a steroid ligand and fold its head group into the K-path entrance (Fig. 1, Introduction) to inhibit proton access. Conversely, the bulky hydrophilic Tween20 head group would not be able to fold into the K-path when its tail is bound in the same position as the DDM tail, suggesting that its stimulating effect on E101A could be due to both removal of inhibitory DDM and provision of a “water sponge” or proton antenna near the K-path entrance. If the presence of a steroid molecule in the pocket can help a detergent molecule bind more strongly and vice versa, this could explain the accentuation by cholesterol of Tween20 stimulation (Fig. 8, Section 3.5) despite cholesterol alone being inhibitory. No such synergistic effects were detectable with cholesterol and DDM (Fig. 7, Section 3.4) because both are inhibitory.

RA has both a long tail and a ring structure, suggesting more complex binding behavior than either a detergent or a steroid. However, modeling of its interaction at the DOC position [20] showed a similar binding mode as the other molecules modeled at this site, with the carboxyl on the end of its isoprene tail oriented close to the K-path entrance in the same position as the carboxyl of the DOC. This would account for its strong activating effect on the E101A mutant. However, RA can also bind to WT RsCcO despite the more hydrophilic WT K-path entrance pocket (compare Fig. 10B and C, below). Although previously reported to have little effect on WT [21], further experiments revealed that RA uniquely stimulated WT activity (Fig. 2, Section 3.1). One hypothesis is that RA increases proton uptake into the WT K-path by adding a second carboxyl near the K-path entrance, thereby augmenting proton availability or water chain organization.

A new crystal structure [PDB:6C10] of the E101A mutant RsCcO provides insights into the ligand binding pocket. The E101A mutant has nearly identical overall structure compared to that of WT (Fig. 10A), so the low activity of this mutant (~50% of WT) is not due to major changes in overall conformation. However, the loss of the carboxyl in the E101A mutant (Fig. 10B) makes the steroid binding pocket at the K-path entrance more hydrophobic as compared to WT (Fig. 10C), thus accounting for the increased affinity of E101A for a variety of hydrophobic ligands. These ligands will be able to bind more deeply into the K-path entry region and impede crucial proton uptake by disrupting the water chain leading into the K-path that is apparent in the crystal structures of WT RsCcO [17,21] and WT PdCcO [16]. Modeling of added tryptophan at either position 101 in subunit II (Fig. 1, Introduction) or position 315 in subunit I (Fig. 10D) predicts a major blockage of the ligand binding pocket, which is consistent with the very low activities of the E101W and P315W/E101A mutants.

Although these studies have clarified the nature of the K-path entrance and the associated ligand binding site, an additional important question is whether these amphipathic ligands or other physiological counterparts could access the steroid binding site to have regulatory effects when the protein is embedded in the native membrane. The effects observed in reconstituted liposomes indicate that they can. Asolectin alone (a complex mixture of lipids; see Materials & Methods) competed with DDM to overcome the inhibition of the E101A mutant (Supplemental Fig. 6). But significantly, E101A reconstituted into lipid vesicles was still strongly activated by RA in the uncontrolled state (uncoupled with FCCP). And importantly, the controlled state was only slightly stimulated and not uncoupled (Fig. 9, Section 3.6), indicating that RA did not act as a proton conductor. These results suggest that the steroid/bile salt binding site found at the mouth of the K-path in both RsCcO and bovCcO may have physiological significance. Various nucleotides, steroid hormones, thyroid hormone and RA have all been predicted to occupy this site [20] and nucleotides at least are expected to be present in cells at mM concentrations in most circumstances. Nevertheless, the question of the identity and effects of physiologically important ligands remains, as well as the suitability of this site as a drug target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Barbara Atshaves and Suzanne Benning for the use of the fluorescence quenching spectrophotometer and Dr. Charles Nait for training in its use, and Drs. Leann Buhrow and Leslie Kuhn for helpful discussions. We thank staff members of LS-CAT for assistance in data collection at APS. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817). This work was supported by NIH R01 GM26916 (to SF-M) and the MSU Foundation Strategic Partnership Grant "Mitochondrial Science & Medicine" (S.F.-M.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbabbio.2018.03.017>.

Abbreviations

bovCcO	CcO from bovine (<i>Bos taurus</i>)
CcO	cytochrome <i>c</i> oxidase
COVs	cytochrome <i>c</i> oxidase-containing lipid vesicles
DDM	dodecyl (lauryl) maltoside
DHA	docosahexaenoic acid
DM	decyl maltoside
DOC	deoxycholate

FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
PDB	Protein Data Bank
PdCcO	CcO from <i>Paracoccus denitrificans</i>
RA	retinoic acid
RsCcO	CcO from <i>Rhodobacter sphaeroides</i>
WT	wild type

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Fig. 1.

Structure of the *R. sphaeroides* CcO based on the WT crystal structure [PDB:3DTU] with the E101W mutation modeled in. Subunit I is gray, subunit II is wheat; hemes are shown as purple sticks. The locations of all three amino acid mutations discussed in the text are shown as magenta spheres, along with a deoxycholate molecule (black sticks) bound in the steroid site, alkyl chains resolved in the crystal structure (green spheres), and the D- and K-paths for proton uptake (black arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Effects of retinoic acid on the activities of WT (solid triangles and line), E101A in 0.01% DDM (open circles, dashed line) and 0.06% DDM (solid circles, dashed line), and E101W (solid squares and line) oxidases. The E101A data for comparison are from [21].

Fig. 3.

Effects of the E101W mutation on the reduction levels of hemes *a* and *a3* during steady-state turnover in 0.01% DDM at pH 7.4. Spectra of CcO are fully oxidized (blue), fully reduced (black), and steady-state (red). Peak positions are indicated above the α and Soret peaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.
Effects of retinoic acid on the activities of WT (open circles) and M350 W (solid circles) oxidases in 0.06% DDM.

Fig. 5. Quenching of fluorescence intensity of WT (open circles) and M350 W (solid circles) oxidases in 0.01% DDM by cholesterol (panel A) and retinoic acid (panel B). Fluorescence intensity of the M350 W tryptophan itself (M350 W data minus WT data) is shown as triangles.

Fig. 6.

Comparison of the effects of retinoic acid on the activities of E101W in 0.01% DDM (solid circles and line), P315W/E101A in 0.01% DDM (open squares, dashed line), and P315W/E101A in 0.06% DDM (solid squares and line).

Fig. 7. Effects of cholesterol on the activity of E101A in 0.01% DDM (open circles, dashed line), in 0.06% DDM (solid circles and line), and in 0.01% DDM with 300 μM DOC (open circles, solid line).

Fig. 8.

Stimulation of the activity of E101A RsCcO by Tween20. Activity was assayed in 0.01% DDM (red), 0.01% DDM + 300 μM deoxycholate (blue), 0.06% DDM (black), and 0.06% DDM with 100 μM cholesterol (green). The data without cholesterol or DOC for comparison is from [21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 9.

Effects of retinoic on the activity of E101A RsCcO reconstituted into lipid vesicles (COVs) versus as free enzyme in detergent solution. COVs were assayed in the coupled condition (squares) and uncoupled with FCCP (triangles), as compared to free enzyme assayed in 0.01% DDM or in 0.06% DDM (shown as dashed lines). The free enzyme data for comparison are from [21].

Fig. 10.

Structures of WT and mutant RsCcOs. Panel A: The overall structure of the E101A mutant (pale blue) nearly completely overlays that of WT (dark cyan). The boxed area shows the ligand binding pocket surrounding E101 (spheres), represented in panels B-D as coulombic surface charge (red, acidic; blue, basic). Green arrows indicate the position of residue 101. Panel B: E101A [PDB:6CI0]; panel C: WT [PDB:3DTU]; panel D: P315W/E101A (P315W modeled into the E101A structure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)