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Review

Implications of ligand binding studies for the catalytic mechanism of cytochrome *c* oxidase

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Abstract

The reaction of oxidized bovine heart cytochrome *c* oxidase (CcO) with one equivalent of hydrogen peroxide results in the formation of two spectrally distinct species. The yield of these two forms is controlled by the ionization of a group with a pK_a of 6.6. At basic pH, where this group is deprotonated, an intermediate called P dominates (P, because it was initially believed to be a peroxy compound). At acidic pH where the group is protonated, a different species, called F (ferryl intermediate) is obtained. We previously proposed that the only difference between these two species is the presence of one proton in the catalytic center of F that is absent in P. It is now suggested that the catalytic center of this F form has the same redox and protonation state as a second ferryl intermediate produced at basic pH by two equivalents of hydrogen peroxide; the role of the second equivalent of H_2O_2 is that of a proton donor in the conversion of P to F.

Two chloride-binding sites have been detected in oxidized CcO. One site is located at the binuclear center; the second site was identified from the sensitivity of $g=3$ signal of cytochrome *a* to chloride in the EPR spectra of oxidized CcO. Turnover of CcO releases chloride from the catalytic center into the medium probably by one of the hydrophobic channels, proposed for oxygen access, with an orientation parallel to the membrane plane.

Chloride in the binuclear center is most likely not involved in CcO catalysis. The influence of the second chloride site upon several reactions of CcO has been assessed. No correlation was found between chloride binding to the second site and the reactions that were examined.

Keywords: Cytochrome *c* oxidase; Hydrogen peroxide; Chloride interaction

1. Introduction

Respiratory oxidases are membrane-bound electron-transfer complexes that catalyze the reduction of dioxygen to water and use the associated free energy changes to generate a transmembrane proton gradient. It has become apparent that most respiratory oxidases, from prokaryotic to eukaryotic organisms, are members of a single family, called the heme–copper oxidase superfamily [1–3]. Despite the differences in electron donor, all members of the family show marked structural [4–6] and functional homology (for reviews, see Refs. [7–9]).

In bovine cytochrome *c* oxidase (CcO), four redox centers are involved in promoting electron transfer (ET) from cytochrome *c* to dioxygen. Cu_A and cytochrome *a* are primary electron acceptors and electrons from these sites are delivered to the binuclear or catalytic center of CcO that consists of cytochrome a_3 and Cu_B. At the binuclear (heme–copper) center, an interaction among electrons, protons, and dioxygen occurs, and dioxygen is converted into two molecules of water. The conversion proceeds through several intermediates; some are already defined while others are putative intermediates (for reviews, see Refs. [7–11]).

The existence of multiple reaction steps and the presence of site–site interactions in CcO offer a variety of approaches to the general objective of establishing the catalytic mechanism. Clearly, the identification and characterization of the

Abbreviations: TX-100, Triton X-100; DM, *n*-dodecyl-β-D-maltoside; CcO, cytochrome *c* oxidase; O, oxidized cytochrome *c* oxidase; P, "peroxy" intermediate; F, ferryl intermediate

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events at the heme–copper center is one of the most important tasks. In this endeavor, it is fortunate that the binuclear center can interact with molecules other than dioxygen and water. Most of these molecules behave as simple ligands although there are some that undergo a chemical reaction at the center [12–14]. Analysis of these chemically various reactions reveals the general characteristics of the center that are potentially relevant to the catalytic mechanism.

Although the binuclear center is an important target for reaction with external ligands, other interacting sites are known to exist. In particular, it has been observed that cytochrome *a* can also interact with ligands [15–21], while the binding of azide in the proximity of cytochrome *a* was recently identified in the crystal structure of oxidized bovine CcO–azide complex [22]. However, the role of these binding sites and interactions at these sites in CcO catalysis has yet to be established.

2. Interaction of H_2O_2 with oxidized CcO: the relationship of the "peroxy" and ferryl forms

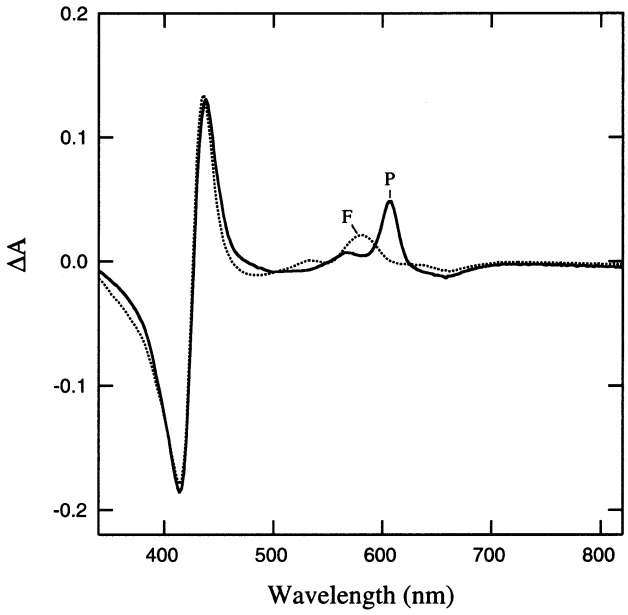
2.1. The "peroxy" form

The simplest reaction of CcO with dioxygen is observed when enzyme is reduced with only two electrons, one electron on Fe_{a_3} and one on Cu_B [23–25]. The enzyme in this redox state is called "mixed valence" CcO (MVCcO). At ambient temperatures, the reaction kinetics of MVCcO with dioxygen is rapid and produces a relatively stable oxy form of CcO [25–27] characterized by an absorbance maximum at 607 nm in difference spectra referenced to oxidized enzyme (Fig. 1A). In the absence of an external electron donor, the fast reactions stop here and the intermediate decays to oxidized oxidase (O) on the minute time scale [28].

It was originally thought that this intermediate is a "peroxy" form (P_M) with an intact O–O bond in the catalytic center (e.g. $Fe^{III}_{a_3}$–O–O–Cu^{II}_B). The same "peroxy" form can be produced by reaction of one equivalent [29] or low concentration of H_2O_2 with oxidized CcO at basic pH [13,30–33] or, in mitochondria, by the energy-driven, two-electron transfer from water present in the catalytic center of oxidized CcO to cytochrome *c* [34,35]. Today, evidence accumulated with both bovine and bacterial oxidases make it clear that the peroxy bond in P_M form is split [13,26,36–42] and that the heme iron in the catalytic center is in ferryl state (e.g. Fe^{IV}=O HO–Cu^{II}_B) [13,26,36–42].

Cleavage of the O–O bond of hydrogen peroxide requires two electrons. One electron is provided by the oxidation of heme Fe^{III} to Fe^{IV} while the source of the second electron has yet to be unequivocally established. Because the interaction of hydrogen peroxide with the binuclear center is known to lead to the formation of free radicals [29,33,43–46], it is assumed that some group in the vicinity of the center is an electron or hydrogen donor.

However, the yield of radicals is low; indeed, in some situations, it is not even detected [29,33,46]. These observations can be explained with either of two assumptions. First, the radical located in the binuclear center is EPR silent



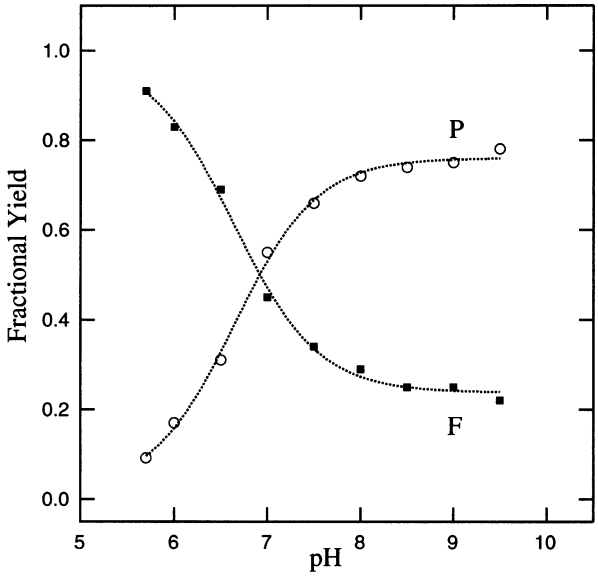


Fig. 1. The "peroxy" (P_M) and ferryl (F) intermediates of bovine heart CcO. (A) The difference optical spectra of P_M and F referenced to oxidized CcO. (B) Dependence of the relative yield of P and F on pH. To make F intermediate, 4.7 μM of oxidized CcO was treated at pH 8.0 (100 mM Tris/0.1% DM) with 5 mM H_2O_2. P_M was obtained from MVCcO complex with CO [38]. The dependence of the maximal yield of P and F on pH was determined via the reaction of 4.1 μM CcO with 20 μM H_2O_2 in 100 mM buffers (Ches, Bicine, HEPES, and Mes) containing 84 mM K_2SO_4 and 0.1% DM. Each point is a fresh sample and the broken lines are fits to a group with a pK_a=6.6.

because of electronic coupling with the paramagnetic heme or copper. Second, the radical is less stable than is the ferryl state of heme a_3 and can migrate within the protein. Then, the observed EPR signals arise from those radicals that have migrated to some site distant from the catalytic center or, less plausibly, to an undefined subpopulation of CcO with an altered binuclear center. Radical migration is indicated by the variable nature of observed radicals [29,33,43–46] and from the modification of an amino acid located in subunit VIIc [47].

Experimental verification of the identity and quantitative presence of a radical in the binuclear center is still missing. Even if the radical is indeed present, it is still an open question as to whether it is part of the regular catalytic cycle. There are already proposals for a radical-based mechanism in CcO catalysis [11,48–50]. To be involved in the catalytic cycle, the rate of radical migration has to be slow compared to enzyme turnover rates and thus also minimizes oxidative damage. Our experiments have indicated that the endogenous quenching of radical at the catalytic center happens within a few minutes after generation of the P_M form [39] and that this process occurs without an observable change in the optical spectrum.

The following scheme summarizes the above discussion for the reaction between MVCcO and O_2 with a possible structure of intermediates at the catalytic center:

$$\mathrm{Fe}^{\mathrm{II}}_{a_3}\ \overset{\mathrm{MV}}{\mathrm{Cu}^{\mathrm{I}}_{\mathrm{B}}} + \mathrm{O_2} \underset{\text{Rapid}}{\Rightarrow} \mathrm{Fe}^{\mathrm{IV}}_{a_3} = \overset{P_M}{\mathrm{O}}\ \mathrm{HO}-\mathrm{Cu}^{\mathrm{II}}_{\mathrm{B}}\mathrm{B}^{0}$$

$$\underset{\text{Slow}}{\rightsquigarrow} \mathrm{Fe}^{\mathrm{IV}}_{a_3} = \overset{P_R}{\mathrm{O}}\ \mathrm{HO}-\mathrm{Cu}^{\mathrm{II}}_{\mathrm{B}}\mathrm{BH}$$

The reaction of MV CcO with dioxygen rapidly produces P_M, characterized by a 607-nm band in the difference spectrum, and with a neutral radical (B^0) at the catalytic center. In the absence of an external electron donor, the radical is less stable than the ferryl state of heme a_3 and is slowly quenched by, so far, unspecified endogenous hydrogen transfer to the catalytic site without a change in the optical spectrum.

In the above scheme, we labeled the intermediate with a quenched radical by the symbol P_R, which is also used now for the "peroxy" form observed during the reaction of fully reduced CcO with oxygen [51–54]. The reason for the same symbol is that we expect the same structure for the catalytic center for both intermediates. When reaction begins with fully reduced (four-electron) oxidase, the transient formation of P_R is associated with an electron transfer from a large fraction of cytochrome *a* into the binuclear center [55–57] and without a proton uptake from solution [58]. With three external electrons available for the reaction, the cleavage of O–O can proceed without radical production and the possible structure of the catalytic center is equivalent to that presented in our scheme.

2.2. The transition of "peroxy" to ferryl form

It was already mentioned that, even in the absence of external electron donors, P_M is unstable and slowly decays to oxidized enzyme. We have studied this transition by optical spectroscopy a few minutes subsequent to P_M formation [28]. The delay was needed to remove carbon monoxide from the solution used for P_M preparation. However, it can be estimated that after that delay, CcO has a catalytic center that is 70% P_R and 30% P_M state [39]. Because both, P_R and P_M forms, have the same or very similar optical spectra, we further call this mixture a P form.

The endogenous transition of P to oxidized CcO can be described by a sequential mechanism with one intermediate [28]:

$$\underset{607\ \mathrm{nm}}{\mathrm{P}} \Rightarrow \underset{575\ \mathrm{nm}}{\mathrm{F}} \Rightarrow 0$$

F, the ferryl form, at acidic pH is characterized by a maximum at 575 nm in the optical difference spectra referenced to oxidized CcO and the conversion of P to F is strongly pH-dependent [28]. The pH dependence of the conversion rate and direct measurement of pH changes in the solution indicated that one proton is taken up from the medium during this transition [28].

From these observations, the net difference between P and F is:

$$\mathrm{P} + \mathrm{H}^{+} \Rightarrow \mathrm{F}$$

We have suggested that in the catalytic center, independent of the presence or absence radical, a proton is utilized for water formation:

$$\mathrm{Fe}^{\mathrm{IV}}_{a_3} = \mathrm{O}\ \ \mathrm{HO}-\mathrm{Cu}^{\mathrm{II}}_{\mathrm{B}} + \mathrm{H}^{+} \Rightarrow \mathrm{Fe}^{\mathrm{IV}}_{a_3} = \mathrm{O}\ \ \mathrm{H_2O}-\mathrm{Cu}^{\mathrm{II}}_{\mathrm{B}}$$

with a consequence of the change in optical spectrum. This conclusion is also in agreement with the transition of P_R to F in the reaction of fully reduced CcO with oxygen. The transition $P_R \Rightarrow F$ occurs concomitant with a proton uptake from the solution without any electron transfer into the catalytic center [55,59–63].

That a proton is involved in the conversion of P to F is also indicated by the study of the interaction of oxidized CcO with hydrogen peroxide. The reaction product of oxidized CcO with single H_2O_2 at acidic pH [29] is ferryl form optically equivalent to the F form observed during the endogenous decay of P [28]. However, at basic pH, a single H_2O_2 produces dominantly the P form [29]. The dependence of the yield of P or F on pH [33,64] suggests that ionization of a group with a pK_a of 6.6 [31,65] controls the reaction product (Fig. 1B). We have suggested that this group could be a proton donor for the reaction of peroxide with the oxidized binuclear center [28].

At basic pH, another, spectrally very similar if not identical, ferryl form is observed following addition of

excess of hydrogen peroxide to oxidized enzyme [29,30,32,33,65] (Fig. 1A). With an excess of peroxide [30,32,65,66], the sequence of observed intermediates for bacterial or bovine oxidases is:

$$O + H_2O_2 \Rightarrow P \Rightarrow F$$

P, transiently made by interaction of oxidized enzyme (O) with one equivalent of hydrogen peroxide, reacts further with the second H_2O_2 to produce F [32,66]. It might be expected that the second molecule of H_2O_2 could be either a hydrogen or an electron donor for P [64,65,67–69]. This hydrogen would annihilate the radical (B^0) in the binuclear center of P with production of superoxide and F as follows:

$$Fe^{III}_{a_3}\,H_2O\;HO - Cu^{II}_B + H_2O_2 \Rightarrow \underset{O}{Fe^{IV}_{a_3} = O}\;\underset{P}{HO - Cu^{II}_B B^0}$$

$$+ H_2O_2 \Rightarrow \underset{F}{Fe^{IV}_{a_3} = O}\;H_2O - Cu^{II}_B B^- + HO^0_2$$

The release of superoxide from the binuclear center is surprising, given the presence of the highly oxidizing oxyferryl state ($Fe^{IV}=O$) [35]. The proposed reaction mechanism needs to answer the question why superoxide is not oxidized to dioxygen in which case the overall reaction would be identical to that catalyzed by catalase.

Superoxide is indeed detected in the reaction of CcO–O with excess of hydrogen peroxide [29,68,69]. Yet the kinetics of superoxide formation more closely resembles the kinetics of bleaching of the CcO optical spectrum than the kinetics of the P⇒F conversion [29]. This leads us to propose that the second equivalent of hydrogen peroxide does not react as an electron/hydrogen donor but serves as a proton donor for P. In this picture, there is no difference in the redox and protonation state of the binuclear centers of F prepared from oxidized CcO by one H_2O_2 at acidic pH or by two H_2O_2 at basic pH.

3. Two sites of chloride binding in CcO: is chloride involved in catalysis?

Several studies have shown that chloride can be bound to the binuclear center of oxidized CcO [70–76]. However, from the sensitivity of optical and EPR spectra of bovine cytochrome oxidase to varying concentrations of chloride, we have established that there are at least two binding sites for chloride in native enzyme [75,77]. As expected, one site is at the binuclear center and chloride binding to this site can be followed by the changes in the optical spectrum of oxidized enzyme [70,75]. Because the binding is pH-dependent [75] and the slope of the dependence of log (K_d) vs. pH is close to 1 (Fig. 2B), it appears that chloride is bound as HCl. This chloride is found in the binuclear center of isolated CcO only when chloride salts are present during enzyme purification. The dissociation of chloride from the binuclear center is extremely slow and is enhanced substantially by binding of cyanide [75], by reduction [76], or by single turnover of the enzyme [75]. The very low affinity of CcO for chloride, the slow kinetics of binding, and the release of chloride from the binuclear center during a single turnover suggests that this chloride is unlikely to participate in catalytic mechanism of CcO.

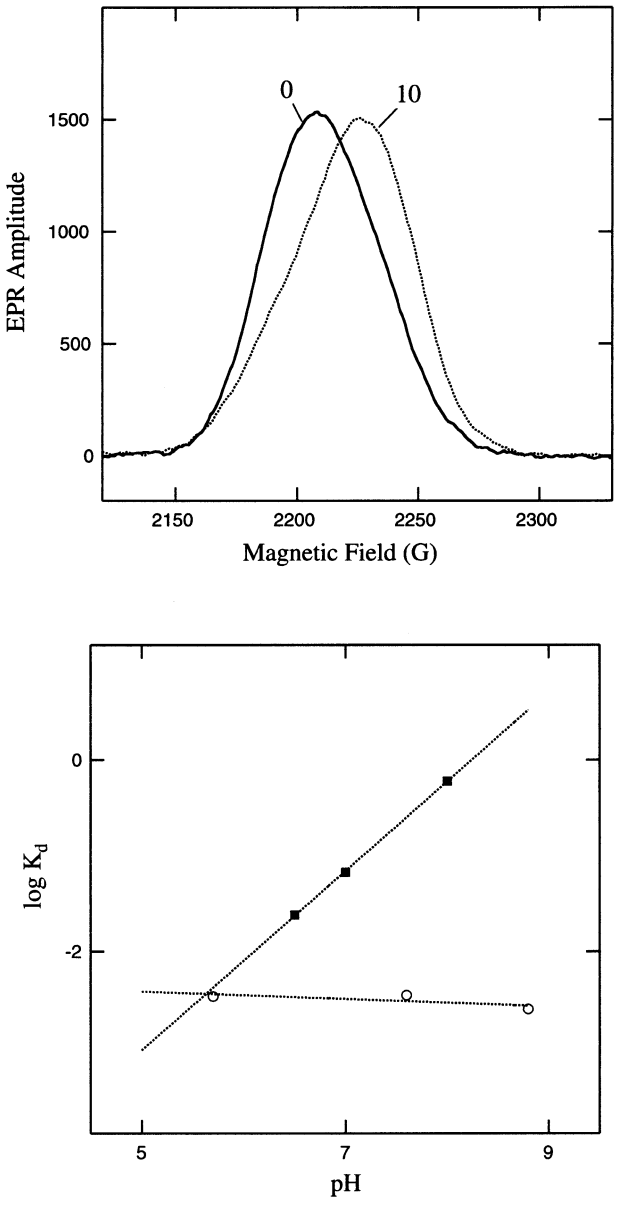


Fig. 2. The interaction of chloride with oxidized bovine heart CcO. (A) The $g=3$ EPR signal of oxidized cytochrome *a* in the absence (0) and presence of 10 mM NaCl (10). (B) pH dependence of the dissociation constants for two chloride binding sites: (■) at the binuclear center and (○) at the new second site. Symbols—experimental data. Lines—linear fits. Data for the binding of chloride to the binuclear center were obtained from the optical spectrum changes [69]. Data for the binding to the second site are based on the sensitivity of the $g=3$ EPR signal to chloride. EPR spectra were recorded on 40 μM CcO in 10 mM HEPES/pH 7.6/0.1% TX 100. Conditions: frequency 9.26 GHz; power 3 mW; modulation amplitude 10 G; the modulation frequency 100 kHz; and temperature 10 °K.

These characteristics can be contrasted with those of the second site for chloride binding. Binding to this site does not induce any detectable changes in the optical spectrum, rather it is the EPR spectrum that is now modified (Fig. 2A). Thus, in the presence of chloride, the $g=3$ EPR signal of cytochrome *a* present in oxidized bovine cytochrome *c* oxidase, both in mitochondria and in purified enzyme, is shifted to higher magnetic field (smaller *g* value) by chloride [15,77]. The dependence of $g=3$ signal shift on chloride concentration shows the presence of a single binding site in native oxidized CcO. Both the association and dissociation of chloride occurs on a substantially shorter time scale than binding of chloride to the binuclear center. The dissociation constant for chloride binding to purified CcO is close to 3 mM and almost pH independent (Fig. 2B). We conclude that, between pH 5.7 and pH 8.8, the chloride anion is bound to a positively charged site. The location of this site is narrowed down by the fact that the site is also accessible to chloride added to mitochondria, implying that chloride access is via the surface of protein located on the cytosolic side of membrane. However, access to this site appears to have some size selectivity because sulfate anions does not affect the $g=3$ signal nor is the dissociation constant for chloride binding changed by the presence of 50 mM Na_2SO_4. We thus expect that the site is partially buried inside the protein and connected to the exterior by a size selective hydrophilic channel.

The available X-ray structures of bacterial or bovine oxidases provide no direct evidence for chloride binding site but it would almost certainly be confused for one or more of the water molecules that are included in the X-ray refinement. So we have to rely in our identification on indirect evidence. The certain fact is that this chloride site is external to the binuclear center, and because chloride does not appear to affect the binding of azide to its second site, it seems that the site for chloride must be elsewhere in the protein.

There are two possible sites in CcO that can satisfy the characteristics described above. The first consists of the conserved Arg-438 and Arg-439 residues of subunit I located in the extramembrane loop at the cytosolic side and connecting the transmembrane helices XI and XII [4,78]. The second possible site is the bound Mg^{2+} in CcO structure. Both Arg-438/Arg-439 and Mg^{2+} are close to the heme propionates of cytochrome *a* [4,78] and electrostatic calculations suggest that there is a strong interaction between these two sites and the propionate side chains [79,80]. The Mg^{2+} lies at the bottom of proposed water/proton channel [78,81] and is coordinated by Asp-369 and His-368 from subunit I, by Glu-198 from subunit II, and by a water molecule [5]. We suggest that water molecule can be displaced by chloride and chloride binding to Mg^{2+} is mediated to the propionates of heme *a*.

The addition of the chloride anion to either of these two sites might be transmitted to the propionate substituents of heme *a* and this leads to changes that influence the $g=3$ EPR signal by the following mechanism. The location of the low-field *g* value of low-spin cytochromes depends on two parameters—the strength of the ligand field provided by the axial ligands and the relative orientations of the imidazole rings with respect to each other. When the axial ligands are neutral histidine residues, the low-field *g* value ranges from ca. 2.95 when the imidazole rings are coplanar to ca 3.8 when they are perpendicular. In cytochrome *a*, the imidazoles are close to coplanar. From a survey of 432 crystal structures of heme proteins, it was concluded that an important factor contributing to the orientation of the imidazole rings is an interaction between the $N_\delta H$ of the imidazole ring and the propionic acid side chains of the heme [82]. Thus, any process that affects the orientation of the propionate groups can affect the orientation of one or both imidazole rings and change the EPR *g* value. In the case of chloride, it would appear that chloride induces the two rings to align more closely with each other.

To examine if there is a catalytic role for the second chloride binding site, we have assessed the redox potential of cytochrome *a*, the catalytic activity of CcO, the rate of cytochrome *a* reduction by ferrocytochrome *c*, and finally the rate of electron transfer (ET) from cytochrome *a* to cytochrome a_3. We found that the redox potential of cytochrome *a* was not influenced by the presence of 50 mM NaCl. The rate of electron transfer to cytochrome *a*, the catalytic activity, and also the rate of intramolecular ET were found to decrease with increasing chloride concentration (at constant ionic strength). However, in each of the three assays, the observed chloride sensitivity was not correlated with the dissociation constant for chloride that was determined by EPR spectroscopy.

Despite these results, there are three indications that chloride might be involved in the CcO catalysis. First, the chloride concentration within a cell is probably close to 25 mM [83]; this is sufficiently high to keep the site in oxidized CcO occupied by Cl.

Second, the rate of intramolecular ET measured in the reaction of fully reduced bacterial quinol *bo* oxidase with dioxygen [84,85] was found substantially higher in the presence of chloride than in its absence leading to the conclusion that Cl modulates the heme–copper binuclear center from a novel binding site [85].

Third, when fully reduced bovine CcO was reoxidized by oxygen in the presence of chloride salts, the initial $g=3$ signal was that characteristic of chloride-free enzyme; however, this changed rapidly to that of chloride-bound enzyme [86].

4. Release of chloride from the binuclear center: orientation of the oxygen channel

The catalytic center of CcO is buried within the protein [4,78] and in the fully oxidized state, there is no free access to the center of the protein from the medium [78]. However,

during catalysis, oxygen and protons must arrive at the binuclear center and water must depart. Consequently, it is widely assumed that there are pathways or channels within the protein to facilitate these movements. Of these channels, the two best-documented channels support the movement of protons. These are called the K and D channels, reflecting the presence of a strategic lysine and aspartic acid, respectively, and connecting the matrix side with the binuclear center (see reviews [8,9,48]).

Possible channels for the movement of oxygen and water release have been suggested on the basis of the crystal structures of oxidized enzyme [4,78]. Three hydrophobic channels, with an orientation parallel to the membrane plane, are plausible routes for oxygen transport to the catalytic center of bovine CcO [78] while only a single channel with the same orientation has been proposed for the bacterial oxidase [87]. Another channel, expected for water or proton release, is presumed to connect the binuclear center with the cytosolic surface of enzyme [4,78].

External ligands can also access the binuclear center and could well utilize the same channel(s) as oxygen and water. Thus, a determination of the access route(s) for a ligand would support the presence of a channel with a specific orientation. This was our rationale for establishing the orientation of the channel used by chloride released from the binuclear center to the medium.

We had previously shown that only a single chloride is bound to the binuclear center and, once bound, its dissociation is extremely slow [75]. Thus, it was possible to make cytochrome oxidase vesicles (COV) containing enzyme with radioactive ^{36}Cl present in the catalytic center with about 40% occupancy. The respiratory control ratio for these COV was 10 or more, and the enzyme was oriented with about 66% having the "right-side out", that is with Cu_A exposed to the external medium. The remainder of the enzyme is assumed to be oriented with the "right-side in".

The dissociation of chloride from the catalytic center of CcO in vesicles was stimulated by anaerobic reduction from the exterior by membrane-impermeant reductants and the amount of enzyme reduced was calculated from the optical spectra. Then, the reduced COV were exposed to air, separated from the external medium by rapid filtration [88], and the filtrate containing the COV used for radioactivity measurement. COV untreated with reductant were used as the control.

If the chloride release channel were oriented uniquely to the cytosolic side, the expected decrease of ^{36}Cl in COV should be about 40% of the amount of CcO that underwent reduction. In three experiments, we find that the average decrease of ^{36}Cl in COV is $28 \pm 3\%$. When the experiments were repeated in the presence of triphenyltin, a membrane-permeant chloride porter, the amount of chloride retained in the COV was substantially reduced. These two sets of observations imply that chloride is partially released to both sides of membrane. The simplest explanation is that the channel is parallel to the membrane surface, and chloride, exiting the catalytic center as HCl [76], can diffuse from the hydrocarbon bilayer to both sides of the membrane. This model is consistent with previous suggestions for the orientation and the hydrophobic nature of the channel that delivers oxygen to the catalytic center [78,87].

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