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Review

Proton pumping by cytochrome oxidase: progress, problems and postulates

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Abstract

The current status of our knowledge about the mechanism of proton pumping by cytochrome oxidase is discussed. Significant progress has resulted from the study of site-directed mutants within the proton-conducting pathways of the bacterial oxidases. There appear to be two channels to facilitate proton translocation within the enzyme and they are important at different parts of the catalytic cycle. The use of hydrogen peroxide as an alternative substrate provides a very useful experimental tool to explore the enzymology of this system, and insights gained from this approach are described. Proton transfer is coupled to and appears to regulate the rate of electron transfer steps during turnover. It is proposed that the initial step in the reaction involves a proton transfer to the active site that is important to convert metal-ligated hydroxide to water, which can more rapidly dissociate from the metals and allow the reaction with dioxygen which, we propose, can bind the one-electron reduced heme-copper center. Coordinated movement of protons and electrons over both short and long distances within the enzyme appear to be important at different parts of the catalytic cycle. During the initial reduction of dioxygen, direct hydrogen transfer to form a tyrosyl radical at the active site seems likely. Subsequent steps can be effectively blocked by mutation of a residue at the surface of the protein, apparently preventing the entry of protons.

Keywords: Oxidase; Cytochrome; Proton; Channel; Oxygen; Enzyme; Peroxidase

1. Introduction: catalytic and chemiosmotic activity

The reaction catalyzed by cytochrome oxidase is deceptively simple, the four-electron reduction of dioxygen to two water molecules [1–5]. Four equivalents of cytochrome *c* provide the electrons and the four substrate protons, H^+, are taken up by the enzyme which end up the product H_2O.

$$4\ \text{cyt}\ c^{2+} + 4\ H^+ + O_2 \rightarrow 4\ \text{cyt}\ c^{3+} + 2\ H_2O \qquad (1)$$

The natural substrates of cytochrome oxidase are cytochrome *c* (reductant) and dioxygen (oxidant). The enzyme mechanism has been studied using the natural substrates as well as a series of artificial substrates. Cytochrome oxidase will oxidize artificial electron donors such as $Ru(NH_3)_6$, TMPD, and PMS, and will also function, albeit slowly, using ferrocyanide, DTT, ascorbate and some foreign cytochromes *c*. Whereas the spectrum of the reductive substrates utilized by cytochrome oxidase is relatively broad, the number of oxidative substrates is limited to dioxygen or its partially reduced forms, such as H_2O_2 and its organic derivatives.

The cytochrome *c* oxidase reaction (EQ. 1) is

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highly exergonic and the free energy liberated by this chemistry is conserved in the form of a protonmotive force across the membrane. The enzyme active site is buried deep within the membrane, and the electrons and protons that are used to form water come from opposite sides of the membrane, resulting in charge separation across the membrane. Bringing the electrons and protons together with dioxygen in the active site of the enzyme results in the movement of four full charges across the membrane and, hence, the generation of a transmembrane electric potential. This simple mechanism is sufficient to make cytochrome oxidase an effective bioenergetic machine. However, there is a second and more fascinating mechanism by which this enzyme also generates a charge separation and protonmotive force during catalysis, discovered by Wikström [6–8]. For each turnover of the enzyme (Eq. 1), four additional protons are pumped across the membrane (Eq. 2).

$$\mathbf{8}\ \mathbf{H}^{+}_{in} + 4\ \text{cyt}\ c^{2+} + O_2 \rightleftarrows 4\ \text{cyt}\ c^{3+} + 2\ H_2O + \mathbf{4}\ \mathbf{H}^{+}_{out} \quad (2)$$

Hence, for each turnover, a total of eight charges are moved across the membrane through the enzyme, eight protons are taken up from the mitochondrial matrix (or bacterial cytoplasm for the prokaryotic oxidase) and four protons are delivered to the mitochondrial intermembrane space (or bacterial periplasm for the prokaryotic oxidase). The apparently simple chemistry that occurs at the active site of the enzyme is driving events that are far from simple and that are at the heart of understanding biological energy transduction and conservation.

The X-ray structures of the bovine heart mitochondrial cytochrome oxidase [9–12] and of the oxidase from the bacterium *Paracoccus denitrificans* [13,14] provide a necessary framework and critical information to design and interpret experiments, but the oxidase structures do not provide sufficient information to define the mechanism and dynamics of the enzyme. These are like snapshots of the enzyme, whereas what is needed is a movie. The central themes and concepts concerning the proton pumping mechanism did not undergo dramatic change when the structures were first presented 4 years ago. However, there are new developments that do call for re-evaluation of some of these ideas [15,16].

There are a number of interrelated issues that must be addressed in order to have a satisfactory understanding of how this enzyme pumps protons. The main questions discussed in this review concern the nature of the catalytic cycle and how proton pumping is coupled to the partial reactions within this cycle. The structures of the intermediates, the energetics of the processes, and the timing of proton uptake, proton release and intra-protein proton transfer are all parts of these questions.

1.1. The redox centers of the oxidase

1.1.1. Metal centers

Cytochrome oxidase has four redox-active metal centers (see [1]). The electron from cytochrome *c* initially goes to Cu_A, which is a bimetallic copper center [17,18]. Electrons are then transferred to heme *a* and, from there, to the heme a_3-Cu_B binuclear center. The heme-copper binuclear center is the active site of the enzyme, where dioxygen binds and is reduced to two water molecules. Heme *a*, heme a_3 and Cu_B are located at about the same 'latitude' in the membrane, which is about midway across the membrane dielectric [19]. Whereas electron transfer from cytochrome *c* to Cu_A does not contribute to the transmembrane voltage [20], the electron transfer from Cu_A to heme *a* results in charge separation across the membrane dielectric [20,21]. After the reduction of heme *a*, subsequent electron transfer from heme *a* to the heme-copper binuclear center does not contribute much to the development of the transmembrane potential [22], since these groups are essentially at the same depth in the membrane. However, there is substantial charge separation that is the result of proton movement through the enzyme that accompanies the transfer of electrons to the heme-copper center [20,21]. This charge separation is due to both the substrate protons delivered to the active site, as well as the pumped protons, moved across the entire membrane, at least for the steps corresponding to the reduction of compounds P and F (see Sections 2.1 and 4).

1.1.2. Amino acids as redox centers

In addition to the four metal redox centers in the oxidase, there are several amino acids near the active site of the enzyme that could conceivably form rad-

icals and, thus, be redox-active. Such reactions are well documented in other redox enzymes [23], such as cytochrome *c* peroxidase [24], photosystem II [25,26], and ribonucleotide reductase [27,28]. Most prominent is tyrosine-288 (*Rhodobacter sphaeroides* numbering), that is close enough to be a potential redox center and/or a direct proton donor in catalyzing the conversion of dioxygen to water [5,11,14,29]. Most intriguing, in the structures of the oxidases from bovine heart and from *P. denitrificans*, this tyrosine is cross-linked to a histidine residue (H284, *R. sphaeroides* numbering) that is also a ligand to Cu_B [11,14]. This tyrosine-histidine pair could be an additional source of electrons during the catalytic cycle, transiently forming a neutral tyrosyl radical. EPR evidence has recently been presented showing the formation of a tyrosyl radical in the enzyme upon oxidation of the binuclear center by peroxide [30]. In addition to Y288, there is a very highly conserved tryptophan (W280 in the *R. sphaeroides* oxidase) that could similarly serve as an electron source during catalysis [31]. At this time, there are no time-resolved experiments directly showing that an amino acid radical is formed during the dioxygen catalytic cycle, although there is an accumulation of circumstantial evidence suggesting that this is likely the case [29,30,32–35].

1.2. Proton-conducting pathways

The catalytic center is buried within the protein and is located about 30 Å distant from the bulk aqueous phase from which protons are taken to form water from dioxygen [10,14]. Hence, just for the purpose of delivering the substrate protons to the activated oxygen species, the enzyme must provide a substantial proton-conducting pathway. In addition, transporting the pumped protons across the membrane requires traversing about 50 Å through the protein. Proton diffusion through the protein consists of hopping along a series of hydrogen bonds [36,37], which can be considered to be a proton wire. Such a proton wire, however, need not be a stable structural element that can be clearly deciphered in the X-ray structure of the protein. The hydrogen bonds might be transient, and there is no need for all the elements of such a wire to be connected at the same time. Also, internal water molecules are almost certainly critical elements of the proton wire, and not all of these may be observed in the X-ray models. For these reasons, the connectivity of the proton wires as they appear in the X-ray models may not be complete.

Three putative proton-conducting input pathways have been identified in the X-ray structures [11,37,38]. These are called the K-channel, D-channel and the H-channel. The names are derived from conserved amino acid residues within each of the putative channels. The H-channel is proposed to be important for conveying pumped protons across the membrane, but only for the oxidases of animals [11]. The lack of conserved residues in the bacterial oxidases, plus mutagenesis data, indicate that this channel is unlikely to be functionally important in prokaryotic oxidases [37,39]. Even though the prokaryotic oxidases lack the H-channel, the efficiency of proton pumping appears to be as high as that of the oxidases from mammals. Perhaps in the eukaryotic oxidases the H-channel has a regulatory role. The K-channel and the D-channel, on the other hand, are observed in both the prokaryotic and eukaryotic oxidase structures [10,11,14], and the importance of these two channels in proton input to the enzyme is supported by extensive functional analysis of mutants.

1.2.1. The K-channel

This leads from the bacterial cytoplasmic surface (mitochondrial matrix) to the region on the heme-copper center [10,14]. The key residue is a highly conserved lysine (K362 in the *R. sphaeroides* oxidase) which, according to electrostatic calculations [40], is in the neutral unprotonated state in both the oxidized and reduced forms of the enzyme. This channel includes T359 and leads to the hydroxy group of Y288 at the active site. Since Y288 (cross-linked to H284, see above) is in a position to be a direct proton or hydrogen donor to the active site, it is reasonable to consider this is a proton pathway for the delivery of at least one of the substrate protons. Analysis of mutants of K362 show that the K-channel is necessary for the reduction of the heme-copper channel prior to the binding of oxygen [41–43]. It has been proposed that the K-channel is not involved in the second half of the catalytic cycle [43], though the experimental evidence during multiple turnover is still not definitive.

1.2.2. The D-channel

This leads from an aspartic acid group (D132, *R. sphaeroides*) near the surface of the enzyme facing the bacterial cytoplasm (mitochondrial matrix) to a buried glutamic acid (E286, *R. sphaeroides*) in the middle of the protein [10,13,14,44,45]. There are a number of internal water molecules within the protein that provide a reasonable pathway for proton diffusion between these two residues, separated by about 25 Å. Mutations in either of these acidic residues result in severe reduction of cytochrome *c* oxidase activity [43,44,46–48]. Residual steady-state activity is not coupled to proton pumping [46–49]. Steps in the catalytic cycle following the binding of dioxygen to the reduced heme-copper center are strongly affected by the mutations in the D-channel. The role of the D-channel, if any, in the steps prior to the binding of O_2 is still an open question. The fate of protons beyond E286 is not clear. It is very likely that the E286 side chain swings between alternate positions to switch the direction of proton flux. In one position, E286 can direct substrate protons to the active site [50], possibly using transient water molecules to provide the necessary hydrogen bond connectivity. Similarly, in another position [51], E286 might direct pumped protons across the membrane via a transient connection with the propionate residues of heme a_3.

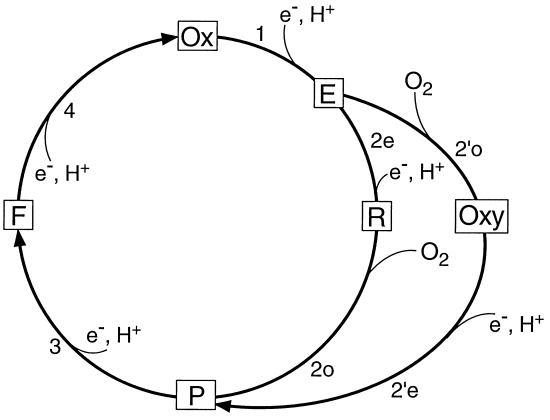

Fig. 1. Two putative pathways for the dioxygen cycle. The cycle starts with the fully oxidized binuclear center denoted as Ox. When the first electron arrives at the active site, species E forms. This reaction is coupled to a proton uptake. Species E represents a branching point on the scheme. Species E can either react with dioxygen, forming an Oxy complex, which is then reduced to form compound P (P_m); or, if species E has a sufficiently long lifetime, it might 'wait' for the second electron to arrive at the binuclear center to form species R, which is well known to react rapidly with dioxygen. There is no information concerning the reactivity of species E towards dioxygen, and it cannot be obtained as a 'stable' form, thus making it difficult to evaluate the role of species E in the catalytic cycle. Therefore, the question about which is the predominant or relevant pathway of the dioxygen catalytic cycle remains open. After compound P formation, the catalytic cycle proceeds by two single-electron steps of reduction of compounds P and F to return to the Ox species.

1.2.3. The exit channel

The D-channel and K-channel provide proton input pathways through the protein to near the level of the heme-copper center. Those protons which are pumped need an exit pathway to the bacterial periplasm (mitochondrial intermembrane space). Once delivered to the region near the heme propionates, there are multiple pathways that can be deciphered in the structures. There is an extensive hydrogen bond network involving several arginine residues and the heme propionates which can in principle provide a proton storage area and a part of the exit pathway [15,40]. Mutations of some of these arginines clearly perturb proton pumping in the cytochrome bo_3 quinol oxidase [52].

2. The dioxygen cycle of cytochrome oxidase

The catalytic cycle is traditionally viewed as consisting of two asymmetric halves. The first part includes the initial reduction of both heme a_3 and Cu_B in the binuclear center, the initial binding of dioxygen, and its reduction to the intermediate called compound P (see Section 2.3.1) The second part of the catalytic cycle includes two single-electron transfer steps, first to convert compound P to compound F, and then converting compound F to the ferric form of heme a_3. These are illustrated in Fig. 1. Intermediates P and F were initially discovered and characterized by Wikström by 'reversing' the oxidase reaction in whole mitochondria about 10 years ago [53].

As discussed in the following sections of this review, the mechanisms of both halves of the catalytic cycle are still uncertain. Below, we consider two putative pathways leading to formation of compound P (Fig. 1), as well as the number of protons translocated concomitant with each step of the cycle.

2.1. *Oxygenated intermediates of cytochrome oxidase and the origin of the work required for oxidative phosphorylation*

In 1989 Wikström [53] demonstrated the strong influence of the presence of a protonmotive force across the mitochondrial membrane on cytochrome oxidase which was catalyzing the oxidation of ferrocyanide ($E_m = +410$ mV). The protonmotive force was generated by ATP hydrolysis due to the combination of the F_1F_0-ATPase and the ATP/ADP translocator. He found that the oxidase reaction could be 'reversed' such that intermediate oxygenated forms of the enzyme could be stabilized in the mitochondrial membrane in sufficient amounts to allow quantitative spectroscopic characterization. In this way, he described two forms of the enzyme, compound P and compound F, by the characteristics of their UV-visible difference spectra (P-minus-Ox; F-minus-Ox), and determined their extinction coefficients. Optical spectroscopy was used to quantify the fraction of the oxidase in the membrane that was in the P (peak at 607 nm) or F (peak at 580 nm) state as a function of the phosphorylation potential. This notable experiment clearly demonstrated that the $P \rightarrow F$ and $F \rightarrow Ox$ steps (Fig. 1) are each coupled to the generation of the protonmotive force.

Furthermore, Wikström extracted from these experiments estimates of the standard state free energy differences between these states and the number of charges translocated across the membrane associated with the conversions $P \rightarrow F$ and $F \rightarrow Ox$. A number of assumptions were used to interpret the results of the 'reverse electron transfer' experiments [53]: (1) that the catalytic intermediates of cytochrome oxidase are in thermodynamic equilibrium; (2) that the hydrolysis of external ATP by mitochondria is coupled to the translocation of four H^+ per ATP molecule (three H^+ from the ATPase and one H^+ from the translocator); (3) that there are no leaks across the mitochondrial membrane and the proton driving force can be calculated given the ADP and ATP concentrations; (4) that the pH values on both sides of the coupling membrane were the same.

It was found that the experimental points in the plots of log(F/Ox) and log(P/F) vs. log(ATP/ADP·P_i) had slopes of 0.75 and 1 respectively. Equating the work of ATP hydrolysis and the work of the $P \rightarrow F$ and $F \rightarrow Ox$ transitions (assumptions 1 and 3), Wikström concluded that each of these steps is coupled to the translocation of $4 \times 1.0 = 4$ and $4 \times 0.75 = 3.0$ (assumption 2) charges (assumption 4), respectively.

Provided that the heme-copper center is located in the middle of the hydrophobic dielectric barrier, Wikström concluded that two charges in each case were translocated due to proton pumping, and the additional three charges (total for $P \rightarrow F \rightarrow Ox$) are due to the transmembrane movement of two electrons to the middle of the dielectric barrier (net one charge) and the four 'chemical' protons from the inner water phase to the heme-copper center (net two charges). Additionally, he found that the plot of log[P/F] or log[F/Ox] versus pH (at a given ADP/ATP ratio and fixed redox buffer potential) supported the point that all protons are taken up during the reduction of compounds P and F.

Subsequent experiments during the past decade demonstrated that two protons are taken up by the enzyme upon reduction of the heme-copper center [54], and have also indicated that there are likely four H^+/ATP driven across the membrane by the ATPase rather than three [55,56]. Whether the system is at equilibrium, the question of proton leaks and the issue about internal pH are all important for the quantitative interpretation of what fraction of the phosphorylation potential can be 'accounted for' by the two steps subsequent to the formation of compound P.

One concern that warrants discussion is whether the system can correctly be considered to be 'at equilibrium'. The working enzyme does not represent a closed system and needs to be considered together with the substrates of the reaction. The different states of the enzyme will be at equilibrium only when ΔG, the differential change in Gibb's free energy of the reaction, is zero. In the system employed, this will require that the free energy of the oxidation of ferrocyanide is matched by the driving counterforce of the membrane protonmotive potential (or the coupled phosphorylation potential). Since the ferro/ferricyanide electrochemical driving force is fixed, then only one point should represent true equilibrium of the system as the phosphorylation potential is varied.

Two important conclusions from the 1989 work that have been verified in subsequent experiments

[20–22,43,57–62] are that (1) the P→F and F→Ox transitions are, indeed, coupled to proton pumping; and (2) the contribution to transmembrane charge separation of each of these steps is equivalent. However, several experiments have shown that the likely stoichiometry is 1 H^+/e^- for each of these steps and that other parts of the catalytic cycle need to considered in the mechanism by which the redox free energy is coupled to the pump.

(1) The prediction that four protons should be pumped out of liposomes containing reconstituted cytochrome *c* oxidase upon oxidation of the fully reduced enzyme has not been realized. The experimental value from two different laboratories is about 1.2–1.3 [16,61].

(2) Direct electrometric measurements have shown that the number of charges translocated across the membrane upon the one-electron reduction of compound F is 2.5 or less [21]. This value is based on the assumptions that heme *a* is located in the dielectric middle of the membrane (0.5 charge translocated due to the electron transfer from Cu_A to heme *a*) and that the electron transfer to heme *a* is irreversible. If one further assumes a contribution of one substrate proton taken up by the enzyme and delivered to the heme-copper site in the middle of the membrane (0.5 charge translocated), this leaves 1.5 protons pumped during the F→Ox step. Possible corrections would reduce even further the estimated charge translocation associated with pumping during this step. If the dielectric distance between Cu_A and heme *a* needs to be corrected, it would be less than 0.5; and the equilibration of an electron between Cu_A and heme *a* results in only about 80% reduction of heme *a*, not 100% [63,64]. These adjustments rationalize the estimated number of charges translocated across the protein attributed to proton pumping during F→Ox to a value closer to 1. One point to note is that the interpretation of these experiments is ambiguous both as regards the calibration of the total number of charges translocated and whether one assigns these charges to the movement of substrate protons or pumped protons.

(3) The Wikström laboratory has very recently claimed proton pumping associated with the reduction of the enzyme [16]. Both proton release from oxidase-containing liposomes and charge translocation measured electrometrically were shown. The peculiar and unexpected aspect of this observation is that this reduction-associated proton pumping is only observed if the enzyme has just been oxidized, suggested an activated form of the oxidized enzyme that is somehow 'triggered' to use stored energy upon reduction. These results will require verification and further analysis, but for the present the importance is the first experimental demonstration of proton pumping coupled in some way to the reductive part of the catalytic cycle.

2.2. *Recent results concerning the first half of the dioxygen cycle: the reduction of the heme-copper center and the initial reaction with O_2*

The considerations described in the previous section make it apparent that the reductive portion of the catalytic cycle, i.e., leading to the formation of compound P (Fig. 1), merits further investigation. The reduction of heme a_3 is clearly necessary for dioxygen to bind to the heme. It is generally assumed [1–5] that it is necessary to form the two-electron reduced heme-copper center before significant binding to O_2 can occur. This is represented by species R in Fig. 1. We would like to suggest that the transient species with only one electron in the binuclear center, although not as yet directly observed, might be capable of binding to O_2 and be an important intermediate during steady state turnover.

2.2.1. *Rate of formation of species R is too slow to support steady-state turnover*

The assumption that the two-electron reduced heme-copper center is required prior to binding of O_2 is based by analogy to the binding of the CO to cytochrome oxidase, considering CO as a substrate analogue for O_2. If, indeed, the two-electron reduction of the binuclear center (species R, Fig. 1) is required, the next step of the catalytic cycle would be the binding of the O_2. This reaction, under a very broad range of O_2 partial pressure, demonstrates a bimolecular behavior with a rate constant of 10^8 M^{-1} s^{-1} to form the Fe^{2+}-O_2 (Oxy) complex (R→Oxy) [1]. The Fe^{2+}-O_2 complex (species A, not shown in Fig. 1) is reduced to compound P within approx. 100 μs, under conditions where heme *a* is oxidized [1].

The kinetics of the initial reductive step of this

reaction sequence, i.e. the formation of the reduced binuclear center (Ox → R), has been investigated by stopped-flow techniques [65–68]. The rate of the anaerobic reduction of the binuclear center found for the bovine enzyme varies over a very broad range. The reaction is biphasic and the rate of the fast phase varies, depending on the preparation of the enzyme, in a range generally within 20–100 s^{-1}. Even the fastest observed rates fall somewhat short in comparison to the turnover rate measured for the enzyme, i.e., 125 O_2/s (500 e/s). Note that the fastest rates are observed under conditions of very low solution electric potential of the reducing substrates (E_h below +70 mV) [68]. The slow phase of the reduction of heme a_3 is about 10-fold slower than the fast phase.

One of the interpretations of the origin of the two phases proposes that the initial reduction of the heme-copper binuclear center represents a branched process and that the first electron goes either to heme a_3 or to Cu_B. Within the framework of this hypothesis, the fast phase represents the one-electron reduction of the binuclear center (i.e., the formation of species E in Fig. 1) in which only that part of the population where the electron settles on the heme is observed optically, while the population with reduced Cu_B does not contribute into the optical response within the first phase. The second phase represents the reduction of heme a_3 in the population of the enzyme in which Cu_B has been reduced within the first phase and, thus, reflects the accumulation of the fully reduced binuclear center.

If this view is correct, then the reductive part of the proposed mechanism becomes kinetically incompetent of supporting the observed turnover rate. Indeed, in this case only the slow phase represents the formation of the enzyme species capable of binding O_2. In contrast, the rate of formation of compound E (Fig. 1) from the form of the enzyme in which both Cu_A and heme *a* are reduced, takes place within 100–200 μs and is kinetically competent (see Section 2.2.4) [69].

2.2.2. Reduction of the binuclear center requires proton delivery via the K-channel

The reduction of the binuclear center is coupled to proton uptake [54,68] and is regulated by the availability of protons to the active site [68]. The rate of reduction of heme a_3 in bovine cytochrome *c* oxidase has been shown to be coupled to proton uptake and to depend on the pH of the medium [68]. Site-directed mutagenesis has revealed amino acid residues that are likely to be important for making the binuclear center readily accessible to protons, most notably K362 (*R. sphaeroides* numbering) [42,70–72]. This residue is located approx. 15 Å from the binuclear center. The K362M mutation effectively eliminates turnover of the enzyme, but does not significantly perturb the properties of the active site. The K362M mutation causes a drastic impedance in the rate of the reduction of the binuclear center. The rate of reduction of the binuclear center in the *R. sphaeroides* K362M mutant, as slow as 1.5 min^{-1}, is approx. 20 000-fold less then that of the wild type, though it is not clear whether the product of the reduction is species E or R (one- or two-electron reduced binuclear center). Thus, the delivery of protons plays a crucial role in regulation of the electron transfer in the enzyme.

2.2.3. H_2O_2 as an alternate substrate: bypassing the K362M block

Cytochrome oxidase can reduce not only the natural substrate, dioxygen, but also hydrogen peroxide [73–77]. Unlike dioxygen, which definitely requires preliminary reduction of at least heme a_3 to interact with the enzyme, H_2O_2 can also react with the oxidized binuclear center, albeit at a much slower rate [59,78]. Hydrogen peroxide reacts with the fully oxidized binuclear center to form compound P [79] (Fig. 2). The rate of H_2O_2 binding to bovine heart cytochrome oxidase exhibits hyperbolic behavior, saturating at molar concentrations of H_2O_2 [69,80]. The second order rate constant for the bovine heart enzyme is approx. 700 M^{-1} s^{-1}. The maximal rate of the H_2O_2 binding is approx. 250 s^{-1} [69]. Interestingly, the V_{max} of the reaction is close to the lifetime of the heme a_3 ferric hydroxide formed at the end of the reaction of the fully reduced enzyme with O_2 [81,82]. Thus, the reaction of H_2O_2 with the fully oxidized enzyme to form compound P may represent not simply a ligand binding reaction, but rather a ligand exchange reaction, possibly rate-limited by the off-rate of hydroxide.

Compound P, formed in the reaction with H_2O_2, has a high midpoint potential and will be reduced by a number of different electron donors which will re-

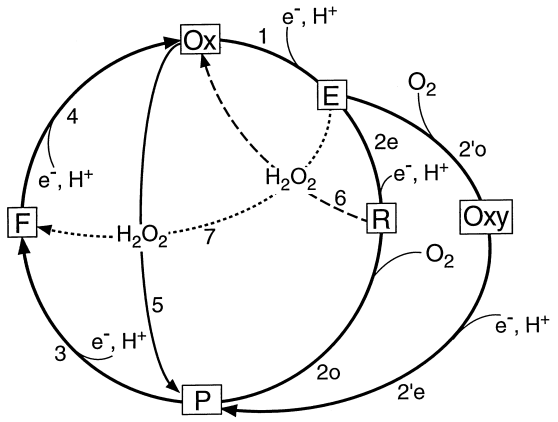

Fig. 2. Interaction of H_2O_2 with the intermediates of the dioxygen catalytic cycle. Hydrogen peroxide interacts with the forms of the binuclear center at all three reduction levels (species Ox, E, R). Reaction of H_2O_2 with the oxidized binuclear center yields compound P (reaction 5). The rate of compound P formation via reaction 5 saturates at submolar concentrations of H_2O_2. The formation of species E increases the reactivity of the binuclear center towards H_2O_2. Species E can be kinetically trapped by H_2O_2, forming compound F and, thus, bypassing formation of compound P. Species R also rapidly reacts with H_2O_2, transiently forming so-called compound F′ (not shown in this figure, but see Fig. 3), which then decays to compound Ox. Thus, there are three mechanisms of H_2O_2 reduction by cytochrome oxidase, via routes 5-3-4, 1-7-4 and 1-2e-6. The operative mechanism is defined by the conditions.

duce compound P into compound F and, further, into the ferric form of heme a_3 (Fig. 2) [78,83,84]. In the presence of cytochrome *c*, the enzyme exhibits cytochrome *c* peroxidase activity [73–76].

$$2\ \text{cyt}\ c^{2+} + 2\ H^+ + H_2O_2 \rightarrow 2\ \text{cyt}\ c^{3+} + 2\ H_2O \qquad (3)$$

Utilization of H_2O_2 becomes a very useful experimental tool when the natural reductive pathway leading to the formation of compound P is blocked (step 1 or 2, Fig. 2). The reduction of heme a_3, as discussed above, requires proton delivery into the active site. The proton delivery can be blocked by carrying out the reaction under alkaline conditions (pH >9.5) [68,85] or by site-directed mutagenesis of residues in the K-channel, such as the K362M mutation [41,42]. In both cases, the natural dioxygen reductive cycle is suppressed due to inhibition of the reduction of the binuclear center (Ox $\rightarrow$ E, Fig. 2), and H_2O_2 significantly accelerates cytochrome *c* oxidation [41,80]. It is important to note that the peroxidase activity of the K362M mutant is independent of pH [85], demonstrating that the step that is blocked by the mutation is the formation of species E (step 1, Fig. 2). The peroxidase activity of the K362M mutant exhibits hyperbolic behavior with respect to the concentration of H_2O_2, and can be described in terms of a V_{max} (25 H_2O_2 oxidized per sec) and K_m (50 mM H_2O_2) [41]. Even at saturating concentrations of peroxide, the peroxidase activity of this mutant appears to reflect the rate of H_2O_2 binding to the fully oxidized binuclear center. In this regard, the K362M mutation has an effect equivalent to limiting both the rate of proton delivery to the active site necessary to generate the dioxygen-reactive species and the rate of binding of H_2O_2 to the fully oxidized binuclear center (about 25 s^{-1} for the K362M *R. sphaeroides* mutant as compared to 250 s^{-1}, measured for the bovine oxidase). Mutations in the D-channel do not seem to have this effect [42], but have a strong influence on steps in the reaction cycle subsequent to the formation of compound P (Fig. 2) [43,44,46–48].

2.2.4. H_2O_2 as an alternate substrate: trapping the one-electron reduced heme-copper center

H_2O_2 also reacts with the fully reduced binuclear center [69,80,86–89]. This reaction (step 6, Fig. 2) is much more rapid than the reaction with the oxidized enzyme, perhaps because water is present as a metal ligand at the binuclear center and is much more readily displaced. The rate of the reaction does not saturate, and at 5 M H_2O_2 the rate of reaction is faster than with 1 mM O_2 (7 μs vs. 10 μs half-time) [69]. There is also strong circumstantial evidence [69] that H_2O_2 can react readily with the one-electron reduced heme-copper center and serve as a very effective kinetic trap for this species (step 7, Fig. 2). By reacting H_2O_2 with the fully reduced oxidase, a species can be transiently generated in which the heme-copper center is oxidized and both heme *a* and Cu_A are reduced (designated species H, for half-reduced). The reaction of species H with a second equivalent of H_2O_2 is much more rapid than the reaction of H_2O_2 with the fully oxidized enzyme. This is presumably due to rapid electron transfer to heme a_3 (i.e., formation of species E (Fig. 2)), accompanied by the protonation of the heme-coordinated hydroxide which is, thus, converted to water and can be more readily displaced. The product of the reaction is compound F, which forms in about 100–200 μs, and the rate

saturates at concentrations of H_2O_2 above 100 mM. A likely explanation for this rate saturation is that the rate is limited by the electron transfer rate from heme *a* to the heme-copper center, an electron transfer reaction which is coupled to proton delivery into the active site.

Under circumstances where no proton transfer is involved, simple electron equilibrium between hemes *a* and a_3 occurs in only 1.5 μs [90–92]. However, under conditions when proton transfer is a required part of this process (i.e., proton uptake accompanying electron transfer from heme $a \rightarrow$ heme a_3), then the equilibration time is increased considerably to about 1 ms [90–92]. Hence, it is reasonable to surmise that the rate limiting step in the reaction of H_2O_2 to form compound F (steps 1–7, Fig. 2) is the proton transfer that must accompany the electron (heme $a \rightarrow$ heme a_3) to transiently form species E (step 1, Fig. 2). Without a supply of electrons, the protonation of the hydroxide at the active site, necessary before H_2O_2 can react, would take several milliseconds [81,82].

2.3. The second half of the dioxygen cycle: the peroxidase part of the reaction

The first half of the catalytic cycle ends with the formation of compound P which, in turn, is further reduced in two one-electron steps: P → F and F → Ox. Each of these steps has been shown to be accompanied by the net uptake of one proton in the reaction catalyzed by the soluble oxidase [1,93,94]. These two single-electron steps have been the focus of research because they are undoubtedly coupled to proton translocation.

There are several ways in which compounds P and F can be generated for study in the laboratory. Operationally these have been defined by absorption peaks in the difference spectrum (*minus* fully oxidized enzyme) at about 607 nm (compound P) and 580 nm (compound F). It is now clear that there are multiple forms of these species which have very different properties depending on how they are prepared. This has led to a current state of confusion of both nomenclature and of experimental results. We will refer to oxygenated species at the two-electron reduced redox state as P, regardless of their spectroscopic features. Compounds designated 'F' imply in our nomenclature the three-electron reduced oxygenated enzyme.

2.3.1. Compounds P: species P_m, P_{607}, P_{580} and P_r

Compound P can be generated in the following ways.

(1) Reduction of the oxidase by CO to form the two-electron reduced (mixed-valence) form of the enzyme, followed by oxidation by O_2 [95,96]. Compound P formed in this manner is designated as P_m [97].

$$\mathbf{Ox} + CO + O_2 \rightarrow \mathbf{Compound\ P_m} + CO_2 \quad (4)$$

The reaction can be carried out in two separate steps by first generating the mixed-valence enzyme and then reacting that with O_2.

$$\mathbf{Ox}\ (Fe^{3+}[HO^-]\ Cu^{2+}) + 2\ CO \rightarrow \mathbf{Mixed-valence}\ (Fe^{2+} - CO\ Cu^{+})[H^+] + CO_2 \quad (5)$$

$$\mathbf{Mixed-valence}\ (Fe^{2+} - CO\ Cu^{+})[H^+] + O_2 \rightarrow \mathbf{Compound\ P_m} + CO \quad (6)$$

where one bound proton at an unknown site of the mixed-valence enzyme is indicated [H^+], and we are assuming that the oxidized enzyme (Ox) has a single hydroxide ligand at the heme-copper center. In compound P_m, one of the electrons required to reduce dioxygen presumably comes from Cu_B, which is likely to be Cu_B^{2+} (HO^-) in compound P_m.

(2) Reversal of the oxidase reaction in mitochondria by imposing a large protonmotive force [53].

(3) Adding H_2O_2 to the oxidized enzyme under alkaline conditions [79,98,99]. We will refer to this species as compound P_{607}, and it is probably identical to compound P_m.

$$H_2O_2 + \mathbf{Ox}(Fe^{3+}[HO^-]\ Cu^{2+}) \rightarrow \mathbf{Compound\ P_{607}} + H_2O \quad (7)$$

At more acidic pH (pH 6) the reaction of stoichiometric amounts of H_2O_2 with the fully oxidized enzyme yields a species that has an absorbance peak near 580 nm and is spectroscopically very similar to compound F [99]. We will refer to this species as P_{580}, reflecting the fact that this is a two-electron

reduced species with a spectroscopic peak near 580 nm.

The experimental complications are aptly illustrated by examining the reaction of the oxidized enzyme with excess H_2O_2 [100] (Pecoraro, Konstantinov and Gennis, unpublished observations). At alkaline pH, the initial product is predominantly compound P_{607}. There appear to be two independent routes to obtain a species with an absorbance shifted to 580 nm. One route is the reaction of a second equivalent of H_2O_2, which serves as an electron donor and produces superoxide as a product. This is a one-electron reduction of $P \rightarrow F$. However, there also seems a route to generate the P_{580} species by an intramolecular rearrangement favored at acidic pH. These data can be rationalized by a speculative model where the '607 nm'-type spectrum is due to heme a_3 oxoferryl with hydroxide bound to Cu_B, and the '580 nm'-type spectrum results from the protonation of the hydroxide on Cu_B to water, which may ($P_{607} \rightarrow F_{580}$) or may not ($P_{607} \rightarrow P_{580}$) be accompanied by further reduction of the enzyme.

(4) Transiently, in the reaction of the fully reduced enzyme with dioxygen [1]. This is most typically done in a flow-flash reaction, in which the CO adduct of the fully reduced enzyme is prepared anaerobically and is then mixed with dioxygen. The CO is 'flashed' off of the enzyme photolytically, at which time the reaction with dioxygen is initiated. The sequence of events that occurs as the enzyme is oxidized can be spectroscopically deconvoluted by either using low temperature to slow the process [97] or rapid data acquisition at room temperature and global spectroscopic deconvolution procedures [101]. In this case, the version of compound P that is observed is denoted as compound P_r. In the flow-flash reaction starting with the four-electron reduced enzyme, it is clear that one of the electrons used to reduce dioxygen to compound P comes from heme *a*. For this reason it is supposed that Cu_B remains reduced in compound P_r [97].

The structures of compounds P remain controversial at several levels.

(1) Is the O-O bond already split in compound P? Resonance Raman data on compound P_m formed from the reaction of the mixed-valence form of the enzyme [1,29] and on compound P_{607} formed using hydrogen peroxide [1,34,35] provide strong evidence that the O-O bond is split in at least a significant fraction of the population to yield the heme a_3 oxoferryl species ($Fe^{4+}=O^{2-}$) plus (presumably) water or hydroxide. The O-O bond has also been shown to be cleaved in compound P_m (Eq. 5) by demonstrating isotope release into bulk water of one ^{18}O atom originating from $^{18}O_2$ [32]. The simplest interpretation consistent with the data is that in compounds P_m and P_{607} (and presumably P_{580}) the O-O bond is split. However, time-resolved resonance Raman spectroscopy has failed so far to demonstrate that the O-O bond is split in compound P_r formed during the flow-flash reaction starting with fully reduced enzyme. Although it is tempting to speculate that the O-O bond is also broken in compound P_r, this conclusion would be based on species P_r having the same UV/vis spectrum (607 nm) as P_m and P_{607} (Eqs. 4 and 7, above). This is not necessarily the case.

(2) What is the source of the additional electron required if the O-O bond is split in compound P_m? Both oxygen atoms are at the valence state of water (each two-electron reduced) if the O-O bond is split. Consider the reaction starting with the two-electron reduced enzyme plus dioxygen.

$$\underset{\text{Compound } P_m}{RH\ (Fe^{2+}\ Cu^{+}) + O_2 \rightarrow R^{\bullet}(Fe^{4+}=O^{2-}\ HO^{-}-Cu^{2+})} \quad (8)$$

It is necessary to postulate an additional source of an electron, R, to accomplish this chemical reaction (see Section 1.1.2). Since the reaction to form compound Pm (Eq. 5) does not entail any proton uptake from solution [102], an internal proton must be provided to form hydroxide. Most likely the source of both the proton and electron is the tyrosine at the active site (Y288 in the *R. sphaeroides* oxidase), which is tentatively identified as the species RH (Eq. 8), and both an electron and proton (i.e., a hydrogen atom) are transferred in the reaction. The fact that both a proton and electron transfer are involved in reaction 5 favors the tyrosine as the electron source over the conserved tryptophan (W280) at the active site, since tryptophan oxidation is not accompanied by deprotonation as is the case with tyrosine. The tyrosine likely also forms a neutral radical in compound

P_{607} (Eq. 8), which is supported by evidence of a tyrosine radical in the oxidase following the reaction with hydrogen peroxide [30].

2.3.2. Compound F

In compound F (three-electron reduced state) there are overwhelming spectroscopic data that heme a_3 is in the oxoferryl form ($Fe^{4+}=O^{2-}$) [1]. Again, however, there appear to be multiple forms and ambiguity with respect to the redox status and protonation state of groups in the immediate vicinity of the active site, depending on how compound F is generated. As mentioned in the previous section, part of the confusion is due to what we are referring to as compound P_{580}, which appears spectroscopically similar to compound F, but is only at the two-electron reduced state.

One can prepare compound F in several ways: (1) reversal of the oxidase reaction in whole mitochondria by imposing a large protonmotive force [53]; (2) reaction with the fully oxidized enzyme with excess H_2O_2 [83,98,103]; (3) reaction of the fully reduced enzyme with dioxygen (transiently formed in the flow-flash reaction) [1].

It is significant that the reduction of compound F ($F \rightarrow Ox$) obtained by each of the three methods listed above is coupled to proton pumping. Hence, it seems likely that the products of these three reactions are identical. A version of compound F, but with Cu_B in the reduced state, has also been prepared recently [80], but it is not known whether the conversion of this species to Ox is coupled to pumping (see Section 4).

3. The F → Ox conversion can utilize internal protons

The $F \rightarrow Ox$ redox step has been studied in isolation by generating compound F using hydrogen peroxide and then using a photo-activated reductant to rapidly inject one electron [18,20,21,43,104]. Electrometric studies (see Section 2.2) indicate that apart from the electron transfer from Cu_A to heme *a*, there are at about two charges (maximum) translocated across the membrane [20,21,43]. These presumably result from the movement of protons within the protein. The same reaction has been examined optically [104], monitoring the electron transfer, and the proton movement coincides with the electron transfer from heme *a* to heme a_3. This single-turnover reaction exhibits multiple phases, and is weakly dependent of pH between pH 7.5 and 10 [104]. The rate of the electron transfer reaction manifests a substantial H/D solvent isotope effect of about 4.5 [104]. In the flow-flash reaction of fully reduced enzyme with O_2, the pH dependence of the $F \rightarrow Ox$ conversion is also very weak, and is very similar to that observed in the single-electron experiments [93]. These data indicate the following:

(1) Electron transfer from heme *a* to the heme a_3 oxoferry species (compound F) is accompanied by the movement of at least two protons either partially or entirely across the dielectric barrier.

(2) The electron transfer reaction is most probably rate-limited by the rate of proton movement within the enzyme.

(3) The proton-donating groups are still able to donate protons, at least in part, even when the bulk pH is 10. In compound F (prepared with hydrogen peroxide), so the apparent p*K* values of proton donors must be quite high. Note that these reactions all refer only to partial turnover (half-cycle or quarter cycle) of the enzyme.

3.1. Mutations in the D-channel

The D-channel is defined by two acidic residues: E286 is located near the heme-copper center and D132 is located about 30 Å away near the protein surface (*R. sphaeroides* numbering). Mutations of either of these two acidic residues have large effects on the activity of the enzyme [43–45,47,48,105]. The most severe blocks are observed in steps in the catalytic cycle following the formation of compound P. In the flow-flash reaction of the fully reduced enzyme with O_2, mutations in either of these residues prevent the net proton uptake that is normally observed with the wild-type enzyme accompanying both the $P \rightarrow F$ and the $F \rightarrow Ox$ steps. The reaction of the reduced E286Q mutant with O_2 appears to stop at the formation of the first oxygenated intermediate, presumably compound P. This would suggest that only those protons already at the active site are utilized in the oxygen chemistry, and the reaction can proceed no further. The reaction with the D132N mutation proceeds to form compound F but, unlike the

wild-type enzyme, there is no proton uptake from solution [48]. This indicates that one proton is available within the D-channel, above the level of D132, to be delivered to the active site. Presumably, the proton-donating residue is E286.

4. Possible regulation of proton pumping by the redox state of Cu_B: hypothesis

Although considerable progress has been made concerning the proton input pathways in cytochrome oxidase, very little is known about the mechanism of how the proton pump actually works. Indeed, little is known about the functional role of Cu_B. Possibly, this metal is transiently reduced during each electron transfer to the active site or, at the other extreme, this metal may function only to polarize the O-O bond or stabilize hydroxide binding at the active site. Cu_B has been proposed to play a central role in the proton pumping mechanism [106,107] and has also been proposed to have no direct role in the pump [11,15].

The demonstrated importance of the first part of the catalytic cycle to proton pumping [16], and the possibility that the favored kinetic pathway might be via the one-electron reduced state of the binuclear center provide intriguing possibilities for speculation. Consider the following experimental facts:

(1) A single-electron reduction of the binuclear center substantially accelerates the rate of H_2O_2 binding.

(2) Electric measurements performed using the electron injection technique demonstrate that the total number of charges translocated during the $F \rightarrow Ox$ conversion is equal to or less than 2.5.

(3) In flow-flash experiments using oxidase reconstituted in liposomes with O_2 as the oxidant, the 100 μs phase corresponding to the $P_r \rightarrow F$ conversion is not coupled to the acidification in the external medium. The total number of protons translocated across the membrane in the flow-flash experiments is approx. 1.2.

(4) There is neither membrane potential generation, nor acidification of the extravesicular medium upon reduction of the primary Fe^{2+}-O_2 (Oxy) complex (species A in Fig. 3) into P_r or P_m.

The scheme shown in Fig. 3 depicts possible derivatives of cytochrome oxidase which can participate in the dioxygen reduction cycle. The compounds are sorted in two circles. The inner circle depicts the compounds with oxidized Cu_B^{2+}. The outer circle depicts the derivatives with reduced Cu_B^{+}. The arrows show possible conversions among these compounds. Some of these conversions are parts of the natural dioxygen reduction cycle, and some of them are not. Note that each compound in the presence of reducing equivalents has at least two potential decay pathways. However, not all the reactions shown in this scheme have been observed experimentally.

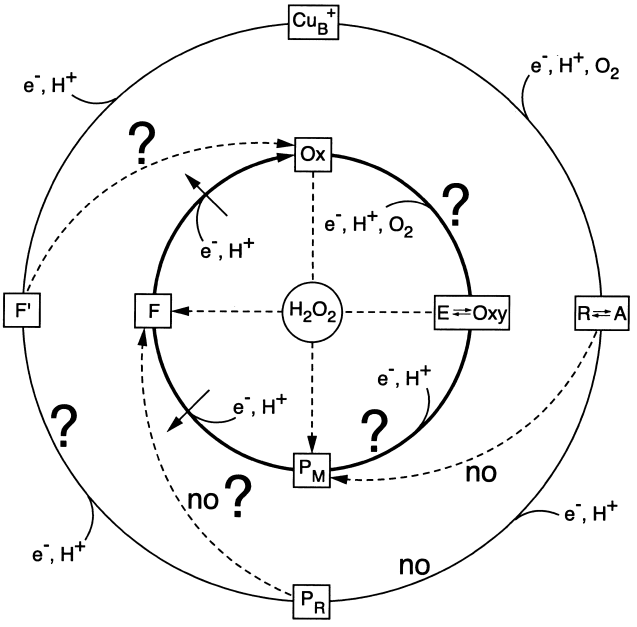

Fig. 3. How might the redox state of Cu_B regulate the productivity of the proton pump in cytochrome oxidase? The forms of cytochrome oxidase are classified in accordance with the redox state of Cu_B. On the inner circle, the derivatives with oxidized Cu_B are shown, while the compounds on the outer circle have Cu_B reduced. Species E and Oxy on the inside circle and species R and A (the historic name for the oxy complex formed in the flow-flash reaction) on the outside circle, are placed in the same boxes, so that each arrow represents an electron transfer reaction. Some, but not all, of the reactions are characterized in terms of coupling to proton pumping. Conversions $P_m \rightarrow F$ and $F \rightarrow Ox$ are coupled to proton pumping, while there is not enough information concerning $Ox \rightarrow E \rightarrow Oxy$ and $E \rightarrow Oxy \rightarrow P_m$. Conversion of species A to either P_m or P_r is not coupled to proton pumping. The coupling of the $P_r \rightarrow F$ conversion to proton pumping remains unclear, though this step does coincide with the generation of membrane potential [60]. Conversion of $F' \rightarrow Ox$ is not characterized in terms of coupling to proton pumping, but is expected to not be coupled in accordance with the hypothesis described in the text.

A useful working hypothesis is that only reactions along the inner cycle are coupled to proton pumping. In other words, the evolution of the intermediates with reduced Cu_B is unproductive. This does not rule out mechanisms involving transient reduction of Cu_B, which is different from the case with pre-reduced metal, since the original distribution of the protons in the proton channels would be different. Indeed, since the reduction of Cu_B is coupled to uptake of a proton, it is reasonable to consider that this might play a crucial role in the regulation of proton pumping machinery.

The importance of Cu_B redox state to proton pumping has been previously suggested in the context of the relevance of the conditions imposed when the enzyme is studied using the flow-flash reaction (i.e., fully reduced) [2].

A few features of the proposed productive dioxygen steady-state cycle should be noted.

(1) The first electron transfer to the oxidized heme-copper center is certainly energetically disfavored. It is necessary to provide two electrons to the enzyme to reduce both Cu_A and heme *a* before there is any significant population of enzyme in which an electron will be found in the heme-copper center. Whether or how this first electron transfer is coupled to proton pumping must remain an open question, and must await further exploration of the activated form of the oxidized enzyme [16].

(2) We propose that the reaction does not proceed through the two-electron reduced heme-copper center, but that there is a rapid conversion from the one-electron reduced Oxy intermediate to compound P_m. As soon as the two input centers (Cu_A and heme *a*) are reduced, there is some electron transfer to the binuclear center which is accompanied by a proton which converts the hydroxide ligand to water, which is immediately displaced by O_2. Although the affinity is not high for O_2, the presence of the second electron distributed between heme *a* and Cu_A assures the rapid conversion to compound P_m. If O_2 kinetically traps species E (the one-electron reduced species), it does so by forming a transient ferrous heme a_3 complex which is rapidly reduced in what would amount to a concerted four-electron reduction to yield heme a_3 oxoferryl plus hydroxide (compound P_m). In this scenario, two electrons come from heme a_3 ($Fe^{2+} \rightarrow Fe^{4+}$), one electron comes from heme *a* (the second delivered to the heme-copper center), and the fourth electron could come from Y288 along with a proton. This would essentially be an irreversible step, favored energetically, and could be easily speculated to be coupled to proton pumping. Note that this sequence of events cannot be observed in the flow-flash reaction, where one starts with reduced Cu_B, or in any reaction of O_2 where one starts with fully reduced enzyme or mixed-valence enzyme. It is reasonable to speculate that the oxidized state of Cu_B is critical to obtain proton pumping as it occurs during steady state turnover.

(3) The third and fourth electrons delivered to the heme-copper center ($P \rightarrow F$ and $F \rightarrow Ox$) are clearly coupled to proton pumping and are energetically favorable. During steady-state turnover, it is very likely that, as in the previous step, Cu_B is oxidized.

This hypothesis is consistent with a proton pumping of three or four protons per cycle, and has a number of features that are experimentally testable. For example, the conversion of $F' \rightarrow Ox$ that was recently observed experimentally [69] is predicted to be unproductive in proton pumping.

5. Several questions to be addressed experimentally

At the end we would like to summarize not the conclusions about our knowledge of cytochrome oxidase, but rather list several of the questions which have to be addressed.

(1) Can dioxygen bind to the one-electron reduced binuclear center or does binding wait until the second electron is present in the heme-copper center?

(2) What is he structure of compound P_r? Which compound, P_r or P_m, is the intermediate of the catalytic cycle, or might both of them be relevant under different reaction conditions?

(3) How many protons are translocated during the overall turnover of the enzyme? In other words, how reliable is the measured stoichiometry of 4 H^+/e^- [108]. Are there conditions where the value might be 3?

(4) What is the nature of the proton pumping that is coupled to the first part of the catalytic cycle [16]? Does the proton pumping observed during single-

turnover conditions starting with the fully reduced enzyme reflect pumping during steady state conditions?

(5) How does the redox state of Cu_B affect the efficiency of proton pumping, if at all?

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