Minireview

Understanding the mechanism of proton movement linked to oxygen reduction in cytochrome *c* oxidase: lessons from other proteins

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Abstract Cytochrome *c* oxidase is a large intrinsic membrane protein designed to use the energy of electron transfer and oxygen reduction to pump protons across a membrane. The molecular mechanism of the energy conversion process is not understood. Other proteins with simpler, better resolved structures have been more completely defined and offer insight into possible mechanisms of proton transfer in cytochrome *c* oxidase. Important concepts that are illustrated by these model systems include the ideas of conformational change both close to and at a distance from the triggering event, and the formation of a transitory water-linked proton pathway during a catalytic cycle. Evidence for the applicability of these concepts to cytochrome *c* oxidase is discussed.

Key words: Cytochrome *c* oxidase; Proton pathway; Redox Bohr; Propionate

1. Introduction

Respiration is achieved by the movement of electrons through a series of redox centers, buried within membrane proteins, which couple this electron movement with the pumping of protons across the membrane. The electrochemical gradient is then utilized by another membrane protein, ATP synthase, to make ATP as an energy source for the cell. The final electron acceptor in the respiratory chain is cytochrome *c* oxidase (C*c*O) which has multiple redox centers: a binuclear Cu_A, heme *a*, heme a_3 and Cu_B. The electrons are consumed in the reduction of oxygen to water at the binuclear active site (heme a_3/Cu_B), a chemical reaction that requires protons to access this buried site. The reduction of a single oxygen molecule requires that C*c*O take up four electrons and four protons to make $2H_2O$, and an additional four protons to be pumped [1]. The exact mechanism of this coupling of proton movement with electron transfer/oxygen chemistry is not fully understood. However, advances in understanding have been greatly aided with the advent of a number of crystal structures of C*c*O [2–5]. These, along with mutagenesis, have helped to identify two uptake paths for protons, the D and K channels, named because of the highly conserved residues, aspartate and lysine respectively, that are required for the function of these proton pathways (Fig. 1). However, neither crystal structures nor mutagenesis have revealed an obvious exit path for protons. Even when proton paths are obvious, knowing the location does not necessarily indicate when and how protons move within them.

The structural and functional complexity of C*c*O presents a formidable challenge, since, presumably, each electron (four) and each proton (eight) entering and exiting the enzyme sees a different redox and/or conformational state. Thus studies on simpler, more well-defined systems may give some insights into underlying principles of how protons can be taken up and released, that can be applied to C*c*O.

In this review we attempt to draw analogies with such systems that take up and release protons in order to address the question of whether C*c*O is likely to employ a *direct* or *indirect* coupling mechanism, or a combination of both. A *direct* mechanism is used here to describe proton movement that depends on protonation/deprotonation of the centers, or direct ligands of the centers, that carry out the redox reactions or oxygen chemistry. Direct mechanisms that have been proposed for C*c*O include the Cu_A ligand exchange model developed by Chan and colleagues [6], and the histidine cycle model proposed by Wikström and coworkers [7]. An *indirect* mechanism would also be triggered by the redox or chemical reactions, but would not use the direct ligands of the centers as proton carriers; rather, conformational and p*K* changes in residues at a distance would lead to proton uptake and release. Examples of indirect mechanisms proposed for C*c*O include that of Yoshikawa and coworkers [8] involving Asp51 (bovine numbering), and versions of the charge neutralization concept initially described by Rich and colleagues [9].

Direct mechanisms of coupling are the guiding principle of the Mitchell hypothesis [10] as illustrated by the Q-cycle in cytochrome bc_1 [11] in which ubiquinone takes up protons when it is reduced and releases them when oxidized. The classic indirect mechanism is the Bohr effect in hemoglobin, where binding of oxygen to the heme iron causes a series of conformational changes that result in proton release at a distance (also reversible, accounting for release of oxygen in the tissues). Given the complexity of C*c*O, a combination of these

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Abbreviations: C*c*O, cytochrome *c* oxidase; MD, molecular dynamics

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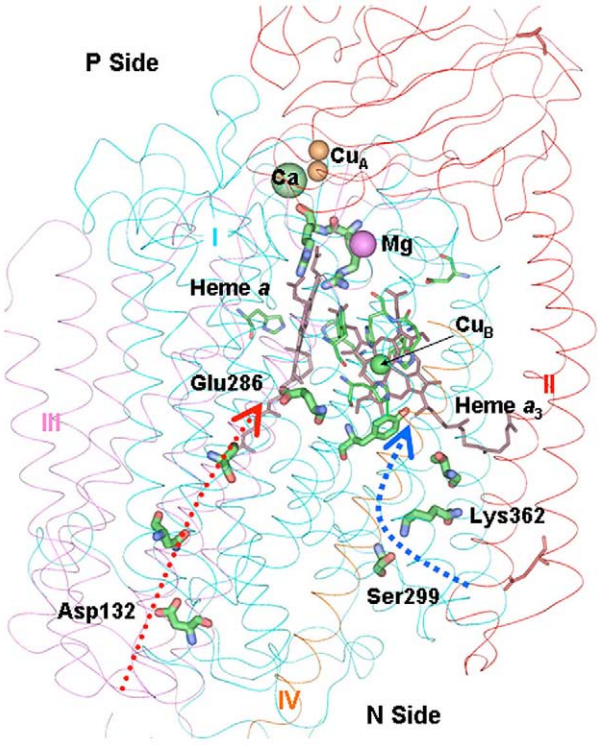

Fig. 1. Diagram of the four subunit structure of *Rhodobacter sphaeroides* C*c*O showing two well-defined proton uptake paths, the D (red) and K (blue) paths. The three redox active centers are labeled: the dinuclear Cu_A center where electrons enter, heme *a*, and the heme a_3/Cu_B active site where oxygen chemistry occurs. There are also two non-redox metal sites shown: Ca and Mg. The backbone structures of subunit I (cyan), II (red), III (magenta) and IV (orange) are shown. The figure was made using Insight II with the coordinates of 1M56 [5].

mechanisms might be expected, and indeed is found in several systems (see below).

2. Bohr effects from ligand binding events – hemoglobin and sensory proteins

Upon oxygen binding to hemoglobin, there are changes in the Fe–His distance and other changes in the heme structure, which are transmitted through the protein to alter the conformation and pK_a values of certain key amino acids at a distance of 15 Å. The resulting release of protons is known as the Bohr effect. Conversely, changes in pH or in surface amino acids, such as tryptophan β37 in human hemoglobin located at α/β subunit interface, alter the Fe–His bonding distance and the affinity of the heme for oxygen [12,13]. Similarly, the sensor protein FixL uses the effect of oxygen or NO binding to initiate different signaling mechanisms [14]. Changes to the heme include the amount of porphyrin ring ruffling and the hydrogen bonding of the porphyrin ring propionates to residues in the protein. Subsequent movement of a flexible loop results in the rotation of an arginine residue into the heme pocket and produces an active 'on' (NO) or 'off' (O_2) conformation for kinase activity in another domain. These examples illustrate that, although processes may be triggered by changes at the heme, they do not have to occur in close proximity.

In C*c*O, the binding of an O_2 ligand to heme a_3 in the heme a_3/Cu_B site, and subsequent chemical changes due to electron transfer events, induce local effects on the metals and their ligands, as observed by resonance Raman and visible spectral changes. It is harder to discern if there are more distant effects. However, there is a covalent connectivity of the histidine ligand of Cu_B, H284 (numbering is for *Rhodobacter sphaeroides* C*c*O), to a key glutamate, E286, which is involved in proton movement. In addition, a tyrosine, Y288, is covalently cross-linked to H284 and proposed to form a tyrosine radical as part of the catalytic mechanism. The unique His–Tyr cross-link creates a circular loop ($H_{284}P_{285}E_{286}V_{287}Y_{288}$) around heme a_3 (Fig. 2). All these critical residues are on helix VI of subunit I, which traverses the membrane at an unusually oblique angle and makes further connections at the outside and inside surfaces of the protein (Fig. 2). Transmission of local changes at the active site to residues at a distance can easily be envisioned as mediated by altered interactions along this helix. In fact, substantial evidence indicates that in the reduced and oxidized states of C*c*O, E286 exists in different conformational states [5,15,16].

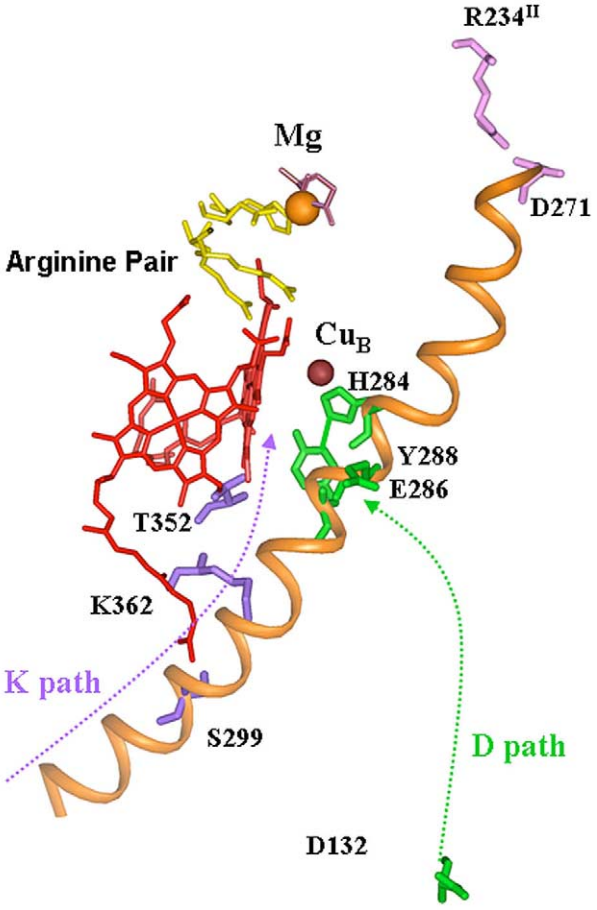

Fig. 2. Helix VI in subunit I of C*c*O contains the residues that make up the PEVY loop, close to the active site, and connected to D132 through the D path (green). It also connects to the interface of subunits I, II and III where D271 at the top end of helix VI electrostatically interacts with R234 of subunit II as well as K103 of subunit III (not shown) and, through a phospholipid, to subunit IV (not shown). Additionally, residue S299 has been shown to play a role in the K pathway. The figure was made by J. Yang and D.A. Mills using Insight II with the coordinates of 1M56 [5].

3. Redox-Bohr effects – the propionates of heme

As in ligand binding events at the heme, the oxidation state of the heme (Fe^{2+}, Fe^{3+}, Fe^{4+}) has effects on its ring substituents and nearby amino acids, and vice versa. In cytochromes *c*-551 of *Pseudomonas*, Leitch et al. [17] saw a pK_a shift of 0.9 pH units of the heme propionate, dependent on the redox state of the heme. It was proposed that a decrease in redox potential correlated with deprotonation of the inner propionic acid (which would stabilize the more positively charged, oxidized heme). Conversely, protonation of a nearby histidine caused an increase in the redox potential (destabilizing the oxidized heme). These results demonstrate that ionization of groups close to the hemes, and particularly the propionate groups, may be important for control of electron transfer or vice versa: redox changes can induce pK_a shifts in nearby residues for proton binding or release.

A similar redox-Bohr effect, dependence of the reduction potential on pH, is observed in the tetraheme cytochrome c_3 of *Desulfovibrio*, which again shows the importance of the heme propionates in redox control [18]. A small conformational change occurs in cytochrome c_3 in response to redox change, which in turn alters the pK_a of a propionate, probably due to a conformationally induced change in solvent exposure of the propionate. Nearby charged residues, lysine, histidine and glutamate, are also affected by the redox state of the heme and appear to be responsible for the cooperativities between the four hemes ('mechano-chemical coupling' [19]).

C*c*O has two hemes with propionate groups that are hydrogen-bonded to two arginine residues that may also be important in the observed cooperativity between the hemes as well as in proton movement [20]. The C*c*O propionates are buried within the protein but in a region that contains crystallographically resolved water. The waters and other propionate ligands may be altered in position and hydrogen bonding during the catalytic cycle, depending on the redox status of the hemes, and could link proton binding and release to electron transfer [20–23]. It has already been observed that changes in hydrogen bonding interactions of the formyl group of heme *a* (more tightly electronically coupled into the porphyrin ring) modulate both the redox and the spectral properties of the heme [24–26].

4. Direct and indirect coupling of proton movement in proton pumps, bacteriorhodopsin and cytochrome bc_1 (and b_6f)

In proteins that act as pumps, protons are moved against the electrochemical gradient using the energy from another process, such as electron transfer or light absorption. Direct coupling of proton movement to the initial event may be readily apparent in some cases. However, indirect coupling can involve small bond rotations or helical motions that are harder to detect except with the highest resolution structures that capture intermediate states. To some extent this has been achieved with bacteriorhodopsin [27].

In bacteriorhodopsin, the energy from light excitation causes conversion of the ligand, retinal, from all-*trans* to 13-*cis*, which results in the deprotonation of its Schiff base connection to the protein and protonation of a specific aspartate (D85) [27]. The initial proton movement is directly coupled to the process of light absorption by the retinal. This initial event is followed by fast proton release (μs) to the extracellular side and slower uptake (ms) on the cytoplasmic side, both dependent on intermediate conformational steps and pK_a changes in an indirectly coupled mechanism. Rearrangements of waters and pK alterations appear to permit rapid proton release in an existing exit channel. But to allow proton uptake for the reprotonation of the Schiff base, more substantial conformational changes are required to create a hydrogen-bonded pathway that did not exist in the resting state [28].

Whether direct or indirect coupling is used, directionality of proton movement requires that the system ensure that the correct pK_a changes and proton accessibility occur at the appropriate steps. Interestingly, proton/ion movement in the bacterial rhodopsins can have an altered directionality under certain conditions [29]. For example, a mutant of bacteriorhodopsin (D85T) has a deprotonated Schiff base with retinal in the all-*trans* state, an altered kinetic dependence of proton transfer and an altered accessibility of a path for the protons, resulting in the proton being taken up, rather than released, on the extracellular side. Similarly, alteration of protonation states and proton path accessibility may be able to explain the reversibility of directional proton movement in mutants of C*c*O such as D132A and E286Q. These mutants prevent the efficient uptake of protons through the D channel, but in the presence of a membrane potential and pH gradient, protons are taken up through the exit path, the reverse direction to normal, which is thermodynamically favored.

Direct coupling of electron/proton movement in cytochrome bc_1 of the respiratory chain occurs using a Q-cycle mechanism [11] as originally proposed by Mitchell [30]. However, pathways for protons through the protein are still necessary to ensure release to the outside, when the quinol is oxidized at the Q_o site, and uptake from the inside when the quinone is reduced at the Q_i site. A molecular dynamics (MD) simulation of the bc_1 complex suggested the formation of such a hydrogen-bonded path upon quinol oxidation, depending on the conformational movement of the iron–sulfur protein domain following electron transfer. This path includes a tyrosine (Y274) hydrogen-bonded to the b_L heme propionate through a water, a glutamate (E272: part of the conserved sequence KPEWY) connected by four waters to an asparagine (N249) that is connected to the protein surface via a water molecule [31]. This mechanism of proton exit is an example of the creation of a proton pathway at the step where it is needed, as in the case of bacteriorhodopsin (above). Similarly, in the b_6f complex of the photosynthetic chain, which is an analog of the bc_1 complex, the glutamate (E78, *Chlamydomonas*) is postulated to undergo a rotation, in response to binding a proton released at Q_o, which places the carboxyl group towards the outside for proton release [32].

In C*c*O, the creation of an exit pathway by the movement of water to bridge regions that otherwise seem to be blocked is appealing [33]. Although the bacteriorhodopsin results [34] and computational methods support this idea [35], high resolution structures at intermediate stages of the catalytic cycle will be needed to substantiate this possibility.

5. Structure of proton pathways in C*c*O

Evidence so far indicates that bioenergetic proton pumps do not have a single continuous proton channel through the protein of hydrogen-bonded water. Rather, the routes for

protons consist of sections of hydrogen-bonded pathways that are formed by waters as well as hydrophilic and hydrophobic amino acids. The K channel has several important hydrophilic residues including an essential lysine (K362) that is flexible in MD simulations and may have varying conformations that allow it to connect to the active site (heme a_3/Cu_B) [35,36]. The D channel in C*c*O is the established route for the uptake of protons that are to be pumped and is observed as a hydrogen-bonded pathway in the crystal structures involving at least 10 water molecules [37]. Seven water molecules were predicted by MD calculations from the first protonation site, D132, to E286 [35]. Both of the carboxyl residues are essential for proton pumping. It has been suggested that E286 acts as a 'switch' similar to retinal in bacteriorhodopsin and can control the directionality of proton movement from the D channel to the exit path or to the binuclear center to supply substrate protons. However, the observed proton path ends at E286 only two-thirds of the way through the protein in both the fully reduced and fully oxidized forms. No forms representing the intermediate states, in which transitory paths might be expected, have been crystallized.

There is no definitive evidence on whether protons move in a direct route through the heme a_3/Cu_B active site, such as via a histidine ligand of Cu_B, or utilize a more indirect path. The Mg^{2+} site, 13 Å above the active site and at the interface of subunits I and II, was proposed as a structural element in a possible path for product water release from the active site [4,38]. It was also conceived that this aqueous region above the hemes could be part of a proton exit path, but the amino acid residues lining the most likely channel indicate a hydrophobic constriction [4] that might function as a proton filter similar to that observed in aquaporin [39], allowing water but not protons to exit.

In both direct and indirect coupling mechanisms, the involvement of the heme propionates of C*c*O in proton movement above E286 has been invoked. To test their role, a pair of arginines (R481/482) that hydrogen-bond with all four propionates have been mutated. Even conservative mutations might be expected to result in changes in the p*K*s of the propionates that could affect their function. Only non-conservative replacements of R481 or mutation of both arginines adversely affect proton pumping (Fig. 2) [20,23], but analysis of the mutant forms is complicated by instability. The conservative mutation of R481 to lysine results in a stable active protein that only shows altered properties in the reconstituted form in the presence of a membrane potential. This may indicate involvement in a proton exit path that only becomes limiting under these controlled conditions. The only other charged residue hydrogen-bonded to a heme a_3 propionate, an aspartate (D407), does not appear to affect activity [40].

Despite the present lack of structural information for a proton exit path in C*c*O, evidence from all other proton pumps suggests that there should be a well-defined, if transitory, hydrogen-bonded path for proton release. As described above, the proton uptake path in bacteriorhodopsin is only formed after a conformational change at later steps in the photocycle, and in the bc_1 complex a possible proton release path was only determined by using MD [41]. Similarly in C*c*O, the release of protons is expected to be carefully controlled by the intermediate steps in the catalytic cycle in order to maintain directionality and coupling (direct or indirect) to the energetic events at the metal centers.

6. Conclusion

The ways in which proton movement can be controlled are extremely variable. The above examples demonstrate how small changes in the hemes, upon ligand binding or alteration of redox state, cause local reorganizations in the heme environment which lead to other changes at a distance. Conformational changes can involve mere bond rotations, helical tilt or whole domain movements. Protonation/deprotonation events occur both at the initial energy-triggering site, such as in the Schiff base of the retinal, and at distant sites.

The insurance that the protons are pumped against a gradient can be provided by control of the accessibility of protons to the inside or outside via transitory pathways. In bacteriorhodopsin a proton cannot be released to the cytoplasmic side in the early stage of the photocycle because no hydrogen-bonded path is available at this step. In the bc_1 complex, the model depicts the hydrogen-bonded pathway for proton release to the outside only forming after a conformational change of the quinone and the iron–sulfur protein. Presumably kinetically competent pathways are formed and unformed as they are needed. Unfortunately, this concept of a proton pump, where protons can move at a distance from the primary energy-linked event through transitory paths, makes it difficult to define the proton pumping mechanism in large complex proteins such as C*c*O. However, the advent of higher resolution structures in different catalytic states should provide the basis for more definitive understanding of this pump and its regulatory properties.

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