Minireview

Structural elements involved in electron-coupled proton transfer in cytochrome *c* oxidase

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Abstract Haem-copper oxidases are the last components of the respiratory chains in aerobic organisms. These membrane-bound enzymes energetically couple the electron transfer (eT) reactions associated with reduction of dioxygen to water, to proton pumping across the membrane. Even though the mechanism of proton pumping at the molecular level still remains to be uncovered, recent progress has presented us with the structural features of the pumping machinery and detailed information about the eT and proton-transfer reactions associated with the pumping process.

Keywords: Haem-copper oxidases; Proton pumping; Membrane protein

1. Introduction

Haem-copper respiratory oxidases catalyse the reduction of dioxygen to water:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Because the electrons and protons are taken up from opposite sides of the membrane, the reaction results in a net charge separation across the membrane corresponding to the translocation of one positive charge from the membrane negative (N) side to the positive (P) side. In addition, in most oxidases studied to date, the reaction is also coupled to proton pumping across the membrane, which results in an overall translocation of two positive charges across the membrane per electron transferred to O_2. In cytochrome *c* oxidases (C*c*Os) the electron donor is cytochrome *c*, which in the enzymes from, e.g., bovine heart, *Rhodobacter sphaeroides* (Fig. 1) and *Paracoccus denitrificans* donates an electron to a dinuclear copper centre (Cu_A). The electrons are then transferred from Cu_A consecutively to a haem group, haem *a*, and to a haem-copper centre (haem a_3-Cu_B), which is the catalytic site of these C*c*Os. Often a tyrosine, Y(I-288)[1], which is presumably redox-active, is also included as being part of the catalytic site (see Fig. 1B).

In bacterial oxidases two proton-transfer (pT) pathways, called the D- and the K-pathway, leading from the cytoplasmic side [the N-side of the membrane] towards the catalytic site, have been identified on the basis of results from studies on mutant oxidases (see e.g., [1–7]) and inspection of the available X-ray crystal structures [8–12] (see Fig. 1).

Results from numerous studies indicate that the D-pathway is used for uptake of both substrate protons and protons to be pumped during the reduction of oxygen, while the K-pathway is presumably used during the reduction of the catalytic site, prior to binding of dioxygen (see e.g., [13–16]).

Here, we review recent results from functional and structural studies on haem-copper oxidases that have provided information about the mechanisms of proton translocation in these enzymes. Specifically, we discuss the temporal and energetic coupling between the electron transfer (eT) and pT reactions associated with oxygen reduction and the translocation of the pumped protons, focussing on the role of specific groups within the D-pathway.

2. Structural features of the proton translocation machinery

Fig. 1B shows selected parts of the structure of C*c*O from *R. sphaeroides*. The four redox-active cofactors are shown together with amino-acid residues in the pT pathways. The so-called D-pathway is named after an aspartate (D(I-132)), which constitutes the entrance point of the pathway. An array of water molecules, lined with polar amino-acid residues, leads to a glutamate (E(I-286)), located ~25 Å "above" the surface, at approximately equal distances from haems *a* and a_3, respectively.

Since the D-pathway is used for uptake of both the substrate protons, which are transferred to the catalytic site, and pumped protons, which are transferred to a proton-accepting group in the exit pathway (see below), there must be a branching point within the pathway. The results from several studies suggest that this branching point is located at E(I-286)

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Abbreviations: C*c*O, cytochrome *c* oxidase; eT, electron transfer; pT, proton-transfer; N-side, negative side of the membrane; P-side, positive side of the membrane

[1] Amino-acid residue and mutant-enzyme nomenclature: N(I-139), Asparagine of subunit I at position 139, ND(I-139), replacement of N(I-139) by aspartic acid Unless otherwise indicated amino-acid numbering is according to the *Rhodobacter sphaeroides* C*c*O sequence.

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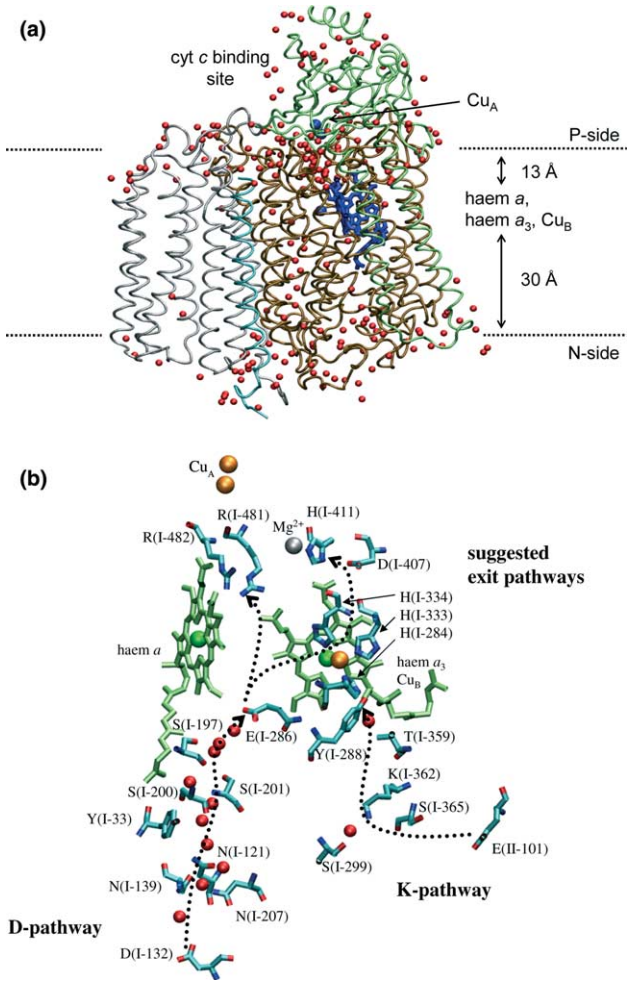

Fig. 1. The crystal structure of C*c*O from *R. sphaeroides* (1m56.pdb in the Protein Data Bank, [11]). The structure of the four-subunit C*c*O together with the redox-active cofactors (blue) and all structurally resolved water molecules (red spheres) are shown in (A). Subunit I is shown in brown, subunit II in green, subunit III in silver and subunit IV in magenta. The approximate location and thickness of the lipid membrane are indicated by the dotted lines. The arrows indicate the depth at which the haems and Cu_B are located in the membrane (all three at the same approximate depth). A more detailed view of the redox-active cofactors and amino acid residues in the pT pathways (dotted arrows) discussed in the text is given in (B). The histidine ligands of Cu_B are also shown. Only the water molecules in the D- and K-pathways are shown, other water molecules are excluded for clarity. The location of the redox-inactive Mg-ion is also indicated. The figures were prepared using the Visual Molecular Dynamics software [74].

[17–20]. Different protonatable groups have been suggested to function as the primary proton acceptor of pumped protons (pump site), e.g., one of the histidines ligated to Cu_B [9,21], or one of the D-ring propionates of the haems [18,19,22,23].

It has been shown that the highly conserved arginines (R(I-481)and R(I-482), see Fig. 1B), which are salt bridged to the D-ring propionates of the haems, are important for proton pumping. In the bo_3 quinol oxidase from *Escherichia coli*, proton pumping was impaired in mutant enzymes in which R(I-481) was substituted by asparagine or leucine [18], while in the C*c*O from *R. sphaeroides* the RK(I-481) mutant enzyme was able to pump protons but the activity was considerably lower than with wild-type C*c*O in coupled vesicles (i.e., during turnover with an electrochemical gradient across the membrane) [22]. Results from FT-IR experiments [23] indicate that the D-ring propionates are stabilised in their anionic state in the oxidised C*c*O, and are able to function as proton acceptors/donors.

From the R(I-481)/R(I-481)/propionates cluster, the protons are probably transferred towards a hydrogen-bonded network of water molecules as well as polar and acidic amino acid residues, located between the haem groups and the p-side of the membrane surface (cf. Fig. 1A). Results from electrostatic calculations suggest that in this region there is an assembly of interacting acidic groups, which could function as proton-shuttling groups [24].

In the crystal structures determined so far there are no water molecules resolved neither in the hydrophobic cavity between E(I-286) and the catalytic site, nor between E(I-286) and the D-ring propionates, and no hydrogen-bonded proton pathway is observed (cf. Fig. 1B). Thus, the question arises as to how protons are transferred through these hydrophobic regions. As evident from theoretical calculations, there is space for water molecules in these regions [17,25]. Results from computer modelling have suggested that the water molecules form a branched chain, connecting E(I-286) with the binuclear centre and the D-ring propionate of haem a_3 [19,20]. Most probably these water molecules are structurally disordered and the hydrogen-bonded water chains may be formed only transiently during catalysis. It was recently suggested by Wikström et al. [19] that a reorganisation of these water molecules could control the pT rate (cf. proton gating) if the orientation of the water molecules could be controlled, through electrostatic interactions with the redox centres.

Another putative pT pathway, the so-called H-pathway, was identified in the structure of C*c*O from bovine mitochondria and suggested by Yoshikawa et al. [26,27] to be used for proton pumping. Results from recent studies, in which the gene for the bovine C*c*O was mutated and expressed in human cell lines, support the idea that one of the key residues in the H-pathway (D(I-51), bovine C*c*O numbering) is involved in proton translocation [28]. However, the H-pathway is only partly conserved in the bacterial oxidases and no effect on proton pumping was observed upon substitution of these residues [6,29]. Thus, at this point, the role of the H-pathway, if any, in the bacterial oxidases is unclear.

3. Transfer of substrate protons through the D-pathway

3.1. The oxygen reduction reaction

The individual steps during oxygen reduction can be studied using the so-called flow-flash technique. Briefly, the reduced, CO-bound C*c*O is mixed with an oxygen-saturated solution in a stopped-flow apparatus. A short time after mixing, the CO ligand is photodissociated using a laser flash. Because oxygen and CO bind to the same site, haem a_3, the oxygen binding is rate-limited by the CO-off rate ($\tau \cong 30$ s). Hence, if the sample is illuminated at $t \ll 30$ s, the light flash sets the starting point of the reaction. The binding of oxygen and its subsequent stepwise reduction (concomitant with the oxidation of the enzyme) can be followed time resolved using spectroscopic techniques. On the basis of the results from these, as well as other measurements, information is

provided on the intermediate states formed during the reaction of the reduced C*c*O with O_2 and the transition times between these intermediates (as summarised in Fig. 2, for review, see [30–32]). Dioxygen binds to ferrous haem a_3 with a second-order rate constant of $\sim 10^8$ M^{-1} s^{-1}, forming state A. Next, the O–O bond is broken forming a oxo-ferryl state at haem a_3 (denoted P_R) and a hydroxide ion at Cu_B with a time constant of ~50 μs. During this transition, an electron is transferred to the catalytic site from haem *a*, whereas the other three electrons and the proton needed to break the O–O bond are taken from within the catalytic site. In the P_R state there is an uncompensated negative charge at the catalytic site, which is neutralised by a proton during the next transition, the formation of the F state with a time constant of ~100 μs (at pH $\leqslant 8$). Concomitantly, with the proton uptake upon F formation the electron on Cu_A equilibrates with haem *a*. The fully oxidised enzyme, denoted O, is formed with a time constant of ~1.2 ms, associated with proton uptake from the bulk solution. Note that this state probably has two hydroxide ions in the catalytic site (cf. [33,34]) and may differ from the relaxed (as isolated) oxidised C*c*O (see [16,35–37] for detailed discussions on this issue). Upon reaction of the reduced C*c*O with O_2, protons are pumped during the $P_R \rightarrow F$ and $F \rightarrow O$ transitions [38,39].

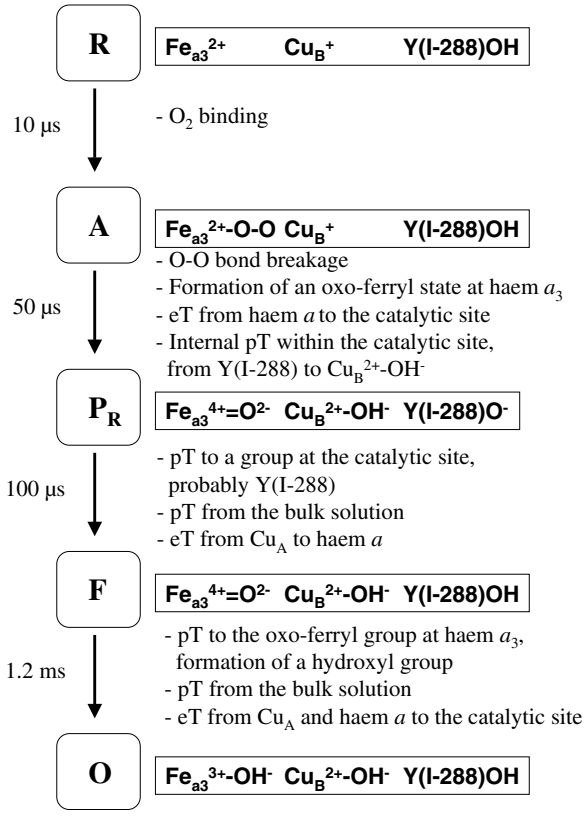

Fig. 2. A scheme illustrating the reaction between fully reduced C*c*O and oxygen. The one-letter codes denote the commonly used abbreviations for the intermediate states. The suggested structures of the intermediate states at the catalytic site are shown in the rectangles to the right (Fe_{a3}: the iron ion of haem a_3, Cu_B: the copper B ion and Y(I-288): a tyrosine close to the haem-copper centre). The time constants given for the transitions are those observed with C*c*O from *R. sphaeroides*.

3.2. The $P_R \rightarrow F$ transition, a two-step pT

The $P_R \rightarrow F$ transition is associated with proton uptake from the bulk solution. Since the electron is transferred to the catalytic site in the previous step, i.e., when P_R is formed ($\tau \cong 50$ μs), studies of the $P_R \rightarrow F$ transition ($\tau \cong 100$ μs at pH $\leqslant 8$) make it possible to specifically investigate pT to the catalytic site through the D-pathway. The protonation event at the catalytic site (presumably of Y(I-288) [40]) results in a shift of the peak in the α-region of the optical absorbance spectrum from 607 to 580 nm. The transition is also associated with equilibration of the electron between Cu_A to haem *a* (see Fig. 2). Hence, the events associated with F formation can be followed in three ways using optical absorption spectroscopy; (1) by monitoring absorbance changes at 580 nm, (2) by measuring the proton uptake from an unbuffered solution using a pH-sensitive dye, or (3) by monitoring absorbance changes associated with the oxidation of Cu_A and reduction of haem *a*.

The glutamate in the upper part of the D-pathway (E(I-286)) and the aspartate (D(I-132)) at the surface are both conserved in a large group of the mitochondrial-type haem-copper oxidases and have been shown to be necessary for the function of these enzymes (see Fig. 1B). Results from mutagenesis studies of C*c*O from *R. sphaeroides* and *P. denitrificans* as well as the quinol oxidase from *E. coli* have shown that, exchanging the glutamate for a non-protonatable group (e.g., glutamine or alanine) results in mutant oxidases with almost no steady-state activity [3,6,7,41,42]. Furthermore, it was shown that these mutant enzymes were not able to form intermediate F [13,43–45]; i.e., when the fully reduced mutant C*c*O reacts with oxygen, the reaction stops at the P_R-intermediate. When E(I-286) was substituted with the protonatable residue aspartate, the steady-state activity was lower than that of the wild-type C*c*O [7], and the $P_R \rightarrow F$ and $F \rightarrow O$ transitions were slowed [46]. Similarly, when E(I-286) was "relocated" to a different position in the D-pathway in C*c*O from *R. sphaeroides* [47,48], the pumping stoichiometry was lowered, the steady-state activity was lowered and the rates of the $P_R \rightarrow F$ and $F \rightarrow O$ transitions were slowed. Taken together, these observations imply that E(I-286) is involved in proton delivery to the binuclear centre upon F and O formation, and presumably also in the translocation of pumped protons.

When D(I-132) at the entrance of the pathway is exchanged for asparagine or alanine, the activity of the mutant oxidases was substantially decreased and the mutant enzymes did not pump protons [1,4,6,7]. The DN(I-132) C*c*O from *R. sphaeroides* does form F upon reaction with oxygen with the same rate as the wild-type C*c*O, but without concomitant proton uptake from the bulk solution [49]. These results indicate that the DN(I-132) mutation inhibits proton uptake from the bulk solution and that the F intermediate can be formed using an internal proton within the D-pathway. In addition, no eT from Cu_A to haem *a* was observed during the F formation, indicating that the eT is controlled by the proton uptake [49]. A similar behaviour was observed with the wild-type C*c*O when the flow-flash experiment was performed with zinc ions present in the C*c*O solution, interpreted in terms of Zn^{2+} binding to the C*c*O surface near the entrance of the D-pathway, resulting in impaired proton uptake from the bulk solution [50].

Taken together, the results discussed above point towards a two-step mechanism of pT to the catalytic site through the D-pathway in which the proton is first taken internally, pre-

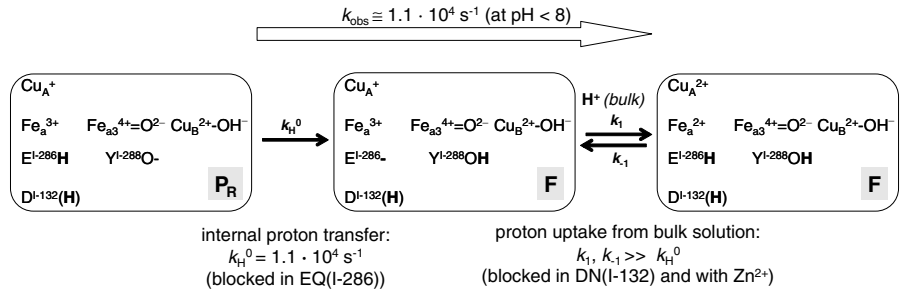

Fig. 3. The two-step mechanism of pT through the D-pathway during the $P_R \rightarrow F$ transition (observed rate constant k_{obs}). The internal pT from E(I-286) to the catalytic site (rate constant k_H^0) is rate limiting for the reaction and E(I-286) is in rapid proton equilibrium with the bulk solution (rate constants k_1, $k_{-1} \gg k_H^0$).

sumably from E(I-286), followed by immediate reprotonation from the bulk solution (see Fig. 3) [49–51]. Hence, the observed rate of F formation is limited by the pT from E(I-286) to the catalytic site, and probably determined by the reorganisation of water molecules or a conformational change of the E(I-286) side chain (see below).

3.3. The pH dependence of the pT reactions

In the mechanism described above, the observed rate of F-formation (i.e., pT to the catalytic site through E(I-286)) depends on the fraction protonated internal proton donor (E(I-286)). Hence, the observed pK_a in the pH-dependence of the rate reflects the apparent pK_a of E(I-286). In the wild-type C*c*O from *R. sphaeroides*, the $P_R \rightarrow F$ rate followed a titration curve with a pK_a of ~9.4 [51] (see Fig. 4), i.e., considerably higher than the pK_a value of a glutamic acid in water solution (~4.5). The increased pK_a is explained by the low dielectric constant in the protein interior, where the uncharged (protonated) form is stabilised. An analysis of the X-ray structure supports the conclusion that E(I-286) is protonated at neutral pH, forming a hydrogen bond with the carbonyl oxygen of M(I-107) [11]. This observation is also consistent with results from experiments using FT-IR spectroscopy, showing high pK_a values (>9) of the corresponding glutamate in haem-copper oxidases from different species [52,53].

In the high-pH range, the pH dependence of the rate of the next transition, $F \rightarrow O$, titrates with a similar pK_a (~8.9) to that observed for the $P_R \rightarrow F$ transition (Fig. 4). However, the pH dependence of the $F \rightarrow O$ transition rate is more complex displaying a titration also in the low-pH region (p$K_a \cong 6.4$). The $F \rightarrow O$ transition rate has been modelled to be a function of the rate of pT to the catalytic site, times the fraction of the F intermediate reduced with another electron [54]. Thus, the higher pK_a is likely to be associated with the pT, reflecting the pK_a of E(I-286), while the lower pK_a could be associated with another titrating protonatable group, interacting with the redox site(s).

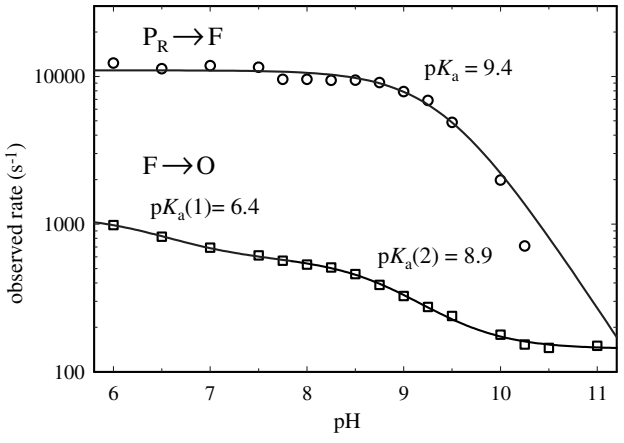

Fig. 4. The pH dependence of the observed rate of the $P_R \rightarrow F$ (circles) (from [51]) and F to O (squares) transitions. The solid lines represent standard titration curves with one and two pK_a values, for the $P_R \rightarrow F$ and $F \rightarrow O$ transitions, respectively. The experimental data of the $P_R \rightarrow F$ transition rate were best fitted to a pK_a value of 9.4 and a maximal rate constant of 1.1×10^4 s^{-1} (i.e., the rate constant with the titrating group fully protonated). The $F \rightarrow O$ data were fitted with two pK_a values, p$K_a(1) \cong 6.4$ and p$K_a(2) \cong 8.9$, a maximal rate constant of 1.1×10^3 s^{-1} and a background rate constant of 140 s^{-1} (i.e., the transition rate with both titrating groups unprotonated). For experimental conditions, see [51].

4. Transfer of pumped protons through the D-pathway

So far, we have focused on the transfer mechanism of substrate protons to the catalytic site through the D-pathway during specific reaction steps and not discussed the fact that during these transitions protons are also pumped. It is interesting to note that the actual reduction of the dioxygen molecule resulting in the O–O bond breaking occurs already in the first step after oxygen binding (i.e., upon P formation, see Fig. 2) and this transition is not coupled to proton pumping. Instead, proton pumping occurs when further electrons are transferred to the catalytic site and groups with high proton affinity are formed. In this way, the free energy available from the oxygen reduction is transiently "stored" in the incompletely protonated oxygen intermediates (see [55]). Below, we discuss a model in which the exergonic transfer of substrate protons to the catalytic site is linked to an energy-requiring structural change, which drives proton pumping.

4.1. Studies on uncoupled mutant forms of CcO

Studies on mutant C*c*Os, which are capable to reduce oxygen to water but do not pump protons, so-called uncoupled mutant C*c*Os, can help us to elucidate the molecular mechanism of proton pumping and its coupling to the driving reaction – the reduction of oxygen. There are two different groups of mutant C*c*Os in which proton pumping is impaired. In one group the transfer of substrate protons to the catalytic site is

inhibited, while in the other group the pT to the catalytic site is not affected (or may even be faster than in the wild-type C*c*O).

One member of the first group is the DN(I-132) mutant C*c*O in which proton uptake through the D-pathway is blocked near the entrance of the pathway (see Fig. 1B). A reversed respiratory-control ratio was observed with this mutant C*c*O, i.e., in contrast to the wild-type C*c*O, the turnover activity was higher in the presence of a membrane potential. This observation was suggested to indicate that protons were taken from the P-side of the membrane [56]. A mutant C*c*O with an aspartate inserted close to E(I-286), in the position of S(I-197), ~7 Å from the carboxylate group of E(I-286), also resulted in an enzyme in which proton pumping was uncoupled from oxygen reduction (Namslauer et al. manuscript in preparation). The SD(I-197) mutant C*c*O showed ~35% steady-state activity of that of the wild-type enzyme and was not able to pump protons. Instead, ~0.2 protons per electron were taken up from the P-side during turnover in experiments in which the mutant C*c*O was reconstituted in liposomes.

The second class of mutant C*c*Os does not exhibit slowed proton uptake. For example, it was shown that when an asparagine, located close to the surface in the D-pathway, was mutated to an aspartate in C*c*O from *P. denitrificans*, the mutant C*c*O (ND(I-139), *R. sphaeroides* C*c*O numbering, see Fig. 1B) exhibited full steady-state activity but was not able to pump protons [57]. The corresponding non-pumping mutant C*c*O from *R. sphaeroides* had approximately two times higher steady-state activity than the wild-type enzyme [58]. In this mutant C*c*O, the rate of the $P_R \rightarrow F$ transition was the same as that of the wild-type C*c*O but the $F \rightarrow O$ transition rate was about two times faster [59]. The $P_R \rightarrow F$ and $F \rightarrow O$ transitions were both associated with proton uptake from the bulk solution also in the mutant C*c*O, and hence the uncoupling was not due to inhibition of proton uptake through the D-pathway. Instead, the mutation resulted in an increase in the observed pK_a in the pH dependence of the $P_R \rightarrow F$ transition rate from ~9.4 to ~11, which was attributed to an increase in the apparent pK_a of E(I-286) [59]. Also the pH dependence of the $F \rightarrow O$ transition rate was shifted to higher pH values. The increase in the apparent pK_a of E(I-286) may be a result of electrostatic interactions between E(I-286) and the inserted aspartate. Alternatively, the insertion of the aspartate results in altering of the structure of water molecules within the D-pathway. The alteration is mediated through the pathway and results in a change in the local environment of E(I-286). Below, we discuss these results in terms of a model for proton pumping by C*c*O (see also [60] and [59]).

4.2. Description of a mechanistic model for transfer of pumped protons

According to the model, E(I-286) constitutes the branching point for substrate and pumped protons. The pump site is a group in the region around the D-ring propionates, R(I-481) and R(I-482) (see above). Initially, E(I-286) is protonated and hydrogen bonded to the carbonyl oxygen of M(I-107). The pump site is stabilised in its deprotonated state, i.e., the pump site has a pK_a that is lower than the pH of the bulk solution on the P-side. The mechanism of proton pumping is described as a sequence of the following steps (see Fig. 5).

(1) A substrate proton is transferred from E(I-286) to a proton acceptor at the catalytic site (B^- in Fig. 5), formed upon eT from haem *a*. The reaction is associated with a structural change of the E(I-286) side chain in which the hydrogen bond to the carbonyl oxygen of M(I-107) is broken. The structural change is transmitted to the region around the pump site (A^- in Fig. 5). The enzyme is now in a "proton-input conformation" in which there is a hydrogen-bonded connection between the pump site and the bulk solution on the N-side. The conformational change results in an increased pK_a value of the pump site. This step is rate-limiting for the overall reaction.

(2) While the enzyme is still in the "proton-input conformation" a proton is rapidly taken up from the bulk solution through the D-pathway and is transferred to the pump site (A^-), which in this state has a pK_a value that is higher than that of E(I-286).

(3) E(I-286) is reprotonated from the bulk solution and the structure relaxes to the initial conformation. The pK_a value of the pump site (AH) decreases and the pumped proton is expelled to the P-side.

It should be noted that there are proton-pumping haem-copper oxidases that lack the glutamate corresponding to E(I-286). It has been suggested that in these oxidases, the role of E(I-286) is taken over by other amino-acid residues, e.g., a glutamate in a different position in the primary structure, but with a similar position in the three dimensional structure [47,61], or by a tyrosine-serine motif [62–64].

A fundamental difference between this model and other suggested models is the order of protonation events. A priori, there is a prerequisite that, in each pumping step, the proton

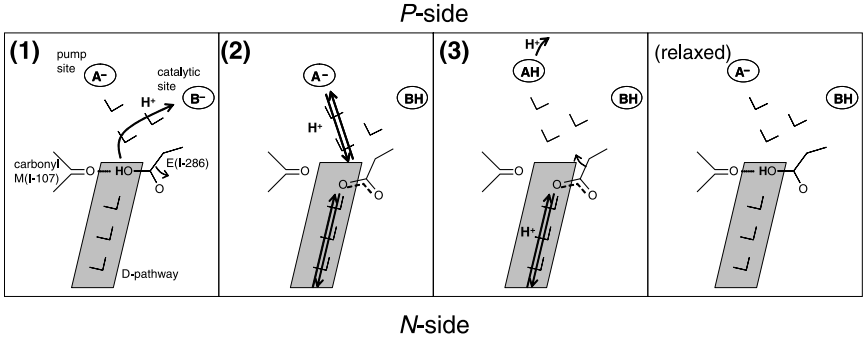

Fig. 5. An illustration of the mechanism of proton pumping discussed in the text. A(H) represents the pump site and B(H) represents the proton acceptor at the catalytic site. The pT is mediated by water molecules (>) through the D-pathway (shaded box) and from E(I-286) to the catalytic site and the pump site.

that is pumped is transferred to the pump site before the substrate proton reaches the catalytic site. If this would not be the case, the energy from the oxygen reduction would be lost. In the model presented here, the substrate proton is transferred to the catalytic site before the pumped proton is transferred to the pump site. The free energy available from the reduction of oxygen is conserved because the exergonic transfer of the substrate proton to the catalytic site is linked to an endergonic structural change. This scenario is supported by the observation that the F intermediate is formed with the same rate in the DN(I-132) mutant (in which proton uptake from the bulk solution is impaired) as in the wild-type C*c*O, which indicates that the internal proton available at E(I-286) is initially transferred to the catalytic site and not to the pump site, at least during the $P_R \rightarrow F$ transition [49,50].

The transfer of the substrate proton to the catalytic site before the transfer of the pumped proton makes the proton pumping sensitive to any changes in the relative rates of protonation of the pump site and reprotonation of E(I-286), which is associated with the relaxation of the structure. Below, various aspects of the model are discussed in more detail and also in terms of experimental data.

4.3. Data supporting the deprotonation of E(I-286) being associated with structural changes

In the crystal structure of wild-type C*c*O from *R. sphaeroides* at pH 6, the OH group of the protonated E(I-286) is hydrogen-bonded to the carbonyl oxygen of M(I-107) [11]. There is also a salt bridge between the D-ring propionate of haem a_3 and R(I-481) that stabilises the propionate in its deprotonated state (i.e., gives it a low pK_a value). In the crystal structure obtained with the wild type C*c*O at pH 10 and that of the EQ(I-286) mutant C*c*O, the hydrogen bond between E(I-286) and M(I-107) is lost and the conformation of the E(I-286) side chain is altered. In addition, there are structural changes in the area around the arginines ((R(I-481) and R(I-482)) and the haem propionates (cf. Fig. 1B). The distance between the D-ring propionate of haem a_3 and R(I-481) is increased and a water molecule enters between the two groups. The movement of this water molecule results in breaking of the salt bridge between R(I-481) and the D-ring propionate resulting in an increased pK_a of the propionate. Hence, there seems to be a structural link between the deprotonation of E(I-286) and the assumed pump site that may result in an increased proton affinity of the D-ring propionate when E(I-286) is deprotonated. It is likely that the same type of structural changes occur transiently during catalytic turnover when a proton is transferred from E(I-286) to the catalytic site. A structural change, linked to the deprotonation of E(I-286), is also supported by results from investigations of the kinetic solvent deuterium isotope effect of the $F \rightarrow O$ transition [46,65]. The kinetic deuterium isotope effect of the transition is large ($k_H/k_D \cong 7$) and the results from D_2O-fractionation experiments indicate that the entire isotope effect is associated with a single protonatable site suggested to be E(I-286), which undergoes a structural change before the pT occurs [46,65].

4.4. How is an increased pK_a value of E(I-286) correlated to the impaired proton pumping

According to the model, the proton pumping efficiency depends on the relative rates of pT from the bulk solution to the pump site and the reprotonation of E(I-286), which is associated with a structural relaxation. These rates depend on the relative pK_a values of E(I-286) and the pump site in the "proton-input conformation". The uptake of a proton to the pump site requires that the proton taken up from the bulk solution in step 2 (see Fig. 5) is transferred to the pump site before reprotonation of E(I-286) and relaxation of the structure.

The proton acceptor at the catalytic site during the $P_R \rightarrow F$ transition presumably has a pK_a value that is $\geqslant 12$ [59]. A high pK_a value of the proton acceptor is expected because the pT to the catalytic site drives the proton pumping. It is assumed that the pK_a value of the pump site is lower. In e.g., the ND(I-139) mutant C*c*O, the increased pK_a value of E(I-286) results in an altered proton equilibrium between E(I-286) and the pump site, i.e., the proton is stabilised on E(I-286). Therefore, E(I-286) becomes reprotonated, allowing the structure to relax, before the pumped proton is transferred to the pump site.

4.5. The functional role of haem a

Several proton pumping models have been suggested in which reduction/oxidation of haem *a* is coupled to protonation/deprotonation of a pump site (see e.g. [36,37,69–73]). It has also been proposed that the redox state of haem *a* controls the structure of the water molecules connecting E(I-286) with the D-ring propionate of haem a_3 and the catalytic site, thereby providing a mechanism of proton gating [19]. Experimental data on proton uptake/release upon haem *a* reduction/oxidation is in many cases contradictory, varying between 0.2 and 1 proton per electron (see e.g. [36,37,69–73]. It has been shown that during the $P_R \rightarrow F$ transition, haem *a* is not reduced by Cu_A unless a proton is taken up from the bulk solution to reprotonate E(I-286), indicating an interaction between haem *a* and E(I-286) [49–51]. In other words, the eT is controlled by the pT. Hence, the role of haem *a* may be to control the timing of eT from cytochrome *c*/Cu_A to the catalytic site, such that eT to the catalytic site is prevented before E(I-286) is reprotonated. Since the pK_a of E(I-286) is $\geqslant 9$, its protonation state does not change upon haem *a* reduction/oxidation when measurements are done below pH ~9, but there may still be a strong electrostatic interaction between E(I-286) and haem *a*, which is observed only transiently when E(I-286) is deprotonated, e.g. during the $P_R \rightarrow F$ transition.

4.6. Summary: proton translocation through the D-pathway

There are two different reasons for uncoupled proton pumping from oxygen reduction: (i) If the proton uptake through the D-pathway from the bulk solution on the N-side is impaired, then rapid protonation of the pump site and E(I-286) is not possible and the enzyme stays in the "proton-input conformation", with E(I-286) deprotonated, after the internal pT to the catalytic site. In this state there may be a higher probability for slow proton uptake, via the pump site and E(I-286), from the P-side of the membrane (cf. results obtained with the SD(I-197) and DN(I-132) mutant C*c*Os, discussed above). The slow uptake of substrate protons results in slowed oxygen reduction and steady-state activities. (ii) If electrostatic and/or structural changes within the D-pathway result in an increased pK_a of E(I-286), then the delivery of substrate protons from the N-side to the binuclear centre can still be rapid and the oxygen reduction rate is not decreased. In this case the proton equilibrium between E(I-286) and the pump site is affected, stabil-

ising the proton on E(I-286), and therefore the effective rate of protonation of the pump site is too slow to maintain proton pumping. We suggest that this is the case with the ND(I-139) [58,59] and ND(I-207) (Namslauer et al. unpublished data) mutant C*c*Os. Thus, efficient proton pumping in C*c*O relies on both rapid proton uptake (through the D-pathway) from the bulk solution on the N-side and finely tuned pK_a values of the proton shuttling groups within the enzyme.

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