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Rapid report

Water-gated mechanism of proton translocation by cytochrome *c* oxidase

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Abstract

Cytochrome *c* oxidase is essential for aerobic life as a membrane-bound energy transducer. O_2 reduction at the haem a_3–Cu_B centre consumes electrons transferred via haem *a* from cytochrome *c* outside the membrane. Protons are taken up from the inside, both to form water and to be pumped across the membrane (M.K.F. Wikström, Nature 266 (1977) 271 [1]; M. Wikström, K. Krab, M. Saraste, Cytochrome Oxidase, A Synthesis, Academic Press, London, 1981 [2]). The resulting electrochemical proton gradient drives ATP synthesis (P. Mitchell, Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin, UK, 1966 [3]). Here we present a molecular mechanism for proton pumping coupled to oxygen reduction that is based on the unique properties of water in hydrophobic cavities. An array of water molecules conducts protons from a conserved glutamic acid, either to the Δ-propionate of haem a_3 (pumping), or to haem a_3–Cu_B (water formation). Switching between these pathways is controlled by the redox-state-dependent electric field between haem *a* and haem a_3–Cu_B, which determines the water–dipole orientation, and therefore the proton transfer direction. Proton transfer via the propionate provides a gate to O_2 reduction. This pumping mechanism explains the unique arrangement of the metal cofactors in the structure. It is consistent with the large body of biochemical data, and is shown to be plausible by molecular dynamics simulations.
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Subunit I of all haem–copper oxidases has a unique arrangement of redox cofactors [4–8]. Haem *a* lies about 1/3 into the membrane domain from the positively charged (P) side. Haem a_3 lies very close, at the same depth, and almost at a right angle to haem *a*. Cu_B is within 5 Å from the haem iron of a_3; together, they form a binuclear site where O_2 is bound and reduced to water (Fig. 1a). All protons pumped across the membrane are transferred across the so-called D-pathway [9], which starts with D132[1] near the negatively charged (N) side of the membrane, and continues into the membrane along polar residues and bound water molecules [9–11], finally connecting to the conserved E286 next to a hydrophobic cavity 10 Å from the binuclear site (Fig. 1b). E286 is essential for proton translocation and for enzyme activity [12,13]. At least three out of the four substrate protons consumed at the haem a_3–Cu_B site per cycle are also transferred through this pathway [9,13,14].

How is chemical energy used for the mechanical translocation of individual protons across the membrane against an electrochemical gradient, in addition to charging up the membrane by selective uptake of electrons and substrate protons from opposite sides? To answer this question, we focus on how protons are transferred further from E286, how pumped protons are distinguished from consumed substrate protons, and how proton translocation is coupled to the driving redox reaction. No proton connectivity beyond E286 is seen in the X-ray structures. The Δ-propionate of haem a_3 (Fig. 1b) has been suggested to initiate the exit path of the pumped protons [4,10], and this has been experimentally supported [15]. Water molecules have been shown to have a key role in light-driven proton pumping by bacteriorhodopsin [16]. Statistical–mechanical calculations [11] predicted that proton transfer could be mediated by three to four water molecules transiently present inside the hydrophobic cavity between E286, the propionate, and the binuclear site (Fig. 1b). During each catalytic cycle, two water molecules are, in fact, produced at

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[1] All numbering of amino acid residues refers to subunit I of cytochrome *c* oxidase from *Rhodobacter sphaeroides*. Amino acids are abbreviated by the one-letter code.

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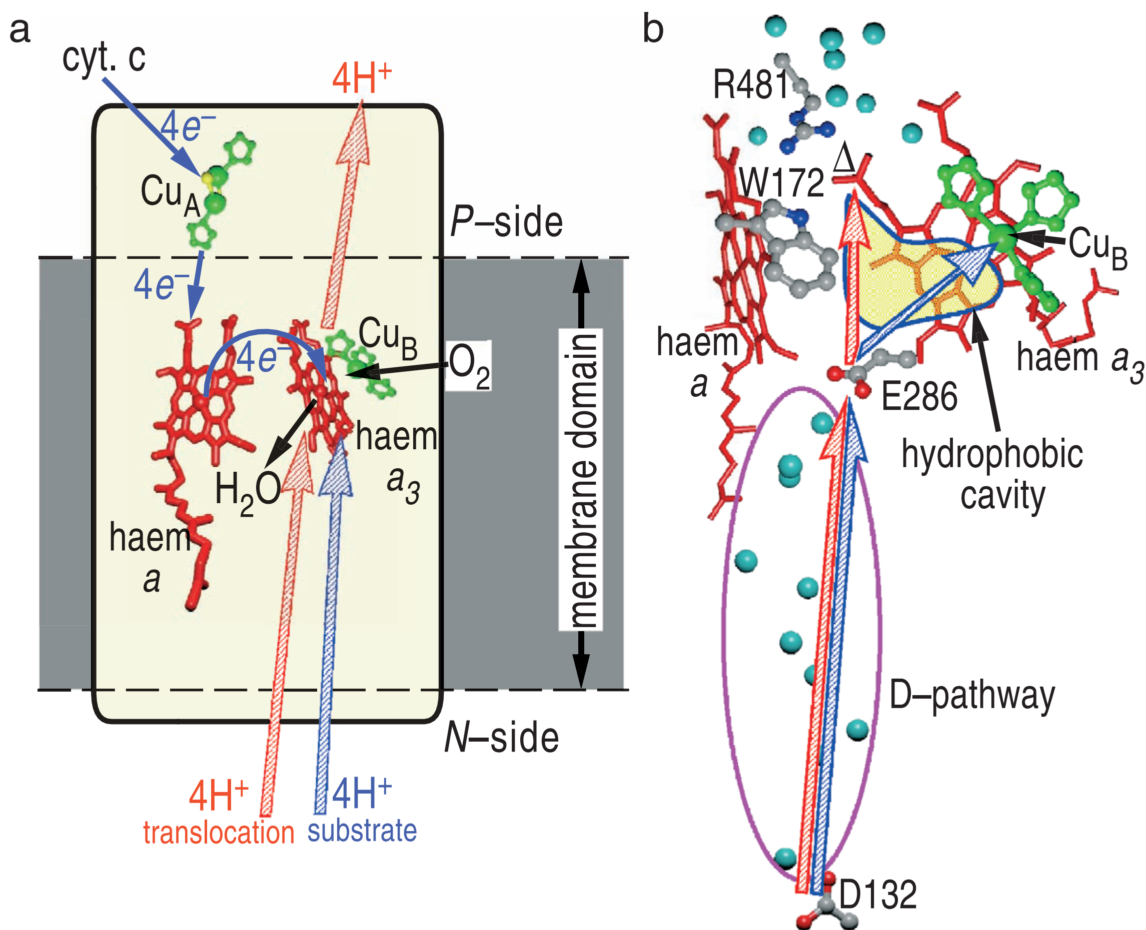

Fig. 1. (a) Overall function of cytochrome *c* oxidase. The blue arrows depict how the chemical reaction of O_2 reduction to water is orientated with respect to the positively charged P-side and the negatively charged N-side of the membrane. The red arrows show proton translocation (pumping) coupled to the chemistry. (b) A view, parallel to the membrane, of key components of the cytochrome *c* oxidase structure (adapted from the crystal structure [8] of cytochrome *c* oxidase from *R. sphaeroides*). The D-pathway transfers protons from the N-side of the membrane (a) via E286, either to be pumped across the membrane (red arrows), or to be consumed to form water at the haem a_3–Cu_B site (blue arrows; see text). Light blue spheres depict water molecules seen in the X-ray structure. The hydrophobic cavity above E286 is predicted to transiently contain three to four water molecules by computational methods [11]. The Δ-propionate of haem a_3 (Δ) is proposed to convey pumped protons to the hydrophilic domain above the haem groups. The programme VMD was used for the molecular graphics [32].

this site as the product of O_2 reduction. Water molecules move in and out of the enzyme during the cycle and this has been shown to limit electron transfer [17]. More recent work indicates that water molecules might indeed provide proton conductivity from E286 to both the propionate and the binuclear site [10,18], consistent with the role of the D-pathway in both translocation and consumption of protons.

Water molecules behave uniquely in narrow hydrophobic cavities [19], and may not only serve as ‘proton wires’, but also as the switch between proton transfer for ‘pumping’ and water formation. Due to the scarcity of interactions with the surrounding structure, and the limited space, they tend to form orientated single file arrays where each water molecule only interacts with its two neighbours. The hydrogen bond orientation of the array determines the allowed direction of Grotthuss proton transfer [20]. Orientated water arrays are excellent unidirectional conductors of single protons [21]; the proton mobility exceeds that in bulk water by a factor of 40. Any reorientation of such an array occurs collectively to maintain the strong water–water hydrogen bonds, hence changing the allowed direction of proton transfer. Thus, alternative water chains from E286 to the propionate, or to the binuclear site, might provide a switch of the proton current to either destination (cf. Ref. [22]). We propose here that the direction of such a switch may be determined by the redox states of the electron donor (haem *a*) and the acceptor (the binuclear centre). This would provide the important link between proton translocation and the redox reaction, and make proton pumping a prerequisite for completion of the oxygen reduction chemistry, assuring tight coupling between the two. The effect of a unit charge at either redox site on a single water molecule in the cavity yields a charge–dipole interaction energy of 0.4–0.8 kcal/mol at an Fe–water distance of 8–10 Å (with $\varepsilon = 4$). Thus, an electric field between haem *a* and the haem a_3–Cu_B site, parallel to the membrane, may orientate the water dipoles in

the cavity between them to form a proton transfer path from the carboxylic group of E286, either to the Δ-propionate of haem a_3, or to the binuclear site.

Fig. 2 summarises the proposed mechanism based on redox-linked switching between proton 'wires' for pumping and oxygen reduction. Electron transfer from haem *a* to the binuclear site does not occur unless accompanied by a charge-compensating proton transfer [23,24]; however, initially, with haem *a* reduced and the binuclear site oxidised, there is no proton connectivity to the latter. Instead, the electric field orientates the water molecules for proton transfer from E286 to the Δ-propionate of haem a_3 (Fig. 2a). Transfer of the proton to the propionate (Fig. 2b) raises the redox potential of the binuclear centre to allow transfer of the electron (Fig. 2c). The electron transfer stabilises the 'pumped' proton electrostatically and inverts the electric field between the redox sites. This reorientates the water molecules to provide a proton path from E286 to the binuclear site (Fig. 2d). The conserved R481 strongly interacts with the propionate [25] (Fig. 1b). Its protonation will therefore cause deprotonation of the arginine, with transfer of the pumped proton to the hydrophilic domain on the P-side of haem a_3 (Fig. 1b), followed by reprotonation of the arginine by the propionic acid (Fig. 2d). Reprotonation of E286 via the D-pathway blocks back-transfer of the pumped proton, and if it fails, as it does in some mutants [9], proton translocation is compromised. The proton on E286 is transferred into the binuclear site to form water where it fully neutralises the charge of the electron (Fig. 2e). As a result, the initially transferred proton is expelled towards the P-side of the membrane by electrostatic repulsion (Fig. 2f).

This mechanism is in accordance with the proton dependence of reduction of the binuclear site [23,24], the apparent dependence of the redox states of the two haems on the same proton-accepting site [2], and the dependence of electron transfer between haem *a* and the binuclear site on the water occupancy of the enzyme [17]. It further explains why haem *a* is positioned very close to the a_3–Cu_B site, which is required for haem *a* to exert its electrostatic effect on the water orientation. Moreover, the short distance ensures a high electron transfer rate constant, which compensates for the presumably rate-limiting initial proton transfer via the propionate. It is noteworthy that the redox-dependent orientation of the water dipoles transfers positive charge from the proton donor (E286) to the acceptor (propionate or binuclear centre) through a displacement current [21], which further enhances the coupling between electron and proton transfer. Involvement of the K-pathway in transferring one of the substrate protons to the binuclear centre [9,13,14] is not contradictory, because this is also expected to cause release of a proton pumped via the D-pathway by the mechanism described here. Finally, the unique positioning of haem *a* and haem a_3–Cu_B in the

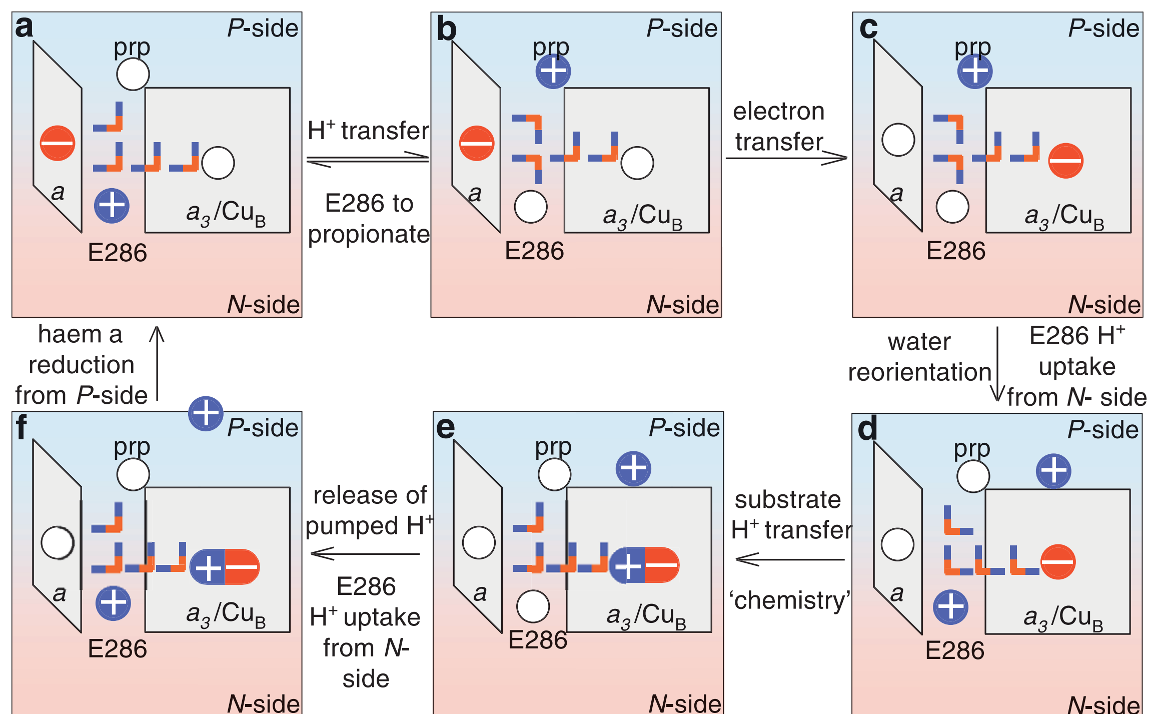

Fig. 2. Schematic of the proposed mechanism. In (a), haem *a* is reduced and the haem a_3–Cu_B site is oxidised. The electric field between the two orientates the water molecules to form a Grotthuss array for proton transfer from glutamic acid-286 to the Δ-propionate of haem a_3. Transfer of a proton from E286 to the propionate (b) is coupled to transfer of the electron to the binuclear site (c). Due to the electron transfer, the electric field switches orientation, and the water dipoles reorientate to provide a proton transfer path from E286 to the binuclear site (d). Uptake of a substrate proton via E286 (d) into the binuclear site to form water (e) forces the first proton out of the membrane, and E286 is again reprotonated (f).

same membrane plane (Fig. 1a) ensures that the transmembrane potential will not affect the electric field between them.

To test the energetic feasibility of the proposed redox-dependent switching of water arrays, we performed a series of molecular dynamics simulations. A 30-Å diameter sphere of structure was chosen around the haem groups in subunit I of cytochrome *c* oxidase from *R. sphaeroides* (access code 1M56, Ref. [8]). The covalent link between H284 and Y288 [5], and the OH^- ligand of Cu_B [26], were constructed using the HyperChem 7.03 programme (HyperCube Inc., Gainesville, FL), and energy-minimised using the MM+ force field. Molecular dynamics simulations were done with Amber99 or Charmm27, as applied in HyperChem. The TIP3P model [27] of water was used. The partial charges of the metal centres and their ligands were from semiempirical ZINDO/1 calculations using HyperChem. The following groups were allowed to move during the simulations: three to five water molecules in the cavity above E286, E286 itself, M107, W172, W280, V287, G283, the Δ-propionate of haem a_3, and the Cu_B–OH structure. Five water molecules were initially arbitrarily positioned in the cavity. Minimisation and initial simulations expelled one water molecule. In most cases, four water molecules were present, of which one was sometimes expelled in 5–200-ps simulations at 300 K with different redox states of haem *a* and Cu_B, keeping haem a_3 oxidised. In many simulations, the E286 carboxyl group was turned to a position towards the P-side [10,28], and tethered to the closest water molecule by constraining the distance between the carboxyl OH and water oxygens to 3.0 Å, applying a harmonic force constant of 7 kcal/mol $Å^2$, and the carboxyl OH...water O angle at 180° with a force constant of 7 kcal/mol deg^2. This prevented excursion of the glutamic acid side chain away from the P-side waters to contact the D-pathway on the N-side (Fig. 1b) [28]. Alternatively, and with similar results, the carboxylic side chain was held in the P-side position by constraining its distance from the haem groups. Similar results were obtained from molecular dynamics simulations including subunits I, II, and III of bovine cytochrome *c* oxidase [5] (access code 2OCC) using AMBER 4.1 (University of California, San Francisco, CA) and the AMBER94 force field [29] without constraints. In these latter simulations, several different charge parameterisations for the redox centres were tested and gave consistent results. Hence, the behaviour of the water arrays seems to be robust and not very sensitive to the precise structure, the force field, or the exact partial charges of the redox centres.

With haem *a* reduced and haem a_3–Cu_B oxidised, two water molecules formed a hydrogen-bonded path within picoseconds from E286 to the propionate (Fig. 3a). The second water molecule donates a hydrogen bond either directly to the propionate, or more frequently to the NH group of W172, which in turn is stably hydrogen-bonded to the propionate. This path remained stable for at least 100 ps. In contrast, when haem *a* is oxidised and Cu_B is reduced, a hydrogen-bonded water array formed within picoseconds from E286 to the OH^- ligand of Cu_B (Fig. 3b). Despite their limitations, these simulations clearly show that the control of water array orientation by redox state is plausible in the way required by the proposed mechanism.

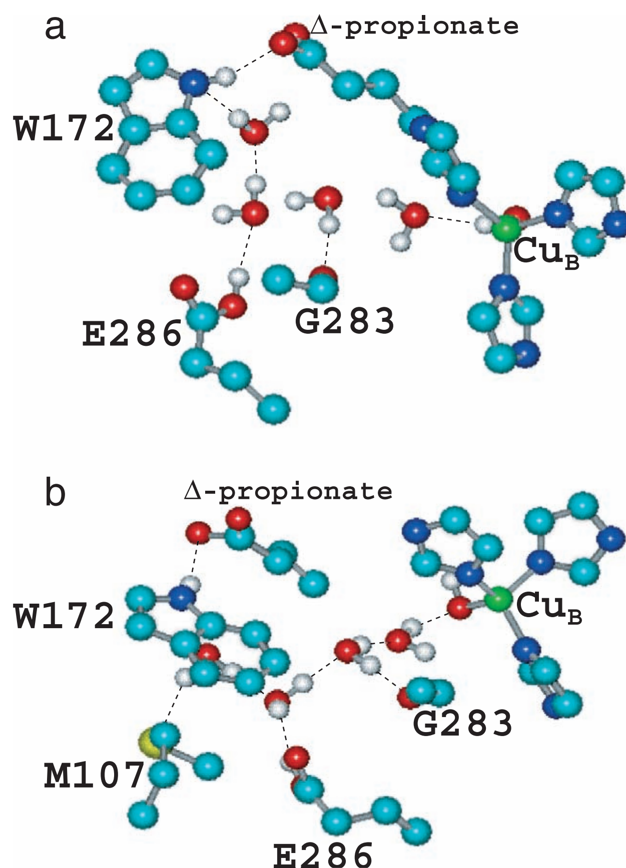

Fig. 3. Proton-conducting, hydrogen-bonded water arrays from E286 to the Δ-propionate of haem a_3 (a), or to the OH^- ligand of Cu_B (b), as obtained from molecular dynamics simulations with (a) haem *a* reduced and Cu_B oxidised, and (b) with haem *a* oxidised and Cu_B reduced (see text). The structure is shown from a slightly different angle in (a) and (b). Note in (a) the orientation of the water molecules next to the OH^- ligand of Cu_B, and the hydrogen bond to G283, and in (b) the orientation of the water molecule below W172, which forms hydrogen bonds to the sulfur of M107, and the water molecule nearest to E286. Colour code: carbon (blue green); copper (Cu_B, green); oxygen (red); nitrogen (dark blue); sulfur (yellow), and hydrogen (white).

It is not surprising that many features of the suggested mechanism are reminiscent of proton translocation models proposed in the past [4,10,18,24,30,31]. However, the unique gating of proton transfer by a redox-state-controlled orientation of water arrays proposed here provides a simple solution to many of the key problems that were previously unsolved. In particular, it explains how the same proton-conducting pathway may be used both for protons to be consumed in formation of water and protons to be translocated.

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