

Redox-driven membrane-bound proton pumps

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In recent years, remarkable progress has been made in our understanding of the structure and function of membrane-bound proton transporters at the molecular level. Perhaps the most challenging and complex of these molecular machines are the haem-copper oxidases. These enzymes are designed to activate the kinetically stable O₂ molecule, to prevent the release of potentially toxic oxygen intermediates and, at the same time, to harness the free energy from O₂ reduction by pumping protons across the membrane. So far, the mechanism of proton pumping has not been determined in any proton pump driven by reduction-oxidation reactions. Although this remains one of the key problems of molecular bioenergetics, recent developments have brought us to its core and closer to its solution.

Proton transporters are membrane-bound proteins that use the free energy provided by light or a chemical reaction to translocate protons across a membrane, thereby maintaining a transmembrane electrochemical gradient of protons. In a living cell, this gradient is used to drive various energy-requiring processes such as the production of ATP. Unlike enzymes in solution, the proton transporters catalyse reactions that not only have direction in time, but also have a well-defined direction in space, which is often referred to as 'vectorial chemistry'. In addition, the reactions catalysed by many transporters are associated with both the exchange of matter and the exchange of free energy. These characteristics make the proton transporters particularly exciting proteins to study, an interest that has been further augmented by the recent determination of the 3D structures of many such transporters.

As described in Box 1, biological systems make use of two basic principles to translocate protons across membranes (reviewed in Ref. [1]). The first principle is 'direct coupling' in transporters, in which proton translocation is associated with oxidation and reduction of the proton carrier itself. For example, in the photosynthetic reaction centre and cytochrome *(bc₁)* complex, the reduction of a quinone is associated with its protonation from one side of the membrane, followed by diffusion of the neutral species across the membrane and release of the proton to the other side of the membrane (Box 1, Figure 1a). In another example of a direct coupling mechanism, the electron and

the proton are donated to an acceptor, A, from different sides of the membrane, resulting in a net charge separation across the membrane (Box 1, Figure 1b, broken blue box).

The second principle is 'indirect coupling' in proton pumps, in which endergonic proton translocation across the membrane is driven by an exergonic reaction (Box 1, Figure 1b). The free energy might be provided by ATP (through reversal of the normal function of ATP synthase), light (in bacteriorhodopsin) or a reduction-oxidation (redox) reaction (e.g. in respiratory haem-copper oxidases).

In this review I describe recent progress on the proton pumps, focusing in particular on those in which proton pumping is driven by redox reactions, for which the detailed mechanism is illustrated by the haem-copper oxidases. This group of enzymes constitutes the last components of the respiratory chain in aerobic organisms and is responsible for processing >90% of the oxygen consumed by living organisms on Earth.

Functional aspects of proton pumps

A proton pump is a membrane-bound protein that translocates protons from one side of the membrane to the other without using any (free) mobile proton carriers. Because the free energy required to move a (positively charged) proton from a water solution to the hydrophobic portion of the membrane is associated with a very large positive change in free energy change – on the order of about one ELECTRONVOLT (see Glossary) – a membrane-bound proton transporter must embody proton-transfer pathways that span the membrane. It is crucial, however, that there is never direct contact between the two membrane sides because this would lead to a short circuit in the electrochemical gradient.

One way in which to control the proton transfer is to introduce a 'switch' in a proton-transfer pathway that provides an 'alternating access' of protons to one or the other side of the membrane (but never both sides simultaneously), thereby defining an INPUT STATE and an OUTPUT STATE (reviewed recently in Refs [2,3]). The switch can be a physical unit, for example, an amino acid residue that alters between two different positions. Alternatively, the switching can be accomplished by modifying the medium through which proton transfer takes place, thereby altering the relative rates of proton transfer to the two sides of the membrane (Box 1, Figure 1c). One way in which to accomplish proton translocation is to couple

Box 1. Mechanisms of charge separation and proton pumping across membranes

Oxido-reduction proton translocators

In an oxido-reduction proton translocator (Figure 1a), the protons are carried across the membrane by diffusible carriers (A_x , where $x = 1, 2, 3$ and so on) that receive electrons from redox sites located near the negative (N)-side surface (r_{xN}) and donate the electrons to redox sites located near the positive (P)-side surface (r_{xP}) of the membrane. In the example shown in Figure 1a, the redox site r_{1N} donates an electron to the carrier A_1 , which is associated with protonation of the carrier from the N -side solution. The neutral species A_1H diffuses across the membrane to a redox site located near the P -side surface. Here, the electron is transferred from A_1H to the redox site r_{1P} and the proton is released to the P -side solution. The electron is then transferred from r_{1P} to the next redox site, r_{2N} , located near the N -side surface.

The process of alternating between the electron-transfer reactions and the diffusion of proton-electron carriers across the membrane results in the translocation of (exactly) one positive charge from the

N -side to the P -side per electron transferred across the membrane. This stoichiometry is obtained because the protons are moved physically by the carrier. An example of a proton-electron carrier is a quinone/quinol, which carries two electrons and two protons at a time.

In Figure 1a, the electron (i.e. negative charge) is transferred across the membrane from the positively to the negatively charged side of the membrane; in other words, it moves against the electrical gradient, thereby contributing to voltage generation. This type of reaction is often referred to as an 'electrogenic' process and in the example shown in Figure 1a it requires progressively increasing midpoint potentials of the redox sites.

Proton pumps

In a proton pump, the proton translocation takes place without the use of carriers that physically move the proton from the N -side to the P -side of the membrane (Figure 1b). Instead, protons are transferred through

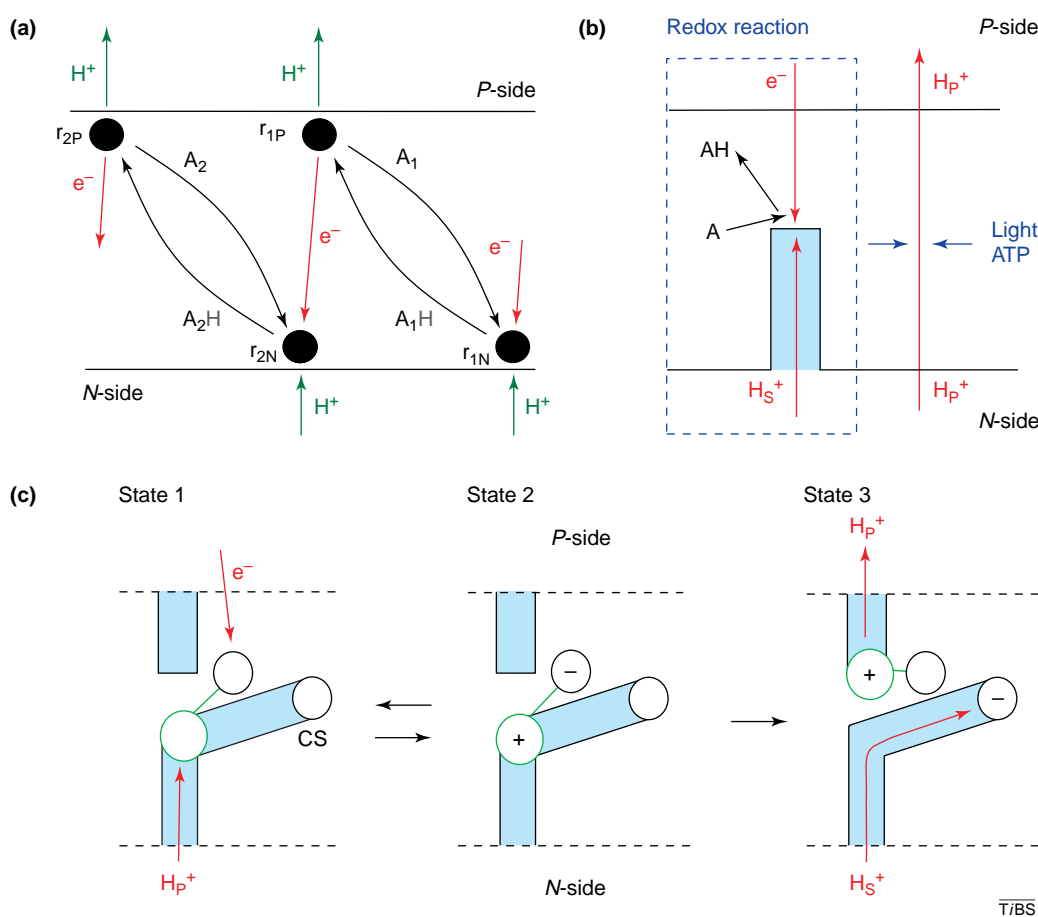


Figure 1. Principles of redox-driven proton translocation across membranes. **(a)** An oxido-reduction proton translocator. Several redox sites, r_{xN} and r_{xP} (where $x = 1, 2, 3$, and so on), are arranged topographically as shown by the filled circles. After reduction of the first redox site, r_{1N} , close to the N -side surface, this redox site donates an electron to a diffusible hydrogen atom carrier (i.e. an electron-proton carrier), A_1 , which also takes up a proton from the N -side solution. The reduced and protonated species, A_1H , diffuses to another redox site, r_{1P} , located near the P -side surface. An electron is transferred from A_1H to r_{1P} , coupled with release of the proton to the P -side. The electron is then transferred across the membrane from r_{1P} to r_{2N} and the cycle is repeated. The electrogenic charge transfer across the membrane is shown in red. **(b)** A proton pump. Proton pumping takes place without using any proton carriers to move the protons physically. The protons are pumped (H_P^+) across the membrane, in an electrogenic reaction, through a proton-transfer pathway spanning the protein (red arrow on right). The free energy needed to transfer the protons can be provided, for example, by light or ATP (right), or by an electron-transfer reaction (shown in the broken blue box on the left). In this last example, the electron transfer takes place from a low-potential donor in solution (not shown) on the P -side to an acceptor A . On reduction, A picks up a substrate proton (H_S^+) from the N -side, where both the electron and the proton transfer are electrogenic (red arrows). **(c)** Mechanistic model of a redox-driven proton pump. Electron transfer from a donor on the P -side of the membrane to an acceptor in a membrane-spanning part of the protein is associated with proton transfer ('pumped proton', H_P^+) from the N -side to a proton acceptor ('proton switch', green circle) in the 'input conformation' (state 1 \rightarrow state 2). Electron transfer to the catalytic site (CS) and the uptake of a substrate proton, H_S^+ , takes place only after the switch moves to the 'output conformation' (state 2 \rightarrow state 3). This reaction results in release of the pumped proton to the P -side. The overall process results in the translocation of two positive charges from the N -side to the P -side per electron transferred to the final acceptor. Red arrows indicate processes associated with the transfer of charge against the transmembrane gradient and perpendicular to the membrane surface (electrogenic reactions). Blue shading represents intraprotein proton-transfer pathways.

specific proton-transfer pathways composed of protonatable residues and water molecules spanning a membrane protein. The free energy needed to transfer the protons can be provided, for example, by light, by ATP or by an electron-transfer reaction from a donor with a low midpoint potential to an acceptor with a high midpoint potential.

For the redox-driven proton pump cytochrome *c* oxidase (CcO), the electron-transfer reaction that drives proton pumping is electrogenic in itself and is associated with an electrogenic uptake of protons, in which the electron and proton-transfer processes result in a net translocation of one positive charge from the *N*-side to the *P*-side per electron transferred from the donor to the acceptor. Because in CcO this process is coupled to proton pumping across the membrane, the overall reaction catalysed by CcO results in the translocation of two positive charges from the *N*-side to the *P*-side per electron transferred to the acceptor. It should be noted that this 2 H⁺/e⁻ stoichiometry cannot be realized by the mechanism shown in Figure 1a.

A functional model of a redox-driven proton pump

Figure 1c shows a functional model of proton pumping driven by a redox reaction (see Ref. [44]). Electron transfer from a redox site on the *P*-side to an acceptor in the protein results in the uptake from the *N*-side of a

proton to be pumped (H_P⁺) by a proton acceptor. This proton acceptor can switch between two conformations in which it can exchange protons with either the *N*-side ('input conformation') or the *P*-side ('output conformation').

In states 1 and 2, the switch is found in the input conformation. Before the reaction proceeds to state 3, however, the switch must move to the output conformation. In state 3, the electron is transferred to the catalytic site, which is the final electron acceptor (see Ref. [53] for a discussion of the control of internal electron transfer in CcO), and the proton H_P⁺ is released to the *P*-side. Reduction of the catalytic site is associated with the uptake of a substrate proton (H_S⁺).

In a 'thermodynamically coupled' pump, reduction or oxidation of the first redox site results in an increase or decrease, respectively, in the p*K*_a of the proton acceptor. In a 'kinetically coupled' pump, the p*K*_a of the proton acceptor is constant and lower than the pH on the *P*-side. The proton acceptor becomes protonated in the input conformation only in a fraction of the population (i.e. the fraction of molecules that reach state 2 in Figure 1c is determined by the p*K*_a of the proton acceptor and the pH on the *N*-side). Switching to the output conformation (the 2 → 3 transition in Figure 1c) takes place only in this fraction and the equilibrium between states 1 and 2 is driven towards state 3.

energetically the reaction that drives proton pumping (e.g. light excitation of a chromophore or an electron-transfer reaction) to changes in the p*K*_a (i.e. the protonation state) of a protonatable group, such that it has a high p*K*_a in the input state and a low p*K*_a in the output state. In other words, the protonatable group picks up a proton when the pump is in the input state and releases the proton when the pump is in the output state.

To illustrate the basic functional principles of proton pumps, below I describe in detail the structure and function of cytochrome *c* oxidases (CcOs). These enzymes constitute a subgroup of the haem–copper oxidase superfamily, which is defined by the presence of a catalytic site consisting of a haem–copper centre in the protein. This subgroup of the haem–copper oxidases uses different types of cytochrome *c* as electron donors. There is also a

second subgroup, the quinol oxidases, which use different quinols as electron donors. The basic functional principles are thought to be essentially the same for these two subgroups.

The haem–copper oxidases are particularly intricate proton pumps because they use the free energy of a redox reaction, which in itself results in transmembrane charge separation through a direct coupling mechanism (see Box 1, Figure 1b, broken blue box), to drive proton pumping across the membrane.

Cytochrome *c* oxidases

The CcO from *Rhodobacter sphaeroides* [4] is composed of four subunits, denoted SU I to SU IV (Figure 1a). In SU I, the largest subunit, are found a haem group (haem *a*) and the catalytic site, which comprises another haem group (haem *a*₃) and a copper ion (Cu_B) located in close proximity (Figure 1b). A di-nuclear redox-active copper centre (Cu_A) is present in SU II. There are no redox-active cofactors in SU III, but this subunit is important for stability of the protein [5]. SU IV consists of one transmembrane helix and its role is unknown.

The structure of this CcO is similar to those of other haem–copper oxidases [6–11]. In particular, the *R. sphaeroides* CcO structure is essentially identical to that of the main core of the mitochondrial bovine heart CcO (SU I to SU III), which is one of the most studied CcOs. The following discussion is centred on the CcOs from *R. sphaeroides* and bovine heart, but the general mechanisms are likely to apply to all proton-pumping haem–copper oxidases.

CcOs are typically found in the inner membrane of mitochondria or the cytoplasmic membrane in bacteria, where they catalyse the four-electron reduction of dioxygen (O₂) to water by four molecules of reduced cytochrome *c*. The reaction is also associated with the uptake of four protons (referred to as substrate protons because they are used as a substrate in the reduction of O₂) as follows:



Glossary

Cytochrome *c* oxidase (CcO) state: The different states of CcO are denoted with one-letter codes as follows: O⁰ or O⁴ (O^{2/4}) oxidized state; E¹, a state in which Cu_B at the catalytic site is reduced; R², a state in which both Cu_B and haem *a*₃ are reduced; P², the peroxy intermediate formed on reaction of R² with O₂; F³, the ferryl intermediate formed on addition of one electron to P². In this review, the superscripts denote the number of electrons that have been added to CcO from an external donor in the different states.

Electronvolt: The energy unit used in this article. A free-energy difference of 1 eV per molecule corresponds to about 100 kJ/mol.

Input/output states: In the input state of a proton pump, a protonatable group in a proton pathway for pumped protons is in rapid equilibrium with the proton input side (*N*-side), but not the output side (*P*-side). In the output state of a proton pump, the situation is reversed. A transition between these states can involve a protein structural change, changes in the configuration of water molecules, or changes in the relative p*K*_a values of protonatable groups in the pathway.

***N*-side/*P*-side:** The (relatively) negatively and positively charged sides of the membrane, respectively.

Redox loop hypothesis: Peter Mitchell's hypothesis describing how protons are translocated across a membrane through a chain of alternating redox centres and hydrogen atom carriers (i.e. electron–proton carriers). Each hydrogen atom carrier picks up an electron from a redox site near the *N*-side membrane surface and a proton from the *N*-side. The carrier then diffuses across the membrane to the *P*-side, where the proton is released and another redox site near the *P*-side membrane surface is reduced. The electron is transferred across the membrane to a third redox site near the *N*-side surface and the cycle is repeated. Because the membrane is impermeable to protons, this sequence of events results in the translocation of one positive charge across the membrane per electron transferred from the *P*-side to the *N*-side.

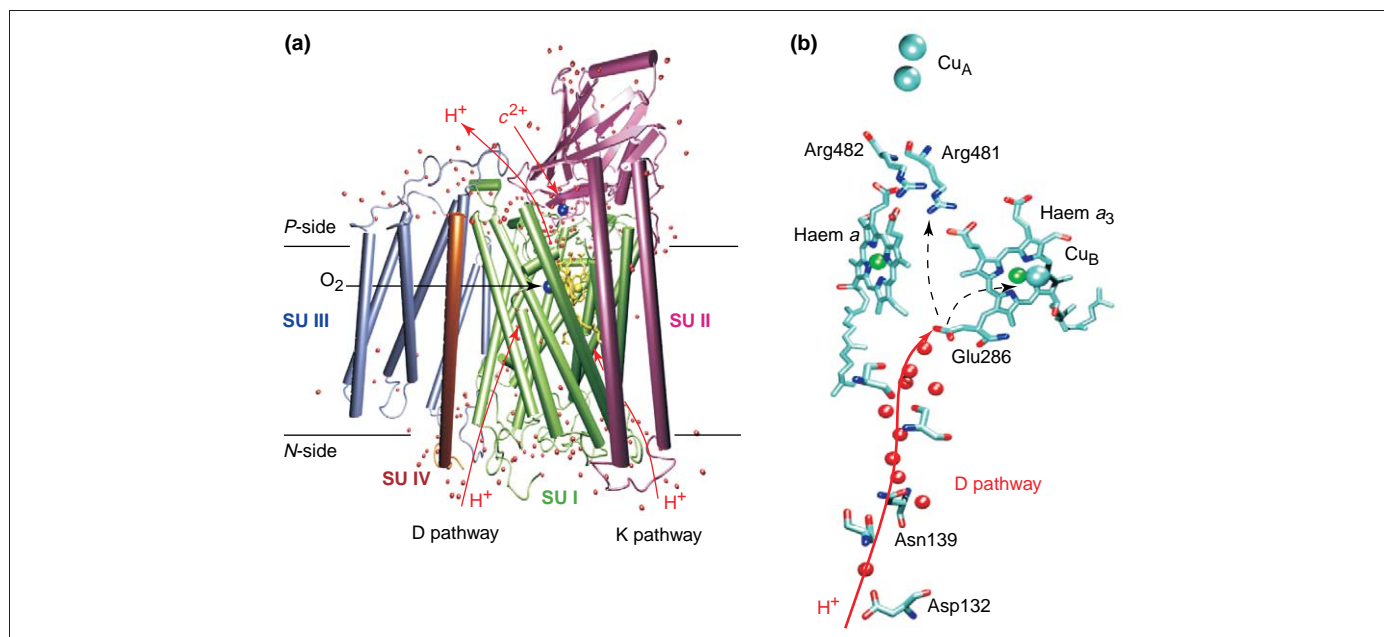


Figure 1. Structure of cytochrome *c* oxidase (CcO). (a) The CcO from *Rhodobacter sphaeroides* (cytochrome *aa*₃) [4] comprises four subunits (SU I to SU IV). Trans-membrane helices are shown as thin rods to visualize the intraprotein cofactors and water molecules. Haem groups are shown in yellow and copper sites in blue. Water molecules that are resolved in the crystal structure are shown as red spheres. The approximate location of the D and K proton-transfer pathways (red arrows) and the membrane (black lines) are indicated. The O₂ molecule most probably enters from the membrane core (black arrow). Electrons from reduced cytochrome *c* (*c*²⁺) are transferred from the positive side (*P*-side) to Cu_A (red arrow). The exit route of the pumped protons, above the haem groups, is not known and it is only shown schematically (red arrow). Water molecules exit towards the top of the molecule (not shown) [54] (b) The D proton-transfer pathway and the cofactors of CcO. Protons are transferred through Asp132 [55] and a chain of intraprotein water molecules (red spheres) to the internal proton donor or acceptor, Glu286. From Glu286, protons are transferred either towards the catalytic site (substrate protons) or towards an acceptor site for the pumped protons (broken arrows). The figure was prepared using the VMD program [56]. Modified, with permission, from Ref. [12].

Here, the subscripts *N* and *P* refer to the electrically negative and positive side, respectively, of the membrane. The (positively charged) substrate protons are taken up from the *N*-Side and the (negatively charged) electrons are donated from the opposite *P*-Side of the membrane. Thus, the chemical reaction catalysed by CcO is arranged topographically in such a way that the reaction results in a charge separation corresponding to the net transfer of one positive charge from the *N*-side to the *P*-side of the membrane per electron transferred to O₂ (Box 1, Figure 1b), which is phenomenologically compatible with Mitchell's REDOX LOOP HYPOTHESIS [1,3,12] (Box 1, Figure 1a).

For the mitochondrial CcO, the free-energy difference for the electron transfer from cytochrome *c* to O₂ at standard conditions is around -550 meV. Because in mitochondria the proton electrochemical potential across the inner membrane is about 200 mV, the charge separation upon moving of one positive charge from the *N*-side to the *P*-side is associated with a free-energy change of roughly 200 meV. Thus, the charge separation in CcO results in conservation of only less than half of the available free energy.

In 1977, Mårten Wikström showed that, in addition to performing this charge separation, CcO is also a proton pump (reviewed in Ref. [3]), whose overall reaction results in the translocation of two positive charges across the membrane (corresponding to a free-energy change of about 400 meV) per electron transferred to O₂ (Box 1, Figure 1b), thereby conserving about 75% of the available free energy of -550 meV. It should be noted that this scheme cannot be explained in terms of Mitchell's redox loop mechanism because, according to the latter mechanism, only one

positive charge can be translocated across the membrane per electron.

Proton-transfer pathways

Proton-transfer pathways are generally composed of networks of hydrogen-bonded water molecules and protonatable and polar amino acid side chains (Figure 1b). Because the rate of a proton-transfer reaction changes substantially even for a 1-Å change in the distance between two neighbouring components of the pathway, these rates can be controlled through minute changes in the positions of amino acid side chains and water molecules [13].

Only a few ordered water molecules are seen below the haem groups in the structures of CcOs (Figure 1a). In the *R. sphaeroides* CcO, an array of water molecules spans the distance between the two conserved residues Asp132, which is located near the protein surface, and Glu286 defining a proton-transfer path called the 'D pathway' (Figure 1b). Between Glu286 and the catalytic site there is a hydrophobic cavity, which is most probably filled with water molecules that are not resolved in the X-ray structure. In addition, it is likely that there are water molecules between Glu286 and the haem propionates located towards the proton output side [14,15] (see also Ref. [16]).

A second proton-transfer pathway, the 'K pathway', that leads from the *N*-side surface towards the catalytic site has been identified [17,18] (Figure 1a). This pathway is used for proton uptake on reduction of the catalytic site of CcO and is not discussed here.

Electron transfer and O₂ reduction by cytochrome c oxidase

The primary electron acceptor of the oxidized CcO state (state O⁰) is the Cu_A centre (Figure 1b). This electron is transferred consecutively first to haem *a* and then to Cu_B at the catalytic site to form the one-electron-reduced CcO state (state E¹), which is associated with the uptake of one proton. The addition of a second electron results in the reduction of haem *a*₃, which is also associated with the uptake of one proton (Figure 2a). When the haem *a*₃-Cu_B catalytic site is reduced by two electrons (state R²), O₂ binds to the haem *a*₃ iron (state A²) and is reduced to form the 'peroxy' state (state P²). The name 'peroxy' is used for historical reasons because initially it was thought that O₂ was reduced by two electrons in this state (one from haem *a*₃ and one from Cu_B), to form the peroxy state Fe_{a₃}³⁺ - O⁻ - O⁻; in other words, the O-O bond was thought to be left intact.

The results from more recent studies indicate that in the P² state the O₂ molecule is already reduced by four electrons, resulting in breakage of the O-O bond and formation of ferryl state [19–22] (Figure 2b). Nevertheless, this state is generally denoted by a 'P' and it appears on reaction of the two-electron-reduced CcO with O₂. Thus, even though in the P² state CcO has received only two electrons from cytochrome *c*, the O₂ molecule receives four electrons after binding to haem *a*₃, where one electron is donated by Cu_B, two are donated by haem *a*₃ and one is presumably donated by a tyrosine residue, as shown in Figure 2b. The R² → P² transition is not linked to proton uptake or pumping, and the free-energy change of the reaction is only 5–10% of that available from the four-electron reduction of O₂ to water [23,24].

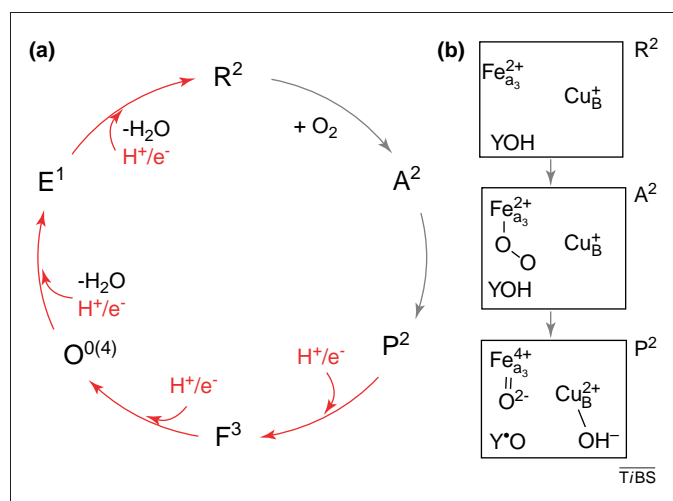


Figure 2. Function of cytochrome *c* oxidase (CcO) illustrated by a schematic reaction cycle. (a) Reduction of the oxidized CcO (state O⁰, the superscript refers to the number of electrons added from cytochrome *c*) results in formation of a one- (state E¹) and two- (state R²) electron-reduced catalytic site. The two-electron reduction is followed by binding of O₂ (state A²). Initially, O₂ is reduced by four electrons to form a ferryl state (state P²). Two electrons are donated by Fe²⁺ (Fe²⁺ → Fe⁴⁺), one by Cu_B⁺ (Cu_B⁺ → Cu_B²⁺) and one presumably by Tyr288 (YOH; Y*O is a tyrosine radical) at the catalytic site (note that in the P² state CcO that started in the O⁰ state has received two electrons from cytochrome *c*). Reduction and protonation of P² results in formation of another 'ferryl' state (state F³). Further reduction and protonation of F³ results in formation of the oxidized CcO (state O⁴ or O⁰). (b) Details of the initial breakage of the O-O bond on reaction of O₂ with CcO in the R² state. For a detailed reaction scheme see Ref. [50]

In the next step, the third electron is transferred to the catalytic site to form a state that is called ferryl (state F³). This reaction is followed by the transfer of the fourth electron to the catalytic site forming the oxidized state (state O⁴ or O⁰) and completing the cycle. Each of the transitions P² → F³ and F³ → O⁴⁽⁰⁾ are associated with proton uptake [25,26] and pumping (the proton pumping is discussed in more detail below), where all protons are transferred through the D pathway ([27–30]; reviewed in Ref. [31]).

This mechanism presents us with a remarkable strategy for preventing the release of potentially toxic intermediates and at the same time conserving free energy for proton pumping [32]. The O₂ molecule is first fully reduced by four electrons to form incompletely protonated water molecules in one, essentially isoenergetic, step (R² → P²), thereby preventing the release of partly reduced oxygen intermediates. The subsequent reaction steps are highly exergonic and associated with proton uptake and pumping.

Energetics and stoichiometry of proton pumping

The knowledge of the 3D structures of CcOs has offered much of the information that is necessary to deduce the molecular mechanism of proton pumping. However, a discussion of this mechanism based solely on an analysis of the structure is more difficult in the case of CcO than for other systems because the reaction that drives proton pumping also involves intramolecular proton-transfer reactions. Detailed mechanistic studies of CcO are therefore crucial for understanding the proton-pumping machinery.

As described in Box 2, Wikström found that each of the transitions P² → F³ and F³ → O⁴⁽⁰⁾ is associated with a change in free energy of about 1 eV (reviewed in Refs [3,24]). Because the total free-energy change on oxidation of four molecules of cytochrome *c*²⁺ and reduction of O₂ to 2 H₂O is -2.2 eV and the process is associated with the pumping of four protons, Wikström suggested that two protons are pumped in each of the two transitions. This scheme was recently questioned by Michel [33,34], who suggested that proton pumping also takes place on the reduction of CcO (O⁰ → R²).

Indeed, results from more recent studies indicate that protons are pumped on the reduction of CcO [35,36], such that during turnover not only the P² → F³ → O⁴⁽⁰⁾ steps, but also the O⁴⁽⁰⁾ → E¹ → R² steps are associated with proton pumping [37]. This scenario means that part of the free energy released during the P² → F³ → O⁴⁽⁰⁾ transitions must be conserved by CcO and used during the steps O⁰ → E¹ → R² [35,37] (Box 2). Although still debated, these hypotheses have opened up new directions in investigations of the proton-pumping mechanism in CcOs.

Identifying the reaction steps that are associated with proton pumping is crucial for dissecting the proton-pumping mechanism at the molecular level. A scheme in which one proton is pumped in each of the transitions, as outlined above, presumably means that a series of identical events are repeated for each of the transitions.

Box 2. Reduction of O₂ in water and by cytochrome oxidase

Even though the reduction of O₂ to water is a highly exergonic reaction, the O₂ molecule is kinetically stabilized against reduction (i.e. the reaction is extremely slow) mainly owing to two factors. In the ground electronic state, the O₂ molecule has two unpaired electrons; in other words, it is in a triplet state, which imposes spin restrictions on many of its reactions. In addition, a positive free-energy change is associated with the one-electron reduction of O₂ (Figure 1a), which imposes a thermodynamic barrier in the initial step of the reduction process.

Cytochrome *c* oxidase (CcO) overcomes these problems by binding O₂ to the reduced haem *a*₃ iron, which also has unpaired electrons. On binding of O₂, the orbitals of Fe²⁺_{a3} and O₂ combine, resulting in new orbitals that are more singlet-like in nature for oxygen. In addition, a second electron donor, Cu_B, is provided in the immediate vicinity of haem *a*₃, making it possible to reduce O₂ by two electrons in one reaction step, thereby bypassing the endergonic one-electron reduction step.

The electron donor, cytochrome *c*, has a midpoint potential of about 0.25 V; therefore, the overall change in free energy for the reaction in Equation 1 (see main text) is about -2.2 eV (four electrons transferred from cytochrome *c* to O₂ in the absence of a proton electrochemical gradient). The $\Delta G'_0$ for each of the first two electrons is about -0.1 eV. Binding of O₂ has been estimated to have a $\Delta G'_0$ of about -0.2 eV.

The two reactions steps associated with the largest changes in $\Delta G'_0$ are the P² → F³ and F³ → O⁴⁽⁰⁾ transitions [24] (Figure 1b, green lines). Results from recent experiments [37] indicate that part of the free energy released in these reaction steps is stored in the enzyme and used during the reduction of CcO in the next cycle (Figure 1b, red lines).

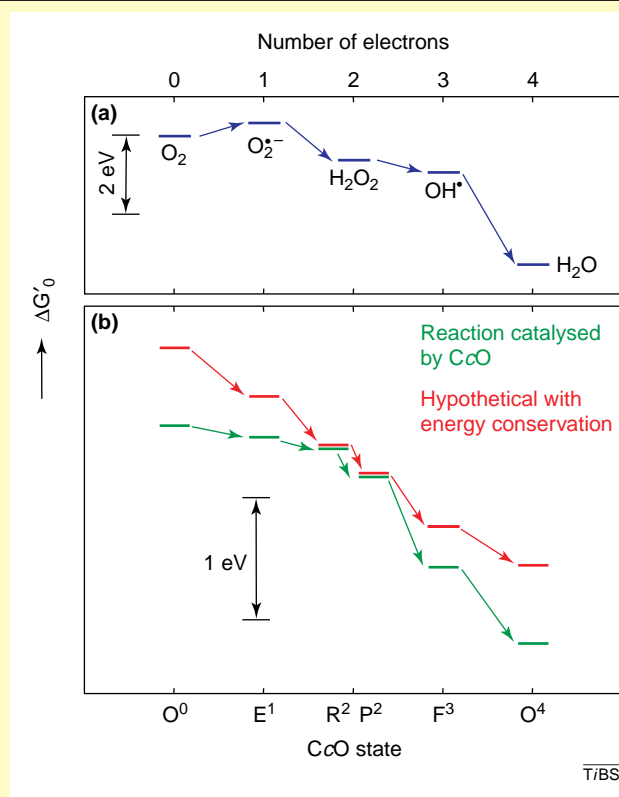


Figure 1. Free-energy changes associated with the step-wise reduction of dioxygen by four electrons to water. (a) Stepwise reduction of O₂ in a solution of water. The overall free-energy change associated with reduction of O₂ to H₂O by four electrons is about 3.2 eV at standard conditions. (b) The green graph shows the free-energy changes associated with transitions between the intermediate states that are formed on addition of electrons to CcO in the presence of O₂. The one-letter code refers to the state, the superscripts refer to the number of added electrons to CcO from cytochrome *c*. The red graph depicts a hypothetical case where part of the free energy provided by the reaction steps P² → F³ and F³ → O⁴⁽⁰⁾ is conserved in the CcO and released during the O⁰ → E¹ and E¹ → R² transitions. The overall free-energy change associated with oxidation of four molecules of cytochrome *c* and the four-electron reduction of O₂ to H₂O is about -2.2 eV. For clarity, the red and green plots have been aligned to overlap in the P² state.

Proton-pumping mechanisms

Because during the P² → F³ and F³ → O⁴⁽⁰⁾ transitions both substrate and pumped protons are taken up through the D pathway, at some point in the pathway there must be a branching point from which the substrate and pumped protons are distributed in different directions. A likely location of this branching point is the Glu286 residue, because it is located at the end of the D pathway [8,38–41] (Figure 1b).

In principle, conservation of free energy in a specific reaction step requires that the proton to be pumped is 'loaded' before the substrate proton is transferred to the catalytic site (Box 1), otherwise the energy would be lost as heat. This proton uptake might occur on reduction of a redox site in CcO [31,33,42–46]. In this context, much focus has been put on haem *a*, reduction of which might be linked to an increase in the pK_a of a protonatable group in the 'pumping pathway', followed by a local structural change of the group (Box 1) and electron transfer to the catalytic site, associated with uptake of a substrate proton.

A molecular model that does not require changes in the pK_a of the proton acceptor group has been presented recently ([14]; see also Ref. [47]). In this model, the reduction of haem *a* results in rearrangement of the intraprotein water molecules to provide a pathway to an acceptor of pumped protons [14]. Protonation results in further rearrangement of water molecules to provide a pathway for transfer of a substrate proton, associated with electron transfer, to the catalytic site.

In terms of addressing the mechanism of proton pumping, a class of mutant CcOs in which the reduction rate of O₂ is unperturbed but proton pumping does not take place is particularly interesting [48–50]. In one such mutant CcO, Asn139 in the D pathway (Figure 1b) has been replaced by an aspartate residue. An investigation of individual electron and proton-transfer reactions on the microsecond timescale has shown that a plausible explanation for the impaired proton pumping in this mutant CcO is an increase in the pK_a of Glu286 [50].

On the basis of this observation, coupled with results from other experiments and analysis of the *R. sphaeroides* CcO structure, a potential scheme for proton pumping by CcO has been proposed [51]. This scheme is shown in Figure 3 to illustrate the basic design principle of a proton pump, but it is only one of many possible models that could explain the molecular mechanism of the membrane-bound proton pump. According to this scheme, electron transfer to the catalytic site (CS in Figure 3) is followed by proton transfer from Glu286 to the catalytic site. The deprotonation of Glu286 is linked to a local structural change around the Arg481–Arg482–haem propionate cluster (defined as the proton acceptor A in Figure 3), which results in an increase in the pK_a of A, such that it becomes protonated from the *N*-side.

In the next step, Glu286 is re-protonated and the pK_a of A relaxes to a low value, which results in release of the proton to the *P*-side. According to this model, the 'proton switch' providing an alternating access of protons to the two sides of the membrane (Box 1, Figure 1c) is located around A. Note that the order of reactions in this model is fundamentally different from the one discussed in Box 1 (Figure 1c) in that the initial step in the sequence of events that lead to proton translocation is an internal proton transfer from Glu286 to the catalytic site, in other words, the transfer of a substrate proton. The free energy of the reaction is conserved by way of changes in the pK_a of A, coupled to deprotonation of Glu286.

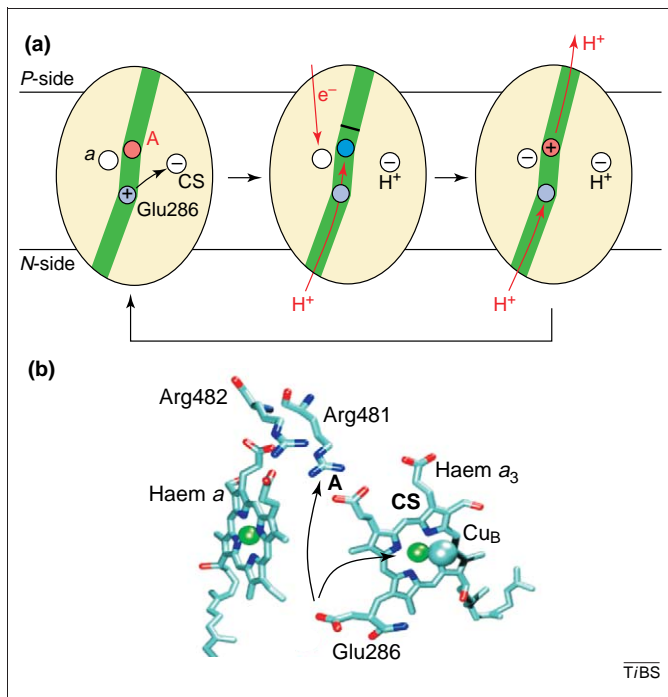


Figure 3. Potential scheme for proton pumping by cytochrome *c* oxidase (CcO) [51]. (a) Left, electron transfer to the catalytic site (CS) is followed by proton transfer from Glu286 ('+' indicates a protonated site). Middle, deprotonation of Glu286 results in a local structural change associated with an increase in the pK_a of the proton acceptor (A), in this case the Arg481–Arg482–haem propionate cluster (red indicates low pK_a , blue indicates high pK_a), and in rearrangement of water molecules around A such that A is now in slow equilibrium with the positive (*P*-side (interruption in the green proton pathway, black bar). Right, protonation of A is followed by re-protonation of Glu286, which is associated with reduction of haem *a* and relaxation of the A conformation to the low- pK_a state in which the group is in rapid equilibrium with the *P*-side. (b) Structure around the haem groups and Glu286 [4].

Concluding remarks

About 40 years ago, Peter Mitchell proposed that the intermediate in energy conversion in biological systems is a proton electrochemical gradient across biological membranes [52]. Since that time, research in bioenergetics has moved gradually from a phenomenological description of the general processes towards a molecular description and visualization of the components involved in energy conversion. These explorations have culminated in the recent determination of 3D structures, at the atomic level, of several of these components. For CcO, these breakthroughs have made it possible to put the results from spectroscopic studies into a framework of specific structural elements of the pump and to apply theory to understand the functional design of the pumping machinery.

The function of CcO as a proton pump requires an accurate timing and a tight energetic coupling between rapid intraprotein electron and proton-transfer reactions. Thus, in future studies aimed at understanding the pumping machinery, it will also be important to identify transient structural states of the proton pump. Because the overall reaction catalysed by CcO can be dissected into elementary processes that are common to most membrane-bound systems involved in energy conservation, studies of these processes in CcO are significant for our understanding of the general functional principles of all of these systems.

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