The structures and functions of hydrophilic channels in electron-transferring membrane proteins are discussed. A distinction is made between proton channels that can conduct protons and dielectric channels that are non-conducting but can dielectrically polarize in response to the introduction of charge changes in buried functional centres. Functions of the K, D and H channels found in A1-type cytochrome c oxidases are reviewed in relation to these ideas. Possible control of function by dielectric channels and their evolutionary relation to proton channels is explored.

1. The tendency for charge neutralization

Intraprotein charge transfers, in particular of electrons or protons (or in some cases sodium ions), are central to the functions of the membrane-embedded enzymes of respiration and photosynthesis. These often occur in buried catalytic sites. They are key components of reaction sequences that result in net movement of ions and charges across the membranes in which the protein complexes are located. The resulting electrical and chemical transmembrane gradients are the components of the proton motive force (PMF) that provides the circuit connection that is central to chemiosmotic energy coupling [1,2].

When such a charge transfer occurs, the environment reacts to minimize the electrostatic costs of local charge imbalances that will be associated with it. In a hydrophobic solid, the dielectric response is small, resulting from induced dipole moments, and in liquid water it is large, owing to the reorientation of permanent water dipoles. For those sites in membrane proteins that have nearby protonatable groups whose pKs are influenced by the charge state (the charge-linked Bohr effect), charge imbalance can be minimized by their (de)protonation. The extents of charge-induced protonation changes are governed by the dielectric constant, which dictates interaction energies, and distances. In addition, induced and permanent dipoles can reorient by limited atomic movements and point charge ions can be displaced. The dominating processes and extents of charge neutralization will depend on the protein structures that house the catalytic centres. For example, in photosystem II, protons are released in response to electron abstraction from the catalytic centre of water oxidation [3] and are taken up when the bound electron acceptor quinone, Qb, is reduced [4,5]. In other structures, the dominant effects may involve reorientation of charge. For example, a prominent feature in the catalytic centre of the F1-ATP synthase is the reorientation of a positive arginine finger towards the negatively charged γ-phosphate of bound ATP [6].

In the mitochondrial respiratory chain, complexes I, III and IV have intricate internal charge-transfer reactions that result in net proton translocation across the membranes in which they are embedded [7]. Complex IV is the terminal enzyme cytochrome c oxidase (CcO). It catalyses intraprotein electron and substrate proton transfer to a buried catalytic centre where oxygen is reduced to water. The exergonic chemistry of oxygen reduction drives additional coupled transmembrane proton transfers. Hence, there are multiple steps involving transient charge changes within buried sites. Here, we review structural and biophysical information on the roles of the hydrophilic channels within CcOs in relation to these charge movements, in particular their possible functions as proton-conducting channels versus ‘dielectric’ channels. The latter term is
used to denote arrays of polarizable groups that are unable to support a continuous current of protons and instead provide a localized structure of high dielectric strength that connects an adjacent aqueous phase to a functional centre that is deeply buried in an otherwise low dielectric environment [8,9]. The array can polarize, possibly with associated charge rearrangements at the aqueous surface, which results in a decreased local net charge change.

2. Proton channels, wells, gates and protonmotive membrane proteins

The Grothuss [10] mechanism of transport of protons in water involves a concerted reorganization of H bonds between adjacent water molecules, followed by the physical movement of atoms by molecular rotations [11]. A similar mechanism is presumably operative in restricted intraprotein proton-conducting channels, involving H-bonded networks of waters with hydrophilic and other protonatable groups (a notion first proposed by Onsager [12,13]). Such ‘proton wires’ have been discussed in detail elsewhere [14].

In order to facilitate a continuous ionic current, protons must, on the timescales of catalysis (often milliseconds or faster), be able to move from/to into sinks at either end of the structure and the channel must be able, after net proton transfer, to revert back to its initial configuration so that transfer of the next proton can proceed. Computational models predict that these physical atomic movements are rate-limiting for multiple proton transfers in water arrays [15] though this has been questioned [11].

If such structures traverse only part of the membrane dielectric barrier and are spatially restricted so as to exclude the dominating ions of the electrolyte, then they function as proton wells as described by Mitchell [2,16]. Proton wells interconvert the ΔpH and ΔΨ components [1] along the well. An electric field that is negative at the bottom of the well relative to the aqueous phase will concentrate protons; a lower pH at the well bottom will generate a positive-out electric field. For example, if the well extends 50 per cent across the membrane dielectric barrier, then a voltage of −120 mV across the membrane will result in a potential of −60 mV, and the local pH at the bottom of the well will be one pH unit more acid than the top. This acidification is a consequence of the proton electrochemical potential being constant from top to bottom, with the proton well converting the Coulombic component of the PMF (Fψ) into the entropic one (−2.303 R.T.pH). Of course, if the structure were sufficiently extensive to be able to accommodate multiple cations and anions of the electrolyte, then it would not act as a proton well and there would be no potential or pH gradient along it.

A proton-conducting structure could extend across the entire membrane-embedded protein so that it connects the aqueous phases on either side of the membrane. Such a channel would catalyse passive proton flow down any electrochemical proton gradient across the membrane. Such channels therefore dissipate PMF and uncouple respiratory/photosynthetic electron flow from ATP synthesis. A well-studied example of this type of uncoupler protein is the gramicidin dimer [17]. In other more complex proton channel proteins, structural changes induced, for example by voltage or pH, can make/break the conducting network, allowing gated control of their uncoupling properties [13].

Because proton channels/wells are not themselves sources of free energy, an active, gated step is required for proton translocation against a transmembrane PMF [18–22]. The reaction must drive protons endergonically from the top of a supply proton well to the bottom of an exit proton well, by coupling to an exergonic reaction. The greater the PMF, the greater the energy required. The gating must prevent uncoupling by preventing passive energy-dissipating proton back-transfer driven by the PMF. Subsequent passage of protons along the proton wells, once the proton transfer driven by the coupled exergonic reaction is completed, will be a passive process unless some kind of conformational strain is retained within the protein that could provide an active energy source [23].

A particularly well-studied ‘protonmotive’ (i.e. PMF-generating) enzyme is light-driven bacteriorhodopsin, in which the pathway of gated proton transfer has been tracked in detail [18,24–26]. Photoisomerization of the photoactive Schiff base disrupts a buried hydrogen bonding network between three water molecules and two aspartates. This results in transfer of a proton from the Schiff base to one of these aspartates (D85), and disruption of H bonding between the structure and an adjacent arginine. This arginine rotates and in turn disrupts a second H-bonded, net protonated, water/glutamate network that causes it to release its proton. The cycle is completed with reprotonation of this network by D85, reprotonation of the Schiff base from the opposite aqueous phase via another water/aspartate proton channel, followed by relaxation of the protonated Schiff base to its ground state conformation. Hence, this bacteriorhodopsin mechanism is a clear example of a protonmotive system composed of two passive proton wells, with proton transfer between them via an active gate formed by the Schiff base. This basic well–gate–well configuration is likely to operate in CrOs (see below), complex I and other protonmotive enzymes.

3. Dielectric channels in proteins

If a proton well connects an aqueous phase to a buried protonatable group whose pK is influenced appropriately by the buried charge change (i.e. the charge is not compensated by other factors [27]) but does not have a further sink for the proton, the well can transfer a bulk proton to the protonatable group when the centre becomes negative [27], and transfer the proton back to the same bulk phase when the negative charge is lost. This classical Bohr (de)protonation will of course lower net buried charge change. However, a buried catalytic centre may also be connected to the protein surface by a hydrophilic channel that cannot conduct protons, or does not connect to a centre that can bind or consume protons. In this case, buried waters, local charges, dipoles and hydrogen bonds may instead respond dielectrically to any charge imbalance at the catalytic site. The more extensive these cooperative changes are, particularly if transmitted to an aqueous surface where surface charge changes can occur, then the greater is the extent of their compensation of the buried charge imbalance. This will alter the free energy ΔG of the transition at the buried site and, therefore, the equilibrium constant/midpoint potential. For multistep electron-transfer reactions, a change in midpoint potential can affect the net rate of reaction. A change in ΔG can also affect intrinsic electron-transfer reaction rate constants because it contributes to the activation free energy in the Marcus equation term...
be formed from arrays of hydrophilic, polarizable groups, most likely with associated bound water molecules. However, dielectric channels will be unable to support a continuous current of protons from an aqueous phase—either because they have little or no intrinsic protonic conductivity and/or because there is no sink to offload the protons and so allow the protonic current to continue (a ‘dead-end’ proton well with a single protonatable group could act in a purely dielectric function by alternately transferring a redox Bohr proton between the aqueous phase and the protonatable group, though with no ability to support a sustained protonic current). Instead, as discussed above, dielectric channels can affect equilibrium midpoint potentials, intrinsic electron-transfer rate constants and net rates of reactions by charge alterations that relax back when the transient intermediate state ceases. If their dielectric properties can be altered, such channels could provide a means to modulate catalytic activities by external factors. This could occur, for example, by binding of effectors close to the aqueous contact surfaces, by allosteric effects on channel components or even by mechanical strain as in the piezoelectric effect. These properties are explored further below.

Proton channels, in contrast, will connect bulk proton sources and sinks and have a high proton conductivity. Key components must be able to flip/rotate/move at sufficient rates that net delivery of protons to the protonatable group(s) is possible on the required timescale of multiple turnovers. In this case, because the process involves atomic movements, H/D exchange in D$_2$O medium is expected to slow down the conduction rate because of the heavier mass of the deuteron [30]. This contrasts with effects of H/D exchange on dielectric channels which, at least in the absence of secondary factors [31,32], should be small [33] because the static and high-frequency dielectric constants of pure liquid H$_2$O and D$_2$O are very similar [34]. However, H/D exchange will inevitably have many small but additive effects on complex protein structures so that interpretation in terms only of effects on proton transfer rates should be treated with caution.

It might also be expected that proton channel conductivity can be blocked by small changes in structure including, for example, replacement with a hydrophobic residue of a single amino acid that is part of the H-bonded network. By contrast, a single replacement in a dielectric channel may be counteracted by additional dielectric polarization of remaining groups and major alteration of function may require more extensive structural changes.

5. Application to cytochrome c oxidase

Mitochondrial cytochrome c oxidase (mtCcO) catalyses the reduction of molecular oxygen to water with electrons from cytochrome c and protons from the mitochondrial matrix. Each catalytic cycle is also coupled to the translocation of four protons across the membrane against the PMF [35]. Hence, conducting proton channels are required for both substrate and translocated protons [36]. Furthermore, because electron-transfer groups are buried, it is possible that some structures may have roles as dielectric channels/wells.

The mtCcOs are members of the A1 subclass of a diverse superfamily of homologous membrane-located enzymes that oxidize cytochrome c or quinol and reduce oxygen or nitric oxide [37]. Atomic structures have been solved of bovine mitochondrial CcO (BtCcO) [38] and the bacterial A1-type
CoOs of *Panacoccus denitrificans* (PdCoO) and *Rhodobacter sphaeroides* (RsCoO) [39,40]. All have a common core of three subunits. Electrons from cytochrome *c* are donated via the dinuclear Cu₃ in the hydrophilic domain of subunit II to haem *a* within subunit I (approx. 16 Å Cu to haem *a* macrocycle edge). Haem *a* then reduces haem *a₃* (approx. 7 Å haem macrocycles edge–edge) of the haem *a₃/Cu₃ dinuclear centre (BNC) where oxygen binds and is reduced. Subunit III has no cofactors but may house a channel through which substrate oxygen diffuses into the BNC.

BtCoO crystallizes as a homodimer with each composed of three core subunits together with 10 additional smaller polypeptides termed supernumerary subunits. Any functional significance of the dimeric state is unknown, and supercomplexes with monomeric CoO have been isolated [41]. Further additional loosely bound subunits may also be associated with the enzyme in some *in vivo* conditions [42]. Homologues of several supernumerary subunits are present in mtCoOs from diverse sources [43]; for example, yeast (*Saccharomyces cerevisiae*) CoO (ScCoO) has homologues of eight of them [44]. The supernumerary subunits do not have direct roles in the proton/electron-transfer reactions catalysed by core subunits I and II. Instead, they may function in assembly, stability, regulation and/or supercomplex formation [45]. Both PdCoO and RsCoO have a fourth, small transmembrane subunit. However, though homologous to each other, they are not related in sequence or in structural location to any of the BtCoO supernumerary subunits.

Oxygen reduction and coupled proton translocation occur in subunit I, with the exergonic oxygen reduction providing the driving force for active, gated proton translocation. Reduction of oxygen to water requires four electrons, together with four ‘substrate’ protons, that must be transferred into the BNC. The multistep catalytic cycle of oxygen reduction has been reviewed extensively elsewhere [7,46,47]. The nomenclature used here for key intermediates of the catalytic cycle is summarized in figure 2. It is widely thought (though see discussion below) that each of the four electron transfers from haem *a* into the BNC is coupled to an active, gated proton transfer between an input proton well connected to the negative phase and an exit proton well connected to the positive phase.

### 5.1. Hydrophilic channels

Subunit I is a tightly packed structure, and conduction of both substrate protons and translocated protons within the protein must be facilitated by proton channels. Three arrays of relatively hydrophilic amino acids and water molecules that could form such channels have been identified in crystallographic structures (figure 3).

#### 5.1.1. The K channel

One array leads from the negative aqueous phase to a tyrosine (Y244) that is covalently linked to a histidine (H240) ligand of Cu₃ of the BNC (figure 4). It is conserved between mitochondrial and bacterial A1 CoOs [38] and was termed the K channel after its central lysine residue (K319). When first identified in the atomic structure, it was suggested to provide the pathway for substrate protons from the N phase into the BNC [39].

In several respects, this is an unusual structure in relation to other, better-defined proton-conducting channels. It leads to Y244 of the BNC via S255, K319, T316 and the hydroxethyl farnesyl group OH of haem *a₃* [49,50]. The only crystallographically identifiable water molecules are (in all CoOs) one that bridges between S255 and K319 and one or several in the haem *a₃* hydroxethyl farnesyl/Y244 region. The connection of K319 to the N phase remains unclear. An entry route via H256/S255 was suggested from the early BtCoO structure [38]. Alternatively, electrostatic calculations [51] predicted that K319 interacts strongly with a glutamic acid (E62₉) that is part of a well-conserved cluster of residues at the N phase interface of subunits I and II. Inhibitory effects of mutagenesis of this residue in *RhCoO* [52] provided support for this proposal. However, mutation of the equivalent residue of *PdCoO*

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**Figure 2.** Nomenclature used for the catalytic intermediates of CoOs. The catalytic intermediates discussed in this text refer only to different states of the oxygen-reducing BNC of CoO and do not take account of the redox states of Cu₃ or haem *a*. Electrons are donated into the BNC from haem *a*. Starting with the catalytically active oxidized O state (ferric haem *a₃* cupric Cu₃), the first and second electron transfers are charge-compensated by substrate proton uptake and produce the one-electron-reduced E state and the two-electron-reduced R state (ferrous haem *a₃* cuprous Cu₃). These 0 → E → R steps are often referred to as the ‘reductive’ steps of the cycle. In the R state, ferrous haem *a₃* binds O₂ to form the unstable oxoferryl A state. If further electrons are not immediately available from haem *a*, A rapidly converts to the relatively stable P₃ state (ferryl haem *a₃* (Fe⁵⁺ = O²⁻), Cu₃⁺ and a neutral tyrosine radical). In the natural cycle, P₅ is reduced when a further electron is available from haem *a*, together with a charge-compensating substrate proton, to form F (ferryl haem *a₃* (Cu₃⁺), tyrosine ground state). If an electron is already available haem *a* when A is formed, A is instead transformed into the P₆ intermediate (ferryl haem *a₃* (Cu₃⁺), tyrosinate)—this is the case in flow-flash experiments described in the text in which fully reduced CoO (the FR state, with all four redox centres reduced) reacts with O₂. P₆ is subsequently converted to F by uptake of a substrate proton. F is converted back into the O state with a further electron from haem *a* together with a charge-compensating substrate proton. The P → F → O steps are often referred to as the ‘oxidative’ part of the reaction cycle. Each electron transfer into the BNC from haem *a* is generally (but see text) thought to be coupled to translocation of a proton across the membrane (not shown). See text for further details. Several different, albeit similar, nomenclatures are now used in the primary CoO literature for these and additional intermediates; those above are most commonly used. However, these oxygen intermediates can be equated with those classically discussed in globins, P450s and peroxidases [48] as follows: A is equivalent to the oxoferryl state of globins and the equivalent ferric-superoxo ‘compound III’ of P450s and peroxidases; P₃ is equivalent to the ferryl-radical ‘compound F’; F and P₅ are equivalent to the ferryl ‘compound II’ with or without a charge-compensating proton, respectively.
had no effect [53] suggesting instead that the entry route may be formed by a more extended region that does not obligatorily involve the E62II residue [49]. This notion has gained support from a recent study that further suggests that this region can bind a range of compounds that may modulate K channel behaviour [54].

Mutations of central K channel residues of bacterial CrOs can result in severely diminished overall catalytic rates, showing that the structure does indeed have a key function [55,56]. More specifically, they can severely inhibit haem a3 reduction from the oxidized state [8,56,57]. This might result from blockage of proton conduction into the BNC because stable reduction of the BNC intermediates (E, R, P, F; figure 2) requires proton uptake [58,59]. However, such mutants have little or no effect on the F → O catalytic step [60] or indeed on the P → F → O steps in the re-oxidation of the fully reduced enzyme with molecular oxygen [61], which is significant because these reactions require pathways for both substrate protons and pumped protons.

A further clear effect of K channel mutants is on the ‘reversed electron transfer’ from haem a3 to haem a that is initiated when CO is photolysed from the mixed valence form of CrOs [62–64]. This reaction has a proton-independent phase at pH < 7. At higher pH values, the extent of reversed electron transfer increases and this additional extent is associated with proton release into the external medium [65,66] that is electrogenic [67]. This high pH component, but not the lower pH one, is inhibited by K channel mutations [61,67].

From the above observations, it was suggested that the K pathway facilitates delivery into the BNC of one [8] or both [56,57] substrate protons that are required for reduction of the BNC metal centres [59] in the ‘reductive’ (O → E → R) part of the catalytic cycle, but is not involved in delivery into the BNC of the substrate protons that are associated with the ‘oxidative’ part of the reaction cycle (R → P → F → O). In addition, it does not appear to provide the route for any of the additional protons that are translocated across the membrane, even though these are associated with both the ‘reductive’ and ‘oxidative’ parts of the catalytic cycle.

This role in enabling protonation change within the BNC during the ‘reductive’ steps is clear. However, it has been suggested that it may achieve this not by conducting protons but instead by acting as a hydroxide (OH−) channel [68]. Arguments were based, in part, on the probability of OH− residing in the K channel being orders of magnitude higher than that of a proton when a PMF is present, and the positive potential surrounding the putative gate to the channel also favouring OH− transport. A channel for hydroxide ions is difficult to distinguish empirically from one for protons. It has been argued that a pH dependency of the high pH component of CO recombination to mixed valence CrO supports its being a proton channel [69]. However, this pH dependency is rather weaker than should be expected and so does not provide a definitive resolution. Besides its H+/OH− transport function, the K channel might also provide an additional function as a dielectric well [8] in some steps, modulating the energetic cost of formation of transient charged intermediates within the BNC. Such a role might only be important for some of the steps of electron transfer into the BNC, depending on which involve an unstable charged intermediate whose activation energy of formation causes its formation to be rate-limiting. It may be pertinent that the O → E → R steps, which require a functional K channel, have little driving force [46] and, if they are both linked to a net proton translocation, would be severely endergonic when a PMF is present. By contrast, the P → F and F → O electron-transfer steps are highly exergonic and do not require a functional K channel. Of further relevance to a possible dielectric channel role is the report that K channel mutants slow down the R → Pk step [70] (but see also [71]).
This latter reaction involves electron transfer from ferrous haem a into the BNC–oxygen complex. There is no proton exchange with the medium, but the reaction does have a small electrogenic component, despite the fact that the electron transfer itself should not be electrogenic. One suggestion is that this electrogenic component arises from a dielectric charge rearrangement within the K channel [70]. It has also been interpreted as arising from internal proton transfer from E242 to a proton trap site that forms part of the route for proton translocation ([72], see below). In this case too, it is possible that the K channel functions as a dielectric channel to aid what would be a proton coupled electron transfer $R \rightarrow P_R$, by reducing the energetic cost of any charge-imbalanced transient intermediate.

All of the above data on K channel mutation effects have come from studies with bCcOs. However, based on the close structural similarities of the K channel in bovine mitochondrial and bacterial CcOs, it seems very likely that its role will be the same in mtCcOs. To date, because of difficulties of introduction of mutations into mtCcOs, the only direct mutation evidence that it does play a functional role in mtCcOs comes from randomly generated K channel mutants of yeast CcO (T316K) that show impaired catalytic activities [73], though introduction of specific mutations into its core subunits is now feasible [74].

5.1.2. The D channel

A second hydrophilic array leads from the negative aqueous phase to a conserved glutamic acid (E242) that is buried in subunit I and roughly equidistant from the two haem edges [39] (figure 5). It was named the D channel after an aspartic acid (D91) at its N phase entrance and initially proposed to conduct translocated protons [39]. It is more extensive than the K channel, involving a network of 10 or more water molecules, H-bonded with asparagine, tyrosine and serine residues, and linking D91 to E242. E242 is part of a pentameric ring of amino acids (H240PEVY244) formed by the covalent bond between H240 and Y244 [38]. This pentamer may provide a structural link between oxygen chemistry events at the BNC and actions at E242 that are linked to gated proton transfer.

Again, evidence for its function comes primarily from studies of bacterial CcOs where mutations of several residues, including D91 and E242, lead to severe impairment of catalytic turnover [75]. Mutation of E242 to a non-protonatable residue also impairs reduction of the BNC when a reducing substrate is provided to the oxidized enzyme [76], the same effect as seen with K channel mutants. Mutations of E242 can also inhibit the $P \rightarrow F$ and $F \rightarrow O$ steps in the reaction of fully reduced CcO with oxygen [60,77] and the small, fast electrogenic phase associated with the $R \rightarrow P_R$ (more specifically the $A \rightarrow P_R$) transition [72]. In a D91N mutant, the electrogenic $R \rightarrow P_R$ reaction still occurred and E242 was observed to become deprotonated [78]. This electrogenic component has been suggested to arise from the transfer of the E242 proton to a trap site that is linked to the exit route for translocated protons, consistent with a role of the D channel and E242 in proton translocation. In a double D91N/Y19F mutant (figure 5), E242 again became deprotonated in the $R \rightarrow P_R$ step but, in addition, the next step of $P_R \rightarrow F$, which involves proton transfer into the BNC, was inhibited [71]. Hence, it appears that the D channel is also functions, at least at this $P_R \rightarrow F$ step, in providing a substrate proton into the BNC (which is provided by Y19 in the D91N mutant). In summary, these observations provide clear support for the D channel being a route both for translocated protons and, at least at the step of $F$ formation, for substrate protons. As noted above, mutation of the lysine of the K channel also affects the $A \rightarrow P_R$ step [70], possibly as a result of its dielectric channel function.

Of particular significance has been the finding that several mutations within the D channel can result in electron transfer and oxygen chemistry at normal rates but with no coupled proton translocation [79–81]. This behaviour is most easily understood if the D channel conducts protons that are destined both for the exit route for proton translocation and for the BNC for water formation. Indeed, further direct evidence for electrogenic transport of substrate protons into the BNC via the D channel has come from studies of a non-pumping mutant of RhCcO [82]. Such mutations then presumably disrupt the strict active gating sequence that controls correct bifurcation between translocation and BNC routes, perhaps by alteration of pK values and relative reaction rates [50].

5.1.3. Possible exit pathways for pumped protons

Although the evidence for transfer of translocated protons through the D channel and E242 is strong, groups that could conduct protons from E242 to pathways that connect to the aqueous $F$ surface or into the BNC are not evident in the atomic structures. Most probably, these connections are made via water molecules that can transiently reorganize to provide alternating protonic connections for translocated protons to the $\delta$-propionate region of haem a and $a_3$ or, for substrate protons, to the BNC [83,84]. These propionates are ion paired with arginines R439/R438 and the R438/haem $a_3$ $\delta$-propionate may itself form a component of the proton exit pathway [85]. A large extended water cluster towards
the P phase is linked to these propionates, from which several routes to the P surface are possible [49,86–88]. Protonic connections are feasible to the H291 ligand of CuB, to the Mg$^{2+}$/Mn$^{2+}$ binding site between the haems and the P phase and to the hydrophilic region around the Ca$^{2+}$-binding site/DS51 region that is part of the proposed exit route of the H channel discussed below [89].

5.2. A model of coupling in bacterial A1-type cytochrome c oxidases

Structural and biophysical studies on bCcOs have indicated that the K channel provides one or both substrate protons required in the BNC in the O → E → R reductive steps. The D channel delivers the final three or two substrate protons to the BNC, and conducts all four translocated protons from the N aqueous phase to E242 where an active gating mechanism moves them to a proton trap site linked to the exit proton channel(s) to the P phase. A model of coupling has emerged [7,21,36,46,90] by combining these deductions with:

— the empirically demonstrated need for minimization of net charge changes within the buried BNC by charge-counterbalancing protonation changes [58,59];
— the likelihood that one proton translocation is associated with each of the four electron transfers from haem a into the BNC in the O → E, E → R, P → F and F → O steps [91,92]; and
— the assumption that all four of these coupled proton transfers occur by the same mechanism involving proton channels connected by an active, gated step as outlined above [93].

The sequence begins with electron transfer (from cytochrome c via CuA) to haem a within subunit I. At this stage, the D channel glutamic acid is protonated, as is known from FTIR data [94]. Electron transfer from haem a into the BNC is associated with gated electrogenic movement of a proton from E242 to the ‘proton trap’ site at the start of the exit route. The positive charge change on the ‘trap’ site electrostatically stabilizes the negative charge introduced into the BNC. However, it is positioned such that the proton cannot act as a substrate proton for subsequent water formation and the gating mechanism prevents its back transfer. The proton and electron transfers must presumably be temporally separated and have a strict sequence and it has been argued recently that protonation must precede electron transfer [21]. In either case, the intermediate will be charge-imbalanced and so will be an unstable transient species. The anionic E242 becomes reprotonated from the N phase via the D channel because its electrostatic environment has returned to that of the starting state. The sequence continues with protonation of the reduced BNC. From evidence summarized above, this proton is most likely provided by the D channel/E242 in the P → F and F → O steps (followed again by reprotonation of the anionic E242) and, most probably (see above), by the K channel in the O → E and E → R step(s). Arrival of the substrate proton into the BNC completes the transformation to the next stable oxygen intermediate. However, it also neutralizes the BNC negative charge and so breaks the electrostatic stabilization with the ‘trap’ proton, which then moves through the exit channel into the aqueous P phase.

Studies of bacterial CcOs have provided substantial experimental support for this coupling mechanism. However, though viable in its physical basis and relative simplicity, significant issues remain unresolved even for bCcOs. The identity of the ‘trap’ site remains uncertain, though an obvious location would be in the network of propionates, hydrophilic, protonatable amino acids and waters immediately ‘above’ the BNC that are linked to the exit routes. Of greater concern is that there is no crystallographically detectable protonic connectivity between E242 and this region and, hence, no definitive gating mechanism that delivers protons alternately to the ‘trap’ site, and the BNC and prevents any backflow that would otherwise cause uncoupling. In principle, correct routing and gating can be achieved through pathways and protonation sites that only function transiently (through chemically and electrostatically controlled pK changes) as the reaction sequence progresses in a temporally organized manner. A promising suggested possibility is that internal water molecules, coordinated to structural changes of the E242, form transiently ordered ‘proton wire’ chains that provide gated conduction pathways alternately to the ‘trap’ or to the BNC sites [46,84,95–99].

This mechanism nevertheless remains unproven. For example, in a comprehensive review of individual catalytic steps, Siletzky and Konstantinov have pointed out the dearth of solid support for protonic coupling at the rather difficult to access E → R step and have raised the possibility that the coupling mechanism may be different at different steps [100,101]. Others favour quite different proposals. For example, one alternative model, based again on kinetic data, is of a conformationally retained strain that drives proton translocation after the oxygen chemistry has occurred [23]. This would require a reordering of the translocated and substrate proton delivery steps of the model outlined above, though the same basis elements are involved. Others favour the key step for translocated proton movements as being haem a oxidation/reduction [102,103], rather than the interhaem electron transfer. Particularly relevant to the present discussion has been the suggestion that vertebrate forms of CcO may translocate protons by a quite different mechanism, involving a third hydrophilic H channel, as described below.

5.3. The H channel

5.3.1. The H channel and coupling mechanism of mitochondrial A1-type cytochrome c oxidases

An additional extensive array of waters and hydrophilic residues that extends across subunit I in the BccOs structure has been termed the H channel [89] (figure 6). It is spatially separate from the BNC and the D and K pathways, with residues provided largely by helices XI and XII. It begins close to the N phase in the vicinity of residue H413. A water cavity/channel links with four serines, a threonine and the haem a hydroxymethyl farnesyl OH group. It continues via R38 that is linked to the haem a formyl and Y371 and Y54 that are close to the haem a α-propionate; these, in turn, are linked by several further waters to the peptide bond between Y440 and S441. It was proposed that this peptide bond acts as the proton transfer gate [104], passing the protons unidirectionally via S205 to DS51 and hence to the P phase. Structural data also indicate that the water-containing cavity/channel ‘below’ haem a can adopt open and closed conformations in different redox and ligand states [105].
Figure 6. The H channel in BtCcO. Component amino acids and associated water molecules (in red) are shown in relation to subunit I structure (grey; PDB code 1V54).

The identification of the H channel in the atomic structure of BtCcO [106] has led to a quite different proposed coupling mechanism in which the H channel acts as the translocated proton conduction pathway, together with a gating mechanism involving the amide bond between Y440 and S441 [104]. Such an extensive structure within a membrane protein is itself remarkable and evidence for a functional role of some type is substantial. Structures of BtCcO in different redox and ligation states show major structural alterations in S382 and the water cavity region of the H pathway, involving a conformational change of helix X that is positioned between the haems and helices XI and XII [105]. There is also a large movement of residue D51 that may provide part of the exit route of protons into the P phase [107]. In addition, in a hybrid bovine/human CcO system, mutations of D51N [89], S441P (to block the proposed gate) or a double mutant V386L/M390W (to block the water channel) [106] resulted in turnover at normal rates that was uncoupled from proton translocation [108]. These features are also absent from bacterial CcOs and, though hydrophilic residues are also found in the region corresponding to the BtCcO H channel, the network is incomplete and more weakly defined. Furthermore, in contrast to the uncoupling effects of H channel mutations in the hybrid bovine/human CcO system, point mutations in the H channel region of bCcOs were without effect on proton or electron transfer [109,110]. In addition, structural and associated water changes between oxidized and reduced states of RsCcO occur primarily in the K and D channels [49,111] though a similar but weaker structural alteration of the equivalent of BtCcO S382 on helix X of RhCcO has been reported recently [112].

Hence, mammalian forms of CcO may, as suggested, have developed a quite different and unique structure for coupled proton translocation [105]. However, it is also quite possible that the H channel is functionally required in all forms of A1-type CcOs, acting not as a channel for coupled proton translocation, but instead providing a dielectric channel, or a pair of dielectric wells. This can enable energetically facile transient electron transfer through the buried haem α cofactor and, possibly, provide a means of modulation of electron transfer through haem a. The observation that single mutations of H channel residues in bCcOs did not have significant effects may be because the remaining residues could still provide an adequate dielectric response; multiple mutations may be required before an observable effect is elicited. It has been shown that the ‘dielectric’ depth of haem a, as measured electrometrically, corresponds roughly to the physical depth indicated in the crystallographic structures [72]. This would suggest that any dielectric well effects of the H channel are roughly equal towards both membrane surfaces.

The structure would move some of the net charge change associated with haem a redox change towards one or both aqueous surfaces. This predicts that properties of haem a could be influenced by surface effects and vice versa. Some evidence for such action at a distance can already be found in the published literature. For example, it is well documented that the binding of H⁺, Ca²⁺ or Na⁺ ions at a specific site close to the P phase exit of the H channel [113] induces a shift in the electronic Soret and α-bands of haem a [114–117] and also causes shifts in the haem a vibrational infrared spectrum [118].Vygodina et al. [119] showed that Ca²⁺ binding alters bovine haem a redox potential and slows internal electron transfer to haem b₃. Haem a is known to have a relatively weak pH dependency of its midpoint potential [103,120]. It was shown that several weakly coupled protonatable groups are responsible and that these are affected by the pH of both the P and N phases [121]. Kirchberg et al. [122] also showed that reduction of Cuₐ results in proton uptake from both sides of the membrane which relaxes back partially when the electron is transferred to haem a. The only protonatable groups within the immediate vicinity of haem a are the haem ring propionates. Although redox-linked protonation of the haem propionates of CcO has been suggested in, for example [123], electrostatic...
calculations indicate that this is unlikely in any functional redox state [124]. Instead, these protonation effects could be explained by protonatable groups at both surfaces whose properties are influenced by dielectric polarization effects transmitted over a distance to the surfaces.

A similar dielectric function has already been proposed for the K channel [8], that may be additional to any H⁺/OH⁻ conduction role, in relation to energy minimization of transient charged intermediates within the BNC and which might, at least in part, explain the inhibitory effects of K channel mutations on the O → E → R and the A → P₆ steps as discussed above. In this case, perhaps a dual role in proton conduction in one or two specific steps, together with a more general dielectric polarization role, is even feasible.

5.3.3. Allosteric modulation of H channel dielectric by supernumerary subunits of mitochondrial cytochrome c oxidases

The roles of the additional supernumerary subunits are a further unresolved issue with mitochondrial forms of CcO. Bovine and human CcOs have 10 such subunits, at least five of which have tissue- or development-specific isoforms. Remarkably, homologues of many of these subunits are found in mtCcOs of evolutionarily distant species. However, there are no known homologues in bCcOs and several must have evolved soon after mitochondrial incorporation into the early eukaryotic cell [43].

Metabolic control analysis of CcO in mammalian cells shows that it has a high control coefficient in vivo [125] (in contrast to complexes I and III that operate close to equilibrium). This means that modulation of its kinetic parameters can potentially control overall respiratory and ATP synthesis rates. Demands on CcO activity can vary considerably in different environments or tissue types. It has been pointed out [43] that vertebrates and most other eukaryotes do not have multiple types of oxidases to deal with different cellular demands, in contrast to bacteria where a range of different types of oxidases are expressed to deal with different conditions. There is increasing evidence that mitochondrial forms of CcO can be controlled to optimize performance in different environments/tissues other than by tight regulation of levels of expression and assembly. Some of the additional subunits have been suggested to house ligand binding and phosphorylation sites [126] that allosterically control core functions. There is also substantial literature on the roles of specific isoforms in optimizing CcO core catalytic function in different tissues or physiological states [43,127]. Additional roles of supernumerary subunits in assembly, stability or supercomplex formation have been proposed.

To date, there is no clear understanding of how supernumerary subunits could exert control effects on the core subunit catalytic functions. One possibility that arises from the above considerations is that the dielectric properties of the H channel might be allosterically modulated by different isoforms of nearby supernumerary subunits, or by effector binding to these subunits. Modulation of its dielectric properties can affect the haem α redox potential and/or its intrinsic electron-transfer rate constants, thereby altering the catalytic parameters of the enzyme.

There is already evidence for core activity modulation by supernumerary subunits. For example, human/bovine subunit IV has tissue-specific isoforms [128,129] and has been proposed to house allosteric regulatory binding sites for adenine nucleotides [130]. The equivalent subunit in ScCcO is subunit Cox5, which also has two isoforms that are expressed at different oxygen tensions and have been shown to alter core catalytic activity [131–133]. Helices XI and XII of subunit I provide the majority of hydrophilic residues of the H channel. In BtCcO, their membrane-facing surfaces bind supernumerary subunit IV (figure 7). Hence, it is possible that changes in subunit IV (Cox5 in ScCcO) could allosterically alter the properties of the H channel and hence provide the mechanism for core activity modulation. Other supernumerary subunits (in particular human/bovine subunits Vb and VIIc, and the equivalent yeast subunits 4 and 8) are also close enough to influence H channel properties. Modulation of H channel properties might also be brought about by components of the PMF, by changes at the Ca²⁺/Na⁺ binding site (located at the ‘top’ of the H channel) or even possibly (because the subunit I external face of the H channel is partly exposed to the membrane) by interaction with other protein complexes or lipids. The recent structural model of a I₁II₂IV₁ ’supercomplex’ indicates that the H channel external face interacts strongly with complex I [41] and this provides another possible means of allosteric modulation of catalytic activity.

6. Wider distribution and evolution of dielectric and proton channels

The notion of conducting versus dielectric channels has been discussed here in relation to the A1-type CcOs. Clearly,
passive proton channels are required where buried reaction sites require chemical protonation changes. However, dielectric channels may also be rather widely distributed in proteins with buried catalytic sites that have charged transient intermediates. One example is a network of buried hydrophilic residues and waters in cytochrome $f$ that has been suggested to act as a proton channel [134,135] and that may instead act as a dielectric channel. This structure is formed with water molecules bonded to hydrophilic, but non-protonatable, groups. They led to the His2 ligand of the cytochrome $f$ haem, which is expected to remain in its neutral imidazole form in both redox forms of the haem. Hence, there is no final sink for a transferred proton and, in any case, cytochrome $f$ has a pH-independent midpoint potential in the physiological pH range and is not known to have any other proton-associated function. Hence, this structure is a candidate for a dielectric channel/well that simply tunes the redox properties of the haem group.

It might be noted that dielectric channels can be formed with relatively unstructured and diverse arrays of amino acids. It is therefore feasible that dielectric channels could provide a stage in the evolutionary pathway for development of more structurally demanding proton conduction pathways and, from there, to the complex actively protonmotrive systems as found in the CrOs. This might provide a clue as to why the D channel is not found universally in more distant subclasses of the superfamily with some apparently developing a gated protonmotive structure based instead on the K channel [81,136]. For example, the earliest forms of CoO would have required substrate protons for water formation. Restriction of routes of transfer of these substrate protons to just one route from the negative phase, possibly in the K channel region, would automatically confer electrogenic properties and partial conservation of energy across the membrane. In some oxidases, this could have further evolved a gated protonmotive function. In other cases, additional dielectric structures could have evolved and developed into the D channel which eventually replaced part of the substrate proton function of the K channel and then evolved its gated proton translocating ability.

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Glossary

BNC binuclear centre
CcO cytochrome c oxidase
BtCcO Bos taurus (bovine mitochondrial) CcO
ScCcO Saccharomyces cerevisiae (yeast mitochondrial) CcO
mtCcO mitochondrial forms of CcO
bCcO bacterial forms of CcO
PdCcO Paracoccus denitrificans CcO
RsCcO Rhodobacter sphaeroides CcO

PMF Protonmotive force

Amino acid sequence numbers are cited relative to the subunit sequences of bovine CcO, even when discussing other forms of CcO. Electronic supplementary material, table S1 lists the corresponding residues in CcOs of other organisms. Electronic supplementary material, figure S1 defines the nomenclature used for the catalytic intermediates.