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Mechanism of proton translocation by the respiratory oxidases. The histidine cycle

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1. Introduction

Most terminal respiratory oxidases conserve energy by proton translocation. The mechanism of proton pumping has been addressed by several groups over a number of years but progress has been limited by insufficient structural and functional information. In the recent years significant advances have been made on the structural frontier by studies of site-directed mutations and by the accumulation of primary structure data from a large number of variants within the enzyme family. This has been accompanied by considerable progress in kinetic studies of the catalytic reaction. In this brief account we will make use of recent experimental information to suggest a concrete model of proton translocation – the Histidine (or H) Cycle. The imidazole side-chain of histidine is capable of transferring *two* H^+ ions, a key feature of the proposed mechanism.

2. Proton translocation by the respiratory oxidases

Many of the respiratory oxidases belong to a family of structurally and functionally related enzymes [1,2]. They are characterised by a binuclear haem$_3$-copper$_B$ oxygen reduction centre [1], and a low-spin haem, which is the electron donor to this centre – all located in the largest subunit (subunit I). Of these enzymes, the quinol oxidases utilise ubiquinol or menaquinol as electron donor. The cytochrome *c* oxidases use ferrocytochrome *c* and contain an additional redox centre, Cu_A in subunit II, which is presumed to function as the electron acceptor from cytochrome *c*. The cytochrome *c* oxidases of the aa_3 type conserve energy by two means: they are redox-driven proton translocators [3,4], and they consume electrons and protons from opposite sides of the membrane in the reduction of dioxygen to water [5]. The quinol oxidases are also reported to be proton translocators [6,7]. Their activity is associated with release of 2 H^+ to the outside of the membrane per transferred electron, while this number is 1 for the cytochrome *c* oxidases. The difference is due to the protons released on oxidation of quinol to quinone, but in both cases the effective charge (q) translocation is expected to be 2 q/e^-. Fig. 1 shows that this charge stoichiometry, well established for cytochrome *c* oxidase (see Ref. [4]), is indeed also true for the quinol oxidase cytochrome bo_3 of *E. coli*.

The quinol-oxidising cytochrome *bd* of *E. coli* is structurally unrelated to the haem-copper family [8]; it lacks copper and does not translocate protons [7].

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[1] Terminology: haem structures (as isolated) are abbreviated by the respective capital letter (A,B,C,D,O); haems in situ by the corresponding lower case letter. The oxygen-reactive haem in the binuclear site is given the subscript '3' irrespective of haem structure in honour of Dr. David Keilin who discovered and named the oxygen-reactive cytochrome a_3. Copper of the binuclear site has the subscript 'B' in honour of Drs. Helmut Beinert and Bob van Gelder, who discovered its functionality and closeness to haem iron.

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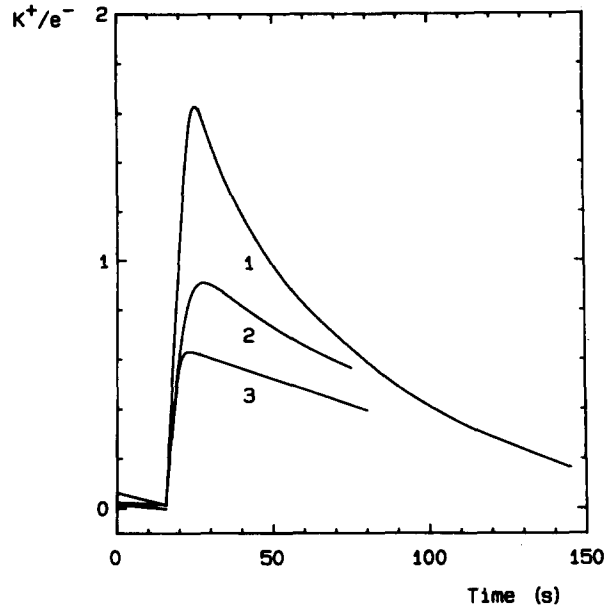


Fig. 1. Charge translocation by cytochromes bo_3 and *bd* in *E. coli* cells. Potassium uptake is determined potentiometrically in oxygen pulse experiments in the presence of succinate and valinomycin. Experiments are shown with the GO103 mutant of *E. coli*, which has cytochrome bo_3 but lacks cytochrome *bd* (trace 1), with the D135N mutant of bo_3 (Ref. [19]; trace 2), and with the GO104 mutant, which contains *bd* but lacks bo_3 (trace 3).

Consistent with this, the q/e^- ratio approaches unity (Fig. 1). Thus cytochrome *bd* shares charge separation of the oxygen chemistry with the members of the haem-copper family, but the molecular machinery of proton pumping is missing.

3. The cytochrome cbb_3-type oxidase

Recently, a quite distinct group of cytochrome *c* oxidases has been characterised. The cytochromes cbb_3, found at least in *Rhodobacter* [9,10] and in *B. japonicum* [11,12], have a binuclear haem-copper centre [13] and a low spin haem, but lack the Cu_A centre. Their subunit I (FixN gene of *B. japonicum*, Ref. [11]) is structurally related to the rest of the family (e.g., all haem- and copper-binding histidines are conserved, cf. Ref. [14]), but the degree of homology is much lower. It is important to assess whether this type of enzyme translocates protons, because this may allow us to determine whether certain structures are required for this process.

In our work with *Paracoccus*, deletion of the genes encoding subunit I of cytochrome aa_3 revealed an alternative cytochrome *c* oxidase (Ref. [16], see also Ref. [15]). Although they lack cytochrome aa_3, the mutant bacteria still exhibited partially myxothiazol-sensitive respiration on succinate (myxothiazol-insensitive respiration is due to the quinol oxidase). More important, the H^+/e^- ratio was close to 3 during succinate respiration, just as in wild type. The ratio was depressed to 2.0 when the bc_1 complex was blocked by myxothiazol, as expected, because then the quinol oxidase becomes responsible for the activity [6]. With ferricyanide replacing O_2 as oxidant, the ratio was also lowered to 2, showing the well-known proton translocation by the bc_1 complex. Hence, the alternative cytochrome *c* oxidase in this mutant is clearly able to function as a proton pump [16].

The *Paracoccus* mutant lacks haems A and O, and partially purified enzyme preparations contain only haems B and C, the CO-reactive unit clearly being a B-type haem [16]. Due to this, and the close relatedness between *Paracoccus* and *Rhodobacter*, it is very likely that this alternative oxidase is of the cbb_3 type (cf. above), which provides the first evidence for proton-pumping activity by this ancient class of respiratory enzymes.

4. Structures in subunit I are involved in proton translocation

Since the mechanism of proton translocation is likely to be the same in all members of the haem-copper enzyme family we may now conclude that the structures responsible for this function must reside in subunit I. The structures of subunits II and III are not conserved in the cbb_3 enzyme [9–11]. This is consistent with the notion that proton translocation is intimately linked to the oxygen chemistry at the binuclear centre [17,18].

Mutation of five highly conserved acidic residues in subunit I of cytochrome bo_3 of *E. coli* to the corresponding amide revealed that one of them (D135) is essential for proton translocation [19]. Fig. 1 shows that charge translocation is depressed to 1 q/e^- in the D135N mutant, confirming the loss of proton translocation. D135 is predicted to reside in a 'loop' between transmembranous helices II and III [20], which may contribute to proton input into the translocation machinery [19]. Although this 'loop' domain is not strictly conserved in the FixN subunit of the cbb_3-type oxidase [11,14], there is some similarity: a D is present with a P at $n + 3$ and an N at $n + 11$. A unique sequence of this segment is apparently not necessary for function: mutations in some of the well-conserved residues, or relocation of the aspartate to certain other positions within the 'loop', can be done with retention of proton translocation (Puustinen, A. and Garcia-Horsman, A., unpublished data). Hence, the variant primary structure of the FixN 'loop' may still be consistent with a role in proton translocation.

The observed proton translocation by the cbb_3-type oxidase leads to an important conclusion:

The pump mechanism does not depend on a large number of specific amino acid side-chain functions.

Among structures that may be involved are, therefore, the six invariant histidines [14,20], bound water molecules, and the axial network of hydrogen bonds in transmembranous α-helices [21]. In addition, serine/threonine hydroxyls are relatively well conserved in cbb_3 [11,14], and may, together with bound water and the acidic residue in the helix II-III loop, contribute to the establishment of proton conduction pathways.

5. A directly coupled proton pump mechanism: outline of the H cycle

In 1978–1979, Wikström and Krab proposed that proton translocation could be brought about by a 'directly coupled proton pump' mechanism involving redox-linked ligand exchanges at a metal centre [4,22]. *A key feature of such a model is the controlled switching of the proton-binding groups between input and output channels coupled to the redox chemistry of the centre.* The most complete description of such a mechanism, to date, is a model proposed in 1986 by Chan and co-workers [23,24]. Although this model assumed coupling at the Cu_A centre, and a uniform 1 H^+/e^- stoichiometry, neither of which is now believed to be the case, the model broke important ground because it applied physical chemisty and electron-transfer theory to a concrete chemical model.

Several features distinguish this type of mechanism from a classical Mitchellian redox loop [25]: instead of conformationally fixed input and output centres, with controlled flux of electrons and hydrogens between them, the proton input and output take place at the same locus, but in different conformations of the system. A 'directly coupled proton pump' mechanism is also distinct from the 'redox Bohr' type mechanism [26], in which oxidoreduction is conformationally linked to uptake and release of protons at a site in the protein distant from the redox centre. A 'redox Bohr' mechanism would likely call for extensive homology in the protein, whereas what has been found in the oxidases is small local areas of high conservation mostly centred on the metal sites themselves.

Present data show that proton pumping is coordinated to the oxygen chemistry of the enzyme [17]. The strongest indication of this is that the steps in proton translocation in which work is done are coupled to only two of the four one-electron steps in the catalytic

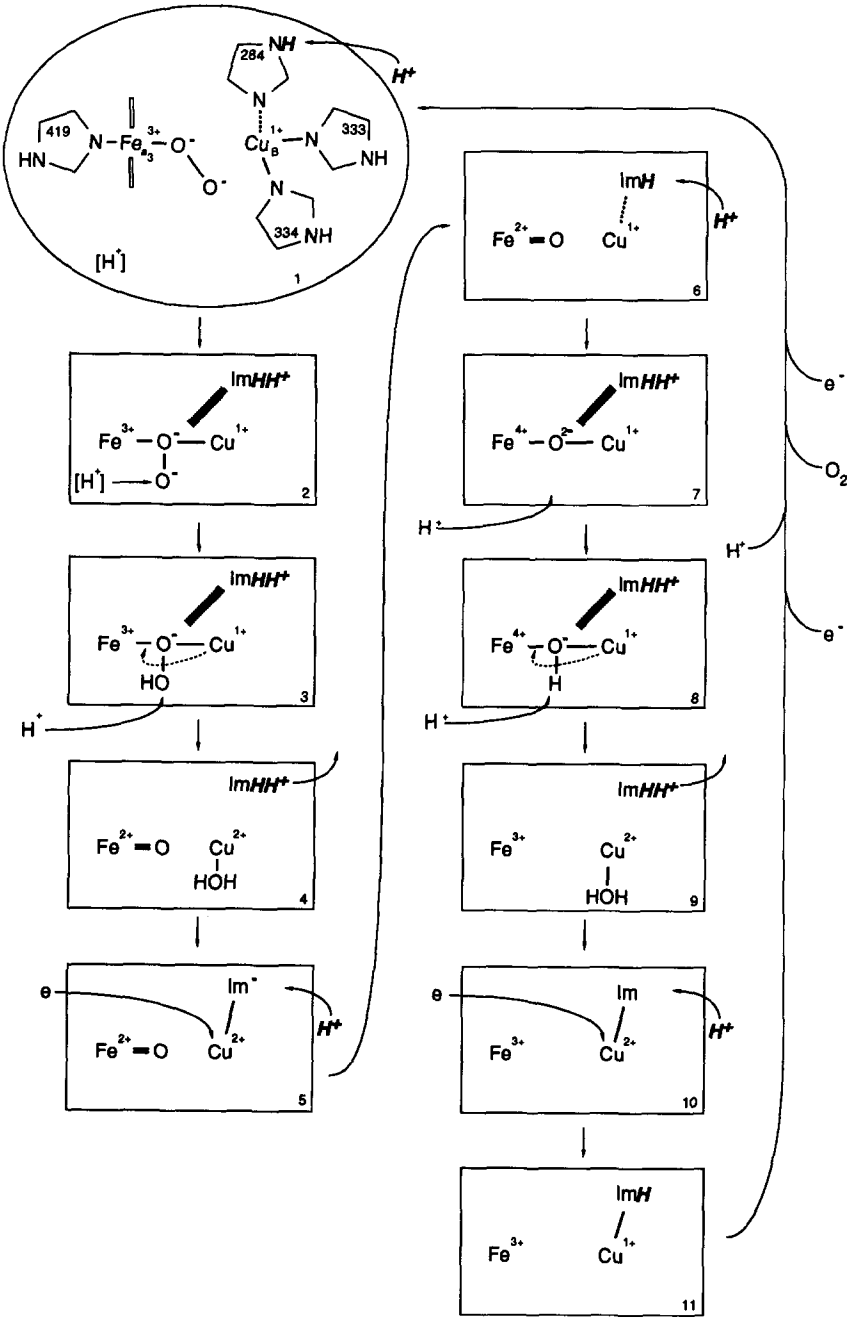
Fig. 2. The H Cycle. Boxes numbered in the lower right corner describe reaction states. State 1 is depicted in more detail structurally; only the changes to it are shown in states 2 through 11. Translocated protons (both input and output) are indicated by boldface on the upper right hand side of the boxes. The scalar uptake of H^+ is indicated by arrows which approach from the left. The crossbar between the oxygenous ligand and the imidazole residue of H284 (states 2, 3, 7 and 8) indicates an electrostatic interaction. The reaction events may briefly be described as follows:
1: This state is formed subsequent to O_2 binding to Fe_{a3}, and the transfer of one (3rd) electron from Fe_a. This is the primary peroxy intermediate, detected spectrophotometrically subsequent to the oxy intermediate [28]. A tentative structure is indicated, while abbreviated in the other states. Note that the reduction of Cu_B and the introduction of negative charge into the Fe_{a3} ligand have the effect of weakening the bond between Cu_B and H284.
2: Protonation of H284 occurs from the input proton pump channel, the Cu_B-H284 bond breaks, and the formed ImH_2^+ is stabilised electrostatically by the negatively charged oxygenous ligand. The state may be further stabilised by bridge formation between the metals.
3: A proton previously taken up on reduction of the binuclear site (far right) protonates bound peroxide which is required for electron transfer to occur from Cu_B.
4: A second (scalar) proton is taken up, which completes scission of the O-O bond and formation of the oxoferryl intermediate. The latter is drawn here as $Fe^{2+}=O$ to emphasize the virtual lack of charge on the oxygen in this compound [30]. As the negative charge on oxygen is annihilated by scalar proton uptake, the electrostatic interaction which stabilised the ImH_2^+ state is lost.
5: The pK_a of ImH_2^+ is lowered due to the loss of electrostatic interaction and formation of Cu^{2+}; The ImH_2^+ is deprotonated, and stabilised by liganding to Cu^{2+} as the Im^- anion, which may be further stablilised by hydrogen bonding to the invariant W-280 in helix VI.
6: Electron transfer (4th electron) from Fe_a reduces Cu_B with uptake of one proton from the input pump channel into H284; the Cu-H284 bond is weakened due to protonation and the geometrical change upon Cu reduction. The electron ends up at copper for thermodynamic reasons: reduction of $Fe^{4+}=O$ is favourable only if protonation is allowed (from the scalar proton channel).
7: Weakening and breaking of the H284-Cu bond causes Cu to approach the oxygenous ligand with possible bridge formation (cf. 1 → 2). This leads to delocalisation of negative charge into the ligand, which stabilises the doubly protonated ImH_2^+ state electrostatically (cf. 2 and 3).
8: Delayed uptake of a scalar proton into 7 is a prerequisite for electron transfer from Cu to Fe_{a3}; the proton greatly increases the E_m of the oxoferryl species.
9: Uptake of another scalar proton to 8 abolishes the negative charge and the electrostatic interaction with H284. Together with the Cu^{2+}, this decreases the pK_a and 2 H^+ are released.
10: The resulting Im^- state of H284 is stabilised by liganding to Cu^{2+}.
11: Electron transfer (1st electron) into the binuclear site occurs to Cu_B (for thermodynamic reasons), and is accompanied by H^+ uptake into H284.
11-> 1: Uptake of the 2nd electron to Fe_{a3}, and H^+ uptake, leads to binding of O_2, first to Cu_B^+ and then to Fe_{a3} (not shown), followed by transfer of the 3rd electron from the low spin haem to yield 1 (see Ref. [28]).

cycle [2]. Importantly, these are the two steps in which reduction of bound oxygen takes place: scission of the O-O bond and reduction of the oxyferryl intermediate [27]. Each of these steps is coupled to the translocation of *two* protons across the osmotic barrier.

Although this suggests that the pumping mechanism is intimately related to the chemistry of the oxygen intermediates, it is difficult to see how the oxygen intermediates themselves could be responsible for carrying the pumped protons, because, in addition to the four protons which are translocated when a molecule of oxygen is reduced to water, this process consumes an equal number of protons chemically. If protons inside of the pump mechanism were available to the oxygen intermediates, they would presumably be consumed preferentially, short-circuiting the pumping process.

Thus, a pump mechanism must fulfil several requirements: (1) energy from the reduction of oxygen must effectively be used to manipulate the energy level

[2] This is not inconsistent with the finding that reduction of the enzyme is associated with uptake of protons [29] as this could occur into an input channel with little work expenditure.



of protons; (2) the access of these protons to the input and output sides of the membrane must be controlled; (3) the entry of subsequent electrons must also be gated, so that the redox reaction does not move ahead of the proton pumping process and thus become uncoupled; (4) although the energy of the oxygen reaction must be brought to bear on the pumped protons, these protons must be effectively insulated from the oxygen intermediates, so that they are not consumed by this process, again uncoupling the pump; (5) the pump must be capable of carrying two protons for each of the electrons in the final stage of the reduction of oxygen.

As a solution to these problems, we propose a model in which redox ligand switching at Cu_B combines with electrostatic effects of the oxygen chemistry to achieve *both* thermodynamic and kinetic control of the proton pump. Cu_B is well placed to have an important role in the proton pump but the Cu_B metal site, in itself, does not appear to change redox potential enough to account for pure thermodynamic coupling. However, proximity of the Fe_{a3} and Cu_B in the oxygen reduction centre suggest the possibility that these two sites could cooperate in the pumping of protons.

Mutagenesis experiments have shown that H333, H334 and H284 are potential Cu_B ligands (Ref. [20], Fig. 2, state 1). Y288 appears to reside near Cu_B as well, and may play a role, although not an essential one because it is replaced by a glycine in the FixN sequence [11]. The imidazole group of any one of the histidines could function as a $2H^+$ carrier alternating between imidazolium (ImH_2^+) and imidazolate (Im^-) states. In our model H284 plays this role, binding preferentially to the oxidised form of Cu_B thus linking the redox events to proton translocation.

We suggest further that the ImH_2^+ state of H284 forms only when stabilised electrostatically by a negative charge at the oxygenous ligand of Fe_{a3}. In order to avoid short-circuiting between vectorial and scalar protons, H284 must not be in protonic contact with the oxygen ligand. Instead, protons arrive here by a separate pathway involving D135 in the helix II-III 'loop'. Protonation of the oxygenous ligand occurs more slowly, and by a separate path (OH groups in helix VIII [14,20]), and leads to kinetically delayed abolition of the negative charge. Together with the oxidation of Cu_B this destabilises the ImH_2^+ state of H284, and Im^- binds to copper with the release of two protons. The binding of protons to the oxygenous ligand results in the destabilisation of the protons on ImH_2^+; thus the energy of binding the chemical protons is used to eject the pumped protons.

Fig. 2 presents a schematic view of the proposed model. It is based on several key postulates, many of which can be tested experimentally:

(1) The mechanism of proton translocation is identical in the two proton-pumping steps, except that the precise oxygen chemistry differs. This is emphasised in Fig. 2 by placing equivalent states as pairs of boxes side by side.

(2) One of the invariant histidine imidazoles on the distal side of Fe_{a3} (H333, H334, H284; here we favour H284) is an intermittent Cu_B ligand, and serves as the vehicle of proton translocation by cycling between Im^-, ImH (Cu_B-bound) and ImH_2^+ states.

(3) Cu_B is oxidised only twice during the proton-translocating catalytic cycle, each time concurrent with a H^+ release phase of proton translocation. This presupposes that the two electrons which reduce oxygen to peroxide come from the enzyme's two haems and that the electron initially in Cu_B is the third electron in the cycle [28]. This would require that the formation of a ferric/cupric peroxy state ('Compound C'), for example in the reaction of two-electron reduced enzyme with O_2 be slower than re-reduction of the low spin haem, and thus not normally part of the catalytic cycle (contrast ref. 18). Its generation from the mixed valence enzyme is not expected to be linked to proton translocation.

(4) A negative electrical charge on the oxygenous Fe_{a3} ligand (L) is essential in that only when it is generated is the ImH_2^+ state sufficiently stable due to an electrostatic interaction (crossbar, Fig. 2) between the oxygenous ligand and the imidazole, which acquires a high pK_a in this state. When introduced, this negative charge will also tend to bring Cu_B^+ closer to L, possibly with bridge formation, and with consequent weakening and breaking of the Cu_B-Im bond.

(5) The postulates in (3) and (4) cause proton translocation to be confined only to the reactions carrying out oxygen chemistry.

(6) Protonic insulation is required between the oxygenous Fe_{a3} ligand and Im; otherwise protons will be transferred from ImH_2^+ to L^- with intrinsic uncoupling as a result.

(7) The input proton pump channel involves D135 in the helix II-III 'loop' and leads to the vicinity of H284. Protonation of L^- (scalar protons) occurs by a separate and slower pathway which may involve OH groups in helix VIII. Proton transfer through this latter channel is regulated; it generally requires a negative charge at L, and is a requirement for electron transfer between Cu_B and Fe_{a3} for thermodynamic reasons. It is also required (together with formation of Cu_B^{2+}) to drastically decrease the pK_a of ImH_2^+ by abolishing its electrostatic interaction with L, which results in H^+ release into an output channel.

(8) If protonation of H284 is impaired, catalysis will be decelerated; if protonation is sufficiently slow, there will be time for proton uptake by L^- before protonation of the histidine and the linkage to proton translocation will be lost, as is apparently the case with the D135N mutant (Ref. [19] and Fig. 1).

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