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Review

Functional proton transfer pathways in the heme–copper oxidase superfamily ☆

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ABSTRACT

Heme–copper oxidases (HCuOs) terminate the respiratory chain in mitochondria and most bacteria. They are transmembrane proteins that catalyse the reduction of oxygen and use the liberated free energy to maintain a proton-motive force across the membrane. The HCuO superfamily has been divided into the oxygen-reducing A-, B- and C-type oxidases as well as the bacterial NO reductases (NOR), catalysing the reduction of NO in the denitrification process. Proton transfer to the catalytic site in the mitochondrial-like A family occurs through two well-defined pathways termed the D- and K-pathways. The B, C, and NOR families differ in the pathways as well as the mechanisms for proton transfer to the active site and across the membrane. Recent structural and functional investigations, focussing on proton transfer in the B, C and NOR families will be discussed in this review. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

In aerobic organisms, electron transfer to oxygen occurs through a series of membrane-bound protein complexes known as the respiratory chain. This energetically downhill electron transfer is coupled to the maintenance of a transmembrane electrochemical proton gradient that is used for energy-requiring processes.

Terminal oxidases catalyse reduction of oxygen to water (see Eq. (1)); the last step of the respiratory chain. These enzymes are located in the mitochondrial inner membrane in eukaryotes or the inner cell membrane in bacteria. Most terminal oxidases belong to the family of heme–copper oxidases (HCuOs), enzymes with a catalytic O_2-binding site which is composed of a heme and a copper ion. All HCuOs have a homologous catalytic subunit with twelve transmembrane helices harbouring six invariant histidines which ligate three cofactors; a high-spin heme and a copper ion in the catalytic site and an additional low-spin heme (for recent reviews on structure and function of the heme-copper oxidases, see e.g. [1–5]). HCuOs conserve the free energy from O_2-reduction by generating a proton electrochemical gradient across the membrane in two ways; first, electrons and protons used to reduce O_2 to H_2O (Eq. (1)) come from opposite sides of the membrane; electrons from donors (often a cyt. *c*) in the 'outside' solution, and protons from the 'inside'. Second, HCuOs are proton pumps, i.e. they translocate protons across the membrane that they are situated in [6]. In A-type HCuOs, four protons are translocated across the membrane for every oxygen reduced to water (i.e. in Eq. (1), $n=4$), but this number is variable (see below).

$$O_2 + 4e^-_{out} + (4+n)H^+_{in} \rightarrow 2H_2O + nH^+_{out} \quad (1)$$

The HCuO superfamily has been classified into three major subfamilies denoted A-, B-, and C-type ([7,8], see [9] for further subdivision). In addition, bacterial NO-reductases (NOR) belong to the HCuO family (see below), where they form their own branch, subdivided into the *c*NOR and qNOR families, indicating that cytochrome *c* or quinol acts as the electron donor [10].

The best known HCuOs are of the type found in mitochondria, the A-type. These oxidases contain, in their catalytic subunit I, a low-spin heme *a* and a high-spin heme a_3. Together with a nearby Cu-ion (Cu_B), heme a_3 forms the catalytic site of oxygen reduction. There is an additional redox cofactor, Cu_A, bound to subunit II, a membrane-anchored protein (see Fig. 1). Cu_A is the acceptor of electrons from the donor, soluble cyt. *c*, and electrons are then transferred via heme *a* to the active site. Protons are transferred through two defined pathways up to the catalytic site, the D- and the K-pathway (see below, and Fig. 4A). These pathways (D- and K-) are used at different times in the catalytic cycle; the D-pathway is used during the oxidation of the reduced enzyme by O_2, both for the chemical and pumped protons, a total of 6–7 H^+ per O_2 turnover. The K-pathway is used for protons (1–2) taken up during the reductive part (see e.g. [11,12]).

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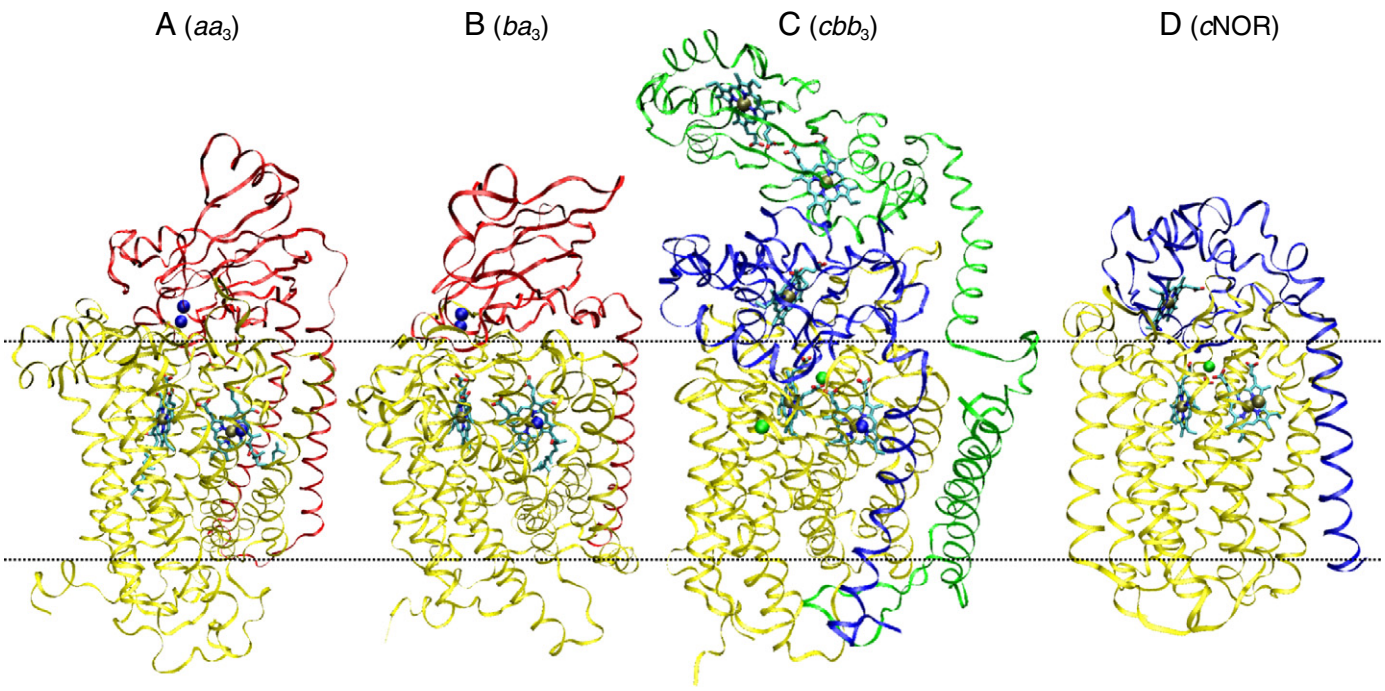

Fig. 1. Overview of the catalytic core structures of A-, B-, C- and *c*NOR heme-copper oxidases. A) The A-type HCuO from *R. sphaeroides* (PDB ID: 1M56 [27]), with subunit I in yellow, and subunit II in red. B) The B-type HCuO from *T. thermophilus* (PDB ID: 1EHK [30]) with the same colour coding as in A. C) The C-type HCuO from *P. stutzeri* (PDB ID: 3MK7 [13]) with CcoN in yellow, CcoO in blue and CcoP in green. D) The *c*NOR from *P. aeruginosa* (PDB ID: 3O0R [14]) with NorB in yellow and NorC in blue. Note that homologous subunits have the same colour. The approximate locations of the membrane surfaces are indicated by the black dotted lines. Heme groups are in light blue, iron ions in gold, copper ions in blue, and calcium ions in green. This (as well as Figs. 3 and 4) figure was made using the VMD software [104].

The other HCuO subfamilies differ from the A-type in that the D-pathway is missing in the sequence and that up to recently, no structural information was available for either the C-type or NOR HCuOs, and little information was available for the proton transfer pathways in the B, C and NOR families. Recent advances, firstly the structural determinations of a C-type HCuO [13] and a *c*NOR [14], and secondly, sequence alignments and functional studies [8,15–22] have improved the knowledge about the mechanism and pathways for proton transfer across the whole HCuO superfamily. This review will discuss these recent advances with focus on the B-, C- and *c*NOR HCuOs.

1.1. The heme-copper oxidase superfamily

The A family HCuOs are widespread in all kingdoms of life (bacteria, archaea and eukarya) [7,23], and among the best studied are the aa_3 (the names, e.g. aa_3 or cbb_3 refer to the types of hemes found in the complexes) enzymes from bovine mitochondria and bacteria such as *Rhodobacter* (*R.*) *sphaeroides* and *Paracoccus* (*P.*) *denitrificans* as well as the bo_3 oxidase from *Escherichia* (*E.*) *coli*. The first structural information for HCuOs came from the A-type; the bovine and *P. denitrificans* aa_3 [24,25], followed by *E. coli* bo_3 [26], and *R. sphaeroides* aa_3 [27,28]. The core functional unit of A-type HCuOs consists of two subunits, shown in Fig. 1A. Subunit I consists of twelve transmembrane (TM) helices and harbours the O_2-binding site as well as the low-spin heme. Subunit II is anchored to the membrane via two TM helices, and its water-soluble domain hosts a dinuclear copper site, Cu_A.

The B-family HCuOs are typically found in archaea [23], although its best studied member is the ba_3 oxidase from the bacterium *Thermus* (*T.*) *thermophilus* (see e.g. [17,29]) which is also structurally defined [30,31], see Fig. 1B. The B-type HCuOs are generally expressed under low O_2 tensions as they have a higher apparent O_2 affinity [15]. Subunit I of the B-type HCuO from *T. thermophilus* consists of thirteen TM helices, where TM helix 1–12 are homologous to subunit I in A-type HCuOs, although the sequence identity is low (<20%, [29]). Subunit II, with one TM helix and a water-soluble domain harbouring a Cu_A site, is homologous to subunit II in A-type HCuOs.

The C (cbb_3) family oxidases are found mainly in bacteria [23], and members of this family have been purified and characterised from e.g. *P. denitrificans* [32], *Bradyrhizobium* (*B.*) *japonicum* [33], *R. sphaeroides* [34,35], *Vibrio* (*V.*) *cholerae* [36], and *Pseudomonas* (*P.*) *stutzeri* [37], where the last one was recently structurally defined at atomic resolution [13], see Fig. 1C. C-type HCuOs were first found in nitrogen-fixing and pathogenic bacteria, expressed under low oxygen tension and, like B-type HCuOs, have a higher apparent O_2 affinity than A-type HCuOs [33]. In the C family, the catalytic subunit CcoN (see Fig. 1C) is related to subunit I of the A- and B-type. CcoN contains the high-spin heme b_3-Cu_B catalytic site and a low-spin heme *b*. CcoO is a membrane-anchored protein containing one *c*-type heme, and is related to NorC in NOR, see below. CcoP, also anchored to the membrane via one trans-membrane helix, contains two *c*-type hemes [37] and has no counterpart in any other HCuOs.

Bacterial NO-reductases (NOR) also belong to the HCuO family [38,39]. However, instead of O_2-reduction, NORs perform a key step in the denitrification process, reducing NO to N_2O (see Eq. (2)).

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (2)$$

NORs are found mainly in bacteria [23] and form two distinct families, the q- and *c*NORs [10], where the letters q and c indicate that they use quinol or soluble proteins such as cyt. *c* as the electron donor. The best studied NORs are the *c*NORs which have been purified and characterised from e.g. *P. denitrificans* [40,41] and *P. stutzeri* [42]. Furthermore, the structure of *c*NOR from *P. aeruginosa* was recently determined [14], see Fig. 1D. Instead of the copper in the catalytic site (Cu_B), NORs contain a non-heme iron (Fe_B). A major difference between the NORs and O_2-reducing HCuOs is that NORs do not couple the exergonic reduction of NO to the generation of a proton gradient,

this reaction is thus non-electrogenic [43–45] and protons (c.f. Eq. (2)) are taken up from the outside (periplasmic) solution [45]. In *c*NORs, the catalytic subunit NorB is related to subunit I of A- and B-type as well as CcoN of C-type HCuOs, although the sequence identity is low. The purified *c*NOR complex additionally contains the cytochrome *c*-containing NorC subunit, which is homologous to the CcoO of C-type HCuOs, but a cytochrome *c*-containing subunit is also found in some A-type HCuOs [46].

Among O_2-reducing HCuOs, the C-type is the closest to NOR in sequence [46] and have been shown to possess significant NO-reduction activities in contrast to the A-type which do not [16,47,48].

2. O_2 reduction in HCuOs

In A-type HCuOs, the catalytic cycle has been extensively studied using the so-called 'flow-flash' technique. In this technique, the fully reduced HCuO with carbon monoxide (CO) bound to the high-spin heme is mixed in a stopped-flow apparatus with an oxygenated solution. A short laser flash is then applied, photolysing the Fe – CO bond and the binding of dioxygen and its subsequent step-wise reduction can be followed using time-resolved spectroscopy. In the A family (the reaction sequence seems identical, and the rate constants similar between the A1 and A2-subfamilies [49], described below), reduction of O_2 studied this way proceeds via a series of intermediates (see Fig. 2); starting with the fully (four available electrons, see green arrows in Fig. 2) reduced, R intermediate, O_2 binds to heme a_3 with a time constant of ~10 μs (at 1 mM O_2) forming the oxygen-adduct or A intermediate. The O – O bond is broken and reduced by four electrons in a single step forming the so-called peroxy or 'P' intermediate with a time constant of ~30 μs in the fully reduced enzyme (see e.g. [50,51]). A proton is then taken up with a time constant of 100 μs, resulting in formation of the ferryl (F) intermediate. Further reduction forms the oxidised (O) enzyme by transfer of the last electron from Cu_A, together with a second proton with a time constant of ~1 ms.

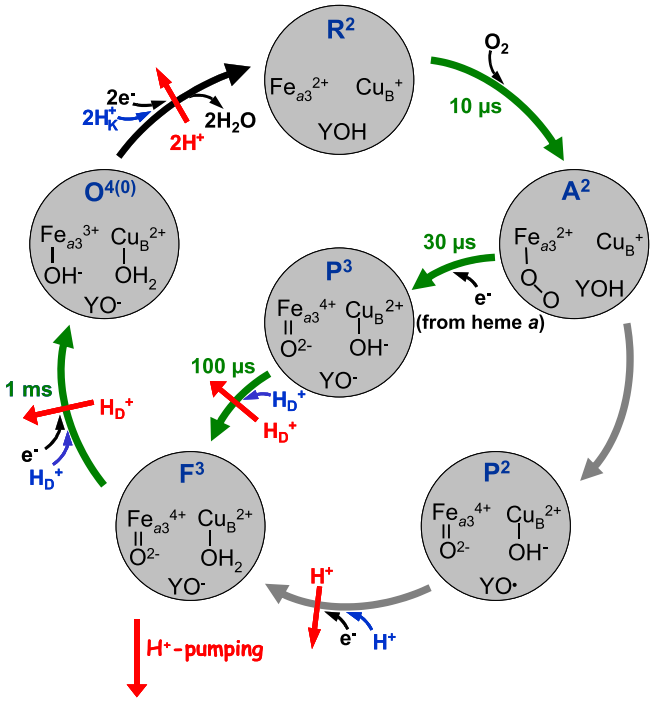

Fig. 2. The catalytic cycle in A(1)-type HCuOs. The reaction studied using the flow-flash technique (with the fully (four-electron) reduced enzyme) is indicated by the green arrows and occurs with the sequence $R^2 \rightarrow A^2 \rightarrow P^3 \rightarrow F^3 \rightarrow O^{4(0)}$, where the superscript indicates the number of electrons transferred to the binuclear site. The YOH is the Tyr cross-linked to one of the His ligands to Cu_B, which acts as the proton donor to the active site in the P^3 state, and also as an electron donor in P^2 (see text for details). Proton uptake from bulk is indicated by blue arrows and the subscripts D and K indicate the use of the D- and K-pathways, respectively. The red arrows across the grey/green arrows indicate which transitions are coupled to proton pumping across the membrane, see text. The grey arrows indicate the reaction sequence when starting with the two-electron (only heme a_3 and Cu_B reduced) enzyme. Note that in forming F^3, we have indicated that the proton goes to the Cu_B-OH^- [105], but the Tyr-O^- is also a possible acceptor.

In the best studied B-type HCuO, the cytochrome ba_3 from *T. thermophilus*, the sequence of events is similar to that with A-type HCuOs [18,20] but with some differences. First, the bimolecular rate constant for O_2 binding is 10 times higher ($10^9\ M^{-1}\ s^{-1}$ [52]) than with the A-type, consistent with the higher apparent O_2 affinity. Second, the F intermediate, with its characteristic peak at 580 nm, is not observed at pH 7.5 [18]. This was interpreted as being due to the P and F intermediates having the same spectrum [18], but in a recent study, we instead suggested that there is an overlap in time of the P→F with the F→O transition [21], linked to a different sequence of events in the proton pumping function (see below).

In C-type HCuOs, the complex behaviour of CO binding [53,54], as well as the overlapping spectra of the three *c*-type hemes, hampers detailed kinetic studies using the flow-flash technique, and consequently, the sequence of oxygen reduction intermediates is largely unknown. However, the A intermediate is presumably formed also in C-type HCuOs [22], and oxidation of all heme groups and proton uptake from solution then occurs with a time constant of ~1 ms [16,22], a reaction possibly limited by the escape of CO from Cu_B (see [53]). Also, in variant cbb_3s (where the residue corresponding to $E49^P$ in Figs. 3 and 4C is modified, see below) where proton transfer through the K-path analogue is slow and rate-limiting, an intermediate with heme *b* oxidised, thus on the same reduction and protonation level as the P^3 intermediate in the A family, is formed [22], before protons are taken up and the *c*-type hemes are oxidised. Presumably in wildtype cbb_3, the lack of identified intermediates is due to the slow rate of P formation ($\tau = 1$ ms), which becomes rate-limiting for subsequent steps including proton transfer (which presumably occurs much faster per se) and the sequence of events could be similar in all O_2-reducing and proton pumping HCuOs.

The *c*NOR from *P. denitrificans* is capable also of O_2-reduction, and this reaction has been studied using the flow-flash technique, mainly for the purpose of investigating proton transfer reactions (see below) because of the experimental advantages of O_2 over NO (see [55], but also [56]). O_2 binds to the heme b_3 of *c*NOR with a $\tau = 40$ μs (at 1 mM O_2), after which oxidation of all heme groups occur with $\tau = 25$ ms (at pH 7.5), with no identified intermediates [55].

In the fully reduced A1 HCuO, the P^3 intermediate (see Fig. 2) forms with three of the four electrons required from the catalytic site (two from heme a_3 which forms a ferryl and one from Cu_B) and the fourth from heme *a*. When starting with the two-electron reduced aa_3 (only heme a_3 and Cu_B reduced, grey arrows in Fig. 2), a P^2 intermediate is formed which presumably contains a radical at a Tyr (Y-288 in *R. sphaeroides* aa_3) close to the active site, as it donates the fourth electron [57]. This tyrosine also acts as the proton donor to cleave the O – O bond in P^3 [58]. The Tyr-288 is cross-linked to one of the His ligands to Cu_B (H-284) [24,59] and considered part of the catalytic core. Such a His-Tyr cross-link with the equivalent residues is also observed in the B-type HCuO from *T. thermophilus* [30,60]. In C-type HCuOs, CcoN lacks the equivalent of the Tyr-288 in the primary sequence, but another Tyr on a different helix fulfills the same role [36,61,62]. A Tyr-His cross-link is thus observed in all major (A, B and C) classes of O_2-reducing HCuOs, but not in the NORs, indicating that it is vital for the O_2-reducing and/or proton-pumping mechanism. One proposed evolutionary scenario for HCuOs [15,23] suggests that the C-family evolved from the A-family and that the positional switch of the active site Tyr is the result of evolutionary migration [61] rather

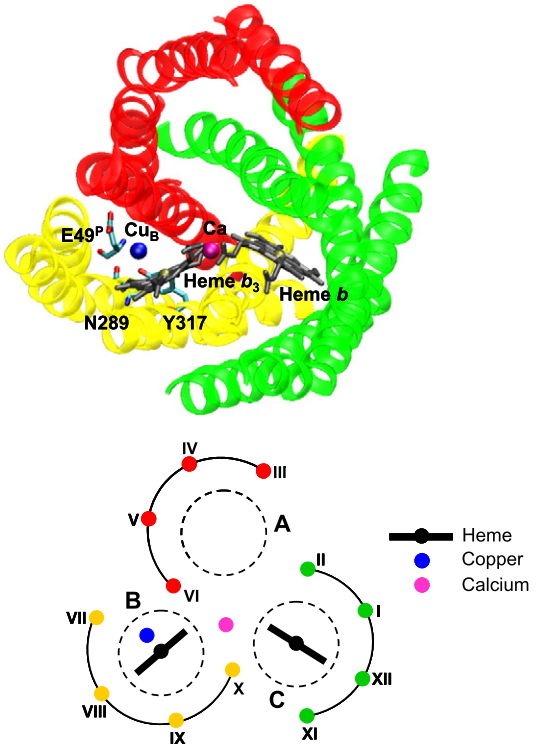

Fig. 3. The symmetrical arrangement of helices (viewed from 'above') in the catalytic subunit of heme–copper oxidases, here exemplified by CcoN in cbb_3 from *P. stutzeri* [13], produces three pores; A (from helices III–VI), B (helix VII–X), and C (helix XI–XII, I–II) [24,25]. The D-pathway in A-type HCuOs is found in pore A. The K-pathway as well as the K-pathway analogues (in the B- and C-type HCuOs) is found in pore B [8], and some of the residues in this path in cbb_3 (see Fig. 4C) are indicated. The $E49^P$ belongs to CcoP and not CcoN. The schematic drawing is based on [24,25].

than 'parallel' evolution. The family of NORs would have evolved from the C-family, and interestingly the position of the Tyr in the A family is occupied by a Glu in NORs, and this Glu is a ligand to the Fe_B that sits at the place occupied by Cu_B in the A family. In this evolutionary scenario, the transition to the NORs thus first involved the migration of the active-site Tyr from helix VI to helix VII (C-family), followed by the change of the Cu_B site to a Fe_B site by the change to Glu in the original position of the Tyr (in A type) and the subsequent loss of the Tyr (in helix VII), since non-heme irons usually prefer oxo-ligands, and the His-Tyr crosslink forces the geometry into one preferred by Cu [14].

3. Proton input pathways in the heme-copper oxidase superfamily

As seen in the crystal structure of the HCuOs, the twelve core transmembrane (TM) helices of the catalytic subunit I are arranged in a pseudo-three-fold rotational symmetry, which produces three pores (A, B and C) each formed by four TM helices [24]. Fig. 3 shows this arrangement in CcoN in cbb_3 [13]. In the A-type HCuOs, the D-pathway for protons is found in pore A, formed from TM helices III–VI and starts at the cytoplasmic surface with a functionally important Asp (see Fig. 4A) (D132, *R. sphaeroides* aa_3 numbering), conserved among A-type HCuOs. The pathway then continues through a network of water molecules stabilised by polar residues (such as N-121, N-139 and S-201) that are non-essential when individually exchanged for non-protonatable groups [63]. The pathway 'ends' at the essential [64] Glu-286, which sits at a ~10 Å distance from Cu_B. Although essential in the A-type HCuOs where it is present (termed A1 [7]), in another A-family subclass (termed A2), the Glu is replaced by a Tyr and a Ser next to each other in sequence [65], and the Glu-286 can be exchanged for non-protonatable amino acids with retained proton pumping if at the same time the YS-motif is introduced [66] (or another Glu introduced on a different helix [67]). The K-pathway for protons, together with the active site, is found in pore B, formed by helices VII–X (Fig. 3), and is named after the essential Lys (K362) found midway up the path. This pathway starts with a glutamate in subunit II ($E101^{II}$) [68,69], which is the only residue in either the D- or K-pathways not found in subunit I (and hence not found by the 'pore analysis'). It then continues up to the Tyr (Y288) cross-linked to one of the His ligands to Cu_B. Site-directed mutagenesis of residues in the K-pathway slows down electron transfer to the active-site heme (see [70] and references therein) in the reductive part of the catalytic cycle (O→R, see

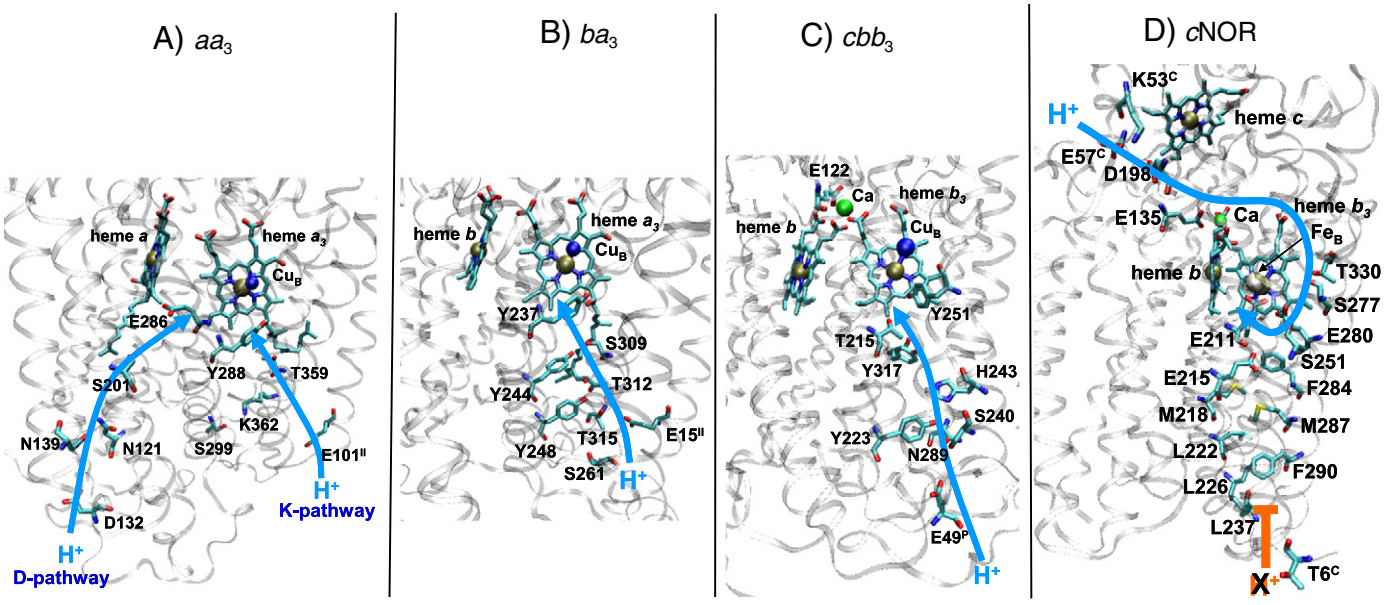

Fig. 4. Enlargement of the suggested proton pathways in the HCuOs in Fig. 1. A) the D- and K-pathways in the A-type HCuO from *R. sphaeroides* [27]. B) The K-pathway analogue in the B-type HCuO from *T. thermophilus* [30]. C) The K-pathway analogue in the C-type HCuO from *P. stutzer* [13]. D) One of the suggested proton pathways from the periplasm to the active site in *c*NOR from *P. aeruginosa* [14]. Also shown in C) and D) is the Ca^{2+} that is ligated by the heme *b* and b_3 propionates (see also Fig. 3) and the Glu-122/Glu-135. The interrupted orange arrow in D) indicates that the area holding the K-pathway (analogues) in the O_2-reducing HCuOs is largely hydrophobic in *c*NOR.

Fig. 2) and have essentially no effect on the oxidative part [71]. Variants with the D-pathway modified, on the contrary, are slow in the oxidative part where proton transfer is involved ($P^3 \rightarrow F^3$, $F^3 \rightarrow O^{4(0)}$ in Fig. 2) [11,72], exemplified by the Glu-286→Gln variant, in which the P^3 state (see Fig. 2) accumulates since the following transitions are very slow [58,64,73]. Moreover, there are D-pathway variants where electron transfer to O_2 is unaffected, but where proton pumping is abolished (termed 'uncoupled' variants), see e.g. [74,75]. These behaviours have led to the designation of the K-pathway as the pathway used for protons taken up during reduction ($1–2H^+/O_2$ turnover) and the D-pathway being used for the protons taken up during oxidation ($2H^+$) and the pumped protons ($3–4H^+$).

It should be noted that in the mammalian A-type oxidases, a different pathway for pumping protons, called the H-pathway, has been suggested (see e.g. [2,76] for details). Some of the H-pathway residues are conserved to bacterial A-type oxidases, but non-functional in proton transfer in these enzymes [77].

In B- and C-type HCuOs, the residues forming the D- and K-pathways in the A-type are missing. The pattern of conserved protonatable residues support the existence of only one proton pathway in both B- [17] and C-type [8] HCuOs. Although proton transfer can be accomplished through a chain of water molecules alone, all known examples of proton pathways in membrane proteins have at least a few participating polar/ionizable side chains, presumably in order for the pathways to be specific for protons rather than other cations [78].

The single proton pathway, composed of conserved amino acid residues, in B- and C-type HCuOs is located in pore B (Fig. 3) and is thus analogous to the K-pathway in the A-family [8,17]. This finding was surprising since it is the D-pathway in A-type HCuOs that delivers most of the protons (including the pumped, see below). In B-type HCuOs, the K-pathway analogue presumably starts at the Glu-15 in subunit II ($E15^{II}$, *T. thermophilus* ba_3 numbering) and continues through polar amino acids such as T312 and T315 up to the catalytic site, see Fig. 4B. The Glu-15 in subunit II is, also in the B-family, the only residue suggested to form part of the proton pathway, that is not located in subunit I. It was further shown that mutations in the K-pathway analogue (of T-312 and T-315) affect the oxidative part ($P^3 \rightarrow F^3$, $F^3 \rightarrow O^4$) of the catalytic cycle [19], i.e. behave as D-pathway mutants in the A-family, supporting the suggestion that this pathway has the role of the D-pathway in the A-family. Furthermore, mutations in the K-pathway analogue were found to inhibit reduction of the active site heme, and in some variants proton pumping was lost [17], supporting this pathway being the only one in the B-family, fulfilling the roles of both the D- and the K-pathway in A-type HCuOs. Variants with modifications in the originally suggested 'D'- and 'Q'-pathways in *T. thermophilus* ba_3 [30] showed no effects on either catalytic activity nor proton pumping [17].

The K-pathway analogue in C-type HCuOs (see Figs. 3 and 4C) was suggested to start at the Glu-49 (*P. stutzeri* cbb_3 numbering [13]) in CcoP. The $Glu\text{-}49^P$ is fairly well conserved in the C family (in a limited alignment (130 sequences), the $Glu\text{-}25^P$ is conserved to 98% (Lee et al., unpublished)). Some amino acids, suggested to form part of the K-pathway analogue [8,13], e.g. Asn-289, Thr-215 and Tyr-317, cannot be replaced without loss of catalytic activity, whereas some, e.g. the Ser-240 and Tyr-223 can be exchanged for a smaller alanine or glycine with retained activity [8]. The His-243 is not conserved in C-type HCuOs, but when absent, it is replaced by small residues possibly providing space for a water molecule, and when exchanged by site-directed mutagenesis for an alanine, the cbb_3 retains catalytic activity [79] (and proton transfer during oxidation of the fully reduced cbb_3 by O_2 is unaffected (Huang et al., unpublished)) but when exchanged for a bulky valine, activity is lost [80].

Recent results from our group showed that proton uptake during oxidation of the fully reduced cbb_3 is severely (up to 500 times) and specifically slowed when the suggested entry point, $Glu\text{-}49^P$ ($Glu\text{-}25^P$ in *R. sphaeroides* cbb_3) is exchanged for a non-protonatable amino acid [22]. These variant cbb_3s thus also behave like D-pathway variants in the A-family, supporting the K-pathway analogue in the C family being the main proton pathway, and that there is no D-pathway analogue made up from non-conserved residues. The Glu-49 in CcoP is also here the only residue in the proton pathway not located in CcoN. We thus note that all major O_2-reducing HCuO families have the entry point to the K-pathway (analogue) in one of the accessory subunits, and that this is the only acidic residue within the pathway. The location of the proton entry point on an accessory subunit might have a functional reason related to the assembly order which for *E. coli* bo_3 (an A-type enzyme) was shown to occur with the subunit II joining the complex last [81]. In the cbb_3, CcoP assembles last [82], and therefore both functional electron transfer (through Cu_A in subunit II, and through the cyt. *c*s in CcoP) is established at the same time as functional proton transfer (through the K-pathway entry points). How assembly occurs, and how fast proton transfer is achieved, in C-type HCuOs that lack the CcoP [83] is not known at present. In this context, it's worth noting that in the wildtype (four-subunit) A-type HCuO from *R. sphaeroides*, there is a strict dependence on the surface aspartate (D-132) for efficient proton transfer into the D-pathway [84,85] that largely disappears in the absence of subunit III [86] as the area around D-132 presumably 'opens up'.

We also note that there is no larger side chain, such as the lysine in the A-type HCuOs, in the K-pathway analogues in the B- and C-type HCuOs. The lysine side-chain has been suggested to change conformation during catalysis [87], possibly in order to 'shut off' the K-pathway during the early stages of O_2-reduction [88] and instead use the D-pathway for proton transfer during the remaining steps in the catalytic sequence (see Fig. 2). This function might thus not be necessary in the B- and C-type HCuOs, which use only their K-pathway analogues for proton transfer throughout the catalytic cycle.

In *c*NOR, even though NO reduction is similarly exergonic to O_2 reduction, the reaction is non-electrogenic and substrate protons are taken from the periplasmic solution [43–45]. The *c*NOR must thus have a proton-transfer pathway leading from the periplasmic (outside) solution into the buried active site. Fig. 4D shows one of the possible pathways identified in the recent structure of *c*NOR from *P. aeruginosa* [14]. In the structure, it was also clear that there are no possible proton relay pathways from the inside in *c*NOR, the area which holds the K-pathway in A-, B- and C-type HCuOs is made up of non-polar amino acids. Before the structure was known, we constructed a model of NorB from *P. denitrificans* based on the homology to the known structures of A- and B-type HCuOs. Based on their conservation, and location in this model, we proposed a possible proton transfer pathway [45]. This pathway started at the equivalent of the Glu-135 (Glu-122 in *P. denitrificans* *c*NOR) in Fig. 4D, which was at the periplasmic surface in our model. Of the residues shown in Fig. 4D, this pathway also contained the equivalents of Asp-198, Ser-277, Glu-211 and Glu-280 (D-185, S-264, E-198 and E-267 in *P. denitrificans* *c*NOR, respectively). Also, another conserved Glu close to the Glu-135, Glu-138, was suggested as part of the pathway. Mutagenesis studies performed before the structure was known, showed that exchanging the Glu-122 (Glu-138 in Fig. 4D) in *P. denitrificans* NOR for either Ala or Gln resulted in severe inhibition of catalytic turnover, whereas mutation to Asp retained ~80% of catalytic activity [89]. These results suggested that the Glu-122 is acting as a proton shuttle. We investigated that proposal by studying the effects by these mutations on the electron transfer coupled to proton uptake ($\tau = 25$ ms phase) during the reaction between fully reduced NOR and O_2. In WT NOR the rate constant of this phase titrates with a single $pK_a = 6.6$, interpreted as the pK_a of an internal proton donor to the active site [55]. Furthermore, the reaction shows a kinetic isotope effect of ~3, consistent with rate-limitation by proton transfer (Krause et al., unpublished). This proton-coupled electron transfer

was severely inhibited in the E122A and Q mutant forms [90], suggested to be due to the involvement of the Glu-122 in the proton pathway. Furthermore, the $\tau = 25$ ms phase is retained in the E122D variant and the perhaps strongest evidence for the involvement of the Glu-122 in proton transfer was the shift in the pK_a of the $\tau = 25$ ms phase from 6.6 (WT) to a p$K_a > 9$ (E122D) [90]. With the structure known, however, the Glu-122 (Glu-135 in Fig. 4D) is shown to be a ligand to a Ca^{2+} ion, additionally ligated by the heme propionates of hemes *b* and b_3 [14]. Such an intricate lattice of interactions presumably affect the midpoint potentials and their pH dependence of both hemes *b* and b_3, making the results with the E122D variant difficult to interpret, but clearly there is a protonatable group important for catalytic function, the pK_a of which shifts dramatically when the Glu-122 is exchanged for an Asp. Further experiments aimed at determining whether the Ca^{2+} is lost in these variants, as well as examining the kinetic effects of other alterations to the Ca^{2+} site is under way in our laboratory.

4. Proton pumping in the heme-copper oxidases

Although they lack the D-pathway that transfers the pumped protons in the A-type HCuOs, the C- and B-type HCuOs have been shown to pump protons, but with a lower stoichiometry than in A-type HCuOs [15,32,91–93], implying that there is a link between having only one pathway and the lower pumping stoichiometry [15]. The lack of a D-pathway in the B- and C-type HCuOs was further suggested to be due to their higher O_2 affinity [15] since the hydrophilic D-pathway overlaps with the suggested hydrophobic path for O_2 [30,94,95]. It thus appears that the A-type is the 'special case', with two specialised proton transfer pathways where the D-pathway can overlap with the gas channel since O_2 delivery need not be as efficient as in the higher affinity B- and C-type HCuOs.

Compared to the well characterised D- and K-pathways for proton transfer into the active site, the output path for protons that are pumped across the membrane is largely unknown, even in A-type HCuOs. Water, and thus possibly protons, exits through the Mg^{2+}-site [96] located above the heme groups in A-type HCuOs. A 'pumping element' (PE), that switches pK_a between high when in contact with the inside, and low when in contact with the outside, is generally assumed to also be located 'above' the active site (see e.g. [97]). If this PE is presumed to be the same across the whole A-, B- and C-type HCuO families, very few candidates exist, and the propionate A of the active site heme has lately been suggested as the pump element from several research groups (see e.g. [17,98]). Also the propionate D of the active site heme was earlier considered as a possible PE [99], but since in the C-type HCuOs (as well as *c*NOR), this propionate is ligated to a Ca^{2+} ion, it presumably stays deprotonated throughout the catalytic cycle, and makes a less likely candidate for the PE [13]. However, the region around the Ca^{2+} in cbb_3 lines a cavity extending towards the periplasm that was suggested to be a possible exit path for protons [13], and a possible input pathway for protons involving the Glu-135 (Glu-122 in *P. denitrificans* *c*NOR, discussed above), one of the Ca^{2+} ligands, was suggested also in *c*NOR [14]. Thus, the Ca^{2+}-ligating Glu could transiently protonate/deprotonate during catalysis. Also, some role (although at present, it is not clear which) for the Glu-135 equivalent in *c*NOR in proton transfer seems likely from the results with the site-directed variants described above.

The exit path for pumped protons in C-type HCuOs is presumably also used for proton uptake when NO reduction is catalysed [16]. This suggestion, that NO reduction uses a different (from 'outside') proton pathway than O_2 reduction in C-type HCuOs, was also consistent with results from the $E25^P$ ($E49^P$ in Fig. 4C) variants in *R. sphaeroides* cbb_3, which showed that NO reduction was not significantly affected by the loss of the protonatable group at $E25^P$ whereas O_2 reduction was severely impaired [22].

The pumping mechanism in the O_2-reducing HCuOs is not known in detail, but several different models have been proposed (see e.g. [100–102]). The basic experimental observations that have to be taken into account are that, in A-type HCuOs, pumping occurs (see Fig. 2) during the $P^3 \rightarrow F^3$ transition [102], the $F \rightarrow O$ transition [72], and during the $O \rightarrow R$ transition [103]. During the $P^3 \rightarrow F^3$ and $F \rightarrow O$ transitions, pumping occurs with the same time constant as the net uptake of a chemical proton (see Fig. 2). In B-type HCuOs, however, the timing is different [18,21] and we recently showed [21] that loading and unloading of the pump element presumably occurs during different transitions, which explains the lower pumping stoichiometry observed (see above). For a detailed discussion of this mechanism, see the contribution of von Ballmoos et al. in this issue. The reason for the difference in the sequence of events could be a different architecture of the pump with only one proton input pathway where it is easier to avoid leakage of protons when the uptake and release of pumped protons are not synchronized.

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