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Mechanism of proton transfer through the K^C proton pathway in the *Vibrio cholerae* cbb_3 terminal oxidase☆

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ABSTRACT

The heme-copper oxidases (HCuOs) are terminal components of the respiratory chain, catalyzing oxygen reduction coupled to the generation of a proton motive force. The C-family HCuOs, found in many pathogenic bacteria under low oxygen tension, utilize a single proton uptake pathway to deliver protons both for O_2 reduction and for proton pumping. This pathway, called the K^C-pathway, starts at Glu-49^P in the accessory subunit CcoP, and connects into the catalytic subunit CcoN via the polar residues Tyr-(Y)-227, Asn (N)-293, Ser (S)-244, Tyr (Y)-321 and internal water molecules, and continues to the active site. However, although the residues are known to be functionally important, little is known about the mechanism and dynamics of proton transfer in the K^C-pathway. Here, we studied variants of Y227, N293 and Y321. Our results show that in the N293L variant, proton-coupled electron transfer is slowed during single-turnover oxygen reduction, and moreover it shows a pH dependence that is not observed in wildtype. This suggests that there is a shift in the pK_a of an internal proton donor into an experimentally accessible range, from > 10 in wildtype to ~8.8 in N293L. Furthermore, we show that there are distinct roles for the conserved Y321 and Y227. In Y321F, proton uptake from bulk solution is greatly impaired, whereas Y227F shows wildtype-like rates and retains ~50% turnover activity. These tyrosines have evolutionary counterparts in the K-pathway of B-family HCuOs, but they do not have the same roles, indicating diversity in the proton transfer dynamics in the HCuO superfamily.

1. Introduction

Aerobic organisms extract the energy required for sustaining life using the reactions that constitute aerobic respiration. The membrane-bound heme-copper oxygen reductases (HCuOs) catalyse the final step of respiration; reduction of oxygen to water. The energy thus released is used to produce and maintain a transmembrane electrochemical pH gradient, used e.g. by ATP synthase to produce ATP. In HCuOs, the gradient is formed both by using protons for water formation exclusively from the inside (bacterial cytoplasm or mitochondrial matrix), and by proton pumping. Protons are transferred through proton input pathways, from the cytoplasmic surface to the buried active site as well as all across the protein. Such proton transfer pathways are composed of hydrogen-bonded chains of protonatable and/or polar residues as well as water molecules, and the transfer of protons across such chains generally follows the Grotthuss mechanism which involves structural rearrangements in the hydrogen-bonded networks (reviewed in [1]). Based on sequence homology and the pattern of conserved amino-acids in the proton pathways, the O_2-reducing HCuOs form three major families: the A-, B- and C-families [2]. A-family HCuOs, the most abundant, are found in bacteria, archaea and eukaryotes. B-family HCuOs (e.g. *Thermus thermophilus* ba_3) are present only in bacteria and archaea. The C-family HCuOs (i.e. cytochrome cbb_3) have so far been reported only in bacteria, including a number of pathogens, and are the most distant relatives to the mitochondrial A-type enzyme.

The A-, B- and C-family HCuOs all have a catalytic subunit that shares a binuclear center consisting of a high-spin heme and a Cu ion (Cu_B), which has a redox-active tyrosine residue covalently linked to one of its histidine ligands [3,4]. A low-spin heme is also present in all catalytic subunits of the HCuOs. Although most residues comprising the proton pathways are located in the catalytic subunit in all HCuOs, they have a different number and composition of their proton pathways

☆ Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulphate; HCuO, heme-copper oxidase, Unless otherwise indicated, residues/mutations are in subunit I or CcoN. The superscript "P" denotes that the residue/mutation is in subunit CcoP; "*Vc*", "*Rs*" and "*Tt*" superscripts indicate *Vibrio cholerae*, *Rhodobacter sphaeroides*, and *Thermus thermophilus*, respectively.

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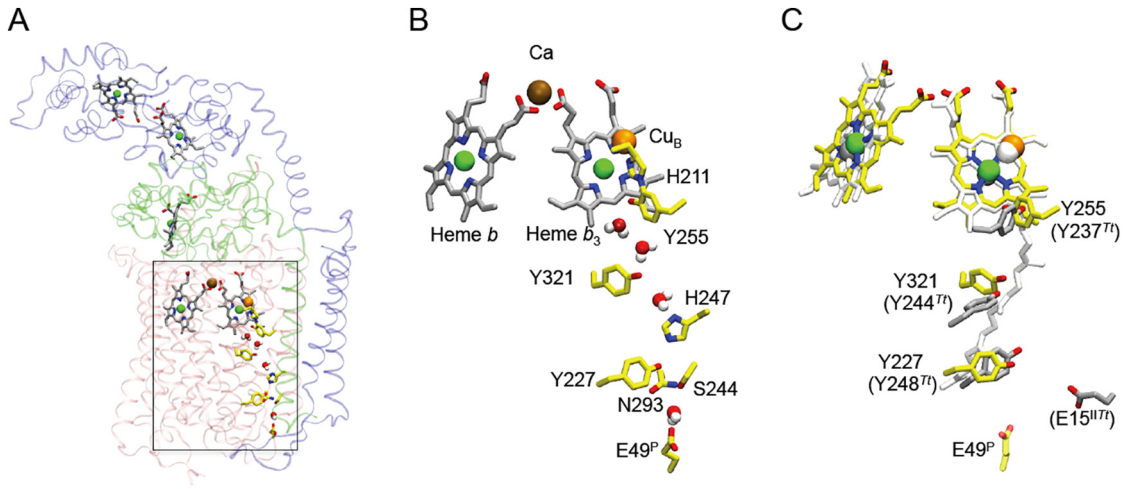

Fig. 1. Structure of cytochrome *cbb*$_3$ from *P. stutzeri*. (A) Ribbon structure with the CcoN in pink, CcoO in green and CcoP in blue, respectively (from PDB ID: 3MK7 [15]). Heme *b*, b_3 and the Ca^{2+} bound at the heme propionates are highlighted along with the residues in the K^C-pathway. The water molecules shown are those observed in previous MD simulations [6]. (B) Enlargement of the K^C-pathway region (C) Structural overlay of the K^C-pathway (using *V. cholerae cbb*$_3$ numbering) in yellow with the K^B-pathway from the *T. thermophilus ba*$_3$ (B-family, PDB ID: 3EH5 [3]) in grey. The structural alignment was made using the MultiSeq module of VMD [17]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(reviewed in [5]), indicating diversity in the detailed proton transfer mechanisms.

The A-family HCuOs have two pathways called the D- and K-pathways, whereas both the B- and C-family HCuOs contain only one functional proton pathway (called K^B- and K^C-pathway, respectively) that is spatially analogous to the K^A-pathway [6–9]. Despite being spatially equivalent, residues lining the K^B- and K^C-pathways are not identical to the K^A-pathway or to each other. In the A-family HCuOs, the D-pathway is used for all pumped protons and most substrate protons, while the K-pathway is only used for transfer of 1–2 substrate protons during the reductive phase of the catalytic cycle [10,11]. The D-pathway starts at D132 (*R. sphaeroides aa*$_3$ numbering) at the N-side surface and ends at E286 near the active site. E286 is a branching point directing protons either to the active site for oxygen reduction or onwards for proton pumping [12–14].

In the C-family HCuOs, electron input from soluble cytochrome *c* occurs first to the CcoP subunit which contains two hemes *c* (see Fig. 1), and then sequentially to the heme *c* in the CcoO subunit. From there, electrons are delivered to the active site via the low-spin heme *b* in CcoN (subunit I). The K^C-pathway, being the only proton pathway, must conduct protons for both pumping and O_2 reduction. Recent studies showed that the K^C-pathway starts from the $E49^P$ (subunit CcoP, *V. cholerae cbb*$_3$ numbering) [6,9], and connects via polar residues and water molecules in CcoN, to Y255 (see Fig. 1) at the active site [6,15]. However, a residue equivalent to the Glu-286 at the 'top' of the D-pathway in the A-family, is absent in the B- and C-family HCuOs. Instead, the $Y321^{Vc}$ was suggested to form such a branch point [6,8,16]. $Y321^{Vc}$, although not conserved to the B-family, is spatially equivalent to $Y244^{Tt}$ of the K^B-pathway [15] (*Thermus thermophilus ba*$_3$ numbering). There is only one conserved residue between the K^C and K^B-pathways: $Y227^{Vc}$ (C-type) equivalent to $Y248^{Tt}$ (B-type, See Fig. 1C), both important for catalysis [6–8].

The reaction of fully reduced A-type HCuOs with O_2 has been extensively investigated by the flow-flash technique. In this technique, the kinetics of oxygen binding and subsequent reactions is monitored by optical spectroscopy after a short laser flash dissociates the photolabile CO bound to the fully reduced enzyme. O_2 binding, forming the so-called A intermediate, is followed by O—O bond breaking by electron transfer from the binuclear center and heme *a*, forming the peroxy (P_R) intermediate (time constant (τ) ~ 30 μs, numbers from the reaction in *R. sphaeroides aa*$_3$ [18]). Next, the ferryl (F) intermediate is formed by internal proton transfer (τ ~ 100 μs), a transition linked to proton uptake and proton pumping [19,20]. The F intermediate decays (τ ~ 1 ms) to form the oxidized (O) state by electron transfer from Cu_A together with a second proton uptake and this transition is also linked to proton pumping [19,20].

In the flow-flash reaction of the fully reduced cytochrome *cbb*$_3$ enzyme (C-family, see [21]), the enzyme is reduced at all six redox centers (heme b_3, Cu_B, heme *b*, and three hemes *c* (in CcoO and CcoP)) and thus capable of fully reducing O_2 to 2 H_2O, leaving two electrons still in the enzyme. Previously, we have shown that in wildtype *cbb*$_3$, all hemes show (partial) oxidation with a time constant of ~1 ms, with no detectable intermediates after formation of the O_2-adduct [6,21]. In the variant $E49^P$A, with the Glu at the entrance of the K^C-pathway (see Fig. 1) exchanged, proton uptake through the K^C-pathway is severely slowed and so is the coupled heme *c* oxidation, whereas the oxidation of heme *b* remained fast [6,9]. This leads to the formation of a partly reduced intermediate not observed with wildtype.

To examine the mechanism of proton transfer in the K^C-pathway in more detail, here we further investigated variants of conserved key residues in the C-family HCuO from *V. cholerae*: Y227, N293 and Y321, by the flow-flash technique. Our results show that exchanging N293 by leucine slows heme *c* oxidation, and also induces a change in the pK_a of an internal proton donor, resulting in possible uncoupling of proton pumping. Furthermore, we report that proton uptake in $Y227^{Vc}$F occurred only slightly slower than in the wildtype whereas proton uptake in $Y321^{Vc}$F was severely inhibited. The phylogenetic and functional importance of these two tyrosine residues ($Y227^{Vc}$ and $Y321^{Vc}$) that also have equivalents in the K-pathway of B-family HCuOs are discussed.

2. Materials and methods

2.1. Site-directed mutagenesis and purification of *cbb*$_3$ oxidase

The mutations were constructed using the QuikChange site-directed mutagenesis kits from Stratagene. DNA oligonucleotides were synthesized at Integrated DNA Technologies. Sequence verification of the mutagenesis product was performed at the Biotechnology Center at the University of Illinois at Urbana-Champaign. The expression, purification, and characterization of the *V. cholerae cbb*$_3$ wildtype and mutants were performed as previously described [6,8].