Is a third proton-conducting pathway operative in bacterial cytochrome c oxidase?

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Abstract
Despite the existence of several three-dimensional structures of cytochrome c oxidases, a detailed understanding of pathways involved in proton movements through the complex remains largely elusive. Next to the two well-established pathways (termed D and K), an additional proton-conducting network (‘H-channel’) has been proposed for the beef heart enzyme. Yet, our recent mutational studies on corresponding residues of the Paracoccus denitrificans cytochrome c oxidase provide no clues that such a pathway operates in the prokaryotic enzyme.

Introduction
Cytochrome c oxidase is the terminal member of the respiratory chain of mitochondria and many bacteria. It catalyses the reduction of oxygen to water, using electrons donated by cytochrome c. The free energy released by this exergonic reaction is harnessed by the enzyme and used to translocate protons across the membrane, contributing to the generation of an electrochemical gradient that is used by ATP-synthase to generate ATP. The mechanism by which haem-copper oxidases couple oxygen reduction to proton translocation is an unsolved problem of major interest, and to this end it is important to identify the pathways through which protons may be translocated. Two such pathways, the so-called K- and D-channels, have been widely accepted based on structural, mutagenic and functional data [1–8]. Some D-pathway mutants have been proposed for the beef heart enzyme. Yet, our recent mutational studies on corresponding residues of the Paracoccus denitrificans cytochrome c oxidase provide no clues that such a pathway operates in the prokaryotic enzyme.

Proton-translocation pathways
Proton translocation through cytochrome c oxidase is energetically coupled to oxygen reduction as summarized in the following equation:

\[ 8H^+_{IN} + 4cyt c^{2+} + O_2 \rightarrow 4H^+_{OUT} + 4cyt c^{3+} + 2H_2O \]

Thus for every dioxygen molecule reduced, four protons are consumed at the binuclear centre and four transferred across the membrane. It was originally thought that two separate channels may exist to serve these distinct processes [1], but it is now understood that whilst the K-pathway feeds two protons to the binuclear centre only [13], the D-pathway supplies protons for both water formation and translocation across the membrane. The D-pathway is named after a conserved aspartate residue (Asp124 in Paracoccus denitrificans), which, in bacteria, is located on the cytoplasmic side of subunit II.

Subunit II is composed of two transmembrane helices and a \( \beta \)-barrel globular domain facing the intermembrane or periplasmic space, and contains the other main catalytic group, CuA, which consists of two electronically coupled copper ions. CuA oxidizes cytochrome c that docks on to the globular domain of subunit II.

Key words: bacterial oxidase, cytochrome c, haem a, H-channel, Paracoccus denitrificans, proton-conducting pathway.

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The putative H-channel

A third proton-translocation channel was first proposed in 1996 based on the high-resolution structure of the bovine enzyme [2], and was later also addressed in the structure from *P. denitrificans* [15]. This channel in the mitochondrial enzyme is spatially distinct from the binuclear centre passing instead by the low-spin haem a group, and it was suggested that redox changes in haem a may provide the driving force for proton translocation through this channel [2,9]. A hydrogen-bonded pathway was shown to extend from Asp407 (bovine numbering) on the internal side of the protein through residues His413, Thr424, Ser461, Ser382, haem b, Ser454, Gln428, Arg38, Asn451 and Tyr443 [2]. This pathway has since been modified to pass from Asn451 to Tyr371, through the proline of haem a to Tyr54, through the peptide bond of Ser441-Tyr440 and then to Asp51 on the external side of the enzyme [9].

In order to investigate the functionality of this channel, a series of experiments were designed in bacterial oxidases in which largely conserved H-channel residues were mutated and the enzymes were tested for proton pumping and catalytic activity [5,10]. With the exception of two positions (Arg38 in *Rhodobacter sphaeroides* and His449 in *P. denitrificans*), which were both thought to cause perturbations in the environment of haem a, all mutants had activities close to wild-type activity. From these experiments it was concluded that the H-channel was not functional, at least in bacterial enzymes.

Recently, the potential role of the H-channel has been reconsidered by the development of a system that allows the testing of site-directed mutants of mitochondrial oxidases [16]. Using this system, three H-pathway mutants of mammalian cytochrome c oxidase were reported, which indicated an uncoupled phenotype [16,17]. The system is based on expression of subunit I of bovine oxidase in a hybrid complex with the other subunits from human oxidase in HeLa cells, in which bovine subunit I is in competition with the native human subunit I in assembly of the oxidase. A D51N (Asp51→Asn) mutation was reported to have lost proton-pumping ability with no change in electron transfer activity, as tested in isolated mitoplasts from these HeLa cells [16]. In addition to this, high resolution structures of the bovine enzyme in the fully oxidized and fully reduced states showed a conformational change in the Asp51 gating residue with corresponding changes in H-bonding networks [16]. This supports the idea that redox-dependent changes in Asp51, caused by changes in the haem a redox state, could act as the driving force for proton pumping through the H-channel. Using the same system, an S441P mutation was constructed, which would block proton transfer through the Ser441-Tyr440 peptide bond, and a V386L/M390W double mutant was made with the intention of blocking essential passage of water molecules through the lower part of the channel [17]. Both these mutations apparently showed an uncoupled phenotype.

We have recently repeated the double mutation in *P. denitrificans*, mutating the conserved Val421 (Val386 in bovine) to leucine and the equivalent Phe425 (Met390 in bovine) to tryptophan, both as single mutations and in tandem. We reconstituted the purified protein into lipid vesicles and tested them for proton pumping using stopped-flow experiments (using methods described in [6]) and found all the mutants to have wild-type electron transfer and proton-pumping activity (results not shown). Once again, this is in conflict with reports from the mammalian oxidase.

A further difficulty with the H-channel proposal is that the critical Asp51, which would be necessary to move protons out from the enzyme, is not conserved amongst lower eukaryotes and bacterial oxidases. Indeed, in several bacterial oxidases, it is replaced by a glycine residue (position 84 in *P. denitrificans*), which could not act as a proton carrier [3,15]. It has been countered that there is a cavity capable of trapping a water molecule directly adjacent to this glycine residue, and that this could be involved in a regulated conformational change, which would control proton exit to the periplasm [18]. This would be an interesting mechanism with no known precedent in proton-transfer pathways studied thus far.

Summary

Evidence has repeatedly shown that the putative H-pathway is not operative in bacterial cytochrome c oxidases. The question remains open whether despite strong similarities in structure and basic properties, bacterial and mammalian oxidases could have evolved divergent proton pathways and consequently catalytic mechanisms [19]. It is likely that the debate will continue for some time, until further studies on other members of the haem-copper oxidases will settle the issue conclusively.

References


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