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Minireview

Cytochrome oxidase evolved by tinkering with denitrification enzymes

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Abstract

The cytochrome *bc* complex which is encoded by the *fixNOPQ* operon in *Bradyrhizobium japonicum*, is the most distant member of the haem–copper cytochrome oxidase family. We have found that its major subunit, FixN, is homologous to the NorB subunit of nitric oxide reductase in a purple bacterium. A second evolutionary link between cytochrome oxidases and denitrification enzymes is the presence of a similar binuclear copper site in cytochrome aa_3 (the mitochondrial oxidase) and nitrous oxide reductase. This centre was probably acquired by a primitive FixN-type oxidase, leading to the evolution of the mitochondrial-type oxidase. These links suggest that the oxygen-reducing respiratory chain developed from the anaerobic, denitrifying respiratory system.

Key words: Cytochrome oxidase; Respiration; Denitrification; Evolution of metabolism

1. Introduction

Cytochrome oxidases are membrane protein complexes that contain haem and copper, reducing oxygen to water as the terminal step in aerobic respiration. Two kinds of homologous respiratory oxidases have been found in bacteria. One type is similar to the mitochondrial cytochrome aa_3 that uses cytochrome *c* as the electron donor, whereas the other takes electrons from quinol. The *Escherichia coli* cytochrome *bo* is an example of the latter [1]. A common structural feature of the oxidase superfamily is the active site where dioxygen is reduced. It is a bimetallic centre, formed by the iron of a pentacoordinated haem and an adjacent copper called Cu_B [2]. These two metals are bound to the major catalytic subunit via four conserved histidines. Subunit I also has the binding site of another haem that is involved in electron transfer to the active site; this haem is axially co-ordinated to two further invariant histidines. The six invariant histidines of subunit I are diagnostic for the family [3–9].

A distinction between the quinol oxidase and cytochrome *c* oxidase branches is the presence of a fourth metal centre in the latter. This is a purple copper site (Cu_A) which appears to be the primary acceptor of electrons coming from cytochrome *c* [10,11]. It is absent in all quinol oxidases studied so far [7]. Cu_A is a unique binuclear centre which contains two copper atoms in a mixed valence configuration. It is located in the membrane-exposed part of subunit II of cytochrome *c* oxidase [12,13].

Terminal oxidases belonging to the family have been found in several eubacteria [7], and also in archaebacteria [14–17]. This suggests that the primitive oxygen-reducing respiratory oxidase was present before the split between eubacteria and archaea. These two domains of life diverged before oxygenic photosynthesis began to release oxygen from water [18]. Therefore, it is likely that the first oxidases were functioning in an environment with a very low concentration of oxygen.

2. The FixN compex is the oldest cytochrome *c* oxidase

A novel cytochrome *c* oxidase has recently been characterized in *Bradyrhizobium japonicum*, *Rhodobacter sphaeroides* and *Paracoccus denitrificans* [19–21]. This enzyme is encoded by the *fixNOQP* operon in *B. japonicum*. It is expressed in the nitrogen-fixing bacteroid living in root nodules. Energy metabolism of bacteroids is supported by oxidative phosphorylation under the conditions in which the concentration of oxygen is very low [22]. It is supposed that the FixN oxidase has an exceptionally high affinity for oxygen [19].

Sequence analysis [23] shows that the FixN complex is the most distant member of the cytochrome oxidase family, but it still shares the six histidine ligands that bind two haems and Cu_B in the catalytic subunit (FixN). The complex contains two other subunits. These are membrane-bound cytochromes *c*, one of which (FixO)

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contains a single haem and the other (FixP) contains two haems in an internally duplicated, membrane-exposed domain [19]. Thus, the FixN complex has a similar active site to the other haem–copper oxidases, but its cytochrome *c*-binding site and the pathway of electron entry must be different from those of the other cytochrome *c* oxidases. The diverged sequence [23] and the high affinity for oxygen in rhizobia suggest that the FixN complex is similar to a primordial cytochrome oxidase which functioned in a microaerobic environment.

3. FixN oxidase is related to the nitric oxide reductase

Denitrification is an anaerobic respiratory process which reduces nitrate to dinitrogen. The pathway involves nitrite, nitric oxidase and nitrous oxide as intermediates. These four nitrous compounds act as electron acceptors in a respiratory chain that utilizes quinol and cytochrome *c* as electron carriers. Cytochrome bc_1 which is a component of the aerobic respiratory chains, is also needed for denitrification [24–26]. Denitrifying species are present among both archaea and eubacteria [24].

Nitric oxide reductase (NOR) is a membrane-bound cytochrome *bc* complex [27–29]. It contains two subunits, NorB and NorC. NorB is an integral membrane protein which contains haem B, whereas NorC is a membrane-anchored cytochrome *c* [30]. The electron donor in the reaction, in which two NO molecules are joined to a N_2O and water is liberated, may be a cytochrome *c*. It is also known that reduction of NO is associated with proton pumping in denitrifying bacterial cells [26].

The cytochrome composition of the NOR complex is similar to the FixN complex. NorB, which is presumably the catalytic subunit in NO reduction, also has twelve trans-membrane segments [30] which is typical of subunit I of cytochrome oxidases [31]. These similarities are extended by a comparison of the NorB and FixN sequences (Fig. 1). It is clear that NorB has all six invariant histidines that bind the haems and Cu_B in subunit I of cytochrome oxidases. It is homologous to the catalytic subunit of haem–copper oxidases, and belongs to the family.

A phylogenetic tree that is based on an alignment of selected bacterial and archaean oxidases with NorB, shows that NorB is the most diverged member of the family (Fig. 2). This supports the idea that the first oxidase evolved from NOR. FixN is the closest branch to NorB in this tree, again suggesting that it is related to the primitive cytochrome oxidase. The haem C-binding FixO subunit of the complex has similar features to the NorC subunit of NOR. The haemopeptide is located about 30 residues after the N-terminal membrane anchor in both cases. The sequence similarity is low (not shown) but the topological features are consistent with diverged evolution of FixO and NorC.

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      1
FixN  MSQPSISKSM TIGESGLAVV FAATAPLCVI AAAKALDAPF AFHAALSAAA
NorB  .......... .......... .......... .......... ..........

      51                                                    I
FixN  SVAAVFCIVN RYFERPAALP PAEINGRPNY NMGPIKFSSF M.AMFWGIAG
NorB  .......... .......... MSSFNPHLKF QSQAVAKPYF VFALILFVGQ
                             *                    *  *

      101                                          II
FixN  FLVGLIIASQ LAWPALNFDL PWISFGRLRP LHTSAVIFAF GGNVLIATSF
NorB  VLFGLIMGLQ YVVGDFLF.. PLLPFNVARM VHTNLLIVWL LFGFMGAAYY
       * ***   *          *   *   *   *   **   *          *

      151                  III
FixN  YVVQKSCRV. ...RLAGDLA PWFVVVGYNF FILVAGTGYL LGV.......
NorB  LIPEESDCEL HSPKLAIILF WVFAAAGVLT ILGYLFVPYA ALAEMTRNDL
            *        **  *    *   *              *

      201                        IV                      V
FixN  ..TQSKEYAE PEWYADLWLT IVWVVYLLVF LATIIKRKEP HIFVANWFYL
NorB  LPTMGREFLE QPTITKIGIV VVALGFLYNI GMTMLKGRKT VVSTVMMTGL
       *   *  *                *   *      *  *              *

      251                                                VI
FixN  AFIVTIAVLH LGNNPALPVS AFGSKSYVAW GGIQDAMFQW WYGHNAVGFF
NorB  IGLAVFFLFA FYNPENLSR. .......... ....DKFYWW FVVHLWVEGV
                 *  *                      *   *    *  *

      301                                          VII
FixN  LTAGFLAIMY YFIPKRA..E RPIYSYRLSI IHFWALIFLY IWAGPHHLHY
NorB  WELIMGAMLA FVLIKVTGVD R.EVIEKWLY VIIAMALITG IIGTGHHFFW
            *        *      *                        *    **

      351                VIII
FixN  TALPDWTQTL GMTFSIMLWM PSWGGMINGL .MTLSGAWDK LRTDPVLRML
NorB  IGAPTVWLWV GSIFSALEPL PFFAMVLFAL NMVNRRRREH PNKAASLWAI
       *        *  **       *        *  *              *

      401  IX                                        X
FixN  VVSV.AFYGM STFEGPMMSI KVVNSLSHYT DWTIGHVHSG ALGWVGFVSF
NorB  GTTVTAFLGA GVW.GFMHTL APVNYYTHGS QLTAAHGHLA FYGAYAMIVM
       * ** *      * *      **   *     *  * *    *

      451                                          XI
FixN  GALYCLVPWA W...NRKGLY SLKLVNWHFW VATLGIVLYI SAMWVSGILQ
NorB  TMISYAMPRL RGLGEAPDAR AQRIEVWGFW LMTISMIAIT LFLTAAGVVQ
          *                    * **   *                * *

      501                                          XII
FixN  GLMWRAYTSL GFLEYSFIET VEAMHPFYII RAAGGGLFLI GALIMAYNLW
NorB  IWLQRIPADG AAM..SFMNT ADQLAIFFWL RFIAGVFFLI GLVCYLYSFR
       *           **  *        *   *  *  *** *     *

      551              571
FixN  MTVRVGEAEV QMPVALQPAE
NorB  QRGRVP.VVV AAPAAA....
       **    *  * *
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Fig. 1. An alignment of NorB and FixN. These proteins are the major subunits of the *Pseudomonas stutzeri* nitric oxide reductase (NorB) [30] and the *Bradyrhizobium japonicum* cytochrome *c* oxidase that is expressed in bacteroids (FixN) [19]. Six invariant histidines are bold, and the trans-membrane spans I–XII are underlined and labelled with Roman numerals. Identical residues are underlined with stars. Note that the NorB sequence contains the pentapeptide GAMLA (alignment positions 306–310). This is the Swedish word for 'old', which further supports the primordial character of NorB.

Nitric oxide was present in the early atmosphere of the earth [32]. Therefore, one can assume that denitrification predates aerobic respiration and that the denitrifying enzymes evolved before the first oxidases. Denitrifying respiratory chains have much in common with the oxygen-reducing aerobic chains [25], and the adaptation from denitrification to respiration may have mainly affected the terminal part of the chain. We propose that the FixN-type oxidase developed from a NOR through a change in the catalytic reaction (Fig. 3). The original enzyme used a bimetallic site for reduction of two NO molecules into N_2O and thereby releasing water. A gene duplication may have led to the development of an O_2-reducing enzyme, e.g. via a change in the distance between the iron and copper atoms. NO binds to the active site of cytochrome oxidase, and it has been shown that

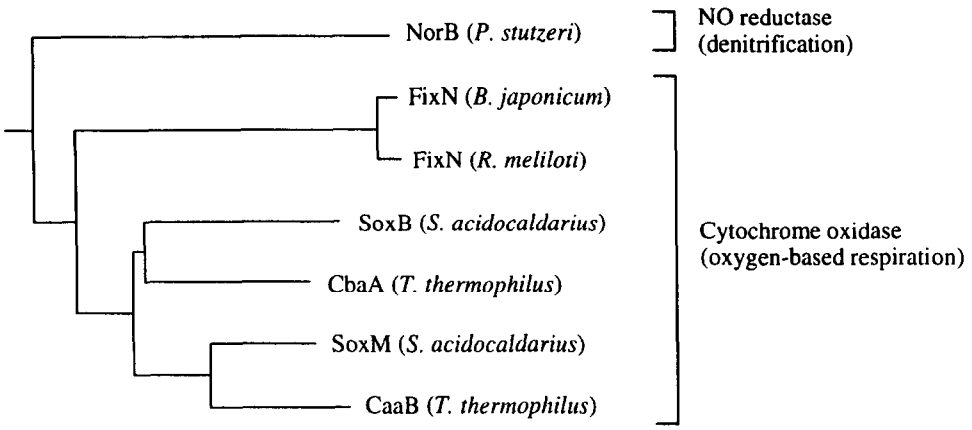

Fig. 2. A phylogenetic tree of subunit I of cytochrome oxidase and the main subunit of nitric oxide reductase. The most divergent members of the oxidase family have been chosen (*T. thermophilus* CaaB is the representative of the rest of the eubacterial and mitochondrial oxidases). The method is based on the neighbor-joining algorithm, as described in [23]. The branching order in the tree is very unstable due to many poorly conserved positions of the multiple alignment. The branch leading to NorB is the longest, and the root of the tree divides it from the other branches. The abbreviations used for the genera are: P., *Pseudomonas*; B., *Bradyrhizobium*; R., *Rhizobium*; S., *Sulfolobus*; T., *Thermus*.

the mitochondrial oxidase is (still) able to reduce NO to N_2O [33].

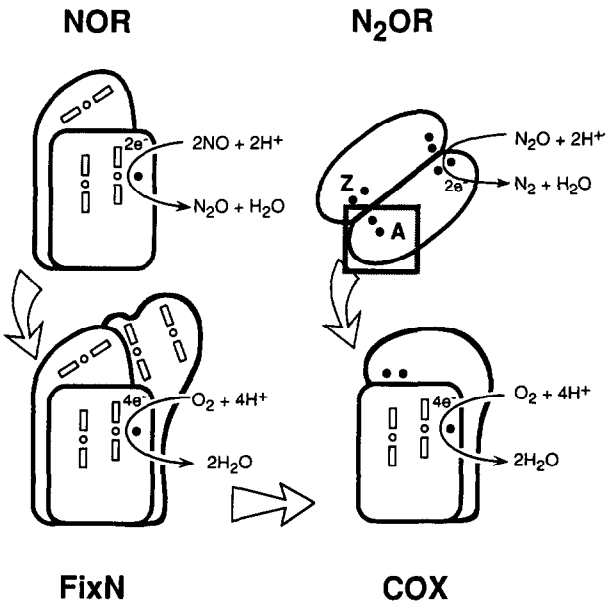

Fig. 3. The proposed evolutionary history of cytochrome oxidase. The first oxidase (FixN) developed from nitric oxide reductase. This adaptation may have required a change in the affinity of the iron/copper active site for NO and O_2. The mitochondrial-type cytochrome *c* oxidase (COX) evolved from the FixN complex which lost the cytochrome *c* domains and acquired the Cu_A-binding domain from the N_2OR lineage. A and Z refer, respectively, to the electron acceptor and active site in N_2OR. The reactions catalysed by the four enzymes are shown. Each enzyme may use cytochrome *c* as electron donor. Haem irons are shown with open circles and coppers with filled circles.

4. Cytochrome aa_3 acquired the Cu_A centre from nitrous oxide reductase

It has been proposed [12] that subunit II of both cytochrome *c* and quinol-oxidizing enzymes contains a domain that has the cupredoxin fold [34]. In the former, this domain is occupied by the binuclear copper site [35,36]. The amino acid sequences of N_2OR C-termini are homologous to this part of the subunit II sequences [12,37]. They may contain the purple copper centre in a three-dimensional fold that is related to the Cu_A domain. The mitochondrial-type cytochrome *c* oxidase and nitrous oxide reductase are the only enzymes known to contain the binuclear copper centre. The similarity between these metal centres is currently supported by a number of biochemical and spectroscopic studies [13,38–40].

The evolution of cytochrome oxidases has included several gene duplication events [23]. At best, there seem to be four homologous haem–copper oxidases in the same bacterial cell [21]. The cytochrome aa_3 complex must be derived from the molecular evolution of one set of duplicated oxidase genes, originating from the FixN lineage. As N_2OR probably existed before the oxidases, it is plausible that the binuclear copper centre of cytochrome *c* oxidase originates from an exchange of genetic material between the genes encoding a primitive oxidase and a nitrous oxide reductase (Fig. 3).

5. Evolutionary tinkering

Evolution never develops new things de novo, but it always uses previous structures and functions to create novelties. As Francois Jacob has written [41]:

'In contrast to the engineer, evolution does not produce innovations from scratch. It works on what already exists, either transforming a system to give it a new function or combining several systems to produce a more complex one. Natural selection has no analogy with any aspect of human behaviour. If one wanted to use a comparison, however, one would have to say that this process resembles not engineering but tinkering, *bricolage* we say in French. While the engineer's work relies on his having the raw materials and tools that exactly fit his project, the tinkerer manages with odds and ends.'

The evolutionary links between cytochrome oxidases and denitrification enzymes are an excellent example of tinker's work, and provide an explanation for the origin of aerobic respiration.

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