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From NO to OO: Nitric Oxide and Dioxygen in Bacterial Respiration

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Nitric oxide reductase (NOR) is a key enzyme in denitrification, reforming the N–N bond (making N_2O from two NO molecules) in the nitrogen cycle. It is a cytochrome *bc* complex which has apparently only two subunits, NorB and NorC. It contains two low-spin cytochromes (*c* and *b*), and a high-spin cytochrome *b* which forms a binuclear center with a non-heme iron. NorC contains the *c*-type heme and NorB can be predicted to bind the other metal centers. NorB is homologous to the major subunit of the heme/copper cytochrome oxidases, and NOR thus belongs to the superfamily, although it has an Fe/Fe active site rather than an Fe/Cu binuclear center and a different catalytic activity. Current evidence suggests that NOR is not a proton pump, and that the protons consumed in NO reduction are not taken from the cytoplasmic side of the membrane. Therefore, the comparison between structural and functional properties of NOR and cytochrome *c*- and quinol-oxidizing enzymes which function as proton pumps may help us to understand the mechanism of the latter. This review is a brief summary of the current knowledge on molecular biology, structure, and bioenergetics of NOR as a member of the oxidase superfamily.

KEY WORDS: Cytochrome oxidase; nitric oxide reductase; respiration; denitrification; evolution.

INTRODUCTION

The composition of the Earth's early atmosphere is still not well defined, but it is thought that CO_2, CO, and N_2 were the main components by the time of the origin of life. In addition, NO could have been produced by shock heating of the atmospheric CO_2 and N_2 during the heavy impacts which were characteristic for this period of the Earth's history, and small amounts of H_2, H_2O, and sulfuric gases could also have been present (Kasting, 1993). It is probable that only trace amounts of O_2 were present but small amounts of oxygen could have been generated from photolysis of water by ultraviolet radiation (Sagan *et al.*, 1993). The level of atmospheric oxygen began to rise about 2400–2600 million years ago and the present concentration of free oxygen was only reached about 500 million years ago (see Stouthamer, 1992). The exact history of oxygen accumulation is not certain, but the general theory is that the emergence of organisms capable of performing oxygenic photosynthesis (probably ancestors of the present-day cyanobacteria) was responsible for this (see Kasting, 1993).

DENITRIFICATION AND RESPIRATION

Denitrification is a respiratory process in which nitrous salts are reduced to dinitrogen by a series of metalloprotein catalysts. It can produce energy and support cell growth. Cytochrome bc_1, cytochromes *c*, and quinols are involved in this electron transfer pathway (Stouthamer, 1992; Zumft, 1993; Berks *et al.*, 1995). There are several molecular links between the evolution of denitrification enzymes and cytochrome oxidases (Castresana and Saraste, 1995; Saraste *et al.*, 1996; Zumft and Körner, 1997). The most important is the homology between nitric oxide reductase (NOR)

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and heme/copper cytochrome oxidases. NOR is a membrane protein complex containing *b*- and *c*-type cytochromes. Although several genes are apparently involved in biosynthesis of NOR, it seems to have only two structural subunits. NorC which is a membrane-anchored cytochrome *c*, and NorB which contains protohemes (Berks *et al.*, 1995).

NOR catalyzes a key reaction of denitrification in which the N–N bond is formed:

$$2\,NO + 2H^+ + 2e^- \rightleftharpoons N_2O + H_2O$$

using electrons which probably derive from cytochrome *c* (Berks *et al.*, 1995).

NorB is homologous to the subunit I of cytochrome oxidases (Saraste and Castresana, 1994; Van der Oost *et al.*, 1994). It is predicted to have the characteristic topology with twelve transmembrane helices, and all six metal-binding histidines that are required for the construction of metal-binding sites in subunit I (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995) are present at the correct locations (Fig. 1a). However, the bimetallic active site of NorB may contain two irons (a heme iron and a non-heme iron) rather than a heme and a copper (Van der Oost *et al.*, 1994; Girsch and Vries, 1997; Kannt *et al.*, 1997; Hendriks *et al.*, unpublished). Another difference is that NOR lacks the residues involved in the aqueous proton channels D and K that connect the heme/copper active site of cytochrome oxidases to the inner surface of the membrane (Iwata *et al.*, 1995; Wikström *et al.*, 1997).

The major catalytic subunit of the extant cytochrome oxidases in archaea, eubacteria, and eukaryotes is remarkably conserved. The basic structure of the heme/copper active site is very similar in all oxidases. This center may have evolved to its present-day configuration prior to the split between archaea and eubacteria, which is thought to have taken place before the emergence of free oxygen in the atmosphere (see Castresana *et al.*, 1994; Castresana and Saraste, 1995; Saraste *et al.*, 1996). This creates a paradox: Did cytochrome oxidase evolve in an environment where dioxygen was only present in local habitats? If this is the case, the ancestor of cytochrome oxidases might have had a function that was different and better adapted to the general circumstances prevailing on the early Earth. The evolutionary links between denitrification and oxygen-based respiration may thus suggest that the latter has evolved from the former, if NO indeed was a more abundant compound in the early atmosphere than O_2.

We tentatively assign the ancestral role in the cytochrome oxidase superfamily to the ancestor of the present-day bacterial nitric oxide reductase, ignoring views that present rather different scenarios suggesting late emergence of denitrification as a part of the nitrogen cycle (Falkowski, 1997). However, the order of events during the evolution of cytochrome oxidases and nitric oxide reductase is not ultimately crucial for the present discussion. The comparison of different enzymes with family links will give important results for understanding of their functional mechanisms.

STRUCTURE OF NITRIC OXIDE REDUCTASE

Purification of an active nitric oxide reductase has been reported from several denitrifying bacteria such as *Pseudomonas stutzeri* (Heiss *et al.*, 1989), *Paracoccus denitrificans* (Carr and Ferguson, 1990; Dermanstia *et al.*, 1991; Fujiwara and Fukumori, 1996; Girsch and de Vries, 1997) and *Achromobacter cycloclastes* (Jones and Hollocher, 1993). In these cases, the purified enzyme contains two polypeptides with apparent molecular weights close to 18 and 38 kDa. They have been identified as the *norC* and *norB* gene products by N-terminal sequencing (Zumft *et al.*, 1994; Hendriks *et al.*, unpublished). Until now, there is no indication for co-purification of additional subunits. However, as will be discussed in the next section, there are several different genes in clusters encoding the NOR activity (Fig. 2), some of which might code for additional structural components of the complex *in situ*.

The ratio of *b*- and *c*-type heams is most likely 2:1 in NOR (Berks *et al.*, 1995; Girsch and de Vries, 1997; Kannt *et al.*, 1997). This is supported by the sequence data which suggests the presence of one binding site for a heme C in NorC and, in analogy to the heme/copper oxidases, two heme-binding sites in NorB (Fig. 1). Metal analysis indicates that NOR contains four or more Fe atoms per monomeric NorB/NorC complex, and that there are no significant amounts of other metals such as Cu, Zn, Co, or Mn (Heiss *et al.*, 1989; Dermanstia *et al.*, 1991; Fujiwara and Fukumori, 1996, Girsch and de Vries, 1997; Hendriks *et al.*, unpublished). Thus, at least one non-heme iron is present per three hemes. EPR spectroscopy of the oxidized enzyme shows that there are no FeS clusters present (Heiss *et al.*, 1989), as expected from the absence of conserved cysteines in the NorBC complex

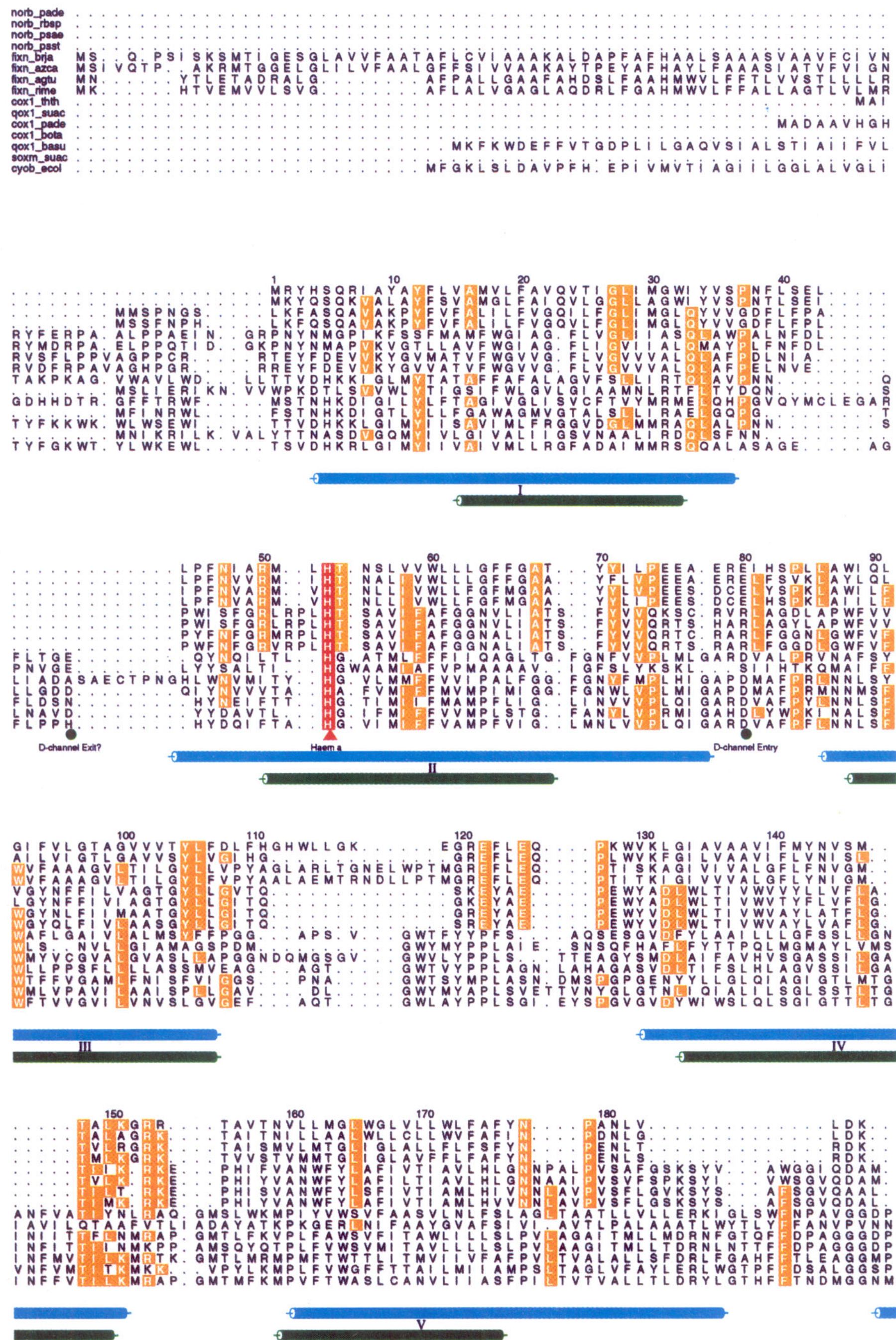


Fig. 1. (a) Sequence alignments of the nitric oxide reductase subunit NorB and subunit I of different cytochrome oxidases. Blue cylinders represent the transmembrane helices of cytochrome *c* oxidase from *Pa. denitrificans* (Iwata *et al.*, 1995). Green cylinders represent the transmembrane helices for NorB from *Pa. denitrificans* as predicted by using the program PHD (Rost *et al.*, 1995). Residues which are identical in more than 50% of the aligned sequences are boxed in orange; conserved residues are boxed in red. Metal-binding sites and selected residues are marked (see text for explanation). The following sequences are aligned: NorB from *Pa. denitrificans* (norb_pade, GenBank accession number U28078), *Rh. sphaeroides* (norb_rbsp, AF000233), *Ps. aeruginosa* (norb_psae, D38133), and *Ps. stutzeri* (norb_psst, Z28384). Subunit I (FixN) of *cbb*$_3$-type oxidases from *Bradyrhizobium japonicum* (fixn_brja, L07487), *Azorhizobium caulinodans* (fixn_azca, X74410), *Agrobacterium tumefaciens* (fixn_agtu, Z46239), and *Rhizobium meliloti* (fixn_rime, Z21854). Subunit I of cytochrome *c* oxidases from *Thermus thermophilus* (cox1_thth, Swiss-Prot accession number P98005), *Pa. denitrificans* (cox1_pade, P98002), and *Bos taurus* (cox1_bota, P00396) and of quinol oxidases from *Sulfolobus acidocaldarius* (qox1_suac (SoxB), P98004, and soxm_suac (SoxM), P39481), *Bacillus subtilis* (qox1_basu, P34956), and from *Escherichia coli* (cyob_ecol, P18401). The alignments were generated using Clustal X (Higgins *et al.*, 1991) with manual adjustments, and the figure was drawn with Alscript (Barton, 1993).

Fig. 1a. Continued

other than the heme *c*-binding motif in NorC (Fig. 1). A small signal in the $g = 2$ region has been ascribed to a radical (Kastrau *et al.*, 1994) or the non-heme iron (Girsch and de Vries, 1997).

The CO difference spectra show the presence of a high-spin *b*-type heme (Fujiwara and Fukumori, 1996; Girsch and de Vries, 1997). Quantitation of the EPR visible hemes accounts for only two hemes/complex (Kastrau *et al.*, 1994; Girsch and de Vries, 1997), and the additional MCD measurements indicate that these have a *bis*-histidine (*b*-type) and histidine/methionine (*c*-type) ligation, respectively (Kannt *et al.*, 1997). These spectroscopic studies propose that the high-spin ferric *b*-heme has a histidine/hydroxide ligation and that it is antiferromagnetically coupled to the non-heme iron (Kannt *et al.*, 1997). However, the presence

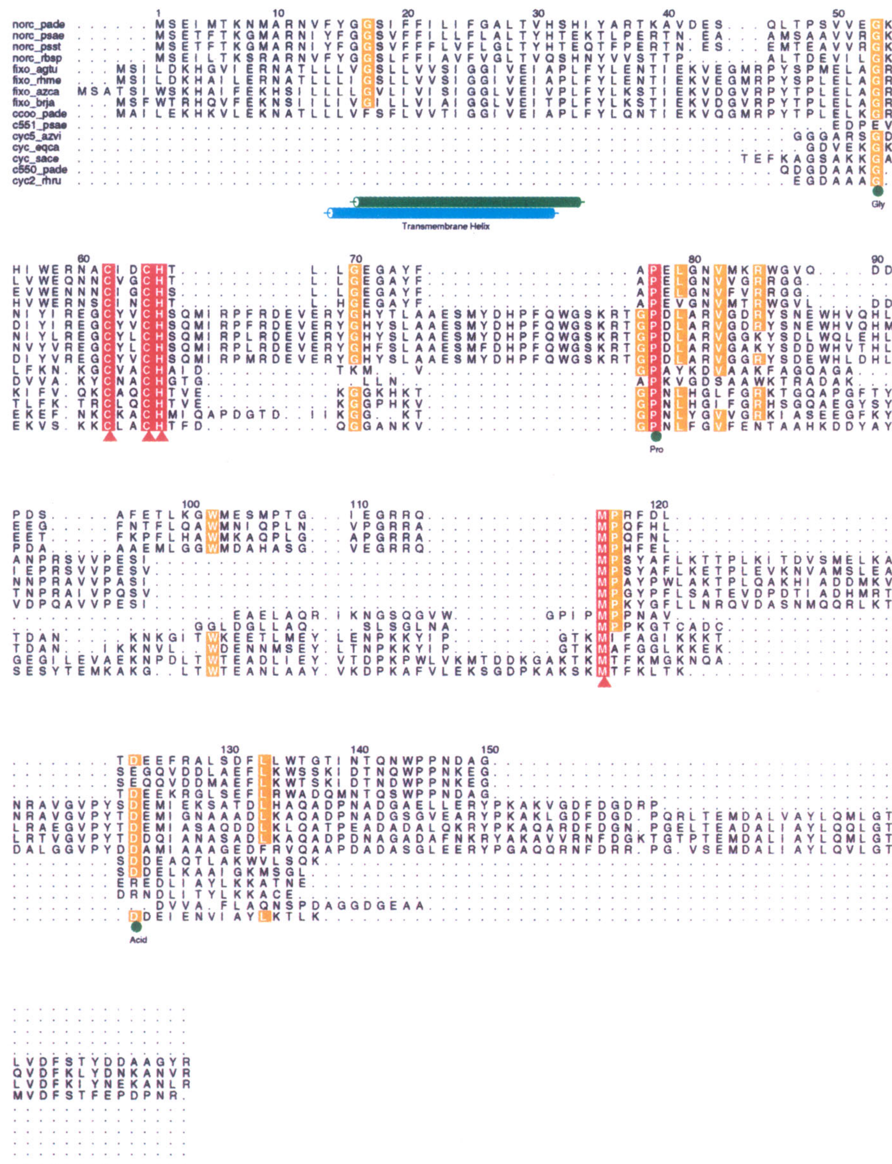


Fig. 1b. (b) Sequence alignment of NorC subunits with FixO subunits of the cytochrome cbb_3-type oxidases and a selection of type-I *c* cytochromes. Green and blue cylinders represent the transmembrane helices for NorC from *Pa. denitrificans* and the cbb_3-type oxidase of *B. japonicum*, respectively, as predicted by PHD (Rost *et al.*, 1995). Residues found in more than 50% of the aligned sequences are boxed in orange; conserved residues are boxed in red. Metal-binding sites and selected residues are marked (see text for explanation). The following sequences are aligned: NorC from *Pa. denitrificans* (norc_pade), *Ps. aeruginosa* (norc_psae), *Ps. stutzeri* (norc_psst), and *Rh. sphaeroides* (norc_rbsp). FixO subunits of the cbb_3-type oxidases are from *A. tumefaciens* (fixo_agtu), *R. meliloti* (fixo rhme), *A. caulinodans* (fixo_azca), and *B. japonicum* (fixo_brja, GenBank; for the accession numbers, see (a)). The included type-I cytochromes *c*'s are the monoheme cytochrome subunit (CcoO) of the cbb_3-oxidase of *Pa. denitrificans* (ccoo_pade, GenBank U34353), cytochrome c_{551} of *Ps. aeruginosa* (c551_psae, SwissProt P00099), and cytochrome c_5 from *A. vinelandii* (cyc5_azvi, P11732), as well as the mitochondrial cytochrome *c*'s from horse (cyc_eqca, P00004) and yeast (cyc_sace, P00044), and large bacterial cytochrome *c*'s cytochrome c_{550} of *Pa. denitrificans* (c550_pade, P00096), and cytochrome c_2 of *Rhodospirillum rubrum* (cyc2_rhru, P00092).

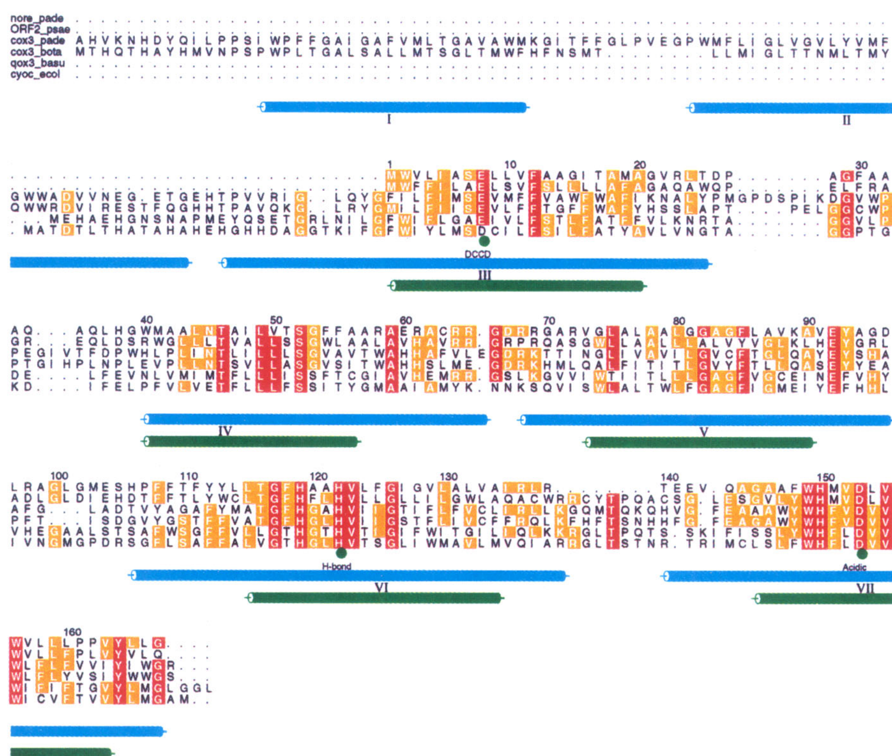
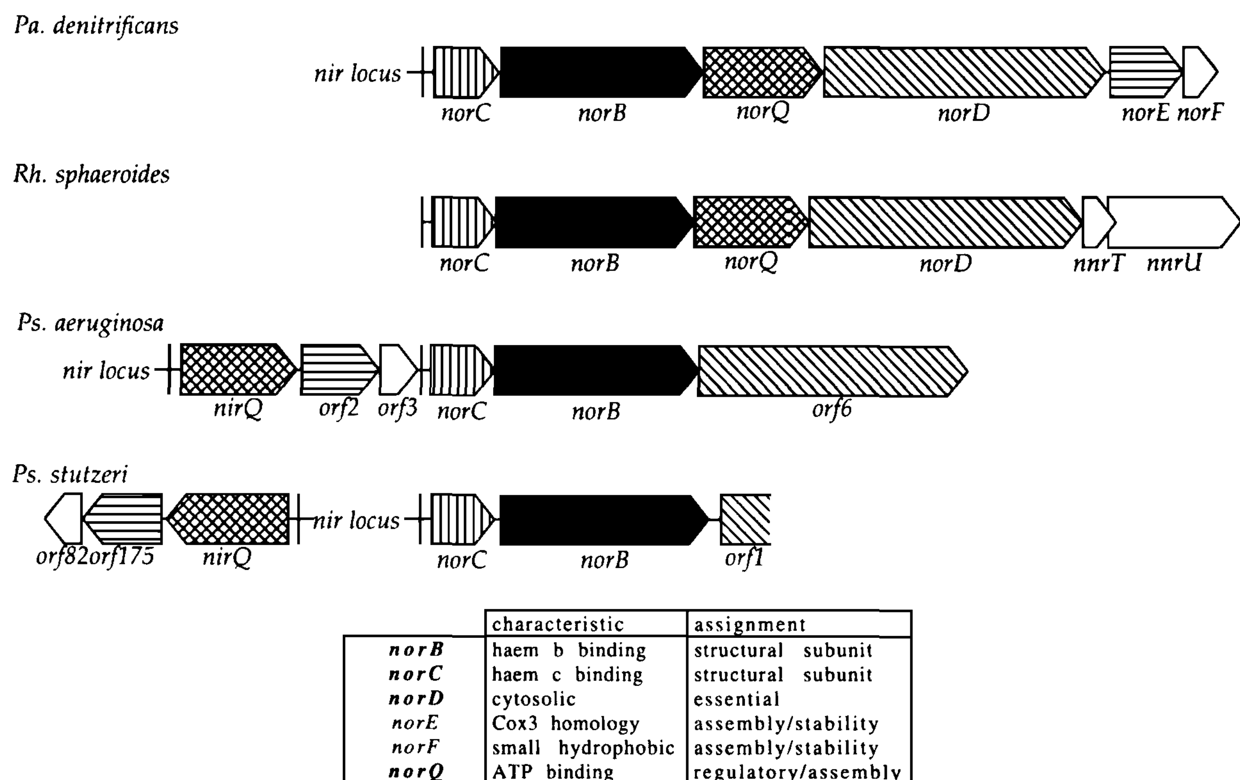


Fig. 1c. (c) Sequence alignment of subunit III from different cytochrome *c* oxidases and NorE. Blue cylinders represent the transmembrane helices of cytochrome *c* oxidase from *Pa. denitrificans* (Iwata *et al.,* 1995). Green cylinders represent the predicted transmembrane helices for NorE of *Pa. denitrificans* using PHD (Rost *et al.,* 1995). Residues found in more than 50% of the aligned sequences are boxed in orange; conserved residues are boxed in red and selected residues are marked (see text). The aligned sequences are NorE of *Pa. denitrificans* (norc_pade, GenBank accession number U28078), the open reading frame 2 which is found between the *nir* and *nor* clusters in *Ps. aeruginosa* (ORF2_psae, Arai *et al.,* 1994), the subunit III of cytochrome *c* oxidases of *Pa. denitrificans* (cox3_Pade, P06030) and *Bos taurus* (cox3_bota, P00415), and the corresponding subunit of the quinol oxidases of *B. subtilis* (qox3_basu, P34958) and *E. coli* (cyoc_ecol, P18402).

of the latter center needs further experimental verification. Along with the metal analysis, this evidence supports the idea that NOR has a heme/non-heme iron binuclear center rather than a heme/copper center. The chemistry of NO reduction may indeed require such an iron/iron site as NO would bind too tightly to a copper ion.

What would be the coordination chemistry of an iron/iron binuclear center? We may assume that the conserved histidines that coordinate the hemes and Cu_B in cytochrome oxidases have identical coordination functions in NOR. The specificity for binding an iron rather than a copper at the "Cu_B site" needs to be created by additional ligands which should be present in NorB. Based on the known structures of non-heme iron sites, one would expect additional coordination to at least one oxygenous ligand that should be close to the three histidine ligands of the canonical Cu_B site. Candidates for such a ligand are carboxylic acids, for instance, the glutamate in the middle of helix VIII (residue 267 in the alignment of Fig. 1a) and the glutamate (residue 198 in Fig. 1a) that is present one helical turn below the histidine ligand in helix VI. Both are peculiar to NO reductases and absent from all heme/copper cytochrome oxidases.

As a cytochrome complex, NOR is reminiscent of the cytochrome cbb_3-type oxidases, which indeed are its closest relatives in the superfamily (van der Oost *et al.,* 1994; Saraste *et al.,* 1996; Kannt *et al.,* 1997). NorC and FixO/CcoO subunits of the latter enzymes are similar membrane-bound mono-heme *c*-type cytochromes. However, apart from the heme-



	characteristic	assignment
norB	haem b binding	structural subunit
norC	haem c binding	structural subunit
norD	cytosolic	essential
norE	Cox3 homology	assembly/stability
norF	small hydrophobic	assembly/stability
norQ	ATP binding	regulatory/assembly

Fig. 2. Schematic discription of genomic loci that code for genes involved in nitric oxide reduction in *Paracoccus denitrificans* (de Boer *et al.*, 1996), *Rhodobacter sphaeroides* 2.4.3 (Bartnikas *et al.*, 1997), *Pseudomonas aeruginosa* (Arai *et al.*, 1994, 1995), and *Pseudomonas stutzeri* (Jüngst and Zumft, 1992; Zumft and Körner, 1997). Vertical lines indicate putative regulator binding sites. Patterns identify homologues genes. The lower part lists characteristics and functional assignment of the genes, using *Paracoccus* names. Essential genes are listed in bold.

attachment site and the axial ligands (a histidine and a methionine), these proteins do not show mutual conservation better than that observed between other cytochrome *c*'s (Fig. 1b).

MOLECULAR BIOLOGY OF NITRIC OXIDE REDUCTION

Molecular genetic studies on genes and operons that encode nitric oxide reductase activity in purple bacteria have shown that the action of several genes is necessary for this. Apart from NorB and NorC, it is currently not clear that any additional gene product might be a structural component of the complex. The genetic data rather suggests that there are at least four gene products which are required for the biosynthesis and assembly of the NOR complex (Fig. 2).

The DNA sequences of four *nor* loci are currently known. The locus invariably begins with the *norC* and *norB* genes. The open reading frame downstream of *norB* in Pseudomonads encodes a protein homologous to the *norD* gene product of *Paracoccus* and *Rhodobacter.* NorD does not contain hydrophobic spans nor a signal sequence, which suggests that it is located in the cytosol. The *norQ* gene that is located between *norB* and *norD* in *Paracoccus* and *Rhodobacter* is very similar to the *nirQ* genes found adjacent to the nitrite reductase (*nir*) locus in the *Pseudomonas* species. They encode a cytosolic protein that contains a P-loop ATP-binding motif (Fig. 2).

In *Paracoccus, norD* is followed by *norE,* which encodes a protein with homology to the subunit III of the heme-copper terminal oxidases. NorE has five transmembrane spans and contains the conserved acidic residues in the first and last hydrophobic span that are characteristics of the subunit III proteins (Fig. 1c). Until now, this protein has not been isolated in a complex with NorB and NorC.

The *norF* gene downstream of *norE* encodes a small protein with two predicted transmembrane helices. In *Pseudomonas,* an open reading frame with

homology to NorE is found downstream of *nirQ*. This *norE*-like gene is also followed by a short open reading frame encoding a small hydrophobic protein. In *Rhodobacter, norD* is directly followed by *nnrT,* again encoding a small protein with two hydrophobic regions. The next gene, *nnrU,* is predicted to have five transmembrane helices, but homology of its product to NorE is not clear.

A recognition site for a family of anaerobic transcription regulators is invariably found upstream of *norC,* implying that it is the transcription start site. Promotor deletion analysis confirms this (Bartnikas *et al,* 1997). *norC* and *norB* are transcribed as a single transcript of 2.0 kb in *Ps. stutzeri* (Zumft *et al,* 1994). This size combined with identification of possible stem and loop structures downstream of *norB* suggests that the operon consist only of *norCB.* In *Ps. aeruginosa,* however, the *norD*-like open reading frame downstream of *norB* is predicted to belong to the operon, since there is only a single base between the stop codon of *norB* and the start codon of *norD* (Arai *et al.,* 1995). In *Paracoccus,* all six genes in the *nor* locus probably form a single multicistronic transcript based on their very close packing and the absence of transcription termination signals (de Boer *et al.,* 1996), whereas in *Rhodobacter,* the two *nnr* genes are thought to have their own internal promotor (Bartnikas *et al,* 1997; see Fig. 2).

In *norB* mutants in *Ps. stutzeri,* which cannot grow by denitrification on nitrate (Braun and Zumft, 1991), the amount of NorC is reduced according to the immunoassays of cell extracts (Zumft *et al.,* 1994). This implies that either the NorC protein is unstable in the absence of NorB or the mutated transcript is degraded. Gene replacement mutants of *norE* or *norF* are able to grow under anaerobic conditions in the presence of nitrate. Heme-containing NorC protein is present at wild-type levels in their membranes, although the NOR activity is highly reduced *in vivo.* This suggests that NorE and NorF play a role in stabilization or assembly of the complex (de Boer *et al.,* 1996), which is similar to the role of the third subunit in the case of cytochrome oxidases (Haltia *et al.,* 1989).

Insertion or gene replacement mutants of *norC, B, Q,* or *D* in *Rhodobacter* (Bartnikas *et al.,* 1997) and *Paracoccus* (de Boer *et al.,* 1996) all are unable to grow on nitrate under anaerobic conditions, similarly to the case of *norD* mutants in *Ps. aeruginosa* (see Arai *et al.,* 1995). No NOR activity could be detected in any of the corresponding *Paracoccus* mutants *in vivo* when these are grown under microaerobic conditions. The amount of heme-containing NorC is highly reduced or virtually absent in the isolated membranes of these mutants (de Boer *et al,* 1996). In a *Ps. stutzeri nirQ* mutant, no NOR activity can be found, although the amount of NorB and NorC detected with antibodies in the total cell extract is doubled as compared to wild type (Jüngst and Zumft, 1992). In *Rhodobacter,* the inactivation of *norQ* also seems not to prevent *nor* expression (see Bartnikas *et al.,* 1997). These data indicate that *norD, norQ,* and *nirQ* are essential for *in vivo* NOR activity and suggest that *nirQ* and *norQ* play a regulatory role in maturation of NOR from translated proteins to a heme-containing, membrane-inserted complex.

NITRIC OXIDE REDUCTASE AND ENERGY CONSERVATION

Nitric oxide reductase catalyzes the reduction of NO to N_2O using cytochrome *c* as electron donor (Berks *et al.,* 1995). NOR is also able to reduce oxygen, although its affinity to O_2 is only in the millimolar range (Fujiwara and Fukumori, 1996). The midpoint potential difference for the reaction catalyzed by NOR is on the order of 1 V. Theoretically, this free energy could be used for work, such as proton pumping performed by the cytochrome oxidases. At the moment, however, the reaction catalyzed by NOR is not believed to be coupled to generation of protonmotive force at all. The protons consumed in this reaction are apparently taken from the periplasm (Fig. 3).

Three lines of evidence support this view (see Berks *et al.,* 1995). First, proton pumping experiments on whole cells of various denitrifiers using oxidant pulses match perfectly with the above-mentioned model, when either endogenous substrates or artificial electron donors are used (Shapleigh and Payne, 1985; Boogerd *et al.,* 1981). Second, the energization state of the *Rhodobacter* cell membranes, as measured by the absorbance shifts of membrane pigments, does not change upon NOR activity in the presence of artificial electron donors (Bell *et al.,* 1992). Third, identical ATP/2e yields have been reported for *Rh. capsulatus* when it is pulsed with NO or NO_3^- using NADH as electron donor (Carr *et al.,* 1989). Reduction of nitrate by NADH involves Complex I (NADH dehydrogenase) and nitrate reductase, which is known to cause charge separation of 2q/2e. Reduction of NO involves Complexes I and III (cytochrome bc_1 complex) and

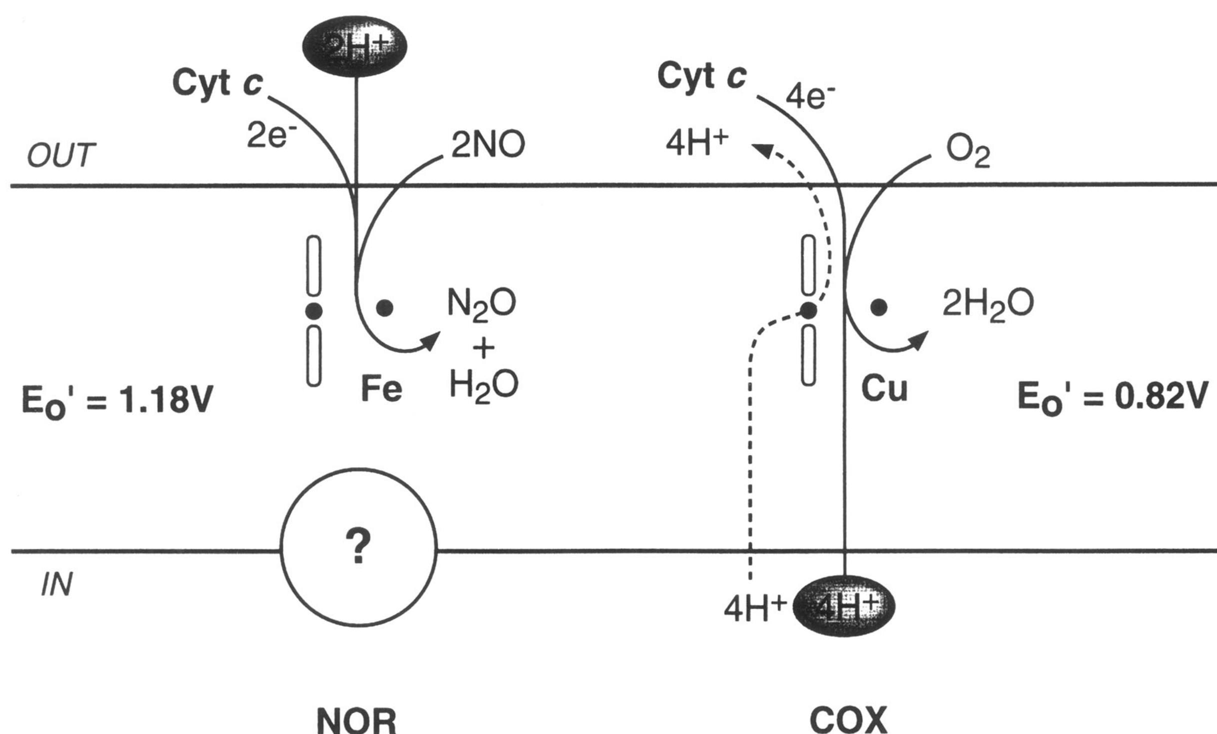


Fig. 3. Energetics of the nitric oxide reductase (NOR) in comparison with the vectorial catalysis of a proton-pumping cytochrome oxidase (COX). In both cases, cytochrome *c* is shown as the electron donor. The midpoint potentials for NO/N_2O and O_2/H_2O couples are indicated on the left and right side, respectively.

NOR. Since the bc_1 complex is coupled to charge separation of $2q/2e^-$, the identical $ATP/2e^-$ yields thus imply that NOR is not involved in charge separation.

Consistent with this view, the critical amino acid residues that are currently assigned to proton translocation in the heme/copper oxidases (Iwata *et al.*, 1995; Brzezinski and Ädelroth, 1997; Wikström *et al.*, 1997) are absent in NorB (Fig. 1a). For instance, the glutamate residue in helix VI, which is part of the conserved HPEVY motif at the end of the D channel, and the conserved hydrophilic residues that align helix VIII in the K channel are not present in NorB.

The lack of proton translocation, and indeed electrogenicity, in the reaction catalyzed by NOR is an intriguing paradox that requires further studies. These studies may help us to understand how its sister enzymes adapted to catalyze reduction of dioxygen to water by subtle changes in the active site, and how they readjusted the topology of vectorial catalysis, and evolved to proton pumps to maximize the energetic gain of the released chemical energy.

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