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Heterologous expression of *Halomonas halodenitrificans* nitric oxide reductase and its N-terminally truncated NorC subunit in *Escherichia coli*

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ABSTRACT

Halomonas halodenitrificans nitric oxide reductase (NOR) is the membrane-bound heterodimer complex of NorC, which contains a low-spin heme *c* center, and NorB, which contains a low-spin heme *b* center, a high-spin heme b_3 center, and a non-heme Fe_B center. The soluble domain of NorC, NorC* (ΔMet1–Val37) was heterologously expressed in *Escherichia coli* using expression plasmids harboring the truncated *norC* gene deleted of its 84 5′-terminal nucleotides. Analogous scission of the N-terminal helix as the membrane anchor took place when the whole *norC* gene was used. NorC* exhibited spectra typical of a low-spin heme *c*. In addition, NorC* functioned as the acceptor of an electron from a cytochrome *c* isolated from the periplasm of *H. halodenitrificans* and small reducing reagents. The redox potential of NorC* shifted ca. 40 mV in the negative direction from that of NorC. Unlike NorC, recombinant NorB was not heterologously expressed. However, recombinant NOR (rNOR) could be expressed in *E. coli* by using a plasmid harboring all genes in the *nor* operon, *norCBQDX*, from which the three hairpin loops (mRNA) were deleted, and by using the *ccm* genes for the maturation of C-type heme. rNOR exhibited the same spectroscopic properties and reactivity to NO and O_2 as NOR, although its enzymatic activity toward NO was considerably decreased. These results on the expression of rNOR and NorC* will allow us to develop more profound studies on the properties of the four Fe centers and the reaction mechanism of NOR from this halophilic denitrifying bacterium.

1. Introduction

Denitrification is a form of anaerobic respiration in which nitrate (NO_3^-) is converted to dinitrogen (N_2) via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) [1]. NO reductase (NOR) protects denitrifying bacteria from NO, which is highly toxic, by converting it to N_2O [2,3]. NORs have been isolated from the inner membrane of denitrifying bacteria such as *Pseudomonas stutzeri* [4], *Paracoccus denitrificans* [5–8], and *Pseudomonas aeruginosa* [9], and from a halophilic bacterium, *Halomonas halodenitrificans* (formerly *Paracoccus halodenitrificans*) [10], and a hyperthermophile, *Thermus thermophiles* [11]. *H. halodenitrificans* NOR, which belongs to the cNOR class, is the heterodimer complex of a smaller subunit containing a heme *c* (NorC), and a larger subunit containing hemes *b* (NorB). Other classes of NORs are classified based on molecular architecture and catalytic center [12–16].

NorC consists of a membrane anchor helix at the N-terminal end and a periplasmic domain that accommodates a low-spin heme *c* center axially coordinated by His and Met residues (His65 and Met115 in *H. halodenitrificans* [17]). NorC functions as the entry point for electrons from the soluble cytochrome *c* to NorB.

NorB is the membrane-spanning subunit. It contains 12 α-helices arranged in a topology analogous to that of subunit I of cytochrome oxidase (COX) [1,18–21]. The structural similarity between NOR and COX has led to the suggestion that the former is an ancestor of the latter [22–24]. NorB contains a low-spin heme *b*, a high-spin heme b_3, and a non-heme iron, Fe_B, which correspond, respectively, to the low-spin heme *a*, heme a_3, and Cu_B in subunit I of COX. A binuclear center is formed by the high-spin heme b_3 and Fe_B bridged with an oxo group, producing an absorption band at 595 nm that is characteristic of resting

Abbreviations: NOR, nitric oxide reductase; cNOR, cytochrome *c*-dependent NOR; NorC, cytochrome *c* subunit of NOR; NorC*, N-terminally truncated NorC (ΔMet1–Val37); NorB, cytochrome *b* subunit of NOR; COX, cytochrome oxidase; Ccm, cytochrome *c* maturation; rNOR, recombinant NOR; SL, stem-and-loop; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; trNorC, N-terminally truncated NorC (ΔMet1–Leu28); DegP, periplasmic endoprotease encoded by *degP*; DegQ, periplasmic endoprotease encoded by *degQ*.

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NOR [6,25–27]. In connection with this, it has been reported from a Mössbauer study of *Pseudomonas nautica* NOR that heme b_3 and Fe_B are bridged by a carboxylate group of a Glu residue located near the binuclear center and the electronic state of heme b_3 is low spin [28], while X-ray crystallography of *P. denitrificans* NOR revealed the oxo-bridged structure [22]. All amino acid residues involved in the coordination of the Fe centers are conserved in NorB and subunit I of COX, although NorB Fe_B is coordinated by three His residues that are conserved in COX Cu_B plus an extra Glu residue [1,16,22,29]. The electron donor for both NOR and COX has been supposed to be a soluble cytochrome *c*, migrating in the periplasm, which carries an electron to the low-spin heme *c* center in NOR and the Cu_A center in COX [22]. It has been proposed that subunit II of COX, in which the Cu_A center is the electron acceptor from the soluble cytochrome *c*, was formed from the fusion of genes for NorC and nitrous oxide reductase, which contains the binuclear Cu_A center [1,18,23,24].

In a previous study [17], we determined the complete nucleotide sequence of *H. halodenitrificans nor* operon, *norCBQDX*, which includes the open reading frames for NorC and NorB (Fig. 1). The *norQ* gene has an ATP-binding sequence motif coding for P-loop (A or G)XXXXGK(S or T) [18], and, accordingly, is believed to be involved in the construction of the tertiary or quaternary structure of NOR [1]. The *norD* gene (1944 bp), located 131 bp downstream of *norQ*, encodes a protein of 647 amino acids with a molecular mass of 73,558 Da. NorD is assumed to be a hydrophilic protein, the C-terminal region of which corresponds to the type A domain of the von Willebrand factor, a eukaryotic metal-binding motif [30]. Therefore, NorD is presumed to be a metal chaperon or a scaffold protein for the construction of the metal centers. The *norX* gene (219 bp), located downstream of *norD* by 39 bp, encodes a protein of 82 amino acids with a molecular mass of 8013 Da. NorX is assumed to be a membrane-bound protein with two transmembrane helices, but has not been characterized in detail in *Halomonas*, *Thioalkalivibrio*, or *Alkalilimnicola*. The *nor* operon (total nucleotide sequence available in gene banks, accession code AB10889) has been supposed to contain the four stem-and-loop (SL) regions, of which SL1 is located between *norB* and *norQ*. Therefore, the *norCB* genes might be transcribed as a two-cistron operon [31,32].

In addition to the gene structure of *H. halodenitrificans* NOR [17], we have characterized the metal centers in NOR using absorption, magnetic circular dichroism (MCD), and electron paramagnetic resonance (EPR) spectra [10,33–35]. Here, we report our attempts to heterologously express NOR (rNOR) and its subunits NorB and NorC in *Escherichia coli* with the aim of elucidating the structure-function relationships of *H. halodenitrificans* NOR. For the expression of NorC, we used both the complete *norC* gene and a truncated gene (*trnor*C), designed to produce the putative soluble domain without the N-terminal membrane anchor (ΔMet1–Leu28).

2. Materials and methods

2.1. Construction of the expression vectors for NorC, NorC*, NorB, NOR, and Ccm

The expression vector (pTAZ-trNorC) for trNorC, which is NorC with Met1 to Leu28 deleted, was constructed by cloning *trnor*C; the azurin signal sequence was attached to the 5′ terminal end to localize trNorC in the periplasmic space (Supplementary Fig. 1). The expression vectors for NorC (p*Trc*-NorC) and NorB (p*Trc*-NorB) were constructed by inserting the *norC* or *norB* gene, respectively, into p*Trc*99A (Pharmacia). The expression vector for NOR (pTVNORdSL) was constructed by cloning *norCBQDX*, from which the three potential SL regions (SL1, SL2, and SL3) were deleted, into the *lacZα* gene in pTV118N (Takara Bio, Fig. 1).

The 6.5-kb fragment containing the operon for cytochrome *c* maturation (*ccmABCDEFGH*) was amplified from the genomic DNA of the strain derived from *E. coli* K-12, using synthetic primers, by Advantage GC Genomic PCR kit (Clontech). The PCR product was cloned using the TOPO XL PCR cloning kit and *E. coli* TOP10 cells (Invitrogen). A 6.6-kb *Eco*RI-*Eco*RI insert was re-cloned into pSTV28 (Takara Bio) (pSTVccm).

2.2. Transformation and cultivation of E. coli

E. coli JCB7120, a strain suitable to express C-type heme (gift from Prof. J. Cole, Birmingham Univ.), was transformed with pTAZ-trNorC harboring the *trnorC* (ΔMet1–Leu28) gene, which attached the coding sequence of secretion signal for azurin at its 5′-terminal, or p*Trc*-NorC harboring the whole *norC* gene. *E. coli* JCB7120 was also transformed with p*Trc*-NorB harboring the *norB* gene. For expression of rNOR, *E. coli* DH10B, which is suitable to maintain a large plasmid, was transformed with pTVNORdSL, together with pSTVccm harboring *ccm* genes. Transformants were cultured as reported previously [10,33–35].

2.3. Purification of NorC*, NorB, and rNOR

NorC*, N-terminally truncated NorC (ΔMet1–Val37) was purified from the periplasmic fraction of the *E. coli* JCB7120 transformants using Toyopearl DEAE 650M (TOSOH), Sephadex G-75 (Pharmacia), and UnoQ-12 (Bio-Rad) columns. NorB and rNOR were purified as reported previously [10,33–35].

2.4. Analytical methodologies and measurements

Protein concentration was determined according to the bicinchoninic acid method with bovine serum albumin as standard. A polyclonal antiserum against the purified NorC* was produced in rabbit

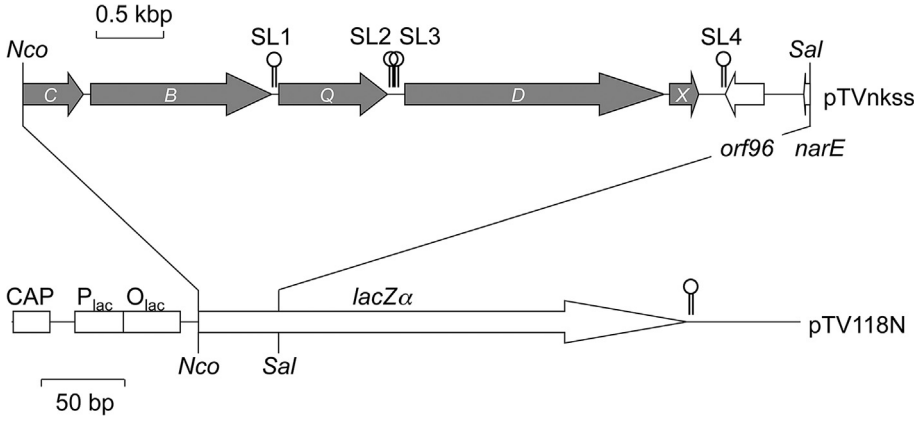

Fig. 1. The *nor* gene cluster of *Halomonas halodenitrificans* cloned into pTV118N. The direction of transcription is shown by the boxed arrows. *Orf96* and part of the *narE* gene in *narXLK2K1.1* gene cluster (Accession no. AB076402) are attached downstream of the *nor* gene. SL1–4 represent the stem-and-loop regions.