BBA - Bioenergetics 1859 (2018) 333–341

Contents lists available at ScienceDirect

BBA - Bioenergetics

journal homepage: www.elsevier.com/locate/bbabio

Pseudomonas aeruginosa overexpression system of nitric oxide reductase for *in vivo* and *in vitro* mutational analyses

Raika Yamagiwa[a,b], Takuya Kurahashi[a], Mariko Takeda[a], Mayuho Adachi[a], Hiro Nakamura[c], Hiroyuki Arai[d], Yoshitsugu Shiro[a,b], Hitomi Sawai[a,b,*], Takehiko Tosha[b,**]

[a] Graduate School of Life Science, University of Hyogo, Hyogo 678-1297, Japan
[b] RIKEN SPring-8 Center, Kouto, Sayo, Hyogo 679-5148, Japan
[c] Division of Structural and Synthetic Biology, RIKEN Center for Life Science Technologies, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
[d] Department of Biotechnology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

ARTICLE INFO

Keywords:
Nitric oxide reductase
Heme
Heme copper oxidase superfamily
Nitric oxide
Pseudomonas aeruginosa
Proton transfer

ABSTRACT

Membrane-integrated nitric oxide reductase (NOR) reduces nitric oxide (NO) to nitrous oxide (N_2O) with protons and electrons. This process is essential for the elimination of the cytotoxic NO that is produced from nitrite (NO_2^-) during microbial denitrification. A structure-guided mutagenesis of NOR is required to elucidate the mechanism for NOR-catalyzed NO reduction. We have already solved the crystal structure of cytochrome *c*-dependent NOR (cNOR) from *Pseudomonas aeruginosa*. In this study, we then constructed its expression system using cNOR-gene deficient and wild-type strains for further functional study. Characterizing the variants of the five conserved Glu residues located around the heme/non-heme iron active center allowed us to establish how the anaerobic growth rate of cNOR-deficient strains expressing cNOR variants correlates with the *in vitro* enzymatic activity of the variants. Since bacterial strains require active cNOR to eliminate cytotoxic NO and to survive under denitrification conditions, the anaerobic growth rate of a strain with a cNOR variant is a good indicator of NO decomposition capability of the variants and a marker for the screening of functionally important residues without protein purification. Using this *in vivo* screening system, we examined the residues lining the putative proton transfer pathways for NO reduction in cNOR, and found that the catalytic protons are likely transferred through the Glu57 located at the periplasmic protein surface. The homologous cNOR expression system developed here is an invaluable tool for facile identification of crucial residues *in vivo*, and for further *in vitro* functional and structural studies.

1. Introduction

Nitrous oxide (N_2O), commonly known as laughing gas, is a greenhouse gas that is 310 times more potent than CO_2 [1]. It also is an ozone-depleting gas [1]. The largest source of global N_2O emissions is microbial denitrification, which is a type of anaerobic respiration that is a step-wise reduction process from NO_3^- to N_2 (*via* NO_2^-, NO and N_2O) catalyzed by four metalloenzymes. In bacterial denitrification, N_2O is produced from nitric oxide (NO) *via* the catalysis of membrane-integrated nitric oxide reductase (NOR) according to the following Eq. (1).

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \quad (1)$$

The NO reduction reaction is catalyzed at the heme/non-heme iron (Fe_B) binuclear active center of NOR.

Based on the homology of amino acid sequences, NOR belongs to a heme copper oxidase (HCO) superfamily [2–4], which is responsible for the aerobic respiration of living systems *via* the four-electron reduction of O_2 at the heme/Cu binuclear active center ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). It is noteworthy that O_2-reducing oxidases pump protons across the membrane to generate an electrochemical gradient for ATP synthesis. On the other hand, despite being a member of the HCO superfamily, the most extensively studied cytochrome *c*-dependent NOR (cNOR) does not pump protons and does not generate an electrochemical gradient [5]. Such functional differences in the enzymes belonging to the HCO superfamily are believed to be related to the

Abbreviations: NOR, nitric oxide reductase; HCO, heme copper oxidase; cNOR, cytochrome *c* dependent NOR; IPTG, isopropyl-1-thio-β-D-galactopyranoside; DDM, dodecyl-β-D-maltoside; AAS, atomic absorption spectroscopy; PMS, phenazine methosulfate; qNOR, quinol-dependent NOR; MD, molecular dynamics

* Correspondence to: RIKEN SPring-8 Center, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan.
** Corresponding author.
E-mail addresses: sawai@sci.u-hyogo.ac.jp (H. Sawai), ttosha@spring8.or.jp (T. Tosha).

https://doi.org/10.1016/j.bbabio.2018.02.009
Received 27 October 2017; Received in revised form 18 February 2018; Accepted 24 February 2018
Available online 27 February 2018
0005-2728/

molecular evolution of respiratory enzymes. Therefore, cNOR has been a good target for examinations into both the mechanisms of catalytic N_2O generation and the molecular evolution of respiratory enzymes. These studies will provide insight into the changes in reactivity that range from NO reduction to O_2 reduction, and into how the proton pumping capability is acquired.

We presented the first crystal structure of cNOR from *Pseudomonas aeruginosa* [6]. cNOR consists of two subunits, a main transmembrane NorB subunit (53 kDa) and a membrane-anchored NorC subunit (17 kDa). The NorB subunit contains low-spin heme *b* and a binuclear active center composed of a high-spin heme b_3 and non-heme Fe_B. The globular hydrophilic domain in the NorC subunit contains heme *c* as an electron entry site. Consistent with the previous view that cNOR has no proton-pumping ability, no proton-transfer pathway from the cytoplasmic side is obvious in the crystal structure. Instead, two possible pathways from the periplasmic side for the transfer of catalytic protons have been proposed based on the structure of *P. aeruginosa* cNOR.

Structure-guided mutational studies are required to elucidate the specific role of each amino acid residue and the structure/function relationships in cNOR. Since the establishment of the *Escherichia coli* overexpression system, almost all the mutational work has been performed using *Paracoccus denitrificans* cNOR [5,7–10]. Before the crystal structure of *P. aeruginosa* cNOR was solved, the Richardson's and Watmough's groups focused on five well-conserved Glu residues based on the amino acid sequence alignment, and examined their mutational effect on the function of *Pa. denitrificans* cNOR [8]. More recently, based on the crystal structure of *P. aeruginosa* cNOR both Ädelroth and co-workers and Gennis and co-workers have prepared several variants of cNOR from *Pa. denitrificans* and *Thermus thermophilus*, respectively, using *E. coli* overexpression systems [9–12]. The amino acid sequence identities of their cNOR with *P. aeruginosa* cNOR are 73% of the cNOR from *Pa. denitrificans* in the Proteobacteria phylum, which is the same phylum of *P. aeruginosa*, and as low as 32% of the cNOR from *T. thermophilus* in the Deinococcus-thermus phylum. Therefore, to elucidate the structure-function relationship in cNOR, it is more desirable to establish the expression system of *P. aeruginosa* cNOR, which has an available crystal structure. However, there was no suitable expression system producing the site-directed variants of *P. aeruginosa* cNOR, and the functional studies based on structure are limited. In order to overcome this issue, we established a homologous expression system for *P. aeruginosa* cNOR. The expression system allowed us to obtain the site-directed variants and to carry out their functional characterization *in vitro*. Using the expression system, we also found that the anaerobic bacterial growth of the cNOR-deficient strain expressing the cNOR variant is a good marker that can be used to explain the functional importance of a mutated residue. Comparing the results from *in vivo* screening with those of *in vitro* characterization showed that the *in vivo* method is useful for a facile screening of important residues in the cNOR function and structure. By using this newly established screening system, we examined the proton transfer pathway in *P. aeruginosa* cNOR.

2. Materials and methods

2.1. Construction of a system for the expression of recombinant wild-type and variants of P. aeruginosa cNOR

The plasmids and bacterial strains used in this study are listed in Table 1. For the expression of cNOR in *E. coli*, DNA fragments containing *norCBD* and *nirQOP* from the *P. aeruginosa* DNA were cloned into the EcoRI-HindIII and NdeI-PacI sites of pET-Duet-1 (Novagen), respectively, which is referred to here as pDuetnor (Fig. 1). For the expression of cNOR in *P. aeruginosa*, we chose a broad-host-range plasmid, pMMB67EH [13], as a vector because previous reports indicate that pMMB67EH can work as an expression vector in *P. aeruginosa* under denitrification conditions [14]. We excised *norCBD* with an N-terminal His-tag in NorC *via* XbaI and HindIII from pDuetnor and cloned it into the multicloning site of pMMB67EH, which is referred to here as pMMnor-His-CBD (Fig. 1). For the construction of an alternative expression vector, a *norCB* fragment was inserted into the EcoRI and HindIII sites of pMMB67EH. Then, a HRV3C protease cleavable site (LEVLFQGP) and a 6 × His-tag were fused at the C-terminus of NorB *via* PCR. This clone is referred to here as pMMnor-CB-His (Fig. 1). Point mutations were introduced by QuikChange site-directed mutagenesis protocol (Agilent) using PrimeStar GXL DNA polymerase (TAKARA BIO INC). Synthetic DNA primers were purchased from Thermo Fisher Scientific. The DNA sequences were confirmed using a DNA sequencing service provided by a Support Unit for Bio-Material Analysis located at the RIKEN Brain Science Institute.

Table 1
Plasmids and bacterial strains used in this study.

Plasmid or strain	Relevant characteristic	Reference or source
Plasmid		
pET-Duet-1	Cloning vector, Ap^r	Novagen
pDuetnor	*norCBD*, *nirQOP* in pET-Duet-1	This study
pMMB67EH	Broad-host-range vector, Ap^r/Cb^r	[13]
pMMnor-His-CBD	*norCBD* in pMMB67EH	This study
pMMnor-CB-His	*norCB* in pMMB67EH	This study
Strain		
PAO1	Used as wild-type *P. aeruginosa*	[31]
RM495	*norCBD*::*tet* (*norCBD* deleted PAO1)	[14]
RM476	*norCB*::*tet* (*norCB* deleted PAO1)	This study

Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance.

2.2. Expression and purification of recombinant cNOR in E. coli

For the expression of recombinant cNOR in *E. coli*, the *E. coli* C43(DE3) cells containing pDuetnor and pEC86 [15], the latter of which contains cytochrome *c* assembly (*ccm*) genes, were grown in LB medium with 50 μg/mL ampicillin and 3 μg/mL chloramphenicol at 37 °C. Expression was induced by the addition of 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) after 4 h cultivation. The membrane fractions were solubilized in the presence of 1% (w/v, final concentration) dodecyl-β-D-maltoside (DDM). The solubilized fractions were obtained by ultracentrifugation (185,300 × *g* for 30 min), and were loaded on a His-tag affinity column pre-equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% DDM) containing 10 mM imidazole. Non-specifically bound proteins were washed with buffer A containing 30 mM imidazole, and the cNOR fractions were eluted by buffer A containing 150 mM imidazole. An eluted sample was further purified using a size exclusion Superdex 200 Increase column (GE Healthcare Bioscience). The protein purity was evaluated by SDS-PAGE.

2.3. Anaerobic growth of P. aeruginosa for evaluation of cNOR expression and in vivo screening

An *norCB*-deficient mutant strain of *P. aeruginosa*, RM476 (PAΔ*norCB*), was constructed by replacing a 1.3-kb SacI-PstI-PstI chromosomal region, which contains portions of *norCB*, with a tetracycline resistance gene, tet^r, according to a previously described method [16]. The cNOR expression vector pMMnor-CB-His and pMMnor-His-CBD were introduced into PAΔ*norCB* and PAΔ*norCBD* (*norCBD*-deficient strain), respectively, *via* electroporation using a MicroPulser (Bio-Rad), and the transformed cells were grown on an LB agar plate containing 200 μg/mL carbenicillin at 37 °C [17]. Colonies were inoculated into 2 mL of LB medium and bacteria were grown aerobically at 37 °C with shaking at 150 rpm for 24 h. Anaerobic culture was then performed in an 8 mL test tube containing 5 mL of LB medium, 150 μg/mL carbenicillin, and 50 mM potassium nitrate. Prior to the anaerobic culture, the test tube was sealed with a rubber septum, and