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Review

Molecular structure and function of bacterial nitric oxide reductase ☆

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ABSTRACT

The crystal structure of the membrane-integrated nitric oxide reductase cNOR from *Pseudomonas aeruginosa* was determined. The smaller NorC subunit of cNOR is comprised of 1 trans-membrane helix and a hydrophilic domain, where the heme *c* is located, while the larger NorB subunit consists of 12 trans-membrane helices, which contain heme *b* and the catalytically active binuclear center (heme b_3 and non-heme Fe_B). The roles of the 5 well-conserved glutamates in NOR are discussed, based on the recently solved structure. Glu211 and Glu280 appear to play an important role in the catalytic reduction of NO at the binuclear center by functioning as a terminal proton donor, while Glu215 probably contributes to the electro-negative environment of the catalytic center. Glu135, a ligand for Ca^{2+} sandwiched between two heme propionates from heme *b* and b_3, and the nearby Glu138 appears to function as a structural factor in maintaining a protein conformation that is suitable for electron-coupled proton transfer from the periplasmic region to the active site. On the basis of these observations, the possible molecular mechanism for the reduction of NO by cNOR is discussed. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Bacterial nitric oxide reductase is a membrane-integrated enzyme that catalyzes the reduction of nitric oxide NO to nitrous oxide N_2O based on the following reaction; $2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$. NOR is involved in bacterial denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), which is a type of anaerobic respiration, in which cyto-toxic NO is immediately decomposed after its production from nitrite NO_2^-, a reaction catalyzed by nitrite reductase [1,2]. In addition, some pathogens utilize NOR to evade destruction by the immune system, as macrophages in mammals use NO as a chemical reagent against infectious agents [3]. In addition to the biological significance of this process, it would be interesting to understand how the N–O bond is cleaved and how the N–N bond is formed in the enzymatic reaction by NOR from a chemical point of view. Furthermore, NOR is important in environmental science studies, since the reactant NO is a type of air pollutant NOx, and the product N_2O is not only a greenhouse gas that is more powerful than carbon dioxide CO_2 by 300 times but is also a main contributor to the depletion of the ozone layer [4]. The largest fraction (~70%) of the N_2O emission on earth is the result of the bacterial breakdown of nitrogen oxides. From these points of view, the structure and function of bacterial NOR should be elucidated at the molecular level.

2. Bacterial nitric oxide reductases

Since the first evidence for the NOR activity in the cellular membrane was obtained from *Alcaligenes faecalis* IAM 1015 in 1971 [5], NOR activities have been reported for *Pseudomonas stutzeri*, *Rhodobacter sphaeroides* IL 106, *Halomonas halodenitrificans* and *Paracoccus denitrificans*. Three types of NORs have been reported thus far (Fig. 1) [6–10]. The first isolated NOR was cNOR which consists of two subunits, namely NorB and NorC. The NorC subunit contains a heme *c* with His and Met residues as axial ligands, and accepts electrons from an external electron donor (such as cytochrome c_{551} or blue-copper azurin) [11–16]. The larger subunit of cNOR, NorB, contains three iron (Fe) centers [17–23], *i.e.*, two *b*-type hemes and one non-heme Fe (Fe_B). The low spin *b*-type heme (heme *b*) mediates the transfer of electrons from heme *c* in the NorC subunit to the binuclear catalytic center constructed from another *b*-type heme (heme b_3) and Fe_B.

A different type of NOR is a single subunit enzyme that contains an N-terminal extension relative to the NorC subunit of cNOR. This type of NOR was found in *Ralstonia eutropha H16* (a gram-negative soil bacterium) [24], *Pyrobaculum aerophilum* (an archaeum) [25], *Synechocystis* sp. Strain PCC6803 (a cyanobacterium) [26], *Neisseria gonorrhoeae* and *Neisseria meningitidis* [27,28]. These NORs are designated qNORs, since a quinol moiety acts as an electron donor in place of the soluble cytochrome *c* in cNOR. Electron(s) from the quinol are transferred to the heme *b* group and subsequently to the same binuclear catalytic center found in cNOR. While cNOR is found only in denitrifying bacteria, many of types of qNOR have been reported in non-denitrifying intracellular pathogens such as *N. gonorrhoeae*. Therefore, the physiological role

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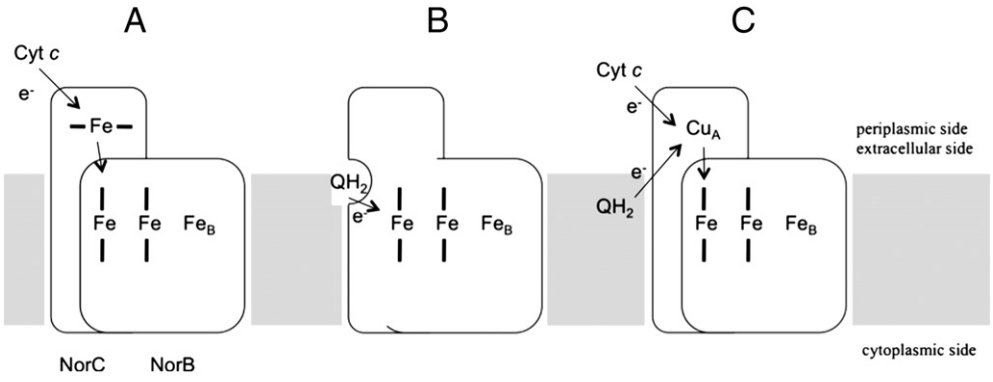


Fig. 1. Schematic models of three NOR: cNOR, qNOR and qCu$_A$NOR.

of qNOR in pathogenic organisms is related to the detoxification of NO that is produced by the defense system of the host organism [3,6,29].

The third type of NOR is qCu$_A$NOR, which can accept electrons from both cytochrome *c* and a quinol moiety [30,31]. The unique feature of qCu$_A$NOR is that the smaller subunit of the enzyme contains two copper atoms (Cu$_A$) like cytochrome oxidase and the amino acid sequence of the N-terminal 15 residues in the small subunit has a 65% identity to the corresponding portion of the cytochrome bo_3 oxidase subunit II. The larger subunit of qCu$_A$NOR contains Fe$_B$ and two heme *b* as is also found in qNOR and NorB of cNOR.

3. Cytochrome *c* dependent NOR (cNOR)

Of these three NORs, the most extensively studied enzyme is the cytochrome *c* dependent NOR, so-called cNOR. The first of this class of cNOR was isolated from *P. stutzeri* [18]. Since then, several cNORs from other bacteria have been purified and characterized by biochemical, molecular biological, spectroscopic and chemical techniques, in attempts to understand their structural and functional properties [7,9,19,21,32–39]. In 1994, NOR was classified as a member of the heme-cooper oxidase (HCO) super-family of proteins [40,41], on the basis of the similarity of the amino acid sequence of the NorB subunit of cNOR to the catalytic subunit of cytochrome oxidases (COX), an aerobic respiratory enzyme that catalyzes the reduction of O_2 ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). NOR is currently believed to share a common ancestor with COX. Regarding the molecular evolution of the respiratory enzyme, one scenario is that the NO reducing enzyme NOR under anaerobic conditions might have been functionally converted into the O_2 reducing enzyme COX, presumably through several structural modifications after the emergence of O_2 on the earth (about 3 billion years ago) [40,42]. However, the opposite scenario, in which NOR could have evolved from COX, is also a possibility, based on the pattern of the distribution of COX and NOR [43,44].

Two equivalent protons and electrons are required in the reaction catalyzed by NOR. The first evidence for this was obtained for the periplasmic consumption of protons during the NO reduction by cNOR in 1985 [45]. The bioenergetics of succinate-dependent NO respiration in membrane vesicles of *Rhodobacter capsulatus* was studied using a carotenoid to monitor the membrane potential [14]. Upon inhibition of the cytochrome bc_1 complex with myxothiazole, both the succinate-dependent NO reduction and the generation of a transmembrane potential are abolished, but NOR activity was restored with ascorbate and the mediator TMPD (N,N,N′,N′-tetramethyl-*p*-phenylenediamine), which donates electrons directly to NOR, but a transmembrane potential was not generated. The result suggested that NOR is non-electrogenic, that is, electrons and protons utilized in the catalytic NO reduction are supplied from the same (periplasmic) side of the membrane. This suggestion was supported by another experiment, in which no electrochemical gradient was detected during the reduction of NO by the cNOR reconstituted into liposomes [39,46]. Proton uptake from the periplasmic side was proposed, based on these electrometric and flow flash experiments for cNOR, but was not established on a structural basis.

When the amino acid sequences of several NORs were compared with one another and with those of COXs, it was found that 6 specific His residues are highly conserved in both NORs and COXs, whereas 5 Glu residues are conserved in NORs, but not in COXs [7,40,41,47–50]. In addition, homology models of the NorB subunit were constructed using either the amino acid sequence of *P. stutzeri* (*P.s.*) cNOR NorB and the coordinates of *P. denitrificans* (*Pa.d.*) cytochrome aa_3 oxidase, or the amino acid sequence of *Pa.d.* cNOR NorB and the coordinates of *R. sphaeroides* cytochrome aa_3 oxidase [7,39]. Both models are comprised of 12 trans-membrane helices, and the heme *b* and the binuclear center (heme b_3 and Fe$_B$) contain 5 His imidazole groups as their ligands are buried in this helix bundle. In these models, the pathway for catalytic protons to the binuclear center is initiated with two conserved Glu residues on the periplasmic face of the enzyme (Glu122 and Glu125 for *Pa.d.* cNOR), and terminate in the region of Glu198 and Glu267, both of which were predicted to be located in close proximity to the active site [39,49–52].

4. *Pseudomonas aeruginosa* cNOR

Fukumori and co-workers first isolated cNOR from the membrane fraction of *P. aeruginosa* (*P.a.*) that was cultivated under anaerobic conditions [19]. The NO reduction activity of *P.a.* cNOR, as measured by the conventional method using a combination of ascorbate and PMS (phenazine methosulfate), was found to be 350 μM-NO/min/μM-enzyme (at [NO] = 10 μM), which is comparable to those of other cNORs, such as the enzymes isolated from *P.s.* and *Pa.d.*, both of which have been extensively studied. The amino acid sequence of *P.a.* cNOR is highly homologous to those of *P.s.* and *Pa.d.* cNORs; the homologies of 95 and 94% for NorC and NorB of *P.s.* cNOR, respectively, and 72 and 75% for NorC and NorB of *Pa.d.* cNOR, respectively. Five conserved Glu residues are also present in *P.a.* cNOR. The optical absorption spectra of *P.a.* cNOR in the fully oxidized, fully reduced and CO-bound forms are shown in Fig. 2A. Comparison on the biochemical and spectroscopic properties suggests that the overall and active site structure of *P.a.* cNOR is similar to those of *P.s.* and *Pa.d.* cNORs.

In 2010, we determined the crystal structure of *P.a.* cNOR in the form of the complex with its monoclonal antibody Fab (Fig. 3) [53]. The antibody Fab interacts with the NorC subunit through electrostatic, hydrogen bonding and hydrophobic interactions, as illustrated in Fig. 4. Numerous water molecules mediate the hydrogen-bonding interactions, while direct hydrogen-bonding was observed as follows; Asn105 (NorC)–Lys31 (Fab light chain) and Arg109 (NorC)–Asp102 (Fab heavy chain)–Thr425 (NorB). Side chains of Trp98, Ala97 and Phe94 in NorC participate in hydrophobic interactions with the phenyl group of Phe56 in the light chain of Fab with distance of 3.8–4.0 Å. In solution, the antibody reversibly inhibits the NO reduction activity of

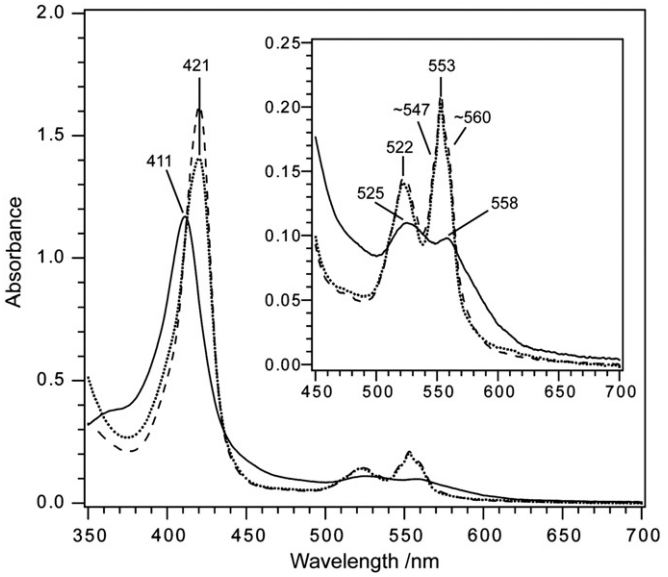


Fig. 2. Optical absorption spectra of *P. aeruginosa* cNOR: resting oxidized state (Fe^{3+}); solid line, dithionite-reduced state (Fe^{2+}); dotted line, and reduced-CO; broken line. The spectra were obtained from 4 μM cNOR in 50 mM Tris buffer at pH 8.0 containing 0.05% DDM.

P.a. cNOR when azurin was used as an electron donor, while the complex is active in the presence of ascorbate and PMS, which directly donates electrons to the active site. This result indicates that *P.a.* cNOR interacts with the antibody in a manner similar to that with proteinous electron donors. For example, the mutation of Asn105 or Arg109 in NorC might result in the loss of NO reduction activity via the inhibition of interactions with the electron donor.

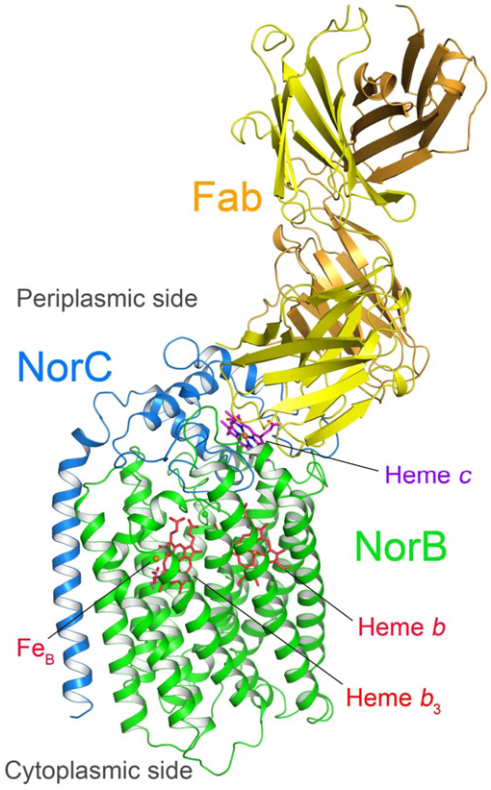


Fig. 3. Structure of *P. aeruginosa* cNOR–Fab complex. Fab recognizes the soluble domain (periplasmic region) of cNOR.

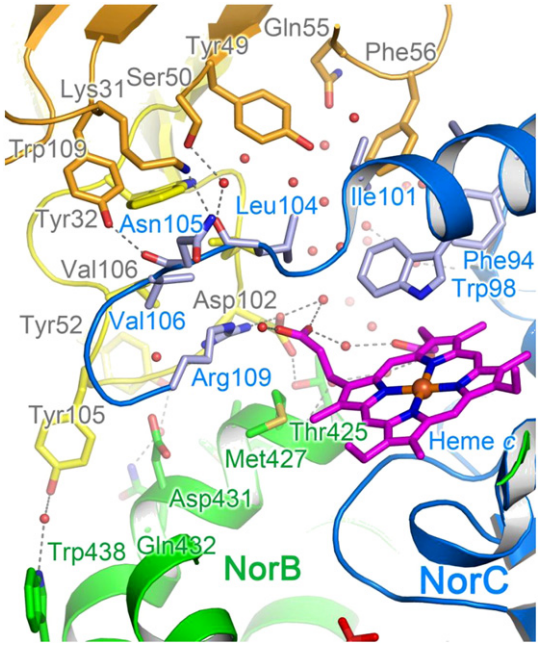


Fig. 4. Interaction between cNOR and Fab. Residues involved in the interaction are shown in a stick model. Interactions are mainly through water-mediated hydrogen bonds. The propionate of heme *c* is involved in the hydrogen-bonding network. In addition, hydrophobic residues and polar and charged residues are also involved in the direct interaction between cNOR and Fab. Light chain and heavy chain of Fab are shown in orange and yellow, respectively. Subunits NorC and NorB are shown in blue and green, respectively. The water molecules are shown as small red spheres.

5. Conserved glutamic acids in *P. aeruginosa* cNOR

As predicted from homology models, the NorB subunit of cNOR consists of 12 trans-membrane helices, and the heme *b* and the binuclear center (heme b_3/Fe_B) are buried in the hydrophobic interior of the trans-membrane region. Among the 6 well-conserved His residues, His60 and His349 serve as axial ligands of heme *b* in the six-coordinate low spin state, His347 is the fifth ligand of heme b_3, and His207, His258, His259 serve as ligands of non-heme Fe_B, in good agreement with previous homology models. On the other hand, the location and putative function of the 5 well-conserved Glu residues are not completely consistent with predictions based on the homology model. Therefore, we mapped the Glu residues on the structure of the *P.a.* cNOR NorB subunit in Fig. 5, and their possible function is discussed, in relation to the catalytic NO reduction reaction of cNOR, based on the structure.

5.1. Glu211

Glu211 is one of the ligands of Fe_B, with a distance of 2.0 Å between Fe_B and the carboxylate oxygen (O) atom. Three conserved His imidazoles (His207, His258, His259) and possibly the oxygen (O) atom of either water or a hydroxide are also ligands of Fe_B, eventually forming a trigonal-bipyramidal coordination structure, as illustrated in Fig. 6. The bond distances are shown in Fig. 6. The coordination structure is comparable to those of mononuclear non-heme iron proteins reported so far, which have 3His, 1Glu (Asp) and a water O, such as FeSOD (Fe-containing superoxide dismutase) [54,55]. It was reported that the mutation of the corresponding Glu in *Pa.d.* cNOR dramatically decreased the enzymatic activity [49], suggesting that it has an important role, presumably in the catalytic formation of N_2O from two NO molecules.

To understand the molecular mechanism of the catalytic N_2O formation on the binuclear center of cNOR, we followed the reaction of the fully reduced *P.a.* enzyme with NO using rapid mixing freeze-quenching techniques [19]. The sample produced at 1 ms after the mixing of the enzyme with NO gave an ESR spectrum, in which

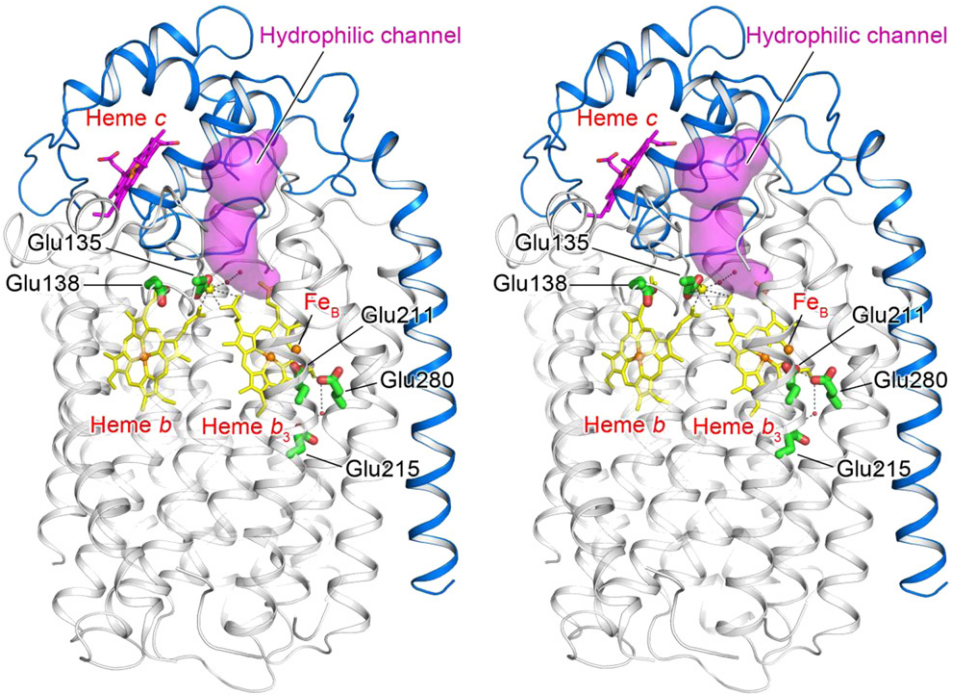


Fig. 5. Stereo diagram showing the position of highly conserved five Glu residues of NorB subunit. Glu in the transmembrane region (Glu211, Glu215, Glu280) or at the interface with NorC subunit (Glu135, Glu138) are shown as green stick models. Main chain of NorB and NorC is shown as ribbon and colored in white and blue, respectively. Hydrophilic channel proposed as a part of proton delivering pathway from periplasmic side is shown as a magenta surface. TM II, III, IV and V are shown in transparent.

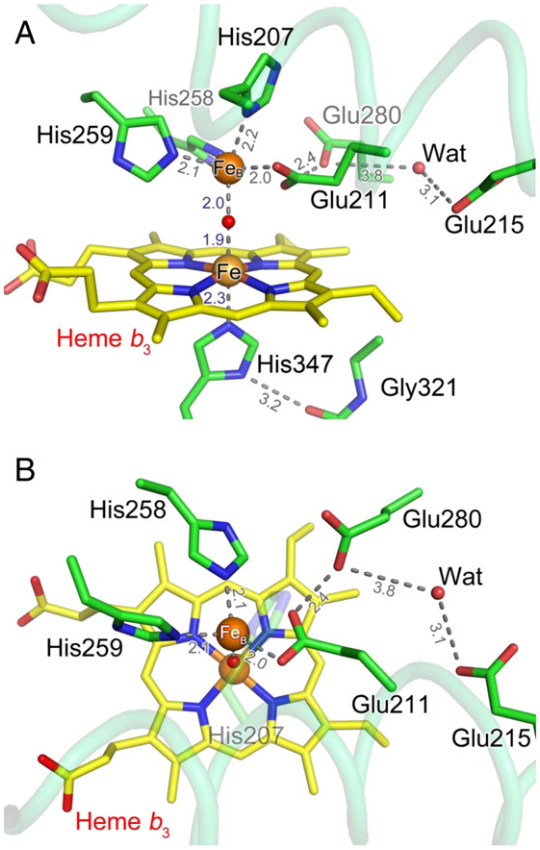


Fig. 6. Binuclear center of cNOR. (A) Side view of heme b_3. Non-heme iron (Fe_B) is coordinated by Glu211, His207, His258, and His259. Residue Glu211 is connected to another conserved Glu280 by hydrogen bond. His207 and Glu211 in cNOR correspond to the His and Tyr, respectively, that are connected by a covalent bond in aa_3, bo_3 and ba_3 oxidases [77–80], so-called A- and B-type COXs. (B) Viewed perpendicular to the heme b_3 plane. Stick model of His207 side chain and ribbon model of TM VI is shown in transparent.

$g=2$ with three-split lines and $g=4.3$ signals are observed. The former signal is originated from the five-coordinated heme b_3 Fe(II)–NO complex [35], and the latter is assigned to the Fe_B(II)–NO complex ($S=3/2$). On the basis of the EPR data, we proposed that two NO molecules are shared with two irons in the binuclear center, and that this species might be the reaction intermediate, which is consistent with the *trans*-mechanism (see Fig. 7). Low redox potential of the heme b_3 iron (60 mV) in *Pa.d.* cNOR [21], as compared with the other redox centers (>300 mV), indicates that the heme b_3 iron is difficult to be reduced (Scheme 1). However, a recent study involving the redox titration in *Pa.d.* cNOR showed that the presence of an exogenous ligand, CO, results in the elevation of the redox potential of heme b_3 to a range similar to that of the other redox centers [56]. Therefore, the binding of NO to heme b_3 iron could facilitate the reduction of the heme b_3 iron (Scheme 1). In the *trans*-mechanism, the electron transfer from both Fe(II) to the two NO ligands, eventually the Fe(III)–NO^- state, would promote N–N bond formation through a disproportionation-type reaction, and two protons then would facilitate N–O bond cleavage to produce N_2O and H_2O

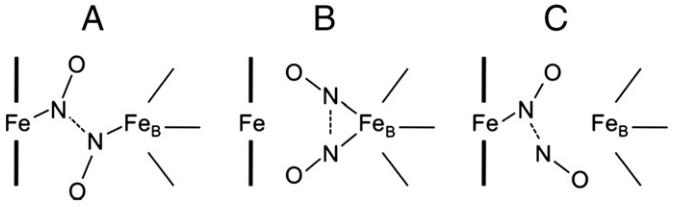
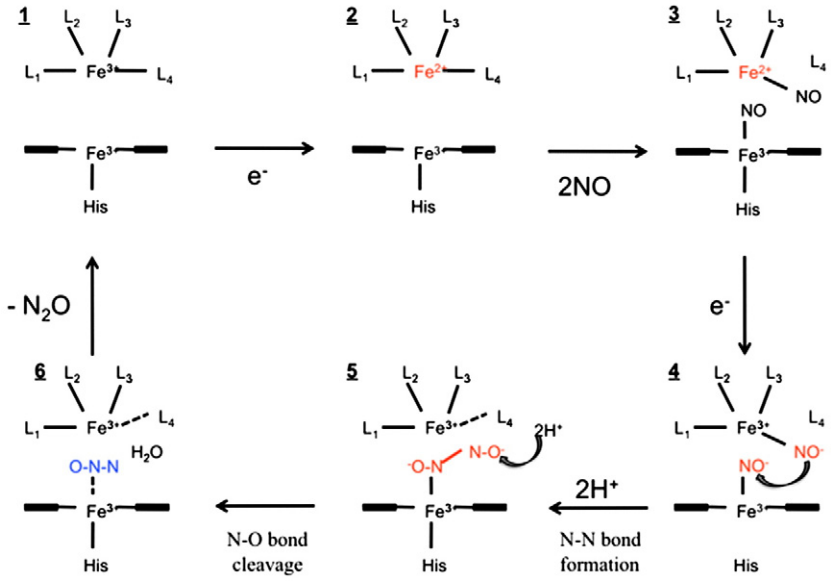


Fig. 7. Proposed structures of hyponitrite intermediate in *trans* and *cis* mechanism. (A) *Trans* mechanism. This mechanism is initiated by the binding of two NO molecules to the heme iron and Fe_B. The two ferrous-nitrosyl species then promotes N–N bond formation by an electrophilic attack of one nitrosyl on the other nitrosyl or by a radical coupling. (B) *Cis*-Fe_B mechanism. Two NO molecules bind at the Fe_B center to form a dinitrosyl species, leading to the formation of the hyponitrite species at Fe_B. (C) *Cis*-heme b_3 mechanism. A heme b_3 nitrosyl species was generated before reacting with a second NO molecule. Free second NO molecule electrophilically attacks heme b_3 nitrosyl species to form the proposed reaction intermediate.



Scheme 1. Proposed *trans*-mechanism. (1 → 2) The resting oxidized enzyme is reduced. On the basis of the reported redox potentials [21], non-heme Fe_B is reduced at first. (2 → 3) Two NO molecules binds to reduced Fe_B and ferric heme b_3 iron. In addition, one of the Fe_B ligands, L_4 in this case, dissociates from Fe_B to accommodate the binding of two NO at the binuclear center. (3 → 4) Heme b_3 iron is reduced, because the binding of NO to heme b_3 possibly elevates the redox potential of the heme b_3 iron [56]. Formation of the Fe_B nitrosyl and the heme b_3 nitrosyl causes the two NO ligands to become closer, and facilitates the electrophilic attack of the Fe_B nitrosyl to the heme b_3 nitrosyl (or the radical coupling reaction of two nitrosyl group). (4 → 5) Hyponitrate intermediate is formed by the N – N bond formation. (5 → 6) Protonations of the oxygen atom of the hyponitrite ligand promote N – O bond cleavage to produce a nitrous oxide and a water molecule.

(Scheme 1). This proposed mechanism was if good agreement with the model study reported by Collman and co-workers, in which NO binds to both heme and non-heme Fe(II) and one N_2O molecule is produced from two equivalents of NO [57]. However, another NOR model with a NO complex is thermally stable and does not react further to give N_2O upon the addition of the proton source [58]. In addition, it is important to bear in mind that the presence of two paramagnetic Fe(II)–NO species in the single binuclear center can lead to spin-coupling and the production of an ESR-silent species [59]. By contrast, two other mechanisms, so-called *cis*-mechanisms, have been also proposed, in which two NO molecules bind to either the heme b_3 iron or Fe_B (see Fig. 7). Thomson and co-workers favor the *cis*-Fe_B mechanism because this model has a vacant heme b_3 site, and a rather stable ferrous heme nitrosyl $\{FeNO\}^7$ species, a potential "dead-end" product, is therefore not formed during the turnover [21,60–63]. In the *cis*-heme b_3 mechanism, the first NO molecule binds to heme b_3 to form a $\{FeNO\}^7$ species which is then electrophilically attacked by the second NO molecule [64,65]. The mechanism appears to be analogous to that proposed for fungal P450-type nitric oxide reductase, P450nor [66–68]. However, in the case of P450nor, the key step is the formation of the short-lived intermediate $\{FeNO\}^8$ by a two-electron (H^-) reduction of the ferric heme–NO complex, while the one-electron reduced form $\{FeNO\}^7$ of P450nor never reacts with another NO.

The molecular mechanism of the NOR catalytic reactions, in particular the coordination and electronic structures of the reaction intermediate, is still controversial, even after our determination of the structure of cNOR. However, it should be noted that, in any proposal, two NO molecules must be accommodated in the space at the heme b_3 and Fe_B binuclear center. An examination of the active site structure of *P.a.* cNOR (Fig. 6) indicates that coordination sphere is quite crowded and highly packed. Therefore, there is no space to accommodate two NO molecules, even after the dissociation of the bridging O ligand. The finding clearly suggests that some conformational changes are needed for the formation of the reaction intermediate, when two NO molecules bind to the binuclear center. One possible change might be elongation of the Fe–Fe distance. Richardson and co-workers proposed the bridging of hyponitrite between heme b_3 and Fe_B as a transient state [69]. In this hypothetical structure, the Fe–Fe distance would need to be at least 4.4 Å, indicating that the irons would need to shift by 0.4 Å from their original (fully oxidized state) positions to create a space sufficiently large to accommodate two NO molecules.

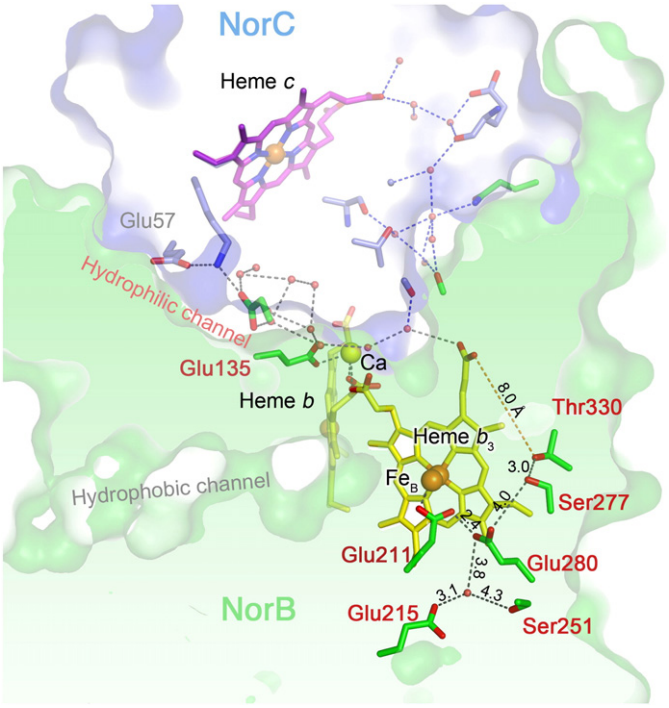


Fig. 8. Possible proton pathways for NO reduction. There is the hydrogen-bonding network involving one of Fe_B ligand, Glu211. Glu280 can be bridging Glu211 and a water molecule, which interacts with another conserved residue Glu215. Ser277 likely interacts with Glu280 and Thr330. Proposed proton pathway starts from Glu57 through Glu135 and the propionate of heme b_3 as indicated by dotted lines. However, there is an 8 Å gap between the propionate and Thr330. In addition, the distances between some residues near the active site are longer than the typical distance for a hydrogen bond.

Another possible change could be dissociation of one of the ligands (His258, His259 or Glu211) from Fe_B to create a space for accommodating the second NO molecule to the binuclear center. Non-heme irons having two His and one Glu/Asp ligands are frequently observed in nature [70,71], and the reversible association/dissociation of a carboxylate ligand from cuprous (Cu^{2+}) having 3 His and 1 Glu was also observed in the crystal structure of quercetin 2,3-dioxygenase [72,73]. By analogy to the latter case, we propose that the Glu211 carboxylate in cNOR could function as the shuttle for catalytic protons from Glu280 (*vide infra*) to the bound-NO. On the basis of the crystal structure, it appears that Glu280 is protonated to form a hydrogen-bond with Glu211, which, because it is a Fe_B ligand, is deprotonated. The transfer of a catalytic proton from Glu280 to Glu211 could induce the dissociation of Glu211 from Fe_B, which is a potential trigger for accommodating the binding of two NO molecules at the binuclear center (Scheme 1).

5.2. Glu280

The carboxylate of Glu280 interacts with the carboxylate of Glu211, in which the distance is 2.4 Å, but does not directly interact with Fe_B. In addition, Glu280 possibly bridges the hydroxyl group of Ser277 and a water molecule, the latter of which is located near Ser251 and the conserved Glu215. Although some of the distances between them are a somewhat longer than the normal hydrogen-bonding distance (Fig. 8), a small conformational change such as the rotation of the side-chain can alter the distances within the range for hydrogen-bonding interactions. Ser277 interacts with the hydroxyl group of Thr330 (Fig. 8), both of which are well conserved residues in all NORs. On the basis of the mutational results of the corresponding residue to Glu280 (Glu267 in *Pa.d.* cNOR) [49], it is likely that the network of Thr330–Ser277–Glu280–Glu211 acts as a delivery pathway for protons that are utilized in the catalytic NO reduction. The crystallographic result is consistent with a previous proposal based on homology models. However, in our *P.a.* cNOR crystal structure, Thr330 is free from any other interactions, and a long gap (8 Å) exists between Thr330 and the heme b_3 propionate. On the other hand, a water cluster is in close proximity to the heme b_3 propionate, and forms a hydrophilic channel with some amino acid side chains (Asp198 (NorB), Lys53 (NorC), Glu57 (NorC)), leading to the periplasmic side through the Ca binding site (*vide infra*). The channel might function as the proton transfer pathway from the periplasmic side to the heme b_3 propionate. Therefore, if one or two crystallographically invisible water molecules were to participate in the interaction between Thr330 and the heme b_3 propionate to connect the long gap, the delivery pathway for the catalytic proton from the periplasmic side to the binuclear center could be constituted, as has been predicted. To assess this prediction, a molecular dynamic simulation should be performed.

5.3. Glu215

Glu215 is located at backside of Glu211, but there is no interaction between them. Glu215 appears to not be directly responsible for the NO reduction reaction by cNOR, since the mutant with the equivalent residue in *Pa.d.* cNOR retained approximately 50% of the activity of the WT enzyme [49,50]. However, a NOR model study using a myoglobin heme pocket by Lu and co-workers [74,75] suggested that, in addition to the Fe_B-coordinated Glu carboxylate, another Glu is indispensable for the activation of NO by NOR. The Glu215 carboxylate group could contribute to the electro-negative environment of the binuclear center of cNOR, and then eventually contribute to the low redox potential of heme b_3 iron (60 mV), which is lower than those of heme *b* (345 mV) and heme *c* (310 mV) in cNOR [21]. In addition, when the K-channel of COXs for proton transfer [76–78] was compared with the same region of cNOR, it was noted that Glu215 is located at the terminal position of the "K-channel" (Fig. 9). It is likely that this residue might reflect the first step in the evolutionary pathway for acquiring a proton delivery pathway from the cytoplasm to the binuclear center in COX.

5.4. Glu135 and Glu138

In *Pa.d.* cNOR, when the corresponding residue of Glu135 was substituted with alanine, glutamine and aspartic acid [51], neither the optical and EPR spectral properties nor the O_2 and CO binding properties were affected in the variants, suggesting that the active site structure of cNOR is not altered by these mutations. However,

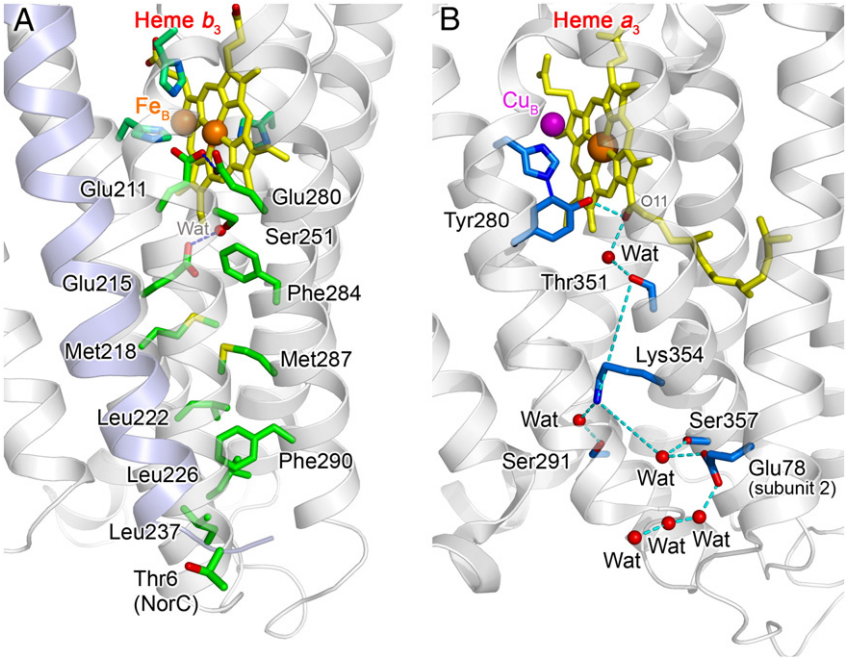


Fig. 9. Comparison of K-pathway of cNOR and COX. (A) The hydrogen-bonding network from cytoplasmic surface (bottom) to binuclear center is blocked by hydrophobic residues (Phe284, Met218, Met 287, Leu222, Phe290, Leu237) in cNOR. (B) K-channel of *P. denitrificans* cytochrome aa_3 oxidase (PDB ID: 3HB3) [81] for delivering protons is shown as cyan dashed line.

proton-coupled electron transfer is severely inhibited in the Ala and Gln mutants, while it was retained in the Asp mutant, but with a significant shift in the p*K*a of the proton donor. On the other hand, the substitution with Asp or Gln for the corresponding Glu138 residue in *Pa.d.* cNOR showed less than 15% of the activity of the WT enzyme. On the basis of these experimental results, Glu135 and Glu138 have been proposed to be located at the entry site of the non-electrogenic proton transfer pathway that delivers catalytic protons from the periplasm to the buried active site in cNOR during catalytic turnover [39,51].

However, in the crystal structure of *P.a.* cNOR, Glu135 and Glu138 are not located at the protein surface, but are present on the loop that connects the transmembrane (TM) helices III and IV (Fig. 10). Glu135 serves as the ligand of Ca, together with the Tyr73 (NorC) phenolate and Gly71 (NorC) main-chain carbonyl. Ca also bridges the two propionates of hemes *b* and b_3, possibly to stabilize the relative configuration of these two hemes. The presence of Ca at the same position was reported for cytochrome cbb_3 oxidase in micro-anaerobic respiration, and a Glu residue is one of the ligands [76]. By contrast, in the aerobic cytochrome oxidases, Ca is replaced with two positively charged Arg residues [77–80]. Furthermore, Glu135 in cNOR interacts with a number of water molecules, which are part of the hydrophilic/water channel that connects the periplasmic side (*vide supra*). Therefore, we propose that the primary role of Glu135 would be ligation to Ca, thereby maintaining the configuration of heme *b* and b_3. In addition to this, Glu135 could assist in the water-mediated proton transfer through the interactions with a number of water molecules.

On the other hand, Glu138 is neither directly involved in the formation of the hydrophilic/water channel nor in the coordination of Ca. However, Glu138 is present on the same loop, as Glu135, which is present in the interface of NorC and NorB. Its carboxylate participates in some interactions with the residues in TM II (Arg57, Thr61 and Asn62) mediated by water molecules, as illustrated in Fig. 10. It is highly plausible that Glu138 could be a key residue for maintaining the unique conformation of the long loop through interactions with the residues in TM II, which would stabilize the coordination of Glu135 to Ca.

From these observations, it is likely that Glu135 and Glu138 are not directly involved in electron-coupled proton transfer in the NOR reaction, but, function as structural factors, contributing to the maintenance of a protein conformation that is appropriate for the transfer of electrons from hemes *b* and b_3 and/or the transfer of protons from the periplasm to the active site. Thus, the mutational results reported so far can be explained as follows. The neutral Ala and Gln side chain in the 135 position cannot stabilize the Ca binding and the Ca ion in cNOR is lost, resulting in a protein conformation that is unsuitable for an electron-coupled proton transfer in the catalytic NO reduction, while the negatively charged Asp at position 135 can retain the Ca binding, but this results in the perturbation of the proton channel, resulting in a shift in the p*K*a but without a loss in enzymatic activity. To clarify the specific roles of the conserved Glu135 and Glu138 in NORs, further structural and functional characterization including the estimation of the Ca content of the conserved Glu variants would be desirable.

In summary, the crystal structure of *P. aeruginosa* cNOR provides us with insights into its structural and functional properties, some of which have been extensively discussed. With respect to the well-conserved glutamic acid residues in NOR, three Glu are located near the catalytic binuclear center, and two are located on the loop that connects TM III and TM IV. In particular, new insights into the roles of Glu135 and Glu138 are reported, both of which possibly contribute to maintaining the proton and electron transfer steps in the NO reduction reaction catalyzed by cNOR. Since it is generally believed that NOR and COX share a common ancestor in the molecular evolution of respiratory enzymes, it would be possible through detailed structural comparison between them to discuss how the NO reducing enzyme could be altered to produce the O_2 reducing enzyme, possibly through structural modification.

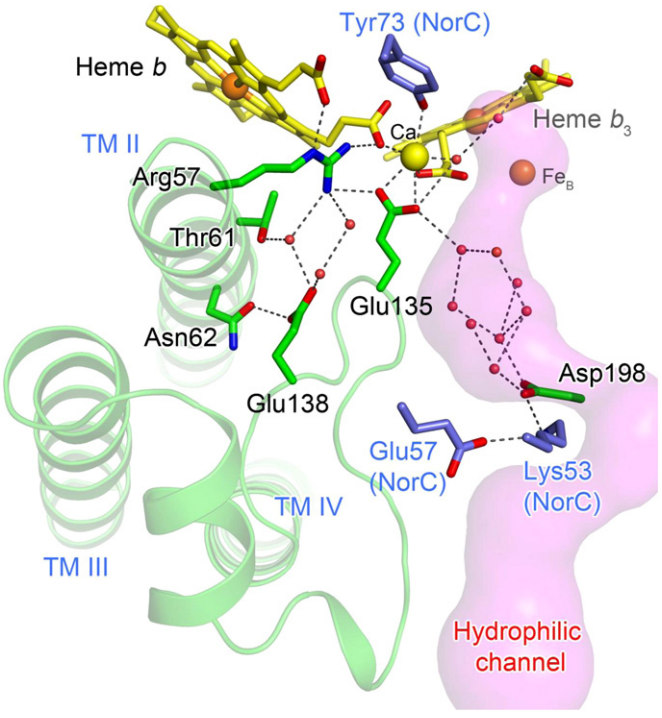


Fig. 10. Glu135 and Glu138 at interface of NorB and NorC. They locate on the loop connecting two transmembrane helices (TM III and IV). Glu135 serves as the ligand of calcium ion that bridges the propionates of heme *b* and heme b_3. Glu138 creates the hydrogen-bonding network with three waters, Arg57, Thr61, Asn62 and Glu135. This network would be important for the conformation of the calcium ligands and the interaction with NorC subunit.

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