# Nitric oxide reductase; a key enzyme in understanding structural and functional conversion of respiratory enzymes in their molecular evolution

Respiration is a physiological process to gain the biological energy, ATP, coupled with a reduction of a terminal electron acceptor with electrons $e^-$ and protons $H^+$. In aerobic respiration, cytochrome oxidase COX pumps $H^+$ from the inside to the outside of cells, coupled with the reduction of molecular oxygen $O_2$ ($O_2+4e^-+4H^+ \rightarrow 2H_2O$). The generated proton gradient across the cellular membrane can be utilized for the ATP biosynthesis by ATP synthase. The corresponding enzyme in anaerobic respiration of a microorganism has been considered to be nitric oxide reductase NOR, which is also a membrane-integrated protein catalyzing reduction of NO ($2NO+2e^-+2H^+ \rightarrow N_2O+H_2O$: eq.1), but does not exhibit the proton pumping ability. Three billion years ago, photosynthesis by cyanobacteria began, producing $O_2$ by water oxidation, and the emergence of $O_2$ on the earth caused a drastic change in the respiration of living systems from anaerobic to aerobic respiration. We determined crystal structures of two bacterial NORs, gram-negative *Pseudomonas aeruginosa* cNOR and gram-positive *Geobacillus stearothermophilus* qNOR, whose diffraction data were collected at beamlines **BL44B2** and **BL41XU** [1,2]. The analysis enabled us to compare structural and functional features of NOR and COX at a molecular level, and provided novel insight into the molecular evolution of respiratory enzymes.

cNOR, which is observed only in denitrifying bacteria, is a cytochrome *c*-dependent enzyme consisting of two subunits, NorB and NorC, whereas qNOR is a related single-peptide and quinol-dependent enzyme that is observed in non-denitrifying pathogenic bacteria as well as in denitrifying bacteria and archaea. The overall structural characteristics of these two NORs, i.e., the configuration of transmembrane (TM) helices as well as the folding of the hydrophilic domain, are almost identical to one another (Fig. 1). A high level of structural similarity of the TM region is found in NORs and COXs, in good consistency with the previous idea that these anaerobic and aerobic respiratory enzymes share the same ancestor protein in their molecular evolution.

On the other hand, the active-site structure of two NORs is somewhat different from that of COX, in which heme iron and copper $Cu_B$ are present, and the His-Tyr linkage in one of the $Cu_B$ ligands is characteristic. In the case of NOR, the catalytic binuclear center is constructed of heme and non-heme irons (Fig. 2). As ligands, the heme $b_3$ iron has one His, and the non-heme iron $Fe_B$ has three His and one Glu, but His-Tyr is absent. Such differences observed in the active-site structure should be responsible for the difference in the catalytic activities of NOR and COX. In combination with the time-resolved spectroscopic results [3], we proposed a possible mechanism of NO reduction by NOR, in which two NO molecules are shared by ferrous heme $b_3$ and $Fe_B$, supporting the so-called trans-mechanism [4].

In the NO reduction reaction catalyzed by NOR (eq. 1), two $H^+$ are required. The highly conserved Glu ligand of $Fe_B$ of NOR has been suggested to be a terminal donor of protons utilized in the catalytic reaction. The $H^+$ can be transferred from bulk water to Glu carboxylate through a channel consisting of the hydrogen-bonding network and/or the water chain.

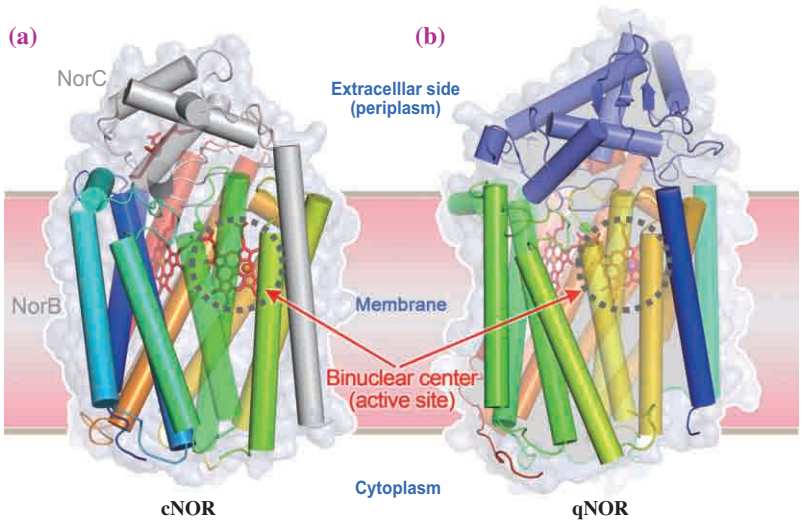


Fig. 1. Overall structures of NORs. **(a)** Crystal structure of *Pseudomonas aeruginosa* cNOR. The helices are represented by cylinders. NorB subunit (colored in rainbow, starting from blue for the N-terminus and ending with red at the C-terminus) contains 12 transmembrane helices with heme *b* and binuclear center (heme $b_3$ and $Fe_B$). NorC subunit (gray) contains one transmembrane helix in its N-terminus and cytochrome *c* domain in the periplasmic side. **(b)** Crystal structure of *Geobacillus stearothermophilus* qNOR in a single peptide is shown. The hydrophilic domain in the extracellular side (light blue) shows cytochorme *c*-like fold but lacks heme *c* and its binding motif.

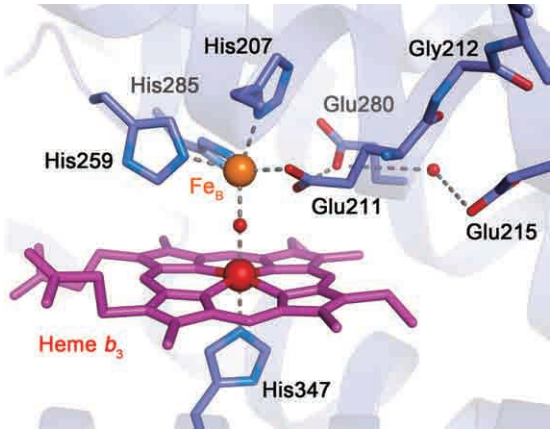


Fig. 2. Binuclear center of *Pseudomonas aeruginosa* cNOR. Heme $b_3$ is shown by red stick model. The ligands for the non-heme metal $Fe_B$ (orange sphere) shows a distorted trigonal-bipyramidal geometry with three His and one Glu.

In cNOR, there are many water molecules and polar residues in the periplasmic side near the binuclear center, but no hydrophilic region in the cytoplasmic region (Fig. 3(a)). The structural observation suggests that the catalytic proton can be delivered from the periplasmic side, consistent with the previous proposal based on electrometric measurement combined with flow-flash methods. On the other hand, in qNOR, we found the water channel connecting the binuclear center with the cytoplasmic side, while there is no channel in the extracelluar (periplasmic) side (Fig. 3(b)). A molecular dynamic simulation based on these two NOR structures supports the suggestion; i.e., although both NORs exhibit the same NO reduction capability, the proton utilized in the catalytic reaction could be supplied in a different way between cNOR and qNOR. It is also interesting to find that the water channel found in qNOR is located in the same region of the "K-channel" of COX, which can act as the catalytic and pumped protons pathway (Fig. 3(c)). The water channel observed in qNOR might be a prototype of the proton-pumping pathway in $O_2$ reduction respiration.

Through denitrification, a kind of anaerobic respiration, NOR, of microorganisms in soil and ocean produces a large amount of nitrous oxide $N_2O$, which is an ozone-depleting and greenhouse gas 310 times more potent than carbon dioxide $CO_2$. It has been predicted that the amount of $N_2O$ will increase yearly in the 21st century [5]. On the other hand, NOR in pathogenic bacteria can detoxify NO for survival, which is produced by macrophages as a chemical weapon against infection agents. Our NOR structures could contribute to a variety of fields including not only biology and chemistry, but also environmental and physiological sciences.

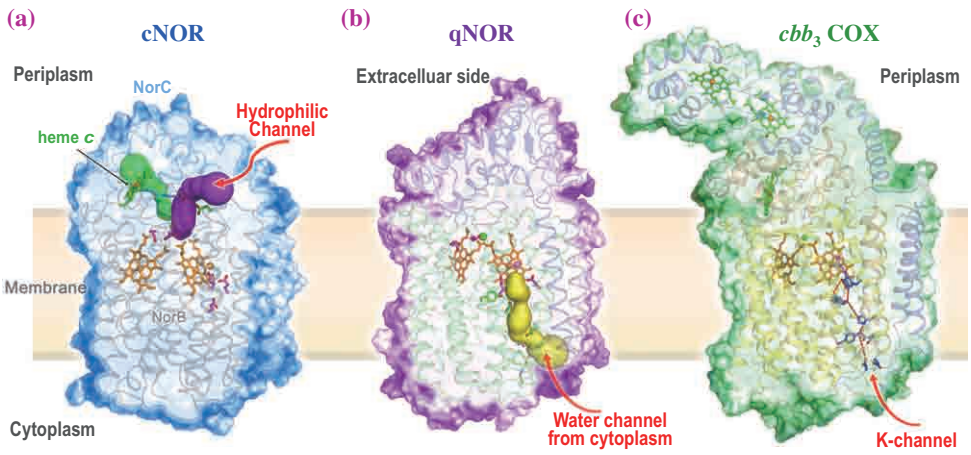


Fig. 3. Possible proton transfer pathways of respiratory enzymes. **(a)** Periplasmic hydrophilic channels observed in crystal structure of cNOR. **(b)** Water channel observed in crystal structure of qNOR. **(c)** K-pathway in $cbb_3$ cytochrome oxidase connecting the cytoplasmic surface with the active site of oxygen reduction.

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**References**

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