Supplementary Information

Crystal Structure of Quinol-Dependent Nitric Oxide Reductase from *Geobacillus Stearothermophilus*

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Supplementary Figure 1 Multiple alignment of amino acid sequences for NORs. Sequence data are from the EMBL and NCBI databases. Secondary structures determined from the structure of *G. stearothermophilus* qNOR are indicated above the alignment with cylinders and arrows for α-helices and β-sheets, respectively. Structurally and functionally important residues are denoted by colored diamonds: red indicates metal ligands; blue indicates residues lining the water channel; white indicates residues at the menaquinol binding pocket; orange indicates calcium ligands; green indicates the heme c binding C-X-X-C-H motif observed in eNOR. The alignment was produced by ClustalX.
Supplementary Figure 2 Comparison of the overall structures and redox centers of the respiratory enzymes. Upper left structures are viewed parallel to the membrane. Lower left structures are viewed from the cytoplasmic side. Hemes are shown as red sticks. Right structures show the configurations of metal and redox centers. (a) *G. stearothermophilus* qNOR. A calcium ion bridges the heme propionates from heme b and b3. (b) *P. aeruginosa* cNOR (PDB code: 3O0R).1 NorB and NorC subunits are shown as green and cyan ribbons,
respectively, in the left structures. A calcium ion and its ligands are located at the same position as they are in qNOR. The residues from the NorB and NorC subunits are shown as green and cyan sticks, respectively, in the right structure. (e) *P. stutzeri cbb₃* oxidase (PDB code: 3MK7). CcoN, CcoO and CcoP subunits are shown as green, cyan and gray ribbons, respectively, in the left structures. A calcium ion is located in a similar position to that in qNOR or cNOR. The conserved calcium ion Glu ligand in the NORs is also observed in *cbb₃* oxidase. The residues from the CcoN and CcoO subunits are shown as green and cyan sticks, respectively, in the right structure. (d) Bacterial *ba₃* oxidase (PDB code: 1XME). Subunit I and II are shown as green and cyan ribbons, respectively, in the left structures. Instead of the calcium ion observed in NORs and *cbb₃* oxidase, two conserved positively charged Arg residues are located between heme propionates from heme b and a₃. The residues from subunit I and II are shown as green and cyan sticks, respectively, in the right structure.
**Supplementary Figure 3** The structural and functional properties of *G. stearothermophilus* qNOR. (a) Optical absorption spectra for *G. stearothermophilus* qNOR in the crystalline and solution states. Spectra (A) and (B) are from a qNOR single crystal before and after data collection, respectively. Spectra (C) and (D) are from oxidized and dithionite-reduced qNOR in solution. The spectrum suggests that heme b was reduced, but not for heme b3 by the irradiation of X-ray. (b) Assignment of the Zn ion at the binuclear center. The anomalous difference Fourier maps calculated from a Zn peak and Zn low-remote data sets are contoured at 4 σ and are shown as red and cyan meshes, respectively. (c) Table for the metal content and enzymatic activity of qNOR and cNOR. The metal contents were determined by ICP-AES measurement. The enzymatic activity, the NO consumption rates, for qNORs and cNOR were evaluated by using Clerk-type NO electrode. (d) Assignment of water molecules interacting with the Zn ion. An |$F_o$|−|$F_c$| omit map contoured at 4 σ, calculated with the qNOR structure excluding two water molecules at the binuclear center, is shown as a gray mesh. (e) Structural rearrangement of TMH VIII including Glu512.
Crystal structure and the structure from MD snapshot are shown with green and yellow, respectively. During MD, we can observe the deformation of TMH VIII, and positional shift of Glu512 which coordinates to Zn$_8$. Multiple alignment of the amino acid sequences of the part of TMH VIII in qNORs and cNORs indicate that there is conserved Gly next to essential Glu.
Supplementary Figure 4 Multiple structural alignment of the cytochrome c folding domains in the respiratory enzymes. (a) Amino acid sequence alignment based on the structures of the cytochrome c folding domains in the respiratory enzymes. Structural data of *P. aeruginosa* cytochrome c551 (PDB code: 351C)\(^4\), *Rhodothermus marinus* cytochrome c (PDB code: 3CP5)\(^5\), *G. stearothermophilus* qNOR, *P. aeruginosa* cNOR (PDB code: 3O0R)\(^1\), and *P. stutzeri* cbb\(_3\) oxidase (PDB code: 3MK7)\(^2\) were used for the alignment. α-Helical regions are highlighted with cyan, and the α-helical regions in qNOR are denoted by TM1 and α1-5 above the alignment. Regions with many deviations in the alignment are indicated by black and red dotted circles for qNOR and the CcoO subunit of cbb\(_3\) oxidase, respectively. The alignment was produced with a multiple 3D alignment by MATRAS\(^6\). (b-d) Superposition of the respiratory enzymes based on the structural alignment. (b) qNOR
(green) and NorC subunit of cNOR (magenta). (e) NorC subunit of cNOR (magenta) and CcoO subunit of $cbb_3$ oxidase (blue). (d) qNOR (green) and CcoO subunit of $cbb_3$ oxidase (blue). The structural differences, denoted by dotted circles, correspond to the regions in panel A.
**Supplementary Figure 5** Comparison of the binuclear centers of the respiration enzymes. (a-d) Structures of the binuclear centers. Key residues are shown as sticks. (a) *G. stearothermophilus* qNOR. (b) *P. aeruginosa* cNOR (PDB code: 3O0R)\(^1\). (c) *P. stutzeri* cbb\(_3\) oxidase (PDB code: 3MK7)\(^2\). (d) Bacterial ba\(_3\) oxidase (PDB code: 1XME)\(^3\). (e) Superposition of the binuclear centers of qNOR (green) and cbb\(_3\) oxidase (magenta). Glu553 in qNOR is substituted with Tyr, which is covalently bound to one of the non-heme metal His ligands in cbb\(_3\) oxidase. (f) Superposition of the binuclear centers of qNOR (green) and ba\(_3\) oxidase (yellow). Glu512 in qNOR is substituted with the Tyr involved in the His-Tyr covalent linkage in ba\(_3\) oxidase.
Supplementary Figure 6 Comparison of the regions of the proposed proton transfer pathways in P. aeruginosa cNOR\(^1\) and corresponding regions in G. stearothermophilus qNOR. (a) and (b) Regions corresponding to channels 1 and 2 of cNOR\(^1\) in qNOR, respectively. The transmembrane core region and hydrophilic domain are shown with green and blue, respectively. The loop between TMH XIII and XIV is shown with magenta in
panel B. (a) Functionally important Asp198 in cNOR is not conserved in qNOR (Ala: 53%, Val: 12%, Ile: 14%, Thr: 10%, Met: 7%). (b) The conformation of the loop between TMH XIII and XIV is different from that of cNOR, and this loop fills the channel 2 cavity. (c) and (d) Proposed proton transfer pathway, designated as channels 1 and 2 in *P. aeruginosa* cNOR, respectively. The NorB and NorC subunits are shown with cyan and blue, respectively. The water molecules located on channels 1 and 2 are shown with yellow balls. The loop between TMH XII and XIII is shown with magenta in panel D.
Supplementary Figure 7 Region between the cytoplasmic surface and the binuclear active center. (a) The water channel in qNOR and the corresponding regions in (b) cNOR\(^1\) and (c) \(cbb_3\) oxidase\(^2\). For qNOR and cNOR, the type of amino acid residue observed in 100 available sequences are summarized on each residue located at the water channel region. In cNOR, the residues corresponding to Gln545 and Glu591 in qNOR are Ile244 and Phe290, respectively, which would collapse the water channel. In \(cbb_3\) oxidase, the “K-pathway” for the proton transfer corresponds to the water channel in qNOR, while the entrance site at the cytoplasmic side is different in qNOR and \(cbb_3\) oxidase. (d) Channels identified in native cNOR. Split hydrophobic channels connecting the binuclear center and the membrane lipid
surface are shown as an orange surface. The channel search was carried out using the native cNOR structure (PDB code: 3O0R)\(^1\) without water molecules. (e) Channels identified in an I244Q/F290E variant of cNOR. In addition to the split hydrophobic channels observed in native cNOR (orange surface), the channel connecting the binuclear center and the cytoplasmic side was identified in the I244Q/F290E variant (magenta surface). The variant was created by computation (Swiss PDB Viewer) using the native structure as a template. The channels were calculated using Caver\(^7\).
Supplementary Movie 1 Dynamics in the water channel in MD simulation. Time-dependent dynamics, such as side chains fluctuations, moving water molecules, and transient hydrogen-bond networks can be seen. Water molecules in the water channel are shown in yellow, and bulk water around the channel entrance is shown as red and white lines. Hydrogen-bonds are shown as dashed green lines.

Supplementary Movie 2 Motion of selected water molecules along the water channel in the MD simulation. Eight individual waters are shown as large spheres and are highlighted in different colors. Other water molecules inside the water channel and in the bulk around the channel entrance are shown as yellow sticks. The selected water molecules come into the water channel via the entry site, exchange with waters inside the channel, travel up to the binuclear active center, and eventually return to the bulk. The water molecules are highly mobile and move easily along the channel on a short timescale. There are several stable positions (“traps”) where water molecules stay near residues for a long time (typically, nanoseconds), before moving further along the channel.
Supplementary Methods

Purification of qNOR from *G. stearothermophilus*. Membranes from anaerobically grown *G. stearothermophilus* were suspended in 50 mM Tris-HCl (pH 8.0) and then concentrated to 10 mg/mL. Triton X-100 (Sigma) was added to the membrane suspension to a final concentration of 10% (v/v). The mixture was centrifuged for 1 h at 125,000 × g. The supernatant was applied to a 250 mL DEAE-Sepharose column (GE Healthcare Bioscience), which was equilibrated with 50 mM Tris-HCl (pH 8.0) buffer containing 0.05% *n*-dodecyl β-D-maltoside (DDM, Dojindo). The column was washed with two column volumes of equilibration buffer, and then the protein was eluted with a linear NaCl gradient (0-0.5 M). Fractions containing qNOR were applied to a 40 mL ceramic hydroxyapatite Type II column, which was pre-equilibrated with 200 mM potassium phosphate buffer (pH 7.5) containing 0.05% DDM. The column was washed with two column volumes of equilibration buffer, and the enzyme was eluted with a linear potassium phosphate gradient (0.2-1.5 M). Fractions containing qNOR were concentrated in an Amicon Ultra 50K concentrator (Millipore) to a final volume of 5 mL. The solution was applied to a Mono Q 16/10 column (GE Healthcare Bioscience), which was pre-equilibrated with Tris-HCl buffer (pH 8.0) containing 0.05% DDM. The sample was eluted with a linear NaCl gradient (0-0.5 M). The peak fractions were concentrated in an Amicon Ultra 50K concentrator (Millipore) to a 5-10 mg/mL protein solution. The enzymes were characterized by the peptide mass fingerprinting method.

Cloning, construction of an NOR expression plasmid, and mutagenesis. *G. stearothermophilus* genomic DNA was used as a PCR template. A sense primer (5′-AATGGTTCCATGGCGTTCTCTCTTTGG-3′) and an anti-sense primer (5′-TATCGCTTTGGCAACGTGAGGTGATCCACGG-3′) were constructed based on *Bacillus kaustophilus* genomic DNA. Using the genomic DNA and the two primers, a 2,700 bp fragment was PCR amplified and cloned into the pCR2.1-TOPO vector (Invitrogen). The cloned qNOR gene was sequenced (DDBJ accession number: AB450501). A DNA
fragment encoding the ORF of qNOR (norZ) was PCR amplified and then ligated between the NdeI and XhoI sites of pET-22b (Novagen). The plasmid was transformed into Rosetta2 (DE3) (Novagen) and BL21-CodonPlus (DE3)-RIL-X (Stratagene) for the expression of the native and selenomethionine (SeMet)-labelled enzymes, respectively. Mutations were introduced using a QuikChange mutagenesis kit (Stratagene) and the pET-22b plasmid harboring the qNOR gene as a template. The mutation was confirmed by sequencing the qNOR gene using an ABI PRISM 310 genetic analyzer (Applied Biosystems).

References