Supporting Information

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SI Materials and Methods

Construction of Expression Plasmids and Site-Directed Mutagenesis. The whole operon, containing the *norC*, *norB*, and an additional ORF following the *norB* gene, from *Thermus thermophilus* PRQ25 strain was cloned into pET22b (Ap^r; Novagen) and into the shuttle vector pMKPbcbgaA (Km^r). For purification purposes, an 8 His-tag was introduced in the C-terminal of the *norB* gene. A Quick Change site-directed mutagenesis kit (Stratagene) was used to construct the different point mutations. The final constructions in pMKPbcbgaA were transformed into *T. thermophilus* HB27 (ATCC BAA-163, Y. Koyama, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan) for homologous expression. For heterologous expression, the constructions in pET22b were transformed into *Escherichia coli* C43 (DE3) strain (Avidis), containing a plasmid carrying tRNA genes needed for reading rare codons (pRARE2) (Km^r) (1) and pEC86 (Cm^r) (2).

Cell Growth, Enzyme Expression, and Purification. The *T. thermophilus* (HB27) strain containing the plasmid to express cNOR (nitric oxide reductase) was grown in rich medium as previously described in (3). *E. coli* C43 cells containing the plasmids to express cNOR were grown in LB medium at 37 °C. The gene expression was induced as previously described (4). All of the purification procedures were carried in 100 mM Tris, pH 8, 150 mM NaCl buffer, and purification was followed as described previously (4). *T. thermophilus* cytochrome c_{552} was prepared as previously described (5).

Protein expression and purification was examined by SDS/PAGE using a 4–20% gradient gel (Nusep). The sample was diluted in the same volume of Tris·glycine SDS sample buffer (Nusep) containing 715 mM β-mercaptoethanol and immediately without heating it was loaded into the gel. Spectra were acquired using an Agilent Technologies 8453 UV-visible spectrophotometer running ChemStation software. Protein concentration of the preparation was determined from reported extinction coefficient ($\varepsilon_{411} = 311\ mM^{-1}{\cdot}cm^{-1}$). Heme staining of the SDS/PAGE gel was used to identify NorC containing covalently attached heme *c* (6).

Steady-State Activity. The NO consumption rates were measured with an ISO-NO electrode equipped with ISO-NO Mark II (World Precision Instruments) in a 2-mL anaerobic reaction chamber at 42 °C. The data were recorded on a Duo 18 (World Precision Instruments). The assay solution contained 50 mM Na citrate, pH 6.0, 0.05% DDM, 5 mM ascorbate, 0.5 or 2.5 mM *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine (TMPD), 10 μM phenazine methosulfate (PMS), or 30 μM cytochrome c_{552}, as indicated. After 20 min of Argon flux, NO-saturated solution was added to the reaction mix at a final concentration of ∼25 μM. Once the background consumption of NO was stable, NO reduction (consumption) was initiated by the addition of cNOR (final concentration was between 0.1 and 0.5 μM). The enzyme turnover (mol electrons/min − mol enzyme) was calculated from the slope of the nitric oxide consumption traces.

Sequence Analysis. Genes encoding NORs were retrieved from the J. Craig Venter Institute, the National Center for Biotechnology Information, and Department of Energy Joint Genome Initiative databases. The sequences were aligned using MUSCLE (7) and manually edited using the cNOR crystal structure (PDB ID code 3O0R) as a guide. Conserved residues were identified with BIOEDIT.

The phylogenetic tree was built using RAxML and MrBayes via the CIPRES portal (8) using 323 cNOR amino acid sequences.

Metal Analysis. The metal content of the protein was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Spectro Genesis spectrometer. The buffer containing the purified cNOR from *T. thermophilus* (*Tt*cNOR) protein was exchanged by dialysis to 100 mM Tris buffer (pH 8) containing 150 mM NaCl, 0.05% DDM, 5% (vol/vol) glycerol, 0.25 mM EDTA, and 0.1 mM EGTA. Metal concentrations were calculated from regression lines of element standards run as samples rather than the regression lines produced by the Spectro software to avoid a 10% underestimation of element concentrations at low levels of the metals.

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3. Schurig-Briccio LA, Gennis RB (2012) Characterization of the PIB-Type ATPases present in *Thermus thermophilus*. *J Bacteriol* 194(15):4107–4113.
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Table S1. Conservation of key residues among cNORs and their predicted function

*Pac*NOR residue	*Ttc*NOR residue	% Conserved (replacements)		Suggested role
NorB subunit				
N54	N55	76	H, D, S, M, G, E, A	Proton pathway?
R57	R58	97	K	
H60	H61	100		Heme *b* Fe axial ligand
E135	E134	>80	E, K, P, H, G, V	Ca ligand
E137	E137	>90	E, D	Proton pathway?; stabilize Ca site
D198	D198	>85	E, Q, S, A	Proton pathway?
H207	H207	100		Fe_B ligand
E211	E211	100		Fe_B ligand
E215	E215	>95	M, V, I	
S277	S277	100		Proton pathway?
E280	E280	>90	Q	Proton pathway?
T330	T330	100		Proton pathway?
N335	N335	100		H-bond to heme b_3 propionate-A
H339	H339	100		H-bond to heme b_3 propionate-A
H347	H347	100		Heme b_3 Fe axial ligand
H349	H349	100		Heme *b* Fe axial ligand
H258	H258	100		Fe_B ligand
H259	H259	100		Fe_B ligand
NorC subunit				
N60	N124	55	S, D, A, G,T, E	Proton pathway?
T66	T130	>80	S, A	Proton pathway?
E57	Q121	75	Q, H	Proton pathway?
Y73	Y137	100		Ca ligand
E77	D141	35	E	Proton pathway?
N80	R144	<10	K, N, H, Q, D, E	Proton pathway?
R84	D148	<10	R, K, Q, T, E, L, H, D	Proton pathway?
C61	C125	100		Heme *c* binding
C64	C128	100		Heme *c* binding
H65	H129	100		Heme *c* Fe axial ligand
E145	D209	>50	N, E, K, R, P	Proton pathway?
M112	M185	93	H	Heme *c* Fe axial ligand

Fig. S1. (*A*) Reduced-minus-oxidized absorption spectra of *Ttc*NOR expressed in *E. coli*. Protein expressed in the presence of the pEC86 plasmid (1) to enhance the assembly of heme *c* (solid line) and in the absence of pEC86 (dashed line). (*B*) SDS/PAGE *Ttc*NOR expressed in *E. coli*. Protein was expressed in a strain with the pEC86 plasmid (lanes 2 and 4) or without pEC86 (lanes 1 and 3). Lanes 1 and 2 were stained with Coomassie Brilliant and lanes 3 and 4 were stained for heme *c* (2).

Fig. S1

1. Arslan E, Schulz H, Zufferey R, Künzler P, Thöny-Meyer L (1998) Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the cbb3 oxidase in *Escherichia coli*. *Biochem Biophys Res Commun* 251(3):744–747.
2. Thomas PE, Ryan D, Levin W (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* 75(1):168–176.

Fig. S2. NO reductase activity of the wild-type and H259F mutant *Ttc*NOR. (*A*) NO reductase activity of *Ttc*NOR. (*B*) Inhibition of the NO reductase activity of *Ttc*NOR by cyanide. (*C*) Demonstration of the lack of NO reductase activity of the H259F mutant of *Ttc*NOR.

Fig. S2

Fig. S3. Phylogenetic tree of different cNOR sequences. The scale bar corresponds to 0.1 changes per amino acid. Blue star: *Pseudomonas aeruginosa* (Gamma-), *Paracoccus denitrificans* (Alpha-), *Pseudomonas stutzeri* (Gamma-), *Halomonas halodenitrificans* (Gamma-) and *Roseobacter denitrificans* (Alpha-) cNORs belong to the *Proteobacteria* phylum. Red star: *Ttc*NOR belongs to the *Thermus* phylum.

Fig. S3

Fig. S4. Oxidized and reduced absorption spectra of different *Tt*cNOR mutants expressed in *E. coli*.

Fig. S4

Fig. S5. Sequence alignment of cNOR family. Residues conserved at the 99% level are marked with an asterisk (*). Residues mutated in this work are identified with a caret (^).

Fig. S5