Characterization of the nitric oxide reductase from *Thermus thermophilus*

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Nitrous oxide (N_2O) is a powerful greenhouse gas implicated in climate change. The dominant source of atmospheric N_2O is incomplete biological dentrification, and the enzymes responsible for the release of N_2O are NO reductases. It was recently reported that ambient emissions of N_2O from the Great Boiling Spring in the United States Great Basin are high, and attributed to incomplete denitrification by *Thermus thermophilus* and related bacterial species [Hedlund BP, et al. (2011) *Geobiology* 9(6)471–480]. In the present work, we have isolated and characterized the NO reductase (NOR) from *T. thermophilus*. The enzyme is a member of the cNOR family of enzymes and belongs to a phylogenetic clade that is distinct from previously examined cNORs. Like other characterized cNORs, the *T. thermophilus* cNOR consists of two subunits, NorB and NorC, and contains a one heme *c*, one Ca^{2+}, a low-spin heme *b*, and an active site consisting of a high-spin heme *b* and Fe_B. The roles of conserved residues within the cNOR family were investigated by site-directed mutagenesis. The most important and unexpected result is that the glutamic acid ligand to Fe_B is not essential for function. The E211A mutant retains 68% of wild-type activity. Mutagenesis data and the pattern of conserved residues suggest that there is probably not a single pathway for proton delivery from the periplasm to the active site that is shared by all cNORs, and that there may be multiple pathways within the *T. thermophilus* cNOR.

proton pathway | thermophilic

Nitric oxide (NO) is important as a signaling molecule in many eukaryotes (animals, plants, and fungi), serves as an immune defense agent in many animals (1), and is also an obligate intermediate of microbial denitrification. NO reduction by prokaryotes is an important environmental process, in part because incomplete denitrification produces nitrous oxide (N_2O) as a final product, which is directly released from cells without being converted into N_2. The unrestrained use of nitrogen fertilizers has led to the release of large amounts of N_2O to the atmosphere as a result of this process. N_2O is a more potent greenhouse gas than CO_2 (2) and contributes to the depletion of the ozone layer (3). In the present work, the enzyme that is at least partially responsible for the N_2O ambient emissions from the Great Boiling Spring (and presumably other hot springs) has been isolated and characterized (4).

In denitrification, NO reduction is carried out by NO reductase (NOR) members of the heme-copper oxidoreductase (HCO) superfamily, which also includes oxygen reductases (quinol and cytochrome oxidases). There are two closely related heme-copper NOR families: the cNOR and qNOR families. These are integral membrane enzymes that catalyze the two-electron reduction of NO to N_2O:

$$2NO + 2e^- + 2H^+ \rightarrow \ N_2O + H_2O$$

The cNORs are composed of two subunits, NorC (~17 kDa) and NorB (~56 kDa). NorC is a monoheme *c*-type cytochrome that possesses an N-terminal transmembrane helix that anchors the heme domain to the periplasmic face of the cytoplasmic membrane. Its function is to relay electrons from the periplasmic donor proteins (cytochromes and cupredoxins) to the catalytic subunit, NorB (5, 6). NorB is structurally related to the catalytic subunit (subunit I) of the respiratory HCOs (5, 7, 8). NorB contains 12 transmembrane helices and binds three metal centers: a low-spin heme *b* and a binuclear active site composed of a high-spin heme b_3, and a nonheme iron (Fe_B). Fe_B is coordinated by three histidine residues that are conserved in all typical HCOs and a glutamate residue that is conserved in all cNORs and qNORs (5). The qNOR family, which receives electrons from quinols, is very similar to the cNOR family, with the exception that NorB and NorC are fused into one subunit and the cytochrome *c* binding site is missing (9). Unlike the oxygen reductases, the cNORs are not electrogenic and are unable to pump protons (10, 11). The protons required for chemistry are delivered from the periplasmic side of the membrane and not from the cytoplasmic side, as in the case of the oxygen reductases.

Enzymes belonging to the cNOR family have been isolated from five denitrifying bacteria: *Pseudomonas stutzeri* (12, 13), *Paracoccus denitrificans* (14, 15), *Halomonas halodenitrificans* (16, 17), *Roseobacter denitrificans* (18, 19), *Pseudomonas nautica* (20), and *Pseudomonas aeruginosa*, for which an X-ray structure has been reported (5, 21). All of these strains are in the *Proteobacteria* phylum. Mutagenesis studies have been done (6, 22), but before the report of the X-ray structure of cNOR from *P. aeruginosa* (5).

Members of the *Thermus* order are thermophilic bacteria of great biotechnological interest as a source of thermostable enzymes (23). The most characterized strains belonging to the *Thermus* genus are strict aerobes; however, a few isolates have been shown to be facultative anaerobes, growing by reducing nitrate to nitrite (nitrate reduction) or by reducing nitrate and nitrite to either N_2O (incomplete denitrification) or N_2 (complete denitrification) (24). Denitrifying *Thermus thermophilus* strains are a contributor of the N_2O released from thermal systems (24), and in this work the operon encoding the enzyme responsible for this has been cloned from the denitrifying strain *T. thermophilus* PRQ25 (25) and has been expressed in both *T. thermophilus* and *Escherichia coli*.

Results

cNOR Expression and Purification. The operon encoding the cNOR from *T. thermophilus* (*Tt*cNOR) was cloned and expressed homologously in *T. thermophilus*. The enzyme was purified by affinity chromatography using a His-tag placed on the C terminus of the NorB subunit. However, because the yield obtained was very low (0.04 mg from 1 L of culture), heterologous expression in *E. coli* was attempted. Expression of cNOR in *E. coli* required the presence in the host strain of a plasmid containing the genes required for the assembly of cytochromes *c*, pEC86 (26), and another plasmid carrying tRNA genes needed for

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reading rare codons (pRARE) (27). This process was successful, and the yield of *Tt*cNOR using the *E. coli* expression system was about 20-times greater than in *T. thermophilus*. SDS/PAGE (Fig. S1*B*) shows the two bands corresponding to NorB and NorC, and heme-staining shows that the NorC subunit contains heme *c*. The reduced-minus-oxidized spectrum has diagnostic peaks for both the heme *c* (553 nm) and low-spin heme *b* (560 nm) components of *Tt*cNOR. Without the pEC86 plasmid in the *E. coli* host strain, the heme *c* component of *Tt*cNOR is absent (Fig. S1*A*) and the relative intensity of the NorC subunit in the SDS/PAGE gel is low (Fig. S1*B*), suggesting that the NorC subunit is unstable if the heme *c* is not properly assembled into the protein.

*Tt*cNOR is active with different electron donors (Table 1). NOR activity could not be measured at 75 °C, the optimum growth temperature for *T. thermophilus* (28), because of limitations of the NO-sensing electrode. Therefore, assays were performed at 42 °C, which is below the optimum growth temperature. The turnover number (k_{cat}) obtained at 42 °C for the *Tt*cNOR is considerably smaller than those reported for other cNORs (5, 6, 13, 16, 18, 20). The highest activity obtained with the *Tt*cNOR was about 9 electrons (e^-)/min (at 42 °C) with the electron donor PMS (phenazine methosulfate) (Table 1), which presumably donates electrons directly to the active site in the NorB subunit without involving the heme *c* in the NorC subunit (5). The turnover number of *Tt*cNOR in the presence of 2.5 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine), which donates electrons to the heme *c* component of the enzyme, was about 5.5 e^-/min (at 42 °C) (Table 1). The recombinant form of the soluble cytochrome c_{552} of *T. thermophilus*, was expressed in *E. coli*, purified as previously described (29), and tested as an electron donor to *Tt*cNOR. In the presence of 30 μM cytochrome c_{552} (plus 0.5 mM TMPD to maintain the cytochrome c_{552} reduced) *Tt*cNOR had a turnover number of about 2.5 electrons per minute (at 42 °C) (Table 1). In the absence of cytochrome c_{552} no activity is observed at this low concentration of TMPD. Unless indicated, all assays of *Tt*cNOR were performed with TMPD (2.5 mM) as the electron donor. The *Tt*cNOR exhibited no detectable oxygen reductase activity.

Regardless of the electron donor, the enzyme activity was completely inhibited by the addition of 100 μM KCN (Fig. S2), demonstrating that the observed NO consumption is enzyme-dependent and not an artifact of the activity assays. The cNOR obtained by heterologous expression in *E. coli* has the same turnover number (6 ± 0.8 e^-/min at 42 °C with 2.5 mM TMPD as electron donor) as the enzyme expressed homologously in *T. thermophilus* (Table 1).

Sequence Analysis. A phylogenetic tree of the cNOR family (Fig. S3), shows that the *Tt*cNOR belongs to a different clade from the previously characterized cNORs (5, 6, 13, 16, 18). The *Tt*NorC is ~75 aa longer than the homologs from *Proteobacteria* and contains an N-terminal sequence with two predicted transmembrane helices instead of one as found in the cNORs from *Proteobacteria* (5). Homologs from other *Thermus* species and from Aquificae also have two predicted transmembrane helices at the N terminus of the NorC subunit. In addition, the *Tt*cNOR operon contains an additional (third) ORF following the *norB* gene, which encodes a hypothetical protein containing 85 amino acids and three putative transmembrane helices. This ORF was included in the constructs used for expression of cNOR from *T. thermophilus*, but the putative third subunit was not detected in the isolated cNOR.

Table S1 lists the most conserved residues in cNORs (over 150 sequences) and their possible functions, inferred in part from the structure of the *P. aeruginosa* cNOR (*Pa*cNOR) (5). Table S1 also includes residues in the NorC subunit that have been postulated to be involved in the proton delivery from the periplasm to the active site (5). These residues are not conserved within the cNORs.

Table 1. NOR activity of the *Tt*cNOR with different electron donors

Electron donors	k_{cat} (e^-/min)	*n*	% Activity
TMPD 2.5 mM	5.5 ± 0.5	15	100
PMS 10 μM	9 ± 0.7	5	163
cyt c_{552} 30 μM + TMPD 0.5 mM	2.5	1	46

Data are expressed as average ± SD of *n* independent experiments.

Site-Directed Mutagenesis of the Calcium Ligands. The crystal structure of *Pa*cNOR shows a single Ca^{2+} that is buried within the protein at the interface between NorB and NorC and ligated by two amino acid side chains, E135 in NorB and Y73 in NorC, in addition to being bound to one propionate each in heme *b* and heme b_3 (Fig. 1) (5). Whereas $Y73^{NorC}$ is totally conserved in cNORs (corresponding to $Y137^{NorC}$ in *Tt*cNOR) (Table S1), E135 (E134 in *Tt*cNOR) is replaced by an uncharged or basic amino acid in about 20% of the cNOR sequences (Table S1). In addition, a second glutamate that is near the Ca^{+2} but not ligated to it, is also present in >90% of the cNORs (E138 in *Pa*cNOR; E137 in *Tt*cNOR), replaced only by aspartate in the remaining sequences.

The Ca^{2+} should be structurally important because it bridges both NorB and NorC as well as the propionates of hemes *b* and b_3, which was confirmed by site-directed mutagenesis (Table 2). The E134Q, E137Q, and $Y137F^{NorC}$ mutants each exhibit less than 25% of the wild-type activity. The E134D and E137D mutants of *Tt*cNOR were apparently not assembled or not stable because no protein was observed in either case. Both the size and charge of the two glutamates are important to maintain the structural integrity of the protein. Although E137 is not directly involved in the coordination of Ca^{2+}, it may be important to maintain the conformation of the loop stabilizing the coordination of E134 to Ca^{2+} (Fig. 1), as previously suggested (30).

Site-Directed Mutagenesis of Glutamic Acids Near the Active Site. In addition to E134 and E137 (*Tt*cNOR), there are three additional glutamates that are conserved to varying degrees in cNORs: E211 (100% conserved), E215 (>95% conserved), and E280 (>90% conserved). According to the *Pa*cNOR crystal structure, E211 is a ligand to Fe_B and, along with the three totally conserved histidines (H207, H258, and H259) form a trigonal-bipyramidal coordination structure (Fig. 2). It has been proposed that the E211 carboxylate in cNOR could also function as the shuttle for protons from E280 to the bound-NO (5, 30). E211 was mutated to D, Q, and A. Both E211D and E211Q mutants have 34% of the wild-type activity and, remarkably, E211A has 68% of the wild-type NO reductase activity (Table 2). Hence, the glutamate ligand to Fe_B is not absolutely essential for function in

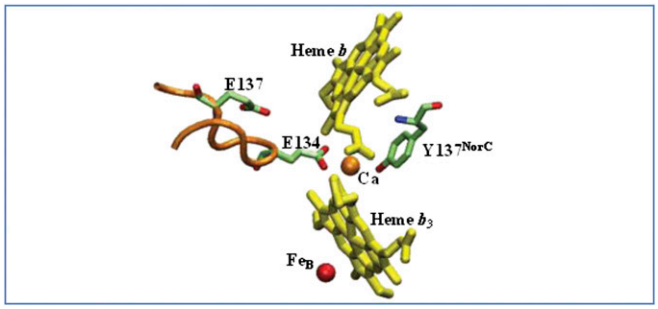

Fig. 1. Calcium binding site at the interface of NorB and NorC. Ca^{2+} is directly ligated to E134 in NorB and to $Y137^{NorC}$ in NorC, along with propionates from heme *b* and from heme b_3. E137 is important to maintain the conformation of the protein loop containing E134 and connecting transmembrane helices III and IV of NorB. The residue numbers are for the cNOR from *T. thermophilus*, and the structure is drawn from the coordinates of the cNOR from *P. aeruginosa* PDB ID code 3O0R (4).

Table 2. NOR activity of *Ttc*NOR mutants and the wild-type enzyme

Site involved	% Conserved	*Ttc* NOR	k_{cat} e^-/min	*n*	% Activity
Wild-type		WT	5.5 ± 0.5	15	100
Sites involved In Ca binding					
E135	>80	E134D	—	—	—
		E134Q	1.0 ± 0.1	2	19
E138	>90	E137D	—	—	—
		E137Q	1.32 ± 0.15	2	24
$Y73^{NorC}$	100	$Y137F^{C}$	0.90 ± 0.05	5	17
Sites near the active site					
E215	>95	E215D	1.70 ± 0.11	3	31
		E215Q	3.1 ± 0.21	9	56
E280	>90	E280D	1.70 ± 0.20	3	31
		E280Q	1.43 ± 0.32	8	26
		E280M	0	4	0
E211	100	E211D	1.87 ± 0.15	7	34
		E211Q	1.88 ± 0.20	7	34
		E211A	3.74 ± 0.35	8	68
H259	100	H259N	0	6	0
Sites in putative proton pathways					
D198	>85	D198E	8.30 ± 1.0	5	151
		D198N	7.50 ± 0.55	4	137
		D198L	—	—	—
$T66^{NorC}$	>80	$T130V^{C}$	0.77 ± 0.16	3	14
$E77^{NorC}$	35	$D141N^{C}$	–	—	—
		$D141L^{C}$	0.99 ± 0.18	4	18
$N80^{NorC}$	<10	$R144M^{C}$	3.30 ± 0.22	4	60
$R84^{NorC}$	<10	$D148L^{C}$	1.11 ± 0.17	3	20
$E145^{NorC}$	>50	$D209E^{C}$	1.92 ± 0.25	5	35
		$D209N^{C}$	1.65 ± 0.23	3	30
		$D209L^{C}$	0	3	0
H339	100	H339F	0	5	0
N335	100	N335L	0.82 ± 0.11	4	15
S277	100	S277L	2.47 ± 0.26	4	45
T330	100	T330L	2.36 ± 0.30	6	43
		T330L/S277L	0	9	0

Data are expressed as the average of ± SD of *n* independent experiments. All mutants had normal UV-visble spectra with the exception of H259N (Fig. S4).

this enzyme. In contrast, when one of the histidine ligands to Fe_B, H259 (Fig. 2 and Table S1), is mutated to an asparagine, the purified H259N mutant has no detectable activity (Table 2).

Metal analysis of two different preparations indicates that the iron of E211A (4 ± 0.32 irons per molecule of protein) is identical to that of the wild-type (4 ± 0.25 irons per molecule of protein), and no zinc or copper was detected. Furthermore, the UV-visible spectra of the wild-type and E211A mutant cNORs are similar [e.g., peak positions and peak ratios of the Soret (418 nm) and α (550 nm) bands]. In comparison, the spectrum of the fully reduced H259N mutant cNOR has a significantly higher A_{418}/A_{550} ratio (Fig. S4), suggesting a significant perturbation of the structure of this mutant.

E280 is hydrogen bonded to E211 (Fig. 2) (5). E280D and E280Q mutations show a significant decrease (to about 30%) in NOR activity, and E280M is totally inactive (Table 2). E215 is near E211 but the two glutamates are not within hydrogen bonding distance (Fig. 2). The E215 carboxylate group could contribute to the electronegative environment of the binuclear center of cNOR and be a factor in determining the low midpoint 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 (31). The E215D and E215Q mutations retain 31% and 56% of the wild-type activity, respectively (Table 2). These results are similar to previous reports of mutating the equivalent residue (E202) in *P. denitrificans* (6, 22).

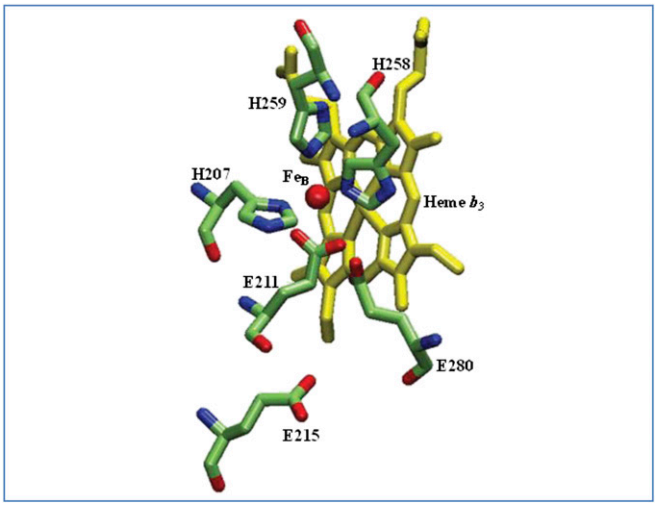

Fig. 2. The binding site of the nonheme iron (Fe_B) in cNOR. Fe_B is coordinated by E211, H207, H258, and H259. Also shown are E280 and E215. These residue numbers are the same for cNORs from *T. thermophilus* and *P. aeruginosa*, and the structure is drawn from the coordinates of the cNOR from *P. aeruginosa* PDB ID code 3O0R (4).

Site-Directed Mutagenesis of Putative Proton Entry Channels. The protons required for chemistry in the NORs are delivered from the periplasmic side of the membrane. Three possible pathways have been proposed from the bulk water in the periplasm to the region of the heme b_3 propionates, and several pathways have been suggested for proton transfer from the heme propionates to the NO bound at the active site.

Bulk water to propionates. Two hydrophilic channels connecting the periplasm to the propionates of heme b_3 were observed in the *Pac*NOR crystal structure (5), and a third channel has been proposed based on molecular dynamics (32). Channel 1 starts from the $E57^{NorC}$ (*Pac*NOR numbering) at the periplasmic surface and includes D198 and E135 (Ca^{2+} ligand), and ends at a propionate-A of heme b_3. However, $E57^{NorC}$ is only about 75% conserved among the cNOR family (Fig. S5), and is a glutamine *Ttc*NOR (Q121 in *Tt*NorC). D198 (same number in *Tt*NorB) is 85% conserved within the cNORs. The D198E, D198N, and D198L mutations were made in *Tt*NorB. D198E and D198N mutants had no deleterious effect in the enzyme activity but D198L was not assembled (Fig. 3 and Table 2).

Channel 2, observed in the *Pac*NOR structure (5), includes polar residues in the NorC subunit: $E145^{NorC}$; $E77^{NorC}$, and $T66^{NorC}$. These residues correspond to $D209^{NorC}$, $D141^{NorC}$, and $T130^{NorC}$ in in *Ttc*NOR (Fig. 3). The putative entrance of channel 2, $E145^{NorC}$ ($D209^{NorC}$ in *Ttc*NOR), is only about 50% conserved and is not an acidic residue in many cNOR sequences (Table S1). $T130^{NorC}$ (*Ttc*NOR) is about 80% conserved in cNORs (often replaced by a serine). $E77^{NorC}$ ($D141^{NorC}$ in *Ttc*NOR) is either a glutamate or an aspartate in the corresponding position in all other cNORs (Fig. S5 and Table S1). In addition, $N80^{NorC}$ and $R84^{NorC}$ are in the same region of the *Pac*NOR channel 2, although they are both very poorly conserved (<10%); these correspond to $R144^{NorC}$ and $D148^{NorC}$, respectively, in *Ttc*NOR (Fig. 3).

Substitutions for $D209^{NorC}$, the putative channel entrance, did reduce NOR activity: $D209E^{NorC}$ (30%), $D209N^{NorC}$ (30%), and $D209L^{NorC}$ (20%) (Table 2). Mutations $T130V^{NorC}$, $D141L^{NorC}$, and $D148L^{NorC}$ similarly exhibited less than 20% activity, whereas $D141N^{NorC}$ was not assembled. $R144M^{NorC}$ retained about 60% of the wild-type activity (Table 2). Aside from $D141N^{NorC}$, which is not assembled, all of the mutations have SDS/PAGE patterns and UV/vis spectra that are similar to the wild-type enzyme. This pattern of phenotypes suggests that the selected residues are important for the full function of the cNOR. It is noted that

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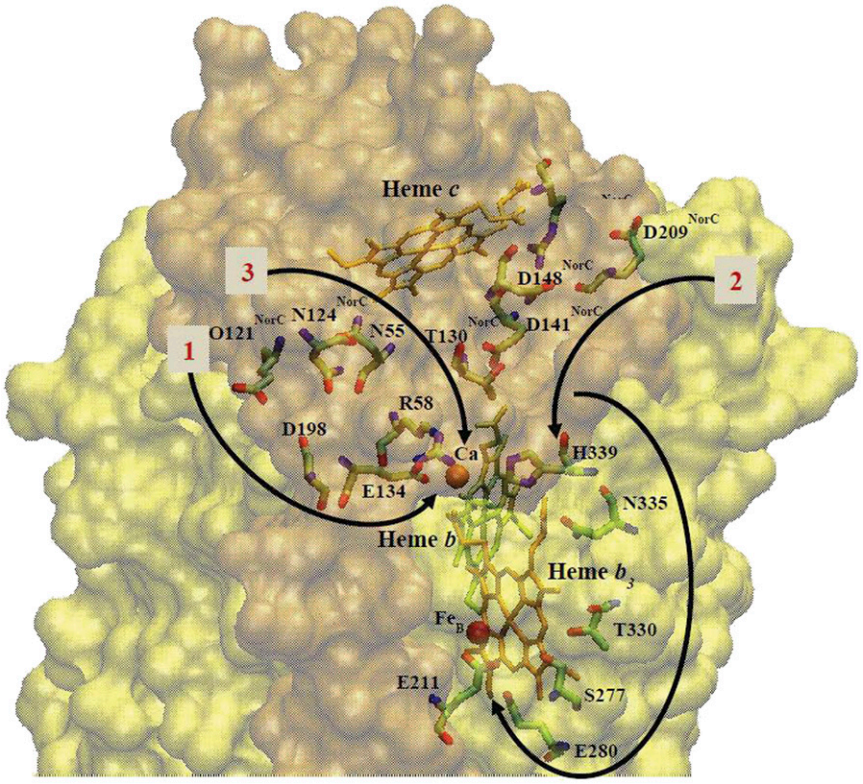

Fig. 3. Residues putatively involved in facilitating proton transfer from the periplasm to the active site (4, 32). The residue numbers are for the cNOR from *T. thermophilus*, and the structure is drawn from the coordinates of the cNOR from *P. aeruginosa* PDB ID code 3O0R (4). The surfaces of NorC and NorB subunits are shown in orange and yellow, respectively. Arrows illustrate the proposed proton pathways.

these residues are near the heme *c* component of the NorC subunit, for example D141^{NorC} (4.86 Å) and R144^{NorC} (2.35 Å).

Channel 3 was proposed based on computational studies (32) and involves N54, N60^{NorC}, E135 (Ca^{2+} ligand), E138, and R57 in *Pac*NOR; these correspond to N55, N124^{NorC}, E134, E137, and R58 in *Ttc*NOR. There are internal water molecules interacting with E135, E138, and R57 (*Pac*NOR), which connect to the bulk water when N54 and N60^{NorC} (*Pac*NOR) move away from their observed positions in the crystal structure (5) and assume an "open" conformation (32). N54 and N60^{NorC} (*Pac*NOR) are 76% and 55% conserved, respectively, within cNORs (Table S1). E138 (*Pac*NOR) is always either E or D in other cNORs, whereas R57 (*Pac*NOR) is either R or K. The only mutations testing channel 3 were those in the two glutamates, E134 and E137 in *Ttc*NOR, which are also implicated in Ca^{2+} binding (Table 2), where it is clear that they are each important for both stability and for function.

Propionates-to-active site. Four different pathways have been suggested for protons to get to the metal-bound NO and bound to the heme b_3 Fe or Fe_B (32). Two pathways involve only internal water molecules, one involves the H259 ligand to Fe_B (which is an essential residue), and the fourth channel involves several amino acid residues that, along with internal water molecules, could facilitate proton transfer from propionate-D of heme b_3 to E211, which is ligated to Fe_B: T330→ S277→ E280 → E211. Propionate-D of heme b_3 is hydrogen-bonded to N335 and H339, which could also be part of this pathway.

S277, T330, N335, and H339, are totally conserved in all cNORs, and E280 is found in >90% of cNORs (Fig. 3 and Table S1). These residues are present in the *Ttc*NOR and have the same residue numbers as in the *Pac*NOR. In the NorB subunit, N335 and H339 are each hydrogen-bonded to propionate-D of the active-site heme b_3 and are fully conserved in all cNORs. The N335L and H339F mutants were assembled but exhibited less than 15% NOR activity. The S277L and T330L mutations each retained about half of the wild-type activity, whereas the double-mutant was inactive (Table 2). The S227L/T330L double-mutant was also obtained in very low yield, suggesting that this mutant is unstable. As previously discussed, mutations of E280 substantially reduced the activity, E280Q (26%), E280D (31%), and E280M (0%), whereas E211A is 68% active (Table 2).

Discussion

The cNOR from *T. thermophilus* belongs to a different clade within the cNOR family than the proteobacterial cNORs previously characterized (Fig. S3) (5, 12–19, 21). The recombinant *Ttc*NOR has the same properties whether it is isolated from an *E. coli* or from a *T. thermophilus* expression system. The purified enzyme has two subunits, NorB and NorC, and has the same heme and metal content as the proteobacterial cNORs: one heme *c* in the NorC subunit, and two hemes *b* plus a nonheme Fe_B in the NorB subunit. As with the proteobacterial cNORs, the *Ttc*NOR is active with the artificial electron donors TMPD and PMS, but is also active with purified *T. thermophilus* cytochrome c_{552} (33), which is likely to be a natural substrate. The k_{cat}, measured at 42 °C is low, about 0.15 e^-/s, compared with the cNOR from *P. aeruginosa*, which has been reported to have a k_{cat} of 7 e^-/s with PMS (5, 6). The activity of the *T. thermophilus* enzyme could not be measured at the optimum growth temperature of the organism (75 °C) because of the limitations of the NO-sensing electrode.

The enzyme activity is the same for initial NO concentrations between 10 μM and 30 μM, demonstrating that the K_M is less than 10 μM and also that there is no substrate inhibition by NO within this concentration range. Although an accurate measure of the K_M for NO was not attempted, the steady-state rate clearly dropped during assays as the NO concentration went from 10 μM to 1 μM. Hence, the K_M for NO at 42 °C is in the low micromolar range. This range is much higher than the K_M reported for the cNOR from *P. stutzeri* (1–2 nM) (13).

The pattern of conserved residues shared within the proteobacterial cNORs is maintained in the *Ttc*NOR. Site-directed mutagenesis was used to address several questions about the roles of specific residues.

Ca^{2+} Binding. $Y137^{NorC}$ (100% conserved), E134 (80% conserved), and E137 (90% conserved) are important for the stability of *Tt*cNOR, probably by stabilizing the binding of Ca^{2+}. Both $Y137^{NorC}$ and E137 are direct ligands to Ca^{2+}, whereas E137 probably stabilizes the protein conformation near the bound Ca^{2+} (30). It is interesting that the nonligating E137 is always an acidic residue (E or D), whereas a significant number of cNORs have nonacidic residues at the corresponding location of the ligating E134. This finding suggests that there are alternative ways to stabilize the Ca^{2+} at this site (or to stabilize the protein without Ca^{2+}).

There is a single Ca^{2+} in the same location in the homologous qNORs (9) and also in the C-family oxygen reductases (cbb_3 oxygen reductases) (8). Site-directed mutagenesis experiments have also concluded that the pair of glutamic acids corresponding to E134 and E137 in *Tt*cNOR is required for structural stability of both the qNORs (9) and the cbb_3 oxygen reductases (34). The A and B family of oxygen reductases do not have Ca^{2+} at the equivalent position but, rather, have a pair of arginine residues that appear to have a similar function (35–38).

Fe_B Binding. E211, H207, H258, and H259 are ligands to the nonheme Fe_B in the cNOR active site and each of these residues is totally conserved within cNORs. The NO reductases are distinguished from the O_2 reductases in the heme-copper oxidoreductase superfamily by the presence of Fe_B in the active site, replacing the Cu_B present in all of the oxygen reductases. In the A- and B-family oxygen reductases, the position corresponding to E211 is always a tyrosine, which is covalently cross-linked to the equivalent of H207 (*Tt*cNOR). In the cbb_3 oxygen reductases (C family), the histidine-tyrosine cross-linked pair of amino acids is also present in the active site but the tyrosine originates from a different position in the sequence (39, 40). Remarkably and unexpectedly, E211 can be replaced in the *Tt*cNOR with substantial retention of activity (e.g., E211A is 68% active). It is clearly not essential to have a glutamate, an acidic residue, or even a potential metal-ligating amino acid, at this site.

The equivalent to E211 has been mutated to an alanine in the cNOR from *P. denitrificans*, with very different results showing a virtual elimination of catalytic function (6, 22, 41). In addition, computational (42, 43) as well as modeling studies (44) on NOR activity have emphasized the importance of the negative charge provided by E211 to NOR activity. One explanation for the relatively high activity of the E211A mutant is that the carboxylate group, at least in the *Tt*cNOR, can be replaced by a hydroxyl (or water) ligand. Further work will be needed to test this proposal. In any event, it is clear that the conformation of the cNOR is not dependent on the ligation of E211 to Fe_B. This finding is consistent with the idea that the glutamate ligand may dissociate from Fe_B as part of the catalytic cycle (5, 30).

It should be noted that the cytochrome cbb_3 oxygen reductase from *P. stutzeri* exhibits NOR activity with a $k_{cat} \sim 2\ s^{-1}$ (45), which is comparable to the *P. stutzeri* cNOR, $k_{cat} \sim 2–7\ s^{-1}$ (13). Although the C-family (cyt cbb_3) oxygen reductases are related to the cNORs, the cytochrome cbb_3 contains Cu_B instead of Fe_B at the active site and also lacks the equivalent of the glutamates present in most cNORs (E211, E215, and E280 in *Tt*cNOR). Hence, these glutamates and Fe_B are not essential for catalytic NOR function. However, the K_M values are very different for the NO reductase activity of the *P. stutzeri* cNOR (1–2 nM) (13) and the *P. stutzeri* cbb_3 oxygen reductase (12–14 μM) (45), so the cNOR is clearly catalytically superior in the physiologically relevant range of substrate concentrations.

Pathways for Proton Delivery to the Active Site. There is no question that the protons required for the chemistry catalyzed at the active site of the cNORs are all taken from the bulk solution on the periplasmic side of the membrane. This finding is opposite of the heme-copper oxygen reductases, in which all of the protons are taken from the cytoplasmic side of the membrane. In the case of the oxygen reductases, the proton delivery channels can be identified by patterns of conserved residues, unique to each family (A, B, or C) of enzymes, and confirmed by site-directed mutagenesis (7, 46, 47). The proton-input channels of the oxygen reductases facilitate proton diffusion from the cytoplasmic bulk water along hydrogen bonds formed by internal water molecules and protonatable amino acid side chains. Single point mutations of conserved residues in the proton-input channels in the oxygen reductases can result in the virtual elimination of catalytic function. In contrast, there is no pattern of conserved residues within the cNORs that can be used to define a pathway leading from the periplasm to the vicinity of the region of the propionates of heme b_3 (Table S1). With the exception of the tyrosine that ligates the Ca^{2+}, and the cytochrome *c* binding residues (including a CXXCH motif), no residues in the NorC subunit is anywhere near totally conserved (Table S1). However, the lack of conserved residues in NorC is accompanied by a large number of internal water molecules that are either resolved in the X-ray structure of the *Pa*cNOR (5) or postulated by computational methods (32). These internal water molecules are present both in those portions of the NorB and NorC subunits that separate the heme propionates from the bulk periplasmic water, and are important components in each of the three proposed proton input channels proposed for cNORs. It is very likely, therefore, that there is no proton input pathway that is universally shared by all cNORs, but that small structural differences might open or close different pathways in individual cNOR variants. Multiple proton input pathways might easily be used in each cNOR, so no residue would be absolutely required for this function. This result would be consistent with the mutagenesis data in the current work on the *Tt*cNOR. The lack of conservation of residues in the proposed channels and the abundance of internal water molecules means that conclusions concerning proton pathways in the *Tt*cNOR from mutagenesis may not apply generally to other cNORs. Based on these mutagenesis data (Table 2), channels 2 and 3 may be used in the *T. thermophilus* cNOR, whereas channel 1 seems less likely. It is interesting that molecular dynamics studies suggest that channel 2 is not likely to function to deliver protons in *Pa*cNOR because there is a region in the NorB subunit where the connection between internal water molecules is broken (32). If this is also the case for the *T. thermophilus* cNOR, then the major proton pathway would be that corresponding to channel 3 in *Pa*cNOR. Channel 3 was proposed for *Pa*cNOR based on molecular dynamics studies showing that small side-chain motions would open a pathway from the bulk solution to internal waters near the Ca^{2+} site and the heme propionates. The critical amino acids that anchor the internal water molecules in this region of the protein are also structurally important, most notably the two glutamates required for Ca^{2+} binding (E134 and E137 in *Tt*cNOR). These two glutamates were postulated to be important for proton input before information about the structure of the *Pa*cNOR or their role in Ca^{2+} binding (6, 10, 41).

Several pathways have been proposed for protons to reach their final destination at the bimetallic active site from the region around the heme b_3 propionates (22, 32). The most prominent suggestion is for protons to follow a pathway from propionate-D of heme b_3→(water)→T330→S277→E280→E211 in *Tt*cNorB (Fig. 3). Although these residues are highly conserved (Table S1), mutagenesis does not indicate that any one of these residues is absolutely essential for function, including the Fe_B ligand E211. Mutants do have significantly lower activity: E280Q (26%), E280M (0%), S277L (43%), and T330L (45%). Previously, it was reported that the E267A mutation in the cNOR from *P. denitrificans* (equivalent to E280 in *Tt*cNOR) has 5% activity (6, 22). Hence, this pathway remains a possibility and is not excluded by mutagenesis data. On the other hand, the proximity of these residues to heme b_3 and Fe_B make it equally

likely that mutations might reduce activity independent of any effects on proton transfer.

Materials and Methods

The operon that was cloned for the expression of cNOR from *T. thermophilus* was obtained from genomic DNA from strain PRQ25. This was expressed in *E. coli* strain C43(DE3) using the pET22b vector. All details for the cloning, expression, mutagenesis, cell growth, protein expression, and purification are included in the *SI Materials and Methods*.

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