Defining the Proton Entry Point in the Bacterial Respiratory Nitric-oxide Reductase

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The bacterial respiratory nitric-oxide reductase (NOR) is a member of the superfamily of O₂-reducing, proton-pumping, heme-copper oxidases. Even although nitric oxide reduction is a highly exergonic reaction, NOR is not a proton pump and rather than taking up protons from the cytoplasmic (membrane potential-negative) side of the membrane, like the heme-copper oxidases, NOR derives its substrate protons from the periplasmic (membrane potential-positive) side of the membrane. The molecular details of this non-electrogenic proton transfer are not yet resolved, so in this study we have explored a role in a proposed proton pathway for a conserved surface glutamate (Glu-122) in the catalytic subunit (NorB). The effect of substituting Glu-122 with Ala, Gln, or Asp on a single turnover of the reduced NOR variants with O₂, an alternative and experimentally tractable substrate for NOR, was determined. Electron transfer coupled to proton uptake to the bound O₂ is severely and specifically inhibited in both the E122A and E122Q variants, establishing the importance of a protonatable side chain at this position. In the E122D mutant, proton uptake is retained but it is associated with a significant increase in the observed pKₐ of the group donating protons to the active site. This suggests that Glu-122 is important in defining this proton donor. A second nearby glutamate (Glu-125) is also required for the electron transfer coupled to proton uptake, further emphasizing the importance of this region of NorB in proton transfer. Because Glu-122 is predicted to lie near the periplasmic surface of NOR, the results provide strong experimental evidence that this residue contributes to defining the aperture of a non-electrogenic “E-pathway” that serves to deliver protons from the periplasm to the buried active site in NOR.

Under anaerobic conditions some bacteria can use nitrate instead of oxygen as their terminal electron acceptor in a process called denitrification, in which nitrate is reduced stepwise via nitrite, nitric oxide, and nitrous oxide to dinitrogen. The N₂ gas is released into the atmosphere, thereby counteracting the process of nitrogen fixation. During denitrification, the reduction of nitric oxide (NO)³ to nitrous oxide (N₂O) (as shown in the equation 2 NO + 2 H⁺ + 2 e⁻ → N₂O + H₂O) is catalyzed by a membrane-bound nitric-oxide reductase. For reviews on denitrification see Refs. 1, 2 and on nitric-oxide reductase see Ref. 3.

Bacterial nitric-oxide reductases (NOR) are integral membrane proteins, purified as one- or two-subunit complexes. The large catalytic subunit was shown by homology searches to be a divergent member of the superfamily of O₂-reducing heme-copper oxidases (HCuOs) (4, 5). The NOR from Paracoccus denitrificans is purified as a two-subunit complex, NorBC. NorB is the catalytic subunit and harbors a low spin heme b₁, a high spin heme b₃, and a non-heme iron, Fe₉, which is presumed to take the place of CuB found in the active site of HCuOs (6, 7). The heme b₁ and Fe₉ form a binuclear center, which is the site of NO reduction. NorC is a membrane-anchored protein with a periplasmic domain that contains heme c, which is the site of electron entry from each of the water-soluble electron donors, cytochrome c₅₅₀ and pseudoazurin (3, 8).

The bioenergetics of succinate-dependent NO respiration in membrane vesicles of Rhodobacter capsulatus were studied by monitoring the formation of a transmembrane electrochemical gradient (9). Using ascorbate to donate electrons directly to NOR in membranes with their cytochrome bc₁ complex inhibited did not lead to the generation of a transmembrane potential. These observations led the authors to conclude that, unlike the HCuOs, the highly exergonic NO reduction as catalyzed by NOR is not electrogenic, i.e. not coupled to charge translocation across the membrane. More recent electrometric measurements (10, 11) of NO and O₂ reduction by NorBC reconstituted into proteoliposomes show that neither reaction is electrogenic. Because electrons are supplied to NorBC from the cytochrome bc₁ complex via soluble donors in the periplasm, the lack of electrogenicity means that the protons required by these reactions must also be derived from the periplasm. Consequently, NOR must contain a proton transfer pathway leading...
from the periplasmic side of the membrane into the catalytic site (see Fig. 1).

The molecular nature of the proton uptake route is not known, but one was recently suggested based on a three-dimensional model of the NorB subunit and the conservation of protonatable residues within this subunit (11). In addition, searches for residues conserved in NOR, but not present in all HCuOs, identified five glutamate residues, some of which may be involved in proton movements (12). These glutamates are found at positions 122, 125, 198, 202, and 267, and they have been individually mutated to non-protonatable residues (8, 12). Those residues that are both essential for normal steady-state activity and form part of the proposed proton input pathway are Glu-122, Glu-125, Glu-198, and Glu-267. Both topological and three-dimensional models of NorB (11, 13) predict that these glutamates are located either close to the periplasmic surface or in the middle of the membrane close to the FeB site (FeB-Glu198 and FeB-Glu267).

The P. denitrificans NOR is, in addition to the physiological NO reduction, capable of reducing O2 to water (12, 14, 15). The levels of O2 and NO reductase activity are closely correlated, implying that the same catalytic components are involved. To study the mechanism of proton transfer in NOR, we have previously used the reaction with O2 because the chemical reactivity of NO in aqueous solutions hampers direct pH measurements. We characterized the single-turnover reaction between fully reduced detergent-solubilized NOR and O2 using the flow-flash technique in combination with time-resolved optical spectroscopy (15). Our results showed that oxygen binds to heme b2 with a $\tau = 40\ \mu s$ (at 1 mM O2), after which electron transfer from the low spin hemes b and c to the O2 bound at the binuclear site occurs with a $\tau = 25\ ms$ (at pH 7.5). A slow additional phase of oxidation of hemes b and c with $\tau \sim 1\ s$ presumably completes the reduction of O2 to H2O. The $\tau = 25$-ms phase is coupled to proton uptake from the bulk solution, and the rate constant depends on pH in a way well described by a model in which the rate is limited by proton transfer from an internal protonatable group, in rapid equilibration with the bulk protons, into the active site with a $k_{\text{on}} = 250\ s^{-1}$. The internal group has a $pK_a$ of $\sim 6.6$ and was suggested to be an amino acid located close to the active site (15, 16).

The $\tau = 25$-ms reaction represents a specific step in the catalytic cycle that is limited by the rate of proton transfer. Consequently, mutations that change the properties of the proton uptake pathway are expected to influence this rate. Here, we have studied the reaction between fully reduced NOR and O2 in mutant forms of NOR where the glutamate at position 122 has been exchanged for either alanine, glutamine, or aspartate, with special emphasis on what the effects of the mutations are on the $\tau = 25$-ms phase. Also, the effects of exchanging a nearby glutamate, Glu-125, with alanine or glutamine are examined. Our results provide strong experimental evidence that both glutamates are needed for efficient proton transfer in NOR and that Glu-122 is at the aperture of a non-electrogenic E-pathway5 that serves to deliver protons from the periplasm to the buried active site in NOR.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacteria and Purification of NOR**—The NOR used in this study was from P. denitrificans, expressed in Escherichia coli JM109 using the expression system described in Ref. 12. Growth of bacteria, the preparation of cytoplasmic membranes, and the subsequent purification of NOR was as previously described (12), but with the following modifications. After induction with 1-mM isopropyl-1-thio-β-D-galactopyranoside, batch cultures (800 ml of TB (terrific broth) (18) in a 2-liter un-baffled conical flask) were incubated at 30 °C for 5 h with shaking (180 rpm). The cultures were incubated for a further 5 h at 18 °C without agitation before being returned to 30 °C with shaking (180 rpm) for 8 more hours prior to harvesting the cells.

After application of the sample to the Q-Sepharose HP column, it was washed with 300 ml of buffer A (20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 0.05% β-dodecyl maltoside (DDM)) that contained 15% (v/v) Buffer B (20 mM Tris-HCl, 1 M NaCl, and 0.05% DDM). The column was then developed with a 750-ml gradient of 15–55% buffer B, and the eluent was collected in 5-ml fractions. These conditions yield enzyme that is sufficiently pure for spectroscopic experiments without the need for further purification on chelating Sepharose. After concentration and dialysis into 500 mM Tris-HCl, 0.05% DDM, pH 7.6, the protein was stored at $\sim 80^\circ$C until it was needed.

**Spectroscopic Characterization of the Glu-122 Mutant Forms of NOR**—Each preparation of the mutant forms of NOR was characterized by both UV-visible and CW-X band EPR spec-

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4 Numbering refers to the P. denitrificans NorB sequence.

5 This pathway should not be confused with the E-pathway hypothesis for fumarate reductase (17).
troscopy. UV-visible spectra were recorded between 350 and 700 nm using an Hitachi U3100 spectrophotometer. The concentration of oxidized NorBC was calculated using the molar extinction coefficient \( (\epsilon_{411} = 3.11 \times 10^4 \text{M}^{-1} \text{cm}^{-1}) \) (19).

EPR spectra were recorded with a Bruker ESP 300 X-band spectrometer equipped with an ESR-900 liquid helium cryostat manufactured by Oxford Instruments. Bruker ESP software version 2.2 was used to manipulate the spectra.

Sample Preparation for Flow-flash Studies—The NOR stock was diluted to 5–10 \( \mu \text{M} \) in 100 mM Hapes, pH 7.5, 50 mM KCl, 0.05% DDM in a modified Thunberg cuvette, air was exchanged for nitrogen on a vacuum line, and the enzyme was reduced by adding 2 mM ascorbate and 0.2 \( \mu \text{M} \) N-methylphenazinium methosulfate. Sometimes, small amounts (10–80 \( \mu \text{M} \)) of sodium dithionite were also added to ensure complete reduction. Nitrogen was then exchanged for 100% CO, and CO recombination to the high spin heme was studied by flash photolysis on a setup described in Ref. 20. Before the flow-flash experiments, the CO concentration was lowered to avoid CO recombination interfering with \( \text{O}_2 \) binding (see Ref. 15). The appropriate CO concentration was judged by measuring the apparent CO recombination rate constant.

Flow-flash Measurements—Experiments were performed as in Ref. 15 on a setup described in Ref. 20. Briefly, fully reduced CO-bound NOR was mixed 1:5 with an oxygenated buffer in an appropriate CO concentration was judged by measuring the Soret and visible regions. Typically, at each wavelength and initiation of the reaction. The time course of the reaction was studied as a probe of the integrity of the catalytic site. In the WT formulation, the \( \tau \sim 10 \text{ ms} \) phase in E122D was fitted to a simple Hender-son-Hasselbalch titration of one protonatable group using the equation \( k_{\text{obs}} = k_{\text{max}}/(1 + 10^{pH-pK_a}) \). The data for WT were fitted the same way but with the addition of a slow background rate constant, \( k_{\text{ip}} \), of 1–3 s\(^{-1}\).

RESULTS

The Spectroscopic and Ligand Binding Properties of the NOR Glu-122 Variants—It has previously been shown from studies of E122A, E122Q, and E122D in whole cells and membranes of \( P. \text{denitrificans} \) that the Ala and Gln variants are essentially inactive but that the E122D variant retains close to WT activity (8). To establish the molecular basis for these observations, the spectroscopic and CO binding properties of the purified NOR variants were assessed. The UV-visible and EPR spectra of the oxidized Glu-122 mutants were essentially indistinguishable from WT recombinant enzyme (supplemental Fig. S1). The CW-X band EPR spectra recorded at 10 K (supplemental Fig. S1) showed that the preparations had the expected resonances of the low spin ferric \( c \)-type and \( b \)-type hemes in NOR (see Ref. 12 for a description of the EPR features of recombinant WT NOR). The features arising from high spin ferric heme \( b_3 \) (\( g = 6.0 \)) and ferric \( Fe_b \) (\( g = 4.3 \)) were relatively small, suggesting that the binuclear center (>95%) remains intact with heme \( b_3 \) and \( Fe_b \) magnetically coupled and therefore EPR-silent.

CO recombination to the reduced high spin heme \( b_3 \) after flash photolysis of the CO-bound fully reduced NOR was also studied as a probe of the integrity of the catalytic site. In the WT recombinant NOR, at 1 mM CO, CO recombination is biphasic, with time constants of 5–10 and 50–100 \( \mu \text{s} \) and \( \sim 50\% \) contribution (time constants and relative amplitudes varying slightly between preparations) from each phase (15). No significant changes in CO recombination time constants or relative amplitudes were observed at 1 mM CO in any of the Glu-122 (Ala, Gln, or Asp) variants (data not shown).

Further evidence for the integrity of the active site of NOR was forthcoming from monitoring oxygen binding in the flow-flash reaction between fully reduced NORs and \( \text{O}_2 \). Previous work with WT recombinant NOR has established that \( \text{O}_2 \) binds to heme \( b_3 \) with \( \tau = 40 \text{ ms} \) (at 1 mM \( \text{O}_2 \)). Fig. 2 shows the comparison between wild type, E122A, E122Q, and E122D in the \( \text{O}_2 \) binding phase, demonstrating that none of the mutant NORs show significant changes in binding rate constants or amplitudes. These results taken together with the CO rebind-
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FIGURE 2. A, absorbance changes at 430 nm upon flash photolysis of fully reduced CO-bound WT and mutant NORs in the presence of O2. The traces show the two phases observed in wild type NOR with time constants (τ) of 40 μs and 25 ms and the effects on these phases in the mutant NORs. The WT trace is from Ref. 15. The amplitude of the τ = 25-ms phase varies somewhat between experiments for both WT and E122D; amplitudes have been normalized to the same total amplitude to emphasize the change in time constant (τ = 10 ms in E122D). Experimental conditions were -1 μM reacting NOR in 100 mM Hepes, pH 7.5, 50 mM KCl, 0.05% DDM, [O2] = 1 mM, T = 295 K. The laser flash at t = 0 gives an artifact that has been truncated for clarity. B, the same reaction as in A, studied at 420 nm on a longer time scale. The traces show the τ = 25 ms and the τ = 1–2 s in WT NOR and the effects on these phases in the mutant NORs. Experimental conditions as in A.

FIGURE 3. The pH dependence of the rate constants for the τ = 25 ms (k = 40 s⁻¹) phase in WT (black squares) and the corresponding τ = 10 ms (k = 100 s⁻¹) in E122D (gray circles). The WT data are from Ref. 15. Shown are also the fits (solid and dashed lines) of the data to a simple titration with a single pK_a (see “Experimental Procedures”) giving for WT k_{250} = 250 s⁻¹, pK_a = 6.6, and for E122D k_{250} = 160 s⁻¹, pK_a = 9.1. Experimental conditions as in Fig. 2, except for the buffers used in the pH titration (see “Experimental Procedures”).

Reduction of the b₃²⁺-O₂ Species in the Glu-122 Variants of NOR—In the reaction between fully reduced wild type NOR and oxygen, the second major phase with a time constant of ~25 ms (at pH 7.5) was assigned to the reduction of the b₃²⁺-O₂ species by electron transfer from both the low spin hemes b and c to the catalytic site (15). The comparison between WT and Glu-122 mutant forms of NOR for this phase is shown both at 430 nm (which reports O₂ binding to and oxidation of the ferrous high spin heme b₃) (see Fig. 2A) and 420 nm (which reports oxidation of both low spin hemes) (see Fig. 2B). These experiments clearly show that the τ = 25-ms phase is severely inhibited in the E122A and E122Q mutant forms. At 420 nm no oxidation is observed on this time scale, and at 430 nm there is only a small residual ms phase with <10% of the WT amplitude in E122A and E122Q; see below.

In both the E122A and E122Q mutant NORs, clear oxidation occurs only on the really slow time scale (τ ~ 3 s), showing that the 25-ms phase is either totally absent or severely delayed in these mutants. Before the τ ~ 3-s phase, there are small absorbance changes occurring (Fig. 2B) that cannot be clearly assigned at present. Because only O₂ binding and no oxidation has occurred in these mutants, a possible reaction is the equilibration at the active site between O₂ and the photolysed CO. Another possibility is that the small residual τ = 25-ms phase seen at 430 nm corresponds to fractional oxidation of the binuclear site and that there is fractional re-equilibration of electrons in the mutant NORs on this slower time scale.

In the E122D mutant NOR, however, oxidation is clearly seen at both 420 and 430 nm (Fig. 2, A and B) with a τ ~ 10 ms² at pH 7.5 and an amplitude similar to WT. The τ ~ 10-ms phase in the E122D mutant involves oxidation of both hemes b and c with amplitudes similar to the corresponding τ = 25-ms phase in WT; as studied also at 550 and 560 nm (data not shown).

In WT NOR, there is a slower phase of heme oxidation with a τ = 1–2 s that is presumably due to the final step in the reaction between fully reduced NOR and O₂, producing the fully oxidized NOR and H₂O. In the E122D mutant NOR, this slow reaction occurs similarly to WT (see Fig. 2B). At some pH values in E122D, there is an additional phase with τ ~ 0.1 s that we cannot assign at present but with an amplitude <5% of the total absorbance change. In the E122A and Gln mutant NORs, as the pH was lowered, the τ = 25-ms phase was still absent at 420 nm and no rate constants were fitted to the small residual phase at 430 nm (data not shown).

The Electron Transfer and Coupled Proton Uptake to b₃²⁺-O₂ in E122D NOR—The rate constant for the τ = 25-ms phase in WT NOR has a pH dependence (see Fig. 3) well fitted by a pK_a = 6.6 (+0.06) and maximum rate constant at low pH of 250 (±10) s⁻¹ (15). In the E122D mutant NOR, the pH dependence of the corresponding phase (τ ~ 10 ms at pH 7.5) was significantly shifted compared with WT. Up to pH ~ 8.5, no dependence on pH was observed, whereas at even higher pH the rate constant started to decrease in a way that could be fitted with a pK_a = 9.1 (±0.2) and a maximum rate constant of ~160 (±10) s⁻¹ (see Fig. 3). It should be noted that the pK_a obtained is very approximate because at higher pH (>9.5) the amplitude of the signal started to decrease, possibly as a result of degradation of the protein. Therefore, the pK_a ~ 9 should be considered a

6 For all time (and rate) constants reported, the variation between separate experiments were <20%.
In the E122D mutant NOR, the number “preloaded” in the reduced NOR, see Ref. 15). The discussion on the number of protons taken up during oxidation was studied using the pH-sensitive dye phenol red at 570 nm (for a 20 mM Hepes buffer from the one obtained in the absence of buffer. Experimental conditions: 50 mM KCl, 0.05% DDM, 50 μM EDTA, 40 μM phenol red, [O₂] = 1 mM, T = 295 K. A laser flash artifact around t = 0 has been truncated for clarity.

lower limit, because there is clearly no pH dependence up to pH 8.5. It is also possible that the decrease in amplitude of the τ ~ 10-ms phase seen in E122D at very high pH is a “real” effect (due, for example, to the pKₐ of the intermediate forming at the binuclear site having a pKₐ in this region; see “Discussion”), and the reason we do not observe this amplitude decrease at higher pH in WT is that this phase has become so slow that it overlaps with the τ = 1-s phase.

In WT NOR, ~1 H⁺/reacting NOR was taken up from solution concomitantly with the τ = 25-ms phase (see Fig. 4), as studied using the pH-sensitive dye phenol red at 570 nm (for a discussion on the number of protons taken up during oxidation and the number “preloaded” in the reduced NOR, see Ref. 15). In the E122D mutant NOR, the τ ~ 10-ms phase was also coupled to uptake of protons from the bulk solution (Fig. 4). The net amplitude (see “Experimental Procedures”) of the signal in E122D corresponded to uptake of 0.8 ± 0.2 H⁺/reacting NOR, i.e. approximately the same stoichiometry as in WT.

Reduction of the b₃²⁺ – O₂ Species in the E125 Variants of NOR—
The E125A mutant NOR has been shown to have very low steady-state activity (8, 12). Purification of this variant does not affect the stability or cofactor composition of the enzyme (12). Fig. 5 shows the progress of the reaction between fully reduced NOR and O₂ in the E125A (and E125Q) variants monitored at 430 nm. It is clear from the figure that the O₂ binding (τ = 40 μs) phase occurs with WT rate constant and amplitude in both these mutant forms, indicating that the ligand binding properties of the catalytic site are intact. However, the proton-coupled τ = 25-ms phase is also severely inhibited also in these mutant forms of NOR.

DISCUSSION

Protons needed for the reduction of NO or O₂ in NOR are taken up from the periplasmic (outside) side of the membrane (10, 11, 21). Based on a three-dimensional model of NorB and the conservation of protonatable residues, we recently proposed a possible proton transfer pathway in P. denitrificans NOR (11) (see Fig. 1). This pathway starts at the periplasmic surface with the Glu-122 and Glu-125, located ~30 Å from the heme b₃–Fe₉ active site, continues through the Asp-185 and Thr-243 to Glu-198, with other potential participants being Ser-264 and Glu-267. The Glu-198 is ~9 Å from Fe₈. The Glu-122 and Glu-125 are completely conserved in NOR, and they are furthermore the only conserved protonatable groups that are located at the periplasmic surface of the protein in our model. Mutation of the Glu-122 to either Ala or Gln has been shown to result in severe inhibition of catalytic turnover, whereas mutation to Asp resulted in retention of ~80% of catalytic activity (8). This pattern of inhibition was suggested to be due to the Glu-122 acting as a proton shuttle. Here we investigated that proposal by studying the effects of these mutations on the electron transfer coupled to proton uptake (τ = 25-ms phase) during the reaction between fully reduced NOR and O₂. The results show severe and specific inhibition of this reaction in the E122A and E122Q mutant forms, indicating that the proton-transferring capability of Glu-122 is necessary for the transfer of protons into the active site, and thus confirm its participation in the proton transfer pathway. Furthermore, as shown by the return of the τ = 25-ms phase in the E122D mutant, the functionality of the pathway is retained if the residue at position 122 has a carboxylate side chain.

Furthermore, our spectroscopic characterization of the oxidized state of the Glu-122 variants (supplemental Fig. S1) shows them to be very similar to WT, suggesting that the binuclear center is intact. This is consistent with the wild type-like CO and O₂ binding properties of each of the reduced Glu-122 variants (see “Results”). Consequently, the inhibition we see in the E122A and E122Q variants of the τ = 25-ms phase is not due to perturbations of the active site or impaired ligand binding.

In WT NOR the τ = 25-ms phase is strongly pH-dependent (pKₐ = 6.6) with a rate constant that saturates at low pH. We interpreted this observation in terms of a model in which proton transfer from an internal (within the protein) donor into the active site is rate-limiting for the overall reaction (15) and
where the internal donor is in rapid equilibrium with the bulk solution. The equivalent $\tau \sim 10$-ms phase in the E122D variant shows no pH dependence up to pH 8.5. This behavior made us consider the possibility that in this mutant the reaction was no longer linked to proton uptake from the bulk solution (and the proton(s) needed to reduce $\text{O}_2$ supplied internally from the protein). To address this point, we measured proton uptake from the bulk solution at pH 7.5 using a pH-sensitive dye (Fig. 4). The result clearly shows that proton uptake from the bulk remains coupled to the $\tau \sim 10$-ms phase in E122D. Moreover, the stoichiometry is approximately the same as in WT, $\sim 1$ H$^+$/NOR (15). Therefore, the immediate donor of protons to the active site in the E122D variant is still reprotonated from solution.

The simplest explanation for this changed behavior is that the p$K_a$ of the proton donor is shifted to much higher pH. This is also indicated by the rate constant starting to decrease at pH $\sim 9$ (Fig. 3). The data were fitted to titration of a group with p$K_a = 9.1$, which represents a crude estimate since data recorded above pH 9.5 could not be used (see “Results”). Thus, the p$K_a$ $\sim 9$ should be regarded as a lower limit. The lower $k_{\text{max}}$ in E122D (160 s$^{-1}$ compared with 250 s$^{-1}$ in WT, see Fig. 3) is also consistent with a higher p$K_a$ of the donor, because the smaller the $\Delta pK_a$ between acceptor (here assumed to be the same in the two cases, the O$_2$ intermediate forming at heme b$_5$-Fe$_{35}$ and donor, the lower the expected rate constant (see, for example, Ref. 22).

It seems likely that the proton-accepting intermediate forming at the active site has a p$K_a$ higher than 9.5, because especially in the E122D mutant it is clear that the $\tau \sim 10$-ms phase still occurs at this high pH. It is interesting to note that in many HCuOs, the immediate proton donor to the catalytic site (the Glu-286, Rhodobacter sphaeroides aa$_3$ numbering) has a p$K_a \sim 9$, i.e. much higher than the p$K_a = 6.6$ donor in WT NOR. This could be related to the suggestion that NO chemistry, in contrast to O$_2$ chemistry, does not form high p$K_a$ intermediates (23, 24), which would make it hard to pull protons from a donor with a high p$K_a$.

The question then arises, what is the proton donor with p$K_a = 6.6$ in WT NOR and what is the p$K_a \sim 9$ donor in E122D? Can they be the same species? We previously suggested that the internal proton donor in WT NOR is located close to the active site (11, 15), but the experimental data suggest only that the proton transfer is internal within the protein and say nothing about the distance. The most straightforward explanation would be that it is the Glu-122 itself that is the p$K_a = 6.6$ donor in WT NOR and that the p$K_a \geq 9$ donor in E122D is the Asp-122. However, we consider this scenario unlikely because of the large p$K_a$ shift, which is difficult to envisage for a change from Glu to Asp, especially since this carboxylate is (presumably) located at the protein surface, which is inconsistent with it having such a high p$K_a$.

An alternative possibility is that the p$K_a = 6.6$ donor in wild-type enzyme is an ionizable group that lies close to the active site (15). In this case the substitution of Glu-122 with an aspartate residue might change the rate-limiting step so that transfer of protons beyond the protein surface becomes the slowest step and the observed p$K_a$ reflects the changed donor at the surface. However, we consider this possibility rather unlikely given that the maximum rate constant in E122D changes by less than a factor of two.

Instead, we favor a model in which the p$K_a = 6.6$ donor in wild-type NOR is an ionizable group close to and interacting with Glu-122. Substitution of Glu-122 with an aspartate causes this interaction to be lost. This would in turn result in a drastic change in the p$K_a$ of the donor (to $>9$). Alternatively, the lost interaction could result in another residue, which forms part of the “proton relay network,” taking over the role of proton donor.

To gain some insight into the mechanism of proton uptake in NOR, it is instructive to consider how protons exit bacteriorhodopsin. Substitution of either Glu-194 or Glu-204, both of which lie close to the extracellular surface of bacteriorhodopsin, with glutamine abolishes proton release (25). However, substitution of Glu-194 with an aspartate residue leads to a significant increase in the observed p$K_a$ of the proton release group. This behavior was recently shown to be due to the proton release site in WT bacteriorhodopsin being a delocalized water cluster with an excess proton coordinated between deprotonated Glu-194 and Glu-204 (26). In the E194D mutant, the distance from the H$_3$O$^+$ to the Asp-194 becomes too long for hydrogen bonding and the Glu-204 becomes protonated because of the lost stabilization of the COO$^-$ by H$_2$O$^+$. In E194D, Glu-204 thus becomes the new proton release group with a higher p$K_a$ than the H$_3$O$^+$ ion acting as the donor in WT.

A similar arrangement is proposed in NOR, where the Glu-122 sits close to another conserved glutamate, Glu-125, which has been shown to be crucial for steady-state activity (8, 12). Substitution of Glu-125 with alanine was shown not to affect the stability or cofactor composition of the enzyme (12). As shown in Fig. 5, the $\tau = 25$-ms phase is severely inhibited in both the E125A and E125Q variants. These observations are consistent with Glu-125 also having a role in proton uptake in NOR.

These results are interpreted in terms of a H$_3$O$^+$ ion that is coordinated between Glu-122 and Glu-125 and that acts as the p$K_a = 6.6$ donor in WT NOR. This would require both residues to be ionizable in order to retain proton transfer capability. The large shift in p$K_a$ in E122D (to $>9$) could then be rationalized in terms of the shorter Asp side chain not hydrogen bonding to the H$_3$O$^+$ ion and forcing Glu-125, which is not at the protein surface and could acquire such a high p$K_a$, to take over the role of proton donor. Note that there is also a nearby arginine residue (at position 121, see Fig. 1) that in principle could take over as the proton donor when the interaction between Glu-122 and the H$_3$O$^+$ ion is lost.

In conclusion, we have shown that the Glu-122 forms a crucial part of the proton transfer pathway in NOR and is likely to define the aperture. By analogy with HCuO nomenclature where the D-proton pathway is named after the crucial amino acid residue at its aperture, we term the non-electrogenic proton pathway from the periplasm to the active site in NOR the E-pathway. In addition to defining a role for Glu-122 in this pathway, we have also localized the proton donor as an ionizable group in its immediate vicinity, possibly a protonated water molecule coordinated between Glu-122 and Glu-125. Proton transfer from this donor is rate-limiting for the reaction.
we have studied and possibly for the overall turnover reaction (see Ref. 11). Having established that the proton transfer pathway in NOR includes Glu-122 and possibly Glu-125, it is interesting to note that these residues are conserved in the \( \text{cbb}_3 \)-type HCuOs (see Ref. 27). The \( \text{cbb}_3 \)-type oxidases have, like other HCuOs, been shown to be proton pumps (28, 29) and consume their substrate protons from the cytoplasm. Consequently, if those residues equivalent to Glu-122 and Glu-125 in NOR are involved in proton transfer, it must be to provide an exit pathway for pumped protons. In an evolutionary sense a relationship between the output pathway for pumped protons in HCuOs and the input pathway for substrate protons in NOR is a very attractive proposition.

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