A pathway for protons in nitric oxide reductase from *Paracoccus denitrificans*

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Abstract

Nitric oxide reductase (NOR) from *P. denitrificans* is a membrane-bound protein complex that catalyses the reduction of NO to N_2O ($2NO+2e^-+2H^+ \rightarrow N_2O+H_2O$) as part of the denitrification process. Even though NO reduction is a highly exergonic reaction, and NOR belongs to the superfamily of O_2-reducing, proton-pumping heme-copper oxidases (HCuOs), previous measurements have indicated that the reaction catalyzed by NOR is non-electrogenic, i.e. not contributing to the proton electrochemical gradient. Since electrons are provided by donors in the periplasm, this non-electrogenicity implies that the substrate protons are also taken up from the periplasm. Here, using direct measurements in liposome-reconstituted NOR during reduction of both NO and the alternative substrate O_2, we demonstrate that protons are indeed consumed from the 'outside'. First, multiple turnover reduction of O_2 resulted in an increase in pH on the outside of the NOR-vesicles. Second, comparison of electrical potential generation in NOR-liposomes during oxidation of the reduced enzyme by either NO or O_2 shows that the proton transfer signals are very similar for the two substrates proving the usefulness of O_2 as a model substrate for these studies. Last, optical measurements during single-turnover oxidation by O_2 show electron transfer coupled to proton uptake from outside the NOR-liposomes with a $\tau = 15$ ms, similar to results obtained for net proton uptake in solubilised NOR [U. Flock, N.J. Watmough, P. Ädelroth, Electron/proton coupling in bacterial nitric oxide reductase during reduction of oxygen, Biochemistry 44 (2005) 10711–10719]. NOR must thus contain a proton transfer pathway leading from the periplasmic surface into the active site. Using homology modeling with the structures of HCuOs as templates, we constructed a 3D model of the NorB catalytic subunit from *P. denitrificans* in order to search for such a pathway. A plausible pathway, consisting of conserved protonatable residues, is suggested.
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1. Introduction

Bacterial denitrification is a process in which nitrate is step-wise reduced via nitrite, nitric oxide and nitrous oxide to dinitrogen, which is released into the atmosphere. The reduction of NO to N_2O (see Eq. (1)) is catalyzed by nitric oxide reductase (for reviews, see [1,2]).

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \qquad (1)$$
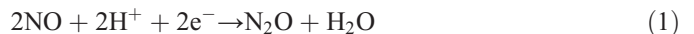
Bacterial nitric oxide reductases (NOR) are integral membrane proteins, and homology searches for the large catalytic subunit (NorB) showed that NOR is a divergent member of the superfamily of O_2-reducing heme-copper oxidases (HCuOs). The HCuOs are characterized by having a large catalytic subunit with six invariant histidines at the same positions in 12 (predicted for NOR) transmembrane helices [3,4]. In the HCuOs, for which crystal structures are known both for the mitochondrial enzyme [5] and for several bacterial enzymes [6–9], these histidines coordinate two heme groups and a copper ion.

In the NORs, for which there is no structural information at atomic detail, in analogy with the HCuOs, two of the conserved histidines coordinate a low-spin heme and one coordinates a high-spin heme. The remaining three histidines, which in the HCuOs coordinate the copper ion, presumably coordinate a non-heme iron in NOR [10,11].

The large subunit of the NORs can be divided into two subclasses, called NorB and NorZ, with high similarity, but with the difference that the NorZ contains a ~ 300 amino acid extension at the N-terminal, which is why these two forms are also called short-chain (sc) and long-chain (lc) NORs. The NorB form is isolated in complex with another protein, the NorC, which contains a c-type cytochrome (cyt.) and receives electrons from water-soluble donors such as cyt. c [1]. The NorZ has been purified in a single subunit form that receives electrons from quinol [12], which has led to the two NOR classes also being termed cNOR (for cyt. c) and qNOR (for quinol).

The NOR from *Paracoccus* (*P.*) *denitrificans* is a cNOR, purified as a complex between NorB and NorC. NorB harbors a low-spin heme b, a high-spin heme b_3, and a non-heme iron, Fe$_B$. The heme b_3 and Fe$_B$ form a binuclear center, which is the site of NO-reduction. NorC is a membrane-anchored protein harboring a low-spin heme c, which is believed to be the site of electron entry from the water-soluble electron donor, in *P. denitrificans* either cyt. c_{551} or pseudoazurin [1].

The HCuOs (for reviews see e.g. [13–16]) catalyze the four-electron reduction of oxygen to water (Eq. (2a)), and use the free energy available from this reaction to generate an electrochemical proton gradient across the membrane (Eq. (2a) and (2b)).

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad (2a)$$

$$4H_{in}^+ \rightarrow 4H_{out}^+ \qquad (2b)$$

The *P. denitrificans* NOR can, in addition to the physiological NO-reduction, also catalyze the reduction of dioxygen (Eq. (2a)) to water [17–19]. The O$_2$- and NO-reduction activities are closely correlated, presumably because the same catalytic components are involved in both processes.

The HCuOs generate an electrical gradient by using electrons and protons derived from opposite sides of the membrane; the protons used for water formation (substrate protons, Eq. (2a)) are derived exclusively from the 'inside' (the mitochondrial matrix or bacterial cytoplasm) and the electrons from the 'outside', e.g. from periplasmic cyt. c. In addition, the HCuOs couple the electron transfer to oxygen ($E'_0 = +0.8$ V) to the translocation of 4H$^+$/O$_2$ through the protein (Eq. (2b)) across the membrane. In HCuOs with high sequence similarity to the mitochondrial oxidase (termed type A1 [15]), protons are transferred from the cytoplasmic surface of the protein into the active site through well-characterised pathways, called the D- and the K-pathway, consisting of protonatable residues and water molecules (for a review on proton transfer in proteins see [20]). The D-pathway is used for both substrate and pumped protons (6–7 in total) during the oxidative part of the catalytic cycle, whereas the K-pathway is probably used for (1–2) pro-

tons during the reductive part (see e.g. [21,22]). The D-pathway starts at the cytoplasmic surface with the Asp-132 (*R. sphaeroides aa$_3$* numbering[2]). It then continues through a network of water molecules stabilised by polar residues up to the essential Glu-286, which sits at a ~ 10 Å distance from Cu$_B$.

In the NORs, these residues, shown in the A1 HCuOs to be crucial for proton transfer/pumping are not conserved (but see Discussion), and available data, from whole cell measurements [23,24] or using an electrometric technique [25] indicate that the two-electron reduction of NO catalyzed by NOR is non-electrogenic, i.e. not coupled to charge translocation across the membrane. This can seem surprising since the free energy available from reducing NO ($E'_0 = +1.2$ V) is even larger than for O$_2$ reduction.

As electrons are supplied by soluble donors from the periplasmic side of the membrane, the lack of electrogenicity in NOR implies that not only is NOR not a proton pump, but also that the substrate protons needed for NO-reduction (see Eq. (1)) must come from the periplasm.

In order to study the mechanism of proton transfer in NOR, we have previously used O$_2$ as the oxidant since the chemical reactivity of NO in aqueous solutions hampers direct measurements of proton consumption using pH-sensitive dyes in unbuffered solutions. We thus characterized the single-turnover reaction between fully reduced detergent-solubilised NOR and O$_2$ using the flow-flash technique in combination with time-resolved optical measurements [19]. Our results showed that oxygen binds to heme b_3 with a $\tau = 40$ μs (at 1 mM O$_2$), after which electron transfer from the low-spin hemes b and c to the O$_2$ bound at the binuclear site occurs with a $\tau = 25$ ms (at pH 7.5). A slow phase of oxidation of hemes b and c with $\tau \sim 1$ s presumably completes the reduction of O$_2$ to H$_2$O. The $\tau = 25$ ms ($k = 40$ s^{-1}) phase is coupled to proton uptake from the bulk solution, and the rate constant shows a pH dependence consistent with limitation by internal proton transfer (with a $k_{max} = 250$ s^{-1} at low pH) into the active site from a protonatable group. This group is in rapid equilibrium with the bulk solution, has a p$K_a = 6.6$ and was suggested to be an amino acid located close to the active site [19,26].

In this work, the aim was to confirm or disprove, by direct measurements, the indication that in NOR, protons are supplied from the periplasmic space. Having established from which side of the membrane the protons originate, our aim was further to search for putative proton transfer pathways into the NOR active site. To this end, we reconstituted NOR into phospholipid vesicles, and studied proton consumption by the liposome-reconstituted NOR (v-NOR) in several different ways. First during several turnovers of O$_2$-reduction using a pH-sensitive dye added to the outside of the vesicles. Second, we studied the single-turnover oxidation of fully reduced v-NOR using the flow-flash technique in combination with electrometric detection in order to compare proton transfer characteristics with O$_2$ as the substrate to those using the physiological substrate NO. Third, we studied the same reaction between fully reduced

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[2] Unless otherwise indicated, for the A1 HCuOs, numbering of residues will from here on be according to the *R. sphaeroides aa$_3$* sequence.