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Proton transfer in the quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus* during reduction of oxygen ☆

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ABSTRACT

Bacterial nitric oxide reductases (NOR) are integral membrane proteins that catalyse the reduction of nitric oxide to nitrous oxide, often as a step in the process of denitrification. Most functional data has been obtained with NORs that receive their electrons from a soluble cytochrome *c* in the periplasm and are hence termed *c*NOR. Very recently, the structure of a different type of NOR, the quinol-dependent (q)-NOR from the thermophilic bacterium *Geobacillus stearothermophilus* was solved to atomic resolution [Y. Matsumoto, T. Tosha, A.V. Pisliakov, T. Hino, H. Sugimoto, S. Nagano, Y. Sugita and Y. Shiro, *Nat. Struct. Mol. Biol.* 19 (2012) 238–246]. In this study, we have investigated the reaction between this qNOR and oxygen. Our results show that, like some *c*NORs, the *G. stearothermophilus* qNOR is capable of O_2 reduction with a turnover of ~3 electrons s^{-1} at 40 °C. Furthermore, using the so-called flow-flash technique, we show that the fully reduced (with three available electrons) qNOR reacts with oxygen in a reaction with a time constant of 1.8 ms that oxidises the low-spin heme *b*. This reaction is coupled to proton uptake from solution and presumably forms a ferryl intermediate at the active site. The pH dependence of the reaction is markedly different from a corresponding reaction in *c*NOR from *Paracoccus denitrificans*, indicating that possibly the proton uptake mechanism and/or pathway differs between qNOR and *c*NOR. This study furthermore forms the basis for investigation of the proton transfer pathway in qNOR using both variants with putative proton transfer elements modified and measurements of the vectorial nature of the proton transfer. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

Bacterial NO-reductases (NOR) are integral membrane proteins that reduce NO to N_2O (Eq. (1)), often as part of the denitrification process in which nitrate is step-wise reduced to nitrogen (for reviews, see Refs. [1–4]).

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \quad (1)$$

The NORs were shown to be divergent members of the superfamily of heme-copper oxidases (HCuOs) where most members are O_2-reducing enzymes terminating the aerobic respiratory chain in mitochondria, bacteria and archea. The HCuO superfamily is characterised by their catalytical subunit having six invariant histidines at the same positions in 12 trans-membrane helices [5,6]. Two of the conserved histidines coordinate a low-spin heme, one a high-spin heme and the remaining three histidines coordinate a copper ion. The high-spin heme and the Cu ion are located in close proximity and together form the active site. In NOR, the copper is replaced by a non-heme iron [7–9].

The HCuO family has been divided into four major classes: A-, B-, and C-type O_2 reducers and NORs [10–12]. The large catalytic subunit of the NORs can be divided into two subclasses, called NorB and NorZ, with the difference that the NorZ contains a 300 amino acid extension at the N-terminal, so that these two forms are also called short-chain (sc) and long-chain (lc) NORs [2]. The NorB is isolated in complex with another protein, the NorC [13], which contains a *c*-type cytochrome (cyt.). The NorC subunit is the entry point for electrons from water-soluble donors such as cyt. *c* [14]. The NorZ is purified as a single subunit, and receives electrons from quinol [15,16], consequently

Abbreviations: NOR, bacterial nitric oxide reductase; *c*NOR, cytochrome *c*-dependent NOR; qNOR, quinol-dependent NOR; HCuO, heme-copper oxidase; DDM, β-D-dodecyl maltoside; PMS, phenazine-methosulfate; MES, 2-Morpholinoethanesulfonic acid; HEPES, 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid; TRIS, Tris-(hydroxymethyl)-aminomethane; BTP, Bis–Tris-propane

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the two NORs are also classified as *c*NOR (for cyt. c) and qNOR (for quinol).

The HCuOs (for recent reviews on structure and function of the heme-copper oxidases, see e.g. Refs. [17–20]) catalyse the four-electron reduction of oxygen to water (Eq. (2)), and use the free energy available from this reaction to generate an electrochemical proton gradient across the membrane.

$$O_2 + 4H^+_{in} + 4e^- + nH^+_{in} \rightarrow 2H_2O + nH^+_{out} \quad (2)$$

This proton gradient is generated by using only protons from the 'inside' (the mitochondrial matrix or bacterial cytoplasm) for water formation (substrate protons). In addition, the HCuOs couple the exergonic O_2 reduction to the translocation of protons (*n* in Eq. (2), where the number varies from 2 to 4 in different HCuOs [21]) across the membrane. In the best-known A-type HCuOs protons are transferred through two pathways (for a recent review on proton transfer pathways across the whole HCuO family, see Ref. [22]) from the cytoplasm (inside) up to the catalytic site; the D- and the K-pathway. These pathways are used during different transitions in the catalytic cycle and the D-pathway is the main pathway used for 6–7 H^+ (of 8 in total, since $n = 4$ in A-type) per O_2 turnover (see e.g. Refs. [23,24]).

The best studied class of NORs are the *c*NORs which have been purified and characterised from e.g. *Paracoccus* (*P.*) *denitrificans* [8,25], *Pseudomonas* (*P.*) *stutzeri* [26] and *P. aeruginosa* [9,27], for which also the crystal structure was determined at 2.7 Å resolution in 2010 [9]. In contrast to the O_2-reducing HCuOs, in *c*NOR the two-electron reduction of NO is non-electrogenic [28–30], i.e. not coupled to charge translocation across the membrane. As electrons are supplied by soluble donors (e.g. cyt. *c*) from the periplasmic side of the membrane, the non-electrogenic reaction in *c*NOR implies that the protons needed for NO reduction (see Eq. (1)) are also taken from the periplasm [30]. This was supported by the structure, showing several putative proton transfer pathways from the periplasm to the active site, but no possible proton pathways from the cytoplasm [9].

In contrast to *c*NORs, which are usually expressed as components of a full denitrification pathway, qNORs are often expressed in the absence of some or all other denitrifying enzymes in pathogenic bacteria [1,2,31]. The major function of these qNORs is presumably to detoxify the NO produced in the immune defense of the host. With the very recent determination of the crystal structure also of a qNOR [32], we now have crystal structures for all major HCuO subfamilies [9,33–36]. In qNOR, the 300 amino acid long extension in NorZ compared to NorB contains a hydrophilic domain homologous to NorC, but with the cytochrome *c*-binding motif absent. The qNOR structure surprisingly revealed a water-containing pathway, lined by polar side-chains, leading from the cytoplasm to the active site [32]. This putative proton transfer pathway has the same spatial location as the K-pathway for proton transfer in the O_2-reducing HCuOs (see Ref. [22]) and is a surprising feature since *c*NORs are known to use protons from the periplasm (see above).

In addition to the physiological NO-reduction activity, several *c*NORs have been shown to catalyse the reduction of dioxygen with turnover numbers on the order of 2–10 electrons s^{-1} [25,37–39]. Studies of mutant forms of *P. denitrificans* NOR have shown that the O_2- and NO-reduction activities are well correlated [25,30].

For the O_2-reducing HCuOs, the catalytic mechanism has been extensively investigated using the so-called 'flow-flash' technique [23,40–44]. Briefly, the fully reduced enzyme with carbon monoxide (CO) bound to the high-spin heme is mixed in a stopped-flow apparatus with an oxygenated solution. The reaction with O_2 is initiated by a short laser flash (~10 ns), dissociating the photolabile Fe–CO bond and allowing binding of O_2 and its subsequent step-wise reduction, which can be followed using time-resolved spectroscopy. This technique has also been used to study the reduction of NO by *c*NOR [29,45]. The reduction of two NO molecules to form N_2O requires two electrons, whereas the fully reduced *c*NOR has four redox-active groups, hence two full turnovers are required to reoxidise the enzyme. Moreover, after oxidation the oxidised heme b_3 can bind NO [46] which presumably causes inhibition [8,45]. These multiple possible reaction paths makes interpretation of results complicated. Therefore, we have also used the reaction with O_2 to study electron/proton coupling as well as possible proton transfer pathways in *c*NOR [37,47,48].

In this work, we have studied the reaction of fully reduced qNOR from *G. stearothermophilus* with O_2 using the flow-flash technique. The aim is two-fold; firstly because of the experimental advantage of O_2 over NO, to use the O_2-reaction to study reactions that are common between NO and O_2 reduction; electron and proton transfer to the catalytic site of qNOR. Secondly, comparisons of the reactions of NORs and the HCuOs with NO and O_2 will provide information about structural elements that determine the substrate specificity. We show that the fully reduced (with three available electrons) qNOR from *G. stearothermophilus* qNOR reacts with oxygen in a reaction where the low-spin heme *b* is oxidised and protons are taken up from solution. The pH dependence of this reaction is markedly different from a corresponding reaction in *c*NOR from *P. denitrificans* [37] indicating that possibly the proton uptake mechanism and/or pathway differs between qNOR and *c*NOR. The implications of these results for the mechanism of action and future studies of qNOR are discussed.

2. Materials and methods

2.1. Growth of bacteria and purification of qNOR

Bacteria were grown and the *G. stearothermophilus* qNOR purified from *E. coli* using Triton X-100 and β-D-dodecyl maltoside (DDM) as described in Ref. [32]. Note that when the enzyme is purified with Triton/DDM, the Fe_B site retains (partly) the iron in the site, whereas the crystal structure, which has zinc in the Fe_B site, was solved with Triton X-100/octyl-glucoside (OG) purified qNOR. UV–vis spectra were recorded on a Cary-400 spectrometer (Varian). The *G. stearothermophilus* qNOR purified from the native host (as described in Ref. [32]) with Triton/DDM contained no bound quinol (Matsumoto, Tosha and Shiro, unpublished data), which means that the fully reduced qNOR (see below) has three available electrons.

2.2. Steady-state O_2-reduction

Oxygen consumption by qNOR was measured using a Clark-type electrode (Hansatech) at 40 °C (313 K). The reaction medium contained ascorbate (3 mM) and phenazine-methosulfate (PMS, 10 μM) as electron donor and mediator in 50 mM Tris, pH 8, 150 mM NaCl. Because of the rather rapid background consumption of O_2 of this buffer system itself, O_2 reduction catalysed by qNOR was difficult to observe. We therefore let the buffer system consume O_2 until ~15 μM O_2 remained before adding the qNOR in order to allow the background rate to diminish (See Supporting Fig. 2).

2.3. Sample preparation for flash photolysis and flow-flash studies

The qNOR was diluted to 5–10 μM in a modified Thunberg cuvette, air was exchanged for nitrogen on a vacuum line, and the enzyme was reduced by adding 2–4 mM ascorbate and 0.2 μM phenazine-methosulfate (PMS). Nitrogen was then exchanged for either 100% CO or different CO/N_2 mixes. A lower CO concentration (~20%) was used for the flow-flash studies to avoid CO recombination interfering with O_2 binding (see Results and [37]). For the flow-flash measurements, also 10–20 U catalase and 50–100 μM dithionite was added to ensure that the sample stayed anaerobic during transfer to the stopped-flow machine. Reduction and ligand binding were followed by UV–vis spectroscopy (Cary-400, Varian).