Biochimica et Biophysica Acta 1817 (2012) 1914–1920

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

Lina Salomonsson [a], Joachim Reimann [a,1], Takehiko Tosha [b], Nils Krause [a,2], Nathalie Gonska [a], Yoshitsugu Shiro [b], Pia Ädelroth [a,]*

[a] *Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden*
[b] *RIKEN SPring-8 Center, Harima Institute, Biometal Science Lab, 1-1-1, Kouto, Sayo, Hyogo 679-5148, Japan*

ARTICLE INFO

Article history:
Received 28 January 2012
Received in revised form 4 April 2012
Accepted 10 April 2012
Available online 17 April 2012

Keywords:
Heme-copper oxidase
Proton transfer pathway
Non-heme iron
Flow-flash
Carbon monoxide

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).

© 2012 Elsevier B.V. All rights reserved.

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

☆ This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

* Corresponding author. Tel.: +46 8 164183; fax: +46 8 153679.
E-mail address: piaa@dbb.su.se (P. Ädelroth).
[1] Current address: Department of Microbiology, Institute of Wetland and Water Research, Faculty of Science, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands.
[2] Current address: Freie Universität Berlin, Experimental Physics: Genetic Biophysics, Arnimallee 14, D-14195 Berlin, Germany.

0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2012.04.007

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).

2.4. Flash photolysis and flow-flash measurements

Flash photolysis and flow-flash measurements were performed at room temperature as in Ref. [37] on a set-up described in Ref. [49]. Briefly, for the flow-flash measurements, fully reduced CO-bound qNOR was mixed 1:5 with an oxygenated buffer in a modified stopped-flow apparatus (Applied Photophysics, U.K.). After a 200-ms delay a 10 ns laser flash (Nd-YAG laser, Quantel) was applied, dissociating CO and allowing O_2 to bind and initiate the reaction. The time course of the reaction was studied from 1 μs to 1 s at different wavelengths. At each wavelength, 10^5 data points were collected, and processed as described [37].

For the pH-dependence measurements, the buffer concentration in the qNOR solution before mixing in the same 1:5 ratio was decreased to 10 mM (HEPES or Bis–Tris Propane (BTP) at pH 7.0–7.5). The oxygenated solution contained 100 mM of BTP (at pH 6–9.5) or Mes (pH 5.5), so that a set of different pH values were measured on the same qNOR sample.

The time-resolved changes in absorbance, measured at different wavelengths, were fitted either separately or globally to a model of consecutive irreversible reactions (e.g. A → B → C) using the program Pro-K (Applied Photophysics, U.K.).

2.5. Proton uptake measurements

In order to record pH changes in solution during the reaction with O_2, the buffering capacity of the sample was lowered by passing it through a PD-10 column (GE Healthcare) equilibrated in 50 mM KCl, 0.05% DDM, 50 μM EDTA, pH ~7.5. Phenol red at 40 μM was then added to the sample which was reduced (with ascorbate/PMS) and CO-equilibrated as above. The O_2-saturated solution contained 50 mM KCl, 0.05% DDM, 50 μM EDTA, and 40 μM phenol red at pH ~7.5. Phenol red in its unprotonated state has a broad pH-sensitive absorbance peak at 560 nm, but absorbance changes were monitored at 570 nm (see Results) to minimise contribution from oxidation of the low-spin heme *b* (which has a maximum at 560 nm). Residual absorbance changes in qNOR at 570 nm were measured in the presence of 100 mM Hepes and subtracted. For further details, see Ref. [37].

3. Results

3.1. Steady-state O_2-reduction by qNOR

Measured with ascorbate/PMS at 40 °C and ~15 μM O_2 (see Material and methods, and Fig. S2), the qNOR displayed an O_2-reducing activity of ~3 $e^{-}s^{-1}$. As controls, we added only buffer and a lower amount of qNOR (giving a correspondingly lower V_0) in order to verify that the qNOR is responsible for the observed O_2-reduction activity.

3.2. Optical spectra

UV–vis spectra of qNOR in the oxidised, reduced and CO-bound forms are shown in Supporting Fig. S1.

3.3. CO recombination to the fully reduced qNOR

As a probe of the active site, and as a prerequisite for determining conditions for flow-flash measurements, we measured CO recombination to the fully reduced *G. stearothermophilus* qNOR at room temperature. After flash-induced dissociation from reduced heme b_3, CO recombines in a major phase contributing 50–60% of the total absorbance change at 440 nm, see Fig. 1) with a time constant of ~5 μs at 1 mM CO. This rate constant is the same as that observed for the *c*NOR from *P. denitrificans* [37,50]. There is also a slower phase with a time constant of ~70 μs contributing ~30–40% of the absorbance change at 440 nm. This is also similar to the situation in *P. denitrificans* *c*NOR. At lower CO (from 0.1 mM–0.5 mM, for 0.2 mM see Fig. 1), the

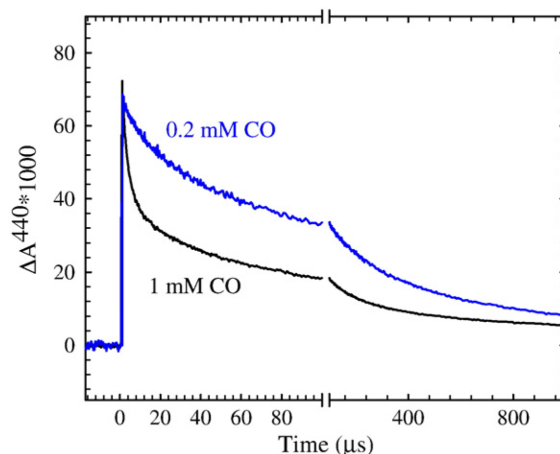

Fig. 1. CO recombination to the fully reduced qNOR studied at 440 m. Experimental conditions: 50 mM Tris–HCl, pH 8.0, 0.05% DDM, T = 298 K. The laser flash at t = 0 gives an artifact that has been truncated for clarity. The black trace shows recombination at 1 mM CO, and the blue trace shows recombination at 20% (or 0.2 mM) CO. The traces have been normalised to the same flash-induced change in absorbance at t = 0.

corresponding rate constants decrease linearly with [CO], giving for the rapid component a k_{on} ~ $2.1 \times 10^8 M^{-1} s^{-1}$. That the reaction is second order indicates that CO recombines from bulk, as in *c*NOR [50]. We note that at 440 nm, there is no significant change in the static absorbance spectrum between the reduced and CO-bound qNOR, whereas there is a substantial signal in the flash-induced CO dissociation, indicating that the state of qNOR after the dissociation of CO is different from the static reduced state. The same behavior is observed with *c*NOR from *P. denitrificans* [37,50], and was interpreted as being due to a ligand bound to the reduced heme b_3, which is then exchanged for CO, responsible for the small change in the static spectrum [50].

The CO concentration was lowered to ~20% before the flow-flash experiments in order to make CO binding slower, and presumably allow for O_2 binding before CO recombination.

3.4. Single turnover O_2-reduction by the fully reduced qNOR

The qNOR from *G. stearothermophilus* reacts with O_2 in a reaction that oxidises the low-spin heme *b* with a major time constant (τ) of 1.8 ms ($k = 560\ s^{-1}$), contributing ~65% of the amplitude (fitted up to 100 ms) at 560 nm, as well as at 420 and 430 nm (reporting also on the high-spin heme), see Fig. 2. There is a second phase with τ = 20 ms ($k = 50\ s^{-1}$) contributing the other 35% of the amplitude. These fitted rate constants varied ~20% between experiments. The spontaneous rate of CO dissociation (at the used measuring light intensity), determined as the rate of oxidation in the absence of a flash occurred with τ = 3 s ($k = 0.3\ s^{-1}$). We did therefore not attempt to resolve any flash-induced reactions on the time-scale of seconds as they would interfere with CO dissociation in the qNOR population that was not photolysed by the flash.

There is also a rapid reaction observed at 430 nm with a time constant of ~30 μs (k ~ 30,000 s^{-1}, see inset in Fig. 2) at 1 mM O_2, that we speculate being due to O_2 binding, but at the current signal-to-noise ratio, both the rate constant and the assignment remain tentative.

Since qNOR has only three available electrons (two in the active site and one on heme *b*) in the fully reduced state, we assume the end product after this phase is in the same oxidation state as the three-electron reduced intermediate in A-type O_2-reducing heme-copper oxidases, i.e. a ferryl state (see Fig. 4). For this intermediate to form in the A-type HCuOs, a proton needs to be taken up from bulk. Proton uptake was therefore measured at 570 nm using the dye phenol red. As seen in the bottom panel of Fig. 2, there is proton

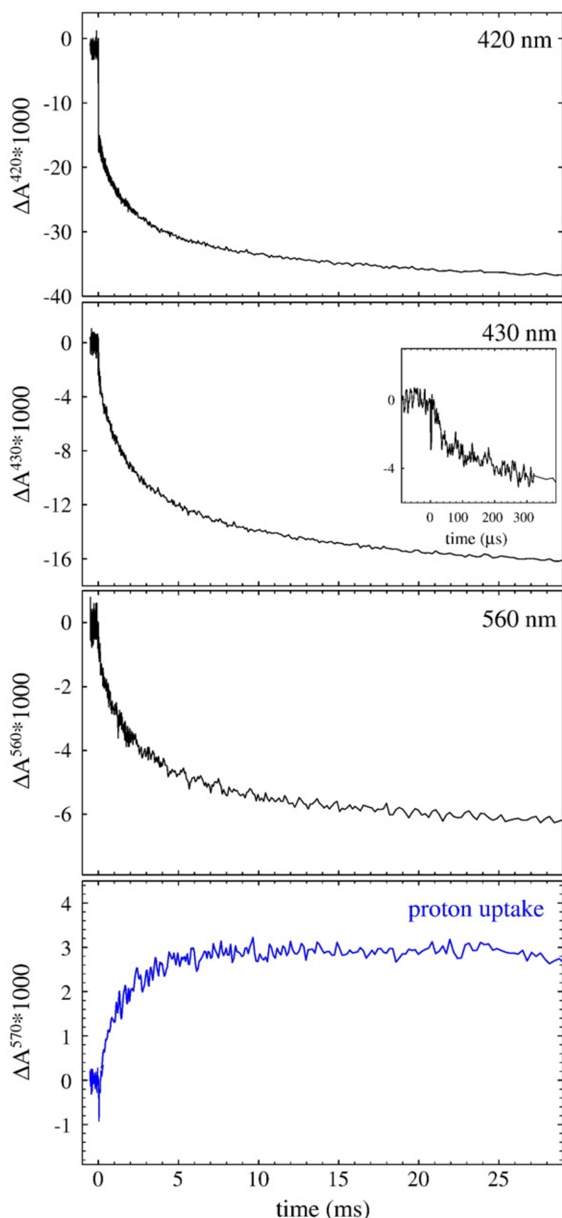

Fig. 2. Absorbance changes during the reaction between the fully reduced qNOR and O_2, at (from top to bottom) 420, 430, 560 (specifically reporting on the low-spin heme *b*) and 570 nm (of phenol red). At 430 nm, also the rapid absorbance changes, tentatively assigned to O_2 binding, are shown as an inset. Experimental conditions: 50 mM Tris–HCl, pH 8.0, 0.05% DDM, 1 mM O_2, T = 298 K. For the proton uptake trace (bottom panel), the trace shown is the response to pH changes of phenol red at 570 nm, i.e. the difference between the trace obtained in unbuffered (50 mM KCl, 0.05% DDM, 50 μM EDTA, 40 μM phenol red at pH ~7.5) solution and the trace obtained after addition of 100 mM Hepes buffer.

uptake from the bulk solution on the same time-scale as the heme *b* oxidation. The obtained trace could be fitted to only one phase with $\tau = 1.7$ ms ($k = 600\ s^{-1}$), i.e. coinciding with the first phase of heme oxidation. Because of the drifts and rather noisy data at 570 nm, we cannot rule out that also the second $\tau = 20$ ms phase is also coupled to proton uptake.

It is also clear from Fig. 2 that CO dissociation is not coupled to proton uptake/release from/to solution, since no changes in dye absorbance are seen faster than the 1.7 ms phase.

3.4.1. pH dependence

Since the $\tau = 1.8$ ms phase of heme oxidation is coupled to proton uptake from solution, we expected the rate constant to show a dependence on the pH of solution, as observed for the corresponding reaction in *c*NOR (see [37] and Fig. 3B). However, as seen from Fig. 3A, in qNOR there is no significant pH dependence of this rate in the pH range from pH 5 to 9 (see Fig. 3B and Discussion).

4. Discussion

NO reductase was first purified from *P. stutzeri* in 1989 [13] and later shown to belong to the family of heme-copper oxidases [6]. With the recent determinations of the crystal structures of a C-type HCuO as well as both *c*NOR [9] and qNOR [32], we now have structural information for all subfamilies of the HCuOs. We are thus in a much better position to understand the catalysed chemistry in these enzymes.

One of the major differences between the O_2-reducing HCuOs and the *c*NORs is the energy conserving properties, O_2-reducing HCuOs conserve the energy available from the exergonic O_2 reaction by translocating protons across the membrane; both by taking electrons and protons from opposite sides of the membrane, and by actively pumping protons through the enzymes. *c*NORs however, were shown not to conserve energy from the equally exergonic NO reduction, since protons and electrons are taken from the same (out)-side and no protons are pumped [29,30,51]. The *c*NOR structure confirmed this finding since the protein region from the cytoplasm to the active site is largely hydrophobic while the region 'above' the active site towards the periplasm contains several putative proton transfer pathways

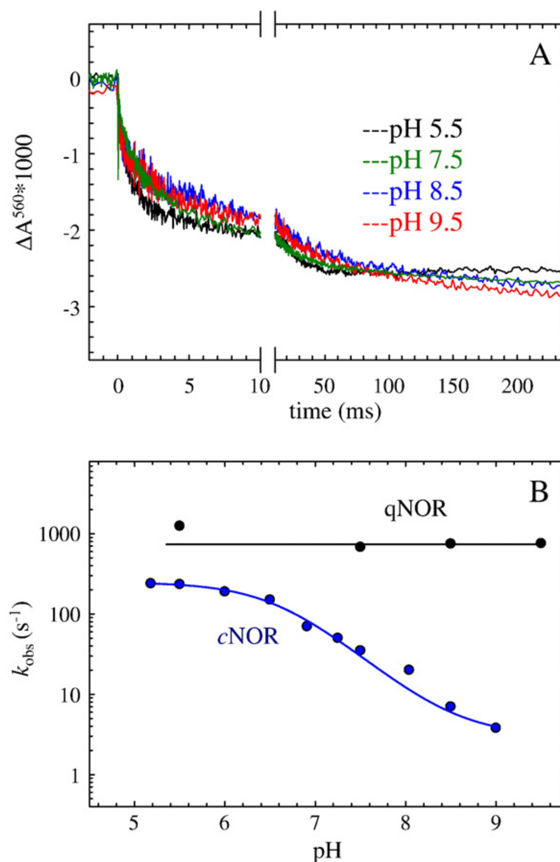

Fig. 3. pH dependence of the proton-coupled electron transfer in qNOR. Absorbance changes at 560 nm at various pH values are shown in panel A, and the fitted rate constant for the major rapid ($k = 560\ s^{-1}$, $\tau = 1.8$ ms at pH 7.5) phase is shown in panel B. Panel B also shows the pH dependence of the corresponding reaction in *c*NOR, data from Ref. [37]. Experimental conditions: pH 7.5–9.5: 100 mM BTP, 50 mM KCl, 0.05% DDM; pH 5.5: 100 mM MES, 50 mM KCl, 0.05% DDM. The traces in panel A were normalised to the same level at 100 ms to aid comparison of rates. A laser artefact at t = 0 has been truncated for clarity.

composed of conserved polar/protonatable amino acids and water molecules [9]. Several possible reasons for the non-electrogenicity of *c*NORs have been discussed; possibly the toxic nature of the NO molecule makes rapid detoxification more important than energy conservation. It is also possible that reaction intermediates during NO reduction have low proton-affinity (low pK_as) [52] making a direct coupling to proton pumping more complicated. The latter explanation was supported by the finding that C-type HCuOs, when reducing NO presumably use protons from the periplasmic space [44].

When the qNOR structure was solved, it was discovered that surprisingly there is a hydrophilic pathway connecting the active site with the cytoplasmic side of the membrane, suggested to be used for proton transfer to the active site [32], see Fig. 5. The location of this proton pathway further overlaps with the K-pathway for protons in the O_2-reducing HCuOs. If this pathway is indeed used for proton transfer in qNOR and the reaction catalysed thus is electrogenic, this must lead to a re-evaluation of the reasons for the non-electrogenicity in *c*NORs, as well as giving a new piece of the puzzle for the evolution of the HCuO superfamily as a whole.

In this study we examined the reaction between the qNOR from *G. stearothermophilus* and oxygen. We show that this qNOR has turnover activity with O_2 of ~3 electrons s^{-1} at 40 °C (with ascorbate/PMS as electron donor) as is found with some *c*NORs [25,37–39]. In *c*NOR, we have used the time-resolved flow-flash reaction with oxygen for studying proton transfer reactions since oxygen is more experimentally suitable for using with pH-sensitive dyes ([37] but see also Ref. [45]) and also, the reaction between fully reduced *c*NOR and O_2 is simpler (comprising one full turnover) than the reaction with NO (comprising two turnovers, see Refs. [29,45]). Studies of mutant forms of *P. denitrificans c*NOR have shown that the O_2- and NO-reduction activities are well correlated [25]. Thus, studying the O_2-reduction mechanism should be relevant also for NO reduction properties. Furthermore, electrogenic events in liposome-reconstituted *c*NOR during oxidation of the fully reduced enzyme are very similar between using O_2 or NO as the oxidant [30].

4.1. Reaction of fully reduced qNOR with oxygen

Using the flow-flash technique in order to time-resolve the reaction between fully reduced qNOR and O_2, we show that qNOR is oxidised in a reaction with $\tau = 1.8$ ms. Concomitantly protons are taken up from solution. The intermediate thus formed at the qNOR active site is at the same reduction level as the F intermediate in O_2-reducing heme-copper oxidases (see e.g. Refs. [53,54]), that is with three electrons transferred to the active site and a proton taken up from solution, and there could thus be a $Fe^{4+}{=}O^{2-}$ at heme b_3 and Fe^{3+}–OH^- at the non-heme iron (see Fig. 4) in analogy with the A-type O_2-reducing HCuOs which have a ferryl ion at heme a_3 and Cu^{2+}–OH^- at the Cu_B in the F intermediate [55].

This study forms the basis for determining the vectoriality of proton transfer in qNOR directly. There are several reasons that the observed proton-coupled electron transfer is ideal for studying the vectoriality of proton transfer; first proton transfer using pH-sensitive dyes is more easily studied using oxygen as there are no proton-coupled side reactions as is the case with NO. Second, the observed reaction with a $\tau = 1.8$ ms is very fast compared to leaking of protons across the membrane, which typically starts to become an experimental problem when the rates are in the range of sub-seconds. Thus studying the $\tau = 1.8$ ms phase in liposomes with pH-sensitive dyes present on either side of the membrane is a possible way to determine vectoriality. Third, and importantly, using the electrometric technique, where only charges moving perpendicular to the membrane [42] are observed, it should be clear from which side this proton is coming, since there should be no contribution from the electron that moves from the heme *b*, at the same 'depth' in the membrane (see Fig. 4).

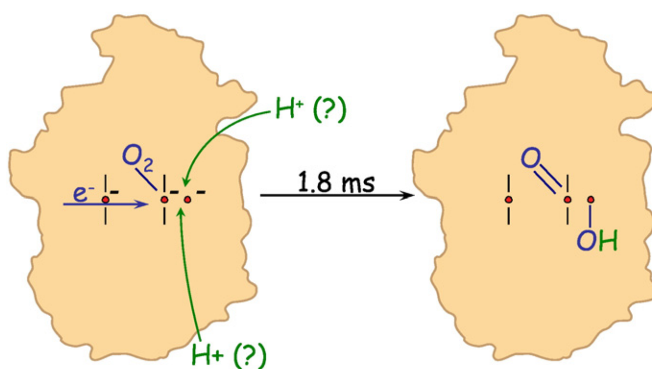

Fig. 4. Schematic illustration of the proton-coupled electron transfer observed when fully reduced qNOR reacts with O_2. A minus sign indicates a reduced site. After flash photolysis of the b_3-CO bond, we assume that the O_2-bound intermediate forms rapidly at the active site (to the right). One electron is then transferred from the low-spin heme *b* (to the left) concomitantly with proton uptake from solution, presumably forming a ferryl intermediate at the heme b_3. The question marks for the proton transfer event indicate that there is no experimental evidence for the directionality of proton transfer in qNOR.

4.2. Proton transfer into the active site in qNOR

The pH dependence of the rate constant for the $\tau = 1.8$ ms phase in the reaction between fully reduced qNOR and O_2 shows very little

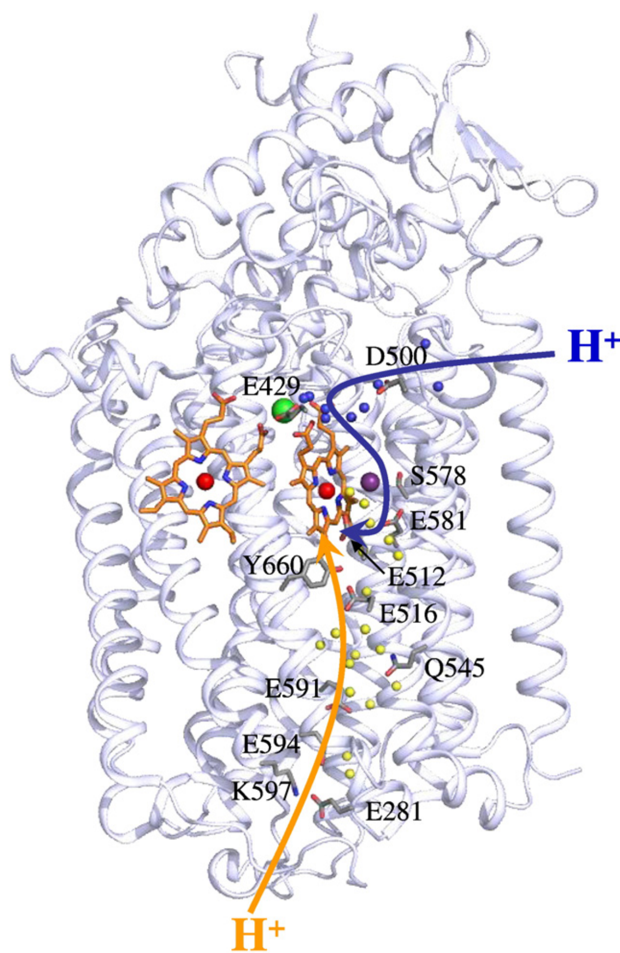

Fig. 5. The structure of qNOR (PDB ID: 3AYF) from *G. stearothermophilus* [32], with possible proton transfer pathways highlighted. The pathway suggested in the structure is marked by an orange arrow and with yellow waters. The blue arrow indicates that it possible that protons are instead taken from outside using some waters (in blue) and amino acids (marked) around the Ca^{2+}-site (green) suggested to be a possible part of the proton pathway in *c*NOR [9], that are conserved to this qNOR. Note that Asp-500 is not conserved to *c*NOR, but the residue preceding it in sequence is an Asp.

variation in the rate between pH 5.5 and pH 9.5, see Fig. 3A,B. This is markedly different from the pH dependence for the corresponding reaction in *c*NOR, which showed a simple Henderson–Hasselbalch titration with a pK_a ~6.6 [37]. This pK_a was suggested to reflect the pK_a of an internal protonatable group acting as a proton donor to the active site [37]. Furthermore, this pK_a was shown to be affected in site-directed variants where residues (now known to be Ref. [9]) close to the Ca^{2+} located above (and interacting with) the heme propionates, were modified [48]. These results indicate that the region around the Ca^{2+}/heme propionates is important for proton transfer in *c*NOR, although the detailed proton transfer path is not defined.

In qNOR, it is possible that there is a pK_a of the reaction (Fig. 3B), but it is at pH<5.5 or pH>9.5 in order to account for the pH independence observed. It is thus clear that the groups/amino acids involved are different between *c*NOR and qNOR, and it is tempting to suggest that this is due to the use of different pathways, with the pathway in *c*NOR leading from the periplasm and the one in qNOR from the cytoplasm, see Fig. 5. However, some residues (but not all) suggested to form part of putative proton transfer pathways in *c*NOR [9,48], notably the Ca^{2+}-ligands, are conserved to the *G. stearothermophilus* qNOR (see Fig. 5). Thus, the use of a proton pathway from the inside in qNOR remains to be proven experimentally, and this study forms the basis also for studying variant qNORs in which amino acids potentially involved in the proton transfer pathway have been modified by site-directed mutagenesis [32].

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.04.007.

Acknowledgements

PÄ is a Royal Swedish Academy of Sciences Research Fellow supported by a grant from the Knut and Alice Wallenberg Foundation. A travel grant from the Royal Swedish Academy of Sciences and the Japan Society for Promotion of Science (JSPS) to PÄ is acknowledged. Studies in PÄ's laboratory are supported by grants from the Swedish Research Council and from the Faculty of Sciences at Stockholm University. T.T. and Y.S. are supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology. We thank Peter Brzezinski for discussions.

References

[1] P. Tavares, A.S. Pereira, J.J. Moura, I. Moura, Metalloenzymes of the denitrification pathway, J. Inorg. Biochem. 100 (2006) 2087–2100.
[2] W.G. Zumft, Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type, J. Inorg. Biochem. 99 (2005) 194–215.
[3] N.J. Watmough, G. Butland, M.R. Cheesman, J.W. Moir, D.J. Richardson, S. Spiro, Nitric oxide in bacteria: synthesis and consumption, Biochim. Biophys. Acta 1411 (1999) 456–474.
[4] T. Hino, S. Nagano, H. Sugimoto, T. Tosha, Y. Shiro, Molecular structure and function of bacterial nitric oxide reductase, Biochim. Biophys. Acta 1817 (2012) 680–687.
[5] M. Saraste, J. Castresana, Cytochrome oxidase evolved by tinkering with denitrification enzymes, FEBS Lett. 341 (1994) 1–4.
[6] J. van der Oost, A.P. de Boer, J.W. de Gier, W.G. Zumft, A.H. Stouthamer, R.J. van Spanning, The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase, FEMS Microbiol. Lett. 121 (1994) 1–9.
[7] J. Hendriks, A. Warne, U. Gohlke, T. Haltia, C. Ludovici, M. Lübben, M. Saraste, The active site of the bacterial nitric oxide reductase is a dinuclear iron center, Biochemistry 37 (1998) 13102–13109.
[8] P. Girsch, S. deVries, Purification and initial kinetic and spectroscopic characterization of NO reductase from *Paracoccus denitrificans*, Biochim. Biophys. Acta 1318 (1997) 202–216.
[9] T. Hino, Y. Matsumoto, S. Nagano, H. Sugimoto, Y. Fukumori, T. Murata, S. Iwata, Y. Shiro, Structural basis of biological N2O generation by bacterial nitric oxide reductase, Science 330 (2010) 1666–1670.
[10] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases, Biochim. Biophys. Acta 1505 (2001) 185–208.
[11] J. Hemp, R.B. Gennis, Diversity of the heme-copper superfamily in archaea: insights from genomics and structural modeling, Results Probl. Cell Differ. 45 (2008) 1–31.
[12] F.L. Sousa, R.J. Alves, J.B. Pereira-Leal, M. Teixeira, M.M. Pereira, A bioinformatics classifier and database for heme-copper oxygen reductases, PLoS One 6 (2011) e19117.
[13] B. Heiss, K. Frunzke, W.G. Zumft, Formation of the N–N bond from nitric oxide by a membrane-bound cytochrome bc complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri*, J. Bacteriol. 171 (1989) 3288–3297.
[14] F.H. Thorndycroft, G. Butland, D.J. Richardson, N.J. Watmough, A new assay for nitric oxide reductase reveals two conserved glutamate residues form the entrance to a proton-conducting channel in the bacterial enzyme, Biochem. J. 401 (2007) 111–119.
[15] R. Cramm, A. Pohlmann, B. Friedrich, Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16, FEBS Lett. 460 (1999) 6–10.
[16] S. de Vries, M.J.F. Strampraad, S. Lu, P. Moënne-Loccoz, I. Schröder, Purification and characterization of the MQH(2): NO oxidoreductase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*, J. Biol. Chem. 278 (2003) 35861–35868.
[17] P. Brzezinski, P. Ädelroth, Design principles of proton-pumping haem-copper oxidases, Curr. Opin. Struct. Biol. 16 (2006) 465–472.
[18] S. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, Proton-pumping mechanism of cytochrome C oxidase, Annu. Rev. Biophys. 40 (2011) 205–223.
[19] M.M. Pereira, F.L. Sousa, A.F. Verissimo, M. Teixeira, Looking for the minimum common denominator in haem-copper oxygen reductases: towards a unified catalytic mechanism, Biochim. Biophys. Acta 1777 (2008) 929–934.
[20] V.R. Kaila, M.I. Verkhovsky, M. Wikström, Proton-coupled electron transfer in cytochrome oxidase, Chem. Rev. 110 (2010) 7062–7081.
[21] H. Han, J. Hemp, L.A. Pace, H. Ouyang, K. Ganesan, J.H. Roh, F. Daldal, S.R. Blanke, R.B. Gennis, Adaptation of aerobic respiration to low O2 environments, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 14109–14114.
[22] H.J. Lee, J. Reimann, Y. Huang, P. Ädelroth, Functional proton transfer pathways in the heme-copper oxidase superfamily, Biochim. Biophys. Acta 1817 (2012) 537–544.
[23] P. Brzezinski, P. Ädelroth, Pathways of proton transfer in cytochrome c oxidase, J. Bioenerg. Biomembr. 30 (1998) 99–107.
[24] M. Wikström, A. Jasaitis, C. Backgren, A. Puustinen, M.I. Verkhovsky, The role of the D- and K-pathways of proton transfer in the function of the haem-copper oxidases, Biochim. Biophys. Acta 1459 (2000) 514–520.
[25] G. Butland, S. Spiro, N.J. Watmough, D.J. Richardson, Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme, J. Bacteriol. 183 (2001) 189–199.
[26] D.H. Kastrau, B. Heiss, P.M. Kroneck, W.G. Zumft, Nitric oxide reductase from *Pseudomonas stutzeri*, a novel cytochrome bc complex. Phospholipid requirement, electron paramagnetic resonance and redox properties, Eur. J. Biochem. 222 (1994) 293–303.
[27] H. Kumita, K. Matsuura, T. Hino, S. Takahashi, H. Hori, Y. Fukumori, I. Morishima, Y. Shiro, NO reduction by nitric-oxide reductase from denitrifying bacterium *Pseudomonas aeruginosa*: characterization of reaction intermediates that appear in the single turnover cycle, J. Biol. Chem. 279 (2004) 55247–55254.
[28] J.P. Shapleigh, W.J. Payne, Nitric oxide-dependent proton translocation in various denitrifiers, J. Bacteriol. 163 (1985) 837–840.
[29] J.H. Hendriks, A. Jasaitis, M. Saraste, M.I. Verkhovsky, Proton and electron pathways in the bacterial nitric oxide reductase, Biochemistry 41 (2002) 2331–2340.
[30] J. Reimann, U. Flock, H. Lepp, A. Honigmann, P. Ädelroth, A pathway for protons in nitric oxide reductase from *Paracoccus denitrificans*, Biochim. Biophys. Acta 1767 (2007) 362–373.
[31] K.R. Barth, V.M. Isabella, V.L. Clark, Biochemical and genomic analysis of the denitrification pathway within the genus Neisseria, Microbiology 155 (2009) 4093–4103.
[32] Y. Matsumoto, T. Tosha, A.V. Pisliakov, T. Hino, H. Sugimoto, S. Nagano, Y. Sugita, Y. Shiro, Crystal structure of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*, Nat. Struct. Mol. Biol. 19 (2012) 238–246.
[33] S. Buschmann, E. Warkentin, H. Xie, J.D. Langer, U. Ermler, H. Michel, The structure of cbb3 cytochrome oxidase provides insights into proton pumping, Science 329 (2010) 327–330.
[34] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 A resolution of cytochrome c oxidase from *Paracoccus denitrificans*, Nature 376 (1995) 660–669.
[35] T. Soulimane, G. Buse, G.P. Bourenkov, H.D. Bartunik, R. Huber, M.E. Than, Structure and mechanism of the aberrant ba(3)-cytochrome c oxidase from *Thermus thermophilus*, EMBO J. 19 (2000) 1766–1776.
[36] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å, Science 272 (1996) 1136–1144.
[37] U. Flock, N.J. Watmough, P. Ädelroth, Electron/proton coupling in bacterial nitric oxide reductase during reduction of oxygen, Biochemistry 44 (2005) 10711–10719.
[38] T. Fujiwara, Y. Fukumori, Cytochrome cb-type nitric oxide reductase with cytochrome c oxidase activity from *Paracoccus denitrificans* Atcc 35512, J. Bacteriol. 178 (1996) 1866–1871.
[39] N. Sakurai, T. Sakurai, Isolation and characterization of nitric oxide reductase from *Paracoccus halodenitrificans*, Biochemistry 36 (1997) 13809–13815.
[40] Q.H. Gibson, C. Greenwood, Reactions of cytochrome oxidase with oxygen and carbon monoxide, Biochem. J. 86 (1963) 541–554.
[41] D.A. Proshlyakov, M.A. Pressler, G.T. Babcock, Dioxygen activation and bond cleavage by mixed-valence cytochrome c oxidase, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 8020–8025.
[42] M.I. Verkhovsky, J.E. Morgan, M.L. Verkhovskaya, M. Wikström, Translocation of electrical charge during a single turnover of cytochrome-c oxidase, Biochim. Biophys. Acta 1318 (1997) 6–10.
[43] I. Smirnova, J. Reimann, C. von Ballmoos, H.Y. Chang, R.B. Gennis, J.A. Fee, P. Brzezinski, P. Ädelroth, Functional role of Thr-312 and Thr-315 in the proton-transfer pathway in ba3 Cytochrome c oxidase from *Thermus thermophilus*, Biochemistry 49 (2010) 7033–7039.

[44] Y. Huang, J. Reimann, H. Lepp, N. Drici, P. Ädelroth, Vectorial proton transfer coupled to reduction of O2 and NO by a heme-copper oxidase, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 20257–20262.

[45] P. Lachmann, Y. Huang, J. Reimann, U. Flock, P. Ädelroth, Substrate control of internal electron transfer in bacterial nitric-oxide reductase, J. Biol. Chem. 285 (2010) 25531–25537.

[46] E. Pinakoulaki, S. Gemeinhardt, M. Saraste, C. Varotsis, Nitric-oxide reductase. Structure and properties of the catalytic site from resonance Raman scattering, J. Biol. Chem. 277 (2002) 23407–23413.

[47] U. Flock, P. Lachmann, J. Reimann, N.J. Watmough, P. Ädelroth, Exploring the terminal region of the proton pathway in the bacterial nitric oxide reductase, J. Inorg. Biochem. 103 (2009) 845–850.

[48] U. Flock, F.H. Thorndycroft, A.D. Matorin, D.J. Richardson, N.J. Watmough, P. Ädelroth, Defining the proton entry point in the bacterial respiratory nitric-oxide reductase, J. Biol. Chem. 283 (2008) 3839–3845.

[49] M. Brändén, H. Sigurdson, A. Namslauer, R.B. Gennis, P. Ädelroth, P. Brzezinski, On the role of the K-proton transfer pathway in cytochrome c oxidase, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 5013–5018.

[50] J.H. Hendriks, L. Prior, A.R. Baker, A.J. Thomson, M. Saraste, N.J. Watmough, Reaction of carbon monoxide with the reduced active site of bacterial nitric oxide reductase, Biochemistry 40 (2001) 13361–13369.

[51] L.C. Bell, D.J. Richardson, S.J. Ferguson, Identification of nitric oxide reductase activity in *Rhodobacter capsulatus*: the electron transport pathway can either use or bypass both cytochrome c2 and the cytochrome bc1 complex, J. Gen. Microbiol. 138 (1992) 437–443.

[52] L.M. Blomberg, M.R. Blomberg, P.E. Siegbahn, A theoretical study on nitric oxide reductase activity in a ba(3)-type heme-copper oxidase, Biochim. Biophys. Acta 1757 (2006) 31–46.

[53] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Redox transitions between oxygen intermediates in cytochrome-c oxidase, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 12235–12239.

[54] M. Karpefors, P. Ädelroth, Y. Zhen, S. Ferguson-Miller, P. Brzezinski, Proton uptake controls electron transfer in cytochrome c oxidase, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 13606–13611.

[55] E.A. Gorbikova, M. Wikström, M.I. Verkhovsky, The protonation state of the cross-linked tyrosine during the catalytic cycle of cytochrome c oxidase, J. Biol. Chem. 283 (2008) 34907–34912.