# Nitric Oxide Reductase from *Paracoccus denitrificans*

## A Proton Transfer Pathway from the "Wrong" Side

**Ulrika Flock**


Stockholm University

© Ulrika Flock, Stockholm 2008

ISBN 978-91-7155-740-7

Frontpage; *"Ullis and Pia in the laser-lab"* by Johan and Erik Flock.
Photographer Per Flock.

Typesetting: Intellecta Docusys
Printed in Sweden by Intellecta Docusys, Stockholm 2008
Distributor: Stockholm University Library

Till Per, Erik och Johan.

# List of publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I **Ulrika Flock** , Nicholas J Watmough , Pia Ädelroth
Electron/Proton Coupling in Bacterial Nitric Oxide Reductase During Reduction of Oxygen
(2005) Biochemistry, **44** (31): p. 10711-10719

II Joachim Reimann, **Ulrika Flock**, Håkan Lepp, Alf Honigmann, and Pia Ädelroth
A Pathway for Protons in Nitric Oxide Reductase from *Paracoccus denitrificans*
(2007) Biochimica et Biophysica Acta (BBA) - Bioenergetics, **1767** (5): p. 362-373

III **Ulrika Flock**, Faye H Thorndycroft, Andrey D Matorin, David J Richardson, Nicholas J Watmough, Pia Ädelroth
Defining the Proton Entry Point in the Bacterial Respiratory Nitric Oxide Reductase
(2008) Journal of Biological Chemistry, **283** (7): p. 3839-3845

IV **Ulrika Flock**, Peter Lachmann, Joachim Reimann, Nicholas J Watmough, and Pia Ädelroth
Exploring the end region of the proton pathway in the bacterial nitric oxide reductase
Manuscript

## Additional publication

**Ulrika Flock**, Joachim Reimann and Pia Ädelroth
Proton Transfer in Bacterial Nitric Oxide Reductase
(2006) Biochemical Society Transactions, **34** (pt1): p. 188-190

# Abstract

Denitrification is an anaerobic process performed by several soil bacteria as an alternative to aerobic respiration. A key-step in denitrification (the N-N-bond is made) is catalyzed by nitric oxide reductase (NOR); $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$. NOR from *Paracoccus denitrificans* is a member of the heme copper oxidase superfamily (HCuOs), where the mitochondrial cytochrome *c* oxidase is the classical example. It is situated in the cytoplasmic membrane and can, as a side reaction, catalyze the reduction of oxygen to water.

NORs have properties that make them divergent members of the HCuOs; the reactions they catalyze are not electrogenic and they do not pump protons. They also have five strictly conserved glutamates in their catalytic subunit (NorB) that are not conserved in the 'classical' HCuOs. It has been asked whether the protons used in the reaction really come from the periplasm and if so how do the protons proceed through the protein into the catalytic site?

In order to find out whether the protons are taken from the periplasm or the cytoplasm and in order to pinpoint the proton-route in NorB, we studied electron- and proton transfer during a single- as well as multiple turnovers, using time resolved optical spectroscopy. Wild type NOR and several variants of the five conserved glutamates were investigated in their solubilised form or/and reconstituted into vesicles.

The results demonstrate that protons needed for the reaction indeed are taken from the periplasm and that all but one of the conserved glutamates are crucial for the oxidative phase of the reaction that is limited by proton uptake to the active site.

In this thesis it is proposed, using a model of NorB, that two of the glutamates are located at the entrance of the proton pathway which also contains two of the other glutamates close to the active site.

# Abbreviations

| | |
|---|---|
| NOR | nitric oxide reductase |
| C*c*O | cytochrome *c* oxidase |
| $cbb_3$ | $cbb_3$ oxidase |
| $aa_3$ | $aa_3$ oxidase |
| HCuOs | heme copper oxidases |
| bR | bacteriorhodopsin |
| heme *c* or only *c* | Denotes heme *c* in the NorC subunit |
| heme *b* or only *b* | Denotes heme *b* in the NorB subunit |
| heme $b_3$ or only $b_3$ | Denotes heme $b_3$ in the NorB subunit |
| $Fe_B$ | Denotes the non-heme iron in the NorB subunit |
| Substitution nomenclature: | E.g. E198A denotes a change of the glutamate at position 198 in NorB for an alanine. |
| cyt. *c* | cytochrome *c* |
| τ | Time constant, 1/rate constant |

# Contents

# 1. INTRODUCTION

Is there anyone in this world who has never stopped, for at least a tiny moment, to ponder or fall silent over the wealth of life around us?

In this thesis, proton pathways in membrane proteins and ideas around the role of glutamates at entrances to and inside these pathways will be discussed. Especially the proton pathway in the c-type nitric oxide reductase (NOR) will be presented and compared to other possibly equivalent proton pathways in the *cbb*$_3$ oxidases and A1 heme copper oxidases.

## 1.1 At the very beginning

Life sprouted on earth as a result of infinite trials and errors, sparked by a fortunate mixture of elements, energy outbursts and a friendly distance to the sun. At this early period in history, about 3.8 billion years ago [1], the atmosphere was primarily composed of carbon dioxide and water vapor, with some nitrogen but virtually no oxygen. Thunderstorms and volcano outbreaks cursed the thin crust around the hot core of iron and it was certainly not a friendly environment from a human point of view.

Today, the atmosphere is roughly composed of (by molar content/volume) ~78 % nitrogen, ~21 % oxygen, ~1 % mixture of gases (mostly argon, but also carbon dioxide, neon, krypton, helium) and water vapor [2].

This atmosphere protects us by reducing temperature extremes between day and night and absorbing ultraviolet solar radiation. In short, the environment has become friendlier compared to the ancient days.

It is interesting that the atmosphere contains about 78 % of nitrogen gas ($N_2$), since all life forms require nitrogen compounds, e.g. for making proteins and nucleic acids. There is plenty of nitrogen gas available, but most organisms can not use nitrogen in this form.

Plants must secure their nitrogen in a "fixed" form, i.e. incorporated into compounds such as: nitrate ($NO_3^-$), ammonia ($NH_3$) and urea $(NH_2)_2CO$ and animals secure their nitrogen from plants (or animals that have fed on plants), but how do the plants “fix” nitrogen?

## 1.2 The nitrogen cycle

Four processes participate in the cycling of nitrogen through the biosphere; nitrogen fixation, nitrogen decay, nitrification and denitrification (figure 1) and microorganisms play major roles in all four of them.

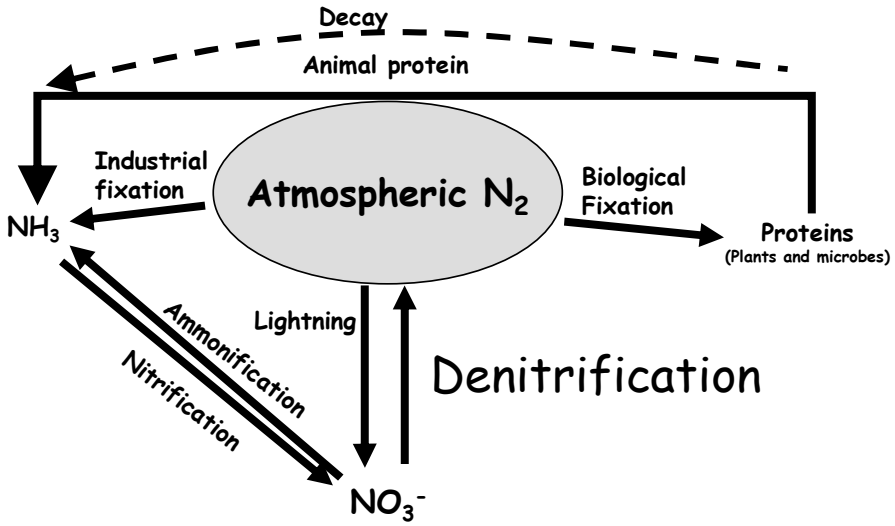


**Figure 1. The nitrogen cycle.** N.B. the only way back into the atmosphere is via denitrification. Modified from;
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/N/NitrogenCycle.html.

*Nitrogen fixation* can be accomplished by biological fixation (~ 68 % of the total fixation), industrial fixation (~ 20 %), combustion (~8 %) and fixation by lightning (~ 4 %) [3]. When it comes to biological fixation, performed by prokaryotic organisms (described in [4, 5]) the most commonly known example is found in leguminous plants, such as pea, lupine or alfalfa. If you tear apart the root nodules you will find parts that are colored pink, where the color is derived from leghemoglobin [6], a heme protein with a very high affinity for oxygen. The enzyme nitrogenase [7] is responsible for catalyzing the fixation of the inert gas $N_2$; $N_2 + 6H^+ + \text{energy} \rightarrow 2NH_3$. Leghemoglobin protects nitrogenase since dioxygen ($O_2$) irreversibly inhibits this enzyme.

For industrial fixation an example can be found in the synthesis of fertilizers used in agriculture, which is produced through the so called Haber process [8]. In this process ammonia is produced by passing a mixture of atmospheric nitrogen and hydrogen over a metallic catalyst at about 600° C and high pressure.

In the process called atmospheric fixation, two unregulated processes are found; one is the phenomenon where $N_2$ is fixed through lightning [9], resulting in $NO_2$ and $NO_X$. In another process, more of an every day one, the driving of cars, the combustion temperature in combination with air results in the fixation of nitrogen to $NO_X$ [3]. This fixation results, later on during rainy weather, in nitric acid, the reason for the expression "acid rain". This process is not a classical industrial fixation process, although a classical event in the industrial world.

*Nitrification* is a two step microbial process where ammonia ($NH_3$) is sequentially oxidized to nitrite ($NO_2^-$) and nitrate ($NO_3^-$) [10, 11], where both processes contribute to the generation of ATP. Now at the level of nitrate (figure 1) in the N-cycle, the next step is denitrification.

When nitrogen has been fixed by some of the processes described above, there is only one way back into the atmosphere and that is through denitrification.

## 1.3 Denitrification - From $NO_3^-$ to $N_2$

Nitrogen is removed from the biosphere through denitrification (figure 1) which is a four step procedure (figure 2) where nitrate in a stepwise manner is converted to gaseous nitrogen via nitrite, nitric oxide and nitrous oxide (also called laughing gas) [12]. Each step is catalyzed by a specific enzyme, starting with nitrate reductase (NAR) for the reduction of $NO_3^-$ to $NO_2^-$, nitrite reductase (NIR) for the reduction of $NO_2^-$ to NO, nitric oxide reductase (NOR) catalyzing the reduction of NO to $N_2O$ and finally the nitrous oxide reductase ($N_2OR$) completing the denitrification process catalyzing the conversion of $N_2O$ to $N_2$.

The release of $N_2O$ during denitrification has been shown to significantly contribute to the global warming process, as $N_2O$ has a warming potential of 320 relative to $CO_2$ [13]. Interestingly, $N_2O$ is converted to NO in the stratosphere[1] and NO plays a role in the destruction of the ozone layer

Denitrification is an important reaction in coastal waterways as it can permanently remove nitrogen from the system as di-nitrogen (figure 2) and therefore it can counteract the eutrophication process[2] [16]. Denitrification is performed by several soil bacteria, e.g. *Rahlstonia eutropha* [17] and *Paracoccus denitrificans* [18, 19], and serves as an alternative to aerobic respiration. In these organisms, the denitrification genes are expressed if the oxygen concentration is low and if N-oxides are present [12].

[1] The second major layer of the atmosphere, from 12 to 50km [14].
[2] Eutrophication is "an increase in the rate of supply of organic matter to an ecosystem".

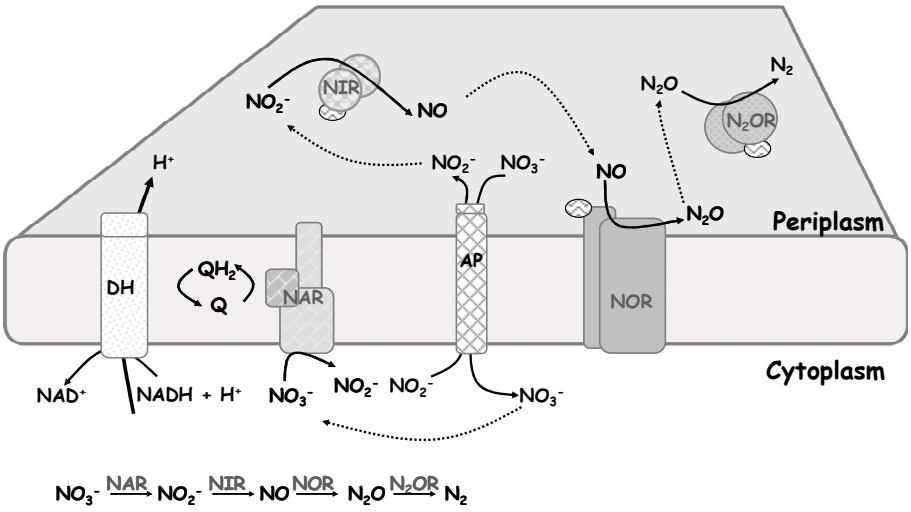


**Figure 2. The enzymes involved in the denitrification process in *P. denitrificans*.** Abbreviations are as follows; DH, NADH dehydrogenase, NAR, nitrate reductase, NIR, nitrite reductase, NOR, nitric oxide reductase, $N_2OR$, nitrous oxide reductase, AP, nitrate/nitrite antiporter, ⊘, cytochrome *c*, $Q/QH_2$, ubiquinone/semiquinone. This picture is modified from an original made by Timothy Paustian, University of Wisconsin-Madison, see: http://lecturer.ukdw.ac.id/dhira/Metabolism/RespAnaer.html.

The enzymes catalyzing the reactions in denitrification are described in several publications; for NAR see [12, 20-22], for NIR see [12, 22-27], for NOR see [12, 27-31] and for $N_2OR$ see [12, 31-33].

### 1.3.1 Denitrification – Noxious intermediates

Two of the intermediates in denitrification are directly harmful to living organisms; nitrite and nitric oxide.

*Nitrite* (figure 3) is ubiquitous as it is used as an additive in food to prevent bacterial growth and as a corrosion inhibitor [34]. Nitrite can be converted into nitrosamine, which is carcinogenic [35], and maybe a little surprising, nitrite are found in high concentrations (50-200 µM) in human saliva [36] probably counteracting bacterial colonization [37].

*Nitric oxide* (figure 3) is a highly reactive, diffusible, and unstable radical. It plays an important role in a wide range of biological processes, including cellular immunity [38], neurotransmission [39-41], as a regulatory factor in cellular respiration [42, 43] and as an intermediate in denitrification [12]. Free NO is a transient molecule with a half-life of about five seconds in vivo

[44] and the reaction of NO with $O_2$ in aqueous solutions produces nitrate and nitrite ions. Moreover, NO can rapidly react with superoxide to produce highly reactive peroxynitrite ($ONOO^-$) [44]. The biological effects of NO are either direct or through other reactive nitrogen intermediates [45, 46].

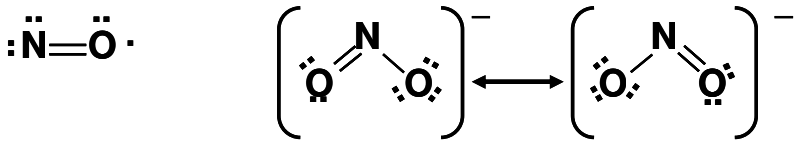

**Figure 3. The Lewis structure of nitric oxide (NO) to the left and nitrite ($NO_2^-$) to the right.** Note the unpaired electron in NO. Nitrite is shown in its two resonance forms.

The *toxicity* of NO is coupled to its reactivity towards transition metal proteins (heme Fe, non-heme Fe, and Cu-containing enzymes are the main targets). NO is also a mutagenic compound, causing changes in the DNA code (C → T, AT → GC, and GC → AT transitions in *Escherichia coli*) due to its nitrosating and deaminating reactivity [47].

In view of the numerous ways that NO and nitrite can exert cytotoxic or genotoxic effects alone or in combination with other reactants, it is surprising that denitrifying bacteria survive during denitrification, but they do[3]. The steady-state concentration of, e.g. free extracellular NO during denitrification, is maintained at a nanomolar level [12].

Now, how is that achieved?

## 1.4 Nitric oxide reductase

NO generation takes place in the periplasm of the bacterium, by the soluble NIR, and the location of nitric oxide reductase (NOR) in the inner membrane forms an efficient sink[4] to prevent NO from reaching the cytoplasm [12].

[3] In this case, the expression; " What does not kill me makes me stronger" is true in an evolutionary point of view [48].

[4] The apparent $K_m$ for NO in NOR is below 1 $\mu$M, which makes NOR a very efficient NO scavenger [49].

### 1.4.1 NOR – What function and where to find it?

The reaction in which the N-N-bond is made (scheme 1) is one of the key-steps of denitrification and is catalyzed by NOR [18]. NOR from *P. denitrificans* can also catalyzes the reduction of $O_2$ to $H_2O$ (scheme 2) [29, 50, 51] as a side reaction. In spite of it being suggested already in 1971 by T. Matsubara and H. Iwasaki [52] it took around 20 years until the character of the last oxidoreductase of denitrification was elucidated [50, 53-56].

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \qquad \text{Scheme 1.}$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \qquad \text{Scheme 2.}$$

NORs are found both in bacteria and fungi. In the fungi the enzyme is a member of the P450 super-family and the NOR from *Fusarium oxysporum* [57, 58] was the first enzyme with NO reduction activity for which the three dimensional (3D) structure was determined [59]. The overall structure is essentially the same as that of the monooxygenase cytochrome P450s, where the most common reaction catalyzed is the insertion of one oxygen atom into an organic substrate. The *F. oxysporum* NOR is a soluble 46 kDa protein consisting of one heme in the active site [60], it receives electrons from NADH and has a turnover numbers of 1000-1200 $e^- s^{-1}$ [60, 61].

This big rate is in contrast to the bacterial NORs that are membrane proteins, with turnover numbers of 40-70 $e^- s^{-1}$ [27] receiving electrons from a variety of donors (cytochrome *c*, pseudoazurin or quinone/semiquinone).

The 3D-structure of the bacterial NORs are yet to be determined[5], but two-dimensional crystals of the c-type NOR have been presented [62], where it also was concluded that the enzyme was purified as a dimer.

The c - and q-type NORs are very similar in the catalytic site [63] and are therefore believed to use the same mechanism for NO reduction.

### 1.4.2 The c-type NOR – Electron flow during anaerobic respiration

cNOR is typically found in bacteria living in soil, such as *P. denitrificans*, [12, 64] and it is expressed during low oxygen conditions when NO is present [18] in concentrations ranging from 5-50 nM (in *Pseudomonas stutzeri* [65]). A knockout mutation for NO reductase is lethal, but the effect can be

[5] Rumors in the scientific community tells that *someone* have diffracting crystals of good quality of a bacterial NOR.

suppressed phenotypically by an additional mutation in *nirS*, i.e. by inactivating the NO generator nitrite reductase [66].

### 1.4.3 Structure

When purified[6], cNOR consists of two subunits (NorC and NorB, figure 4). This "core" complex seems to carry the full enzymatic activity [62], but there is genetic evidence of additional subunits (NorQ, D, E, and F) *in situ*. These may be lost during purification [67] or are only needed for the assembly of the enzyme. cNOR is purified as a dimer, is crystallized as such [62] and it is also present in the membrane as such.

To perform at full activity, NOR requires some lipids, but these can be replaced by detergents [68].

The catalytic subunit (70 kDa) has two components. The smaller one (17 kDa), NorC (figure 4) is a membrane-bound cytochrome *c* (heme *c*, $E_m$= 310 mV [69]) and probably the site where electrons from cytochrome *c* enter. NorC is predicted to have one single trans-membrane helix that anchors the more globular heme *c* containing part, which faces the periplasm.

The larger subunit (54 kDa), NorB (figure 4), binds a heme *b* ($E_m$= 345 mV), a low potential heme $b_3$ ($E_m$= 60 mV) and a non-heme iron, $Fe_B$, ($E_m$= 320 mV) [70], where the two latter are believed to form the active site [71]. NorB is predicted to have 12 trans-membrane helices and belongs to the heme copper oxidase superfamily (HCuOs). The orientation in the membrane of the helices has partly been confirmed by *PhoA* reporter gene fusions [72] for the *P. stutzeri* enzyme. Since *P. stutzeri* NorB has a high sequence similarity to *P. denitrificans* NorB we assume that these results are valid for this protein as well. The coordination of the cofactors has been attributed to histidines. In the *P. denitrificans* enzyme these are for heme *b* His53 (in helix II) and His336 (helix X), for heme $b_3$ His334 (helix X) and for the $Fe_B$ His194 (helix VI) and His245 and His246 (helix VII) (figure 4). Heme $b_3$ is also anti-ferromagnetically coupled to the $Fe_B$ in its oxidized form via a μ-oxo bridge [56, 73].

[6] When eluted from the anion-exchanger, the enzyme has a beautiful strawberry juice color, which makes it a gratifying protein to work with. You see it very well!

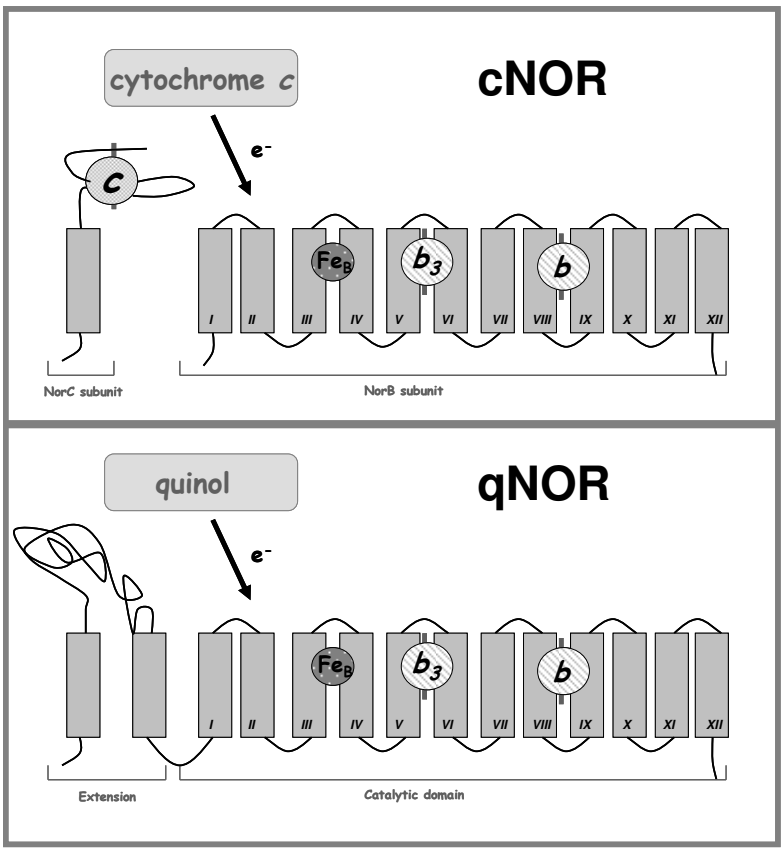


**Figure 4. The structure of c-type and q-type NORs.** c-type NORs receive electrons from cytochrome *c* or pseudoazurin, it is composed of 2 subunits, the NorC and the NorB. q-type NORs receive electrons from quinols and is composed of one subunit, the NorZ. Both enzymes have two *b* hemes and a non-heme iron in the catalytic subunit. The cNORs have also a low-spin heme *c* in the NorC, which is where electrons enter.

Non-heme irons, if not in Fe-S-clusters, demand an octahedral coordination, meaning they need six ligands [62] with histidine and oxo-ligands being the preferred ones. This is seen in e.g. the alternative oxidases (AOX) [74] and in the $O_2$ scavenging flavodiiron protein [75]. The AOX has been shown to contain conserved iron binding motifs (Glu-X-X-His) [76], but no such motifs are found in NOR [77, 78]. Besides the three histidines, a μ-oxo bridge (described in [79]) could function as ligand number four, but two additional ligands are needed. For this purpose three conserved glutamates (described below) have been discussed since models put them in the membranous part of NorB, close to the active site [72, 80].

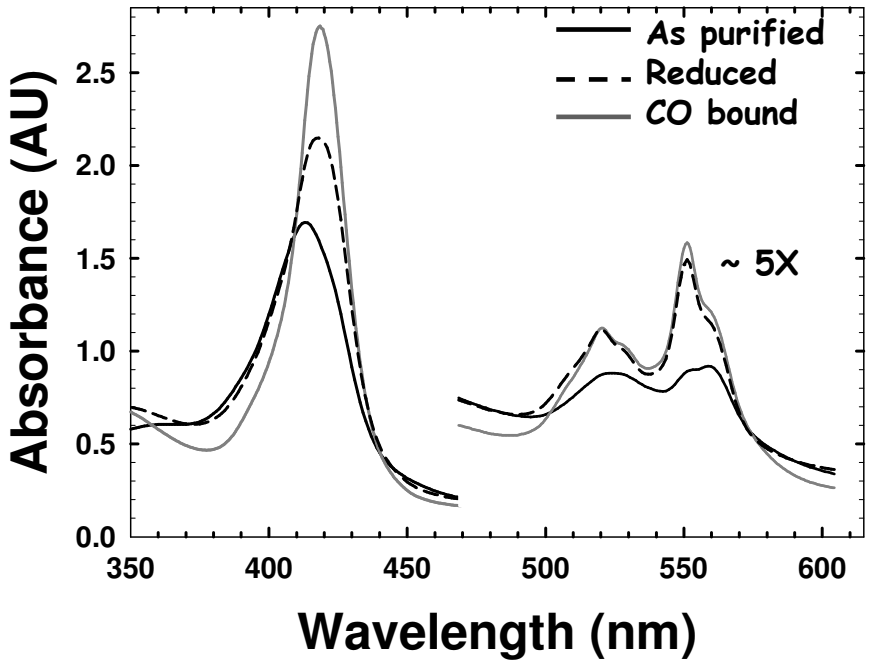


**Figure 5. The UV-VIS spectra for NOR from *Paracoccus denitrificans*.**
The black spectrum is taken of the NOR "as purified", i.e. oxidised by air. The dashed spectrum is taken of the NOR reduced with 1 mM ascorbate. The dark grey spectrum is taken of the NOR with CO bound (100% CO). The reduced heme *c* absorbs at 418.5 nm, 523 nm and 551 nm and the oxidized heme *c* absorbs at 411nm. The reduced heme *b* absorbs at 418.5 nm, 523 nm and 560 nm and when oxidized. The reduced high-spin heme $b_3$ absorbs at 430 nm and the oxidized $b_3$ displays a charge transfer band between 585-605 nm, but see [56]. $Fe_B$ is not detectable in the UV-VIS spectrum. Note that the NOR in the "As purified" spectrum is semi-reduced, harboring 1-2 electrons (Flock *et al.* unpublished data).

Due to the heme and iron content of NOR it has typical spectra in the UV-vis region (figure 5). The different cofactors absorb at specific wavelengths, creating a unique and sensitive "finger-print" of the enzyme. This feature is very useful for studies concerning electron- and proton transfer reactions in the enzyme (see Material and Methods and Results and Discussion).

### 1.4.4 Function - Reactions catalyzed

cNOR catalyzes the reduction of NO to $N_2O$ (scheme 1) with a maximum turnover number of 40-70 $e^- s^{-1}$ for the *P. denitrificans* enzyme [27, 50, 56]. cNOR can moreover catalyze the reduction of $O_2$ to water with a turnover number of ~ 10 $e^- s^{-1}$ [29, 50].

These turnover numbers are very different from the turnover number for reduction of NO by the P450 NOR from (~ 1000 $e^- s^{-1}$) and the turnover number for dioxygen reduction catalyzed by cytochrome *c* oxidase from *Rhodobacter sphaeroide*s (~1500 $e^- s^{-1}$) [81]. The relatively low maximum turnover number of NOR is partially attributed to the inhibitory effect of elevated NO concentrations (> 5 μM). This feature is suggested to be due to the binding of NO to the oxidized enzyme [56].

### 1.4.5 The NO reduction mechanism

Different theories have been presented on how the NO molecules approach the binuclear site. The so-called *cis*-mechanism (i.e. binding of two NO molecules to one of the cofactors, the *cis*-$Fe_B$ or *cis*- $b_3$ mechanisms) and the *trans*-mechanism (i.e. binding of one NO molecule to each cofactor) have been dominating the discussion, see in for example [18, 82].

The *trans*-mechanism has been proposed from steady-state kinetic experiments, where results suggested a sequential binding of the NO molecules [56], and rapid-freeze quench EPR experiments with the fully-reduced NOR [83].

The *cis*-$Fe_B$ mechanism, on the other hand, seems to fit with the very low redox potential of heme $b_3$, which should make the three-electron reduced enzyme prevalent during turnover [70, 84], but theoretical calculations made by Blomberg *et al*. [85] do not favor this scenario, and experimental results demonstrate that NO binds to heme $b_3$ (see [86] and Lachmann *et al.*, unpublished), thereby excluding the *cis*-$Fe_B$ mechanism.

The *cis*-heme $b_3$ mechanism has however found support from observations of the NO reaction in $cbb_3$, where a ferrous (five-coordinated) heme $b_3$-NO complex was seen [87, 88] and theoretical calculations [85].

All in all, no uniform mechanism has been agreed on by the NOR community that brings together all spectroscopic data and structural information available.

### 1.4.6 The q-type NOR– Bacterial saviour and a human nightmare

In contrast to the respiratory cNORs, the qNORs are found mostly in pathogens as a defense against the NO bombardment from the host's immune system.

### 1.4.7 qNORs - Structure and function

The q-type NOR receives electrons from quinol and can be found in pathogenic bacteria [89] such as the *Neisseria* species (e.g. *N. gonorrhea* and *N. meningitis* [90]) and *Mycobacterium bovis*. Here the NOR is more a part of the bacterium's defense against the hosts immune system than part of an alternative respiratory chain, although this point of view has been challenged to some extent recently [91, 92]. It has been shown that *N. meningitis* can support growth by the reduction of $NO_2^-$ via NO to $N_2O$, during oxygen limitations.

qNOR is also found in archeal bacteria such as *Pyrobaculum aerophilum* as part of complete denitrification ($NO_3^-$ to $N_2$) [17].

qNOR is composed of one subunit, NorZ (figure 4). It consists of 14 predicted trans-membrane helices, where twelve of them correspond to the 12 helices of NorB, and the remaining two are an extension, corresponding to NorC and thus the domain where quinol binds and the electrons enter [18]. The part of the qNOR that builds the catalytic core, coordinates one low-spin heme *b*, one high-spin heme *b* and a non-heme iron [18], where the latter two comprise the catalytic site.

The qNOR catalyzes the reduction of NO to $N_2O$, but so far no reports on the $O_2$ reduction reaction have been published. However, presumably the qNORs do reduce $O_2$ to $H_2O$, as do most of the cNORs (although not the cNOR of *P. stutzeri* [54]).

## 1.5 The heme-copper oxidase superfamily

Since the bacterial NORs are considered to be members of the superfamily of *heme-copper oxidases* [17, 93] a description of this large family is suitable.

In order to be a member of the heme-copper oxidase superfamily (HCuOs) the amino acid sequence of the catalytic subunit of the enzyme has to be consistent with twelve trans-membrane helices and possess six invariant histidines that coordinate three cofactors within the subunit (figure 6).

Most of the heme-copper oxidases do contain heme and copper in the active site, but NORs do not (as described above). Moreover, pointed out in figure 6 are five strictly conserved glutamates in the catalytic subunit, NorB, which are not conserved in the other HCuOs.

A classic member of the HCuO family is presented in next section.

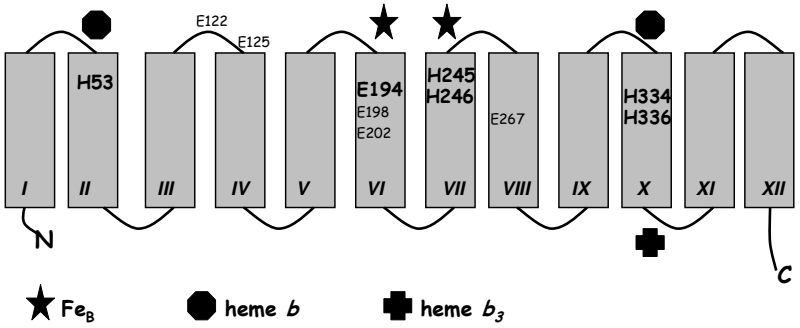


**Figure 6. The properties required to be a member of the heme-copper oxidase superfamily.** Twelve predicted trans-membrane helices in the catalytic subunit and six invariant histidines that coordinate the three cofactors. The *star*, *octahedron* and *cross* denote which helix or helices that participate in the coordination of which cofactors. Also shown are the five glutamates (E122, E125, E198, E202, and E267, *P. denitrificans* numbering) that are strictly conserved among the cNORs.

### 1.5.1 Cytochrome *c* oxidase – structure and function

Cytochrome *c* oxidase (C*c*O) is a very well studied enzyme and it was purified and characterized almost 50 years ago [94-96]. It is situated either in the cytoplasmic membrane of bacteria or in the inner membrane of mitochondria. C*c*O catalyzes the reduction of molecular oxygen to water while pumping protons across the inner membrane, receiving the electrons (from cytochrome *c*) for the reaction from the periplasm (in the bacterium) or the intermembrane space in the mitochondrion. C*c*O thereby contributes to the generation of proton motive force (PMF) that is used by the ATP synthase to produce ATP. C*c*O can not reduce nitric oxide, in contrast to NOR, which

can reduce both NO and oxygen. The 3D-structure has been determined for C*c*O from several sources, for reviews on C*c*O, see [78, 97-99].

### 1.5.2 NOR *vs* $cbb_3$ *vs* $aa_3$ – multi-tasked *vs* specialized

NOR, $cbb_3$ oxidases and $aa_3$ oxidases are all HCuOs; their catalytic subunit has the twelve trans-membrane helices and the six invariant histidines that coordinate the three cofactors (two hemes and a copper/non-heme iron) and they all catalyze the reduction of oxygen to water. Even so, strikingly different features clearly separate these three family members (figure 7).

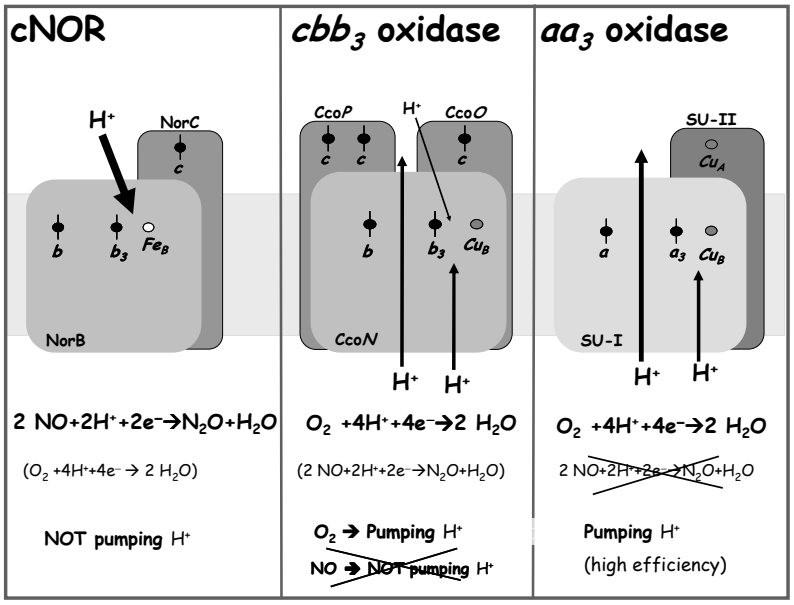


**Figure 7. A comparison between three types of heme-copper oxidases.** The *c*-type NOR from *P. denitrificans* can reduce nitric oxide to nitrous oxide and oxygen to water as a side reaction. It does not pump protons across the membrane with any of its substrates, and substrate protons are derived from the periplasm. The $cbb_3$ oxidase from *V. cholerae* can reduce oxygen to water and nitric oxide to nitrous oxide as a side reaction. It pumps protons (not very efficiently) across the membrane with oxygen as the substrate and derive protons for the chemistry from the cytoplasm. With nitric oxide as substrate the protons are derived from the periplasm and no proton pumping occurs (Huang *et al.*, unpublished). The $aa_3$ oxidase from *R. sphaeroides* can only reduce oxygen to water and during this reaction it pumps protons (with high efficiency) across the membrane. This modified picture, originally made by Pia Ädelroth (Stockholm University), is published with courtesy of Pia Ädelroth.

NOR bears the closest resemblance, as shown by sequence alignments, to oxidases that are considered old[7] (and sometimes primitive) members of the HCuOs family [19, 93, 100], exemplified by the $cbb_3$ and $ba_3$ oxidases.

The two latter reduce NO and $O_2$ *and* pump protons during $O_2$-reduction, whereas NOR does not pump any protons (figure 7).

$cbb_3$ oxidases, interestingly, does not pump protons during NO reduction and takes protons for the NO reaction from the periplasm (Huang *et al*., unpublished) as does NOR [80]. The cytochrome *c* oxidases (C*c*O), e.g. the mitochondria like, can only reduce oxygen (NO inhibits the enzyme and is believed to function as a regulator of respiration [101-103]). It pumps protons very efficiently across the membrane [94, 95] and takes the protons for chemistry *and* pumping from the mitochondria matrix/bacterial cytoplasm.

NOR is considered to be a unique member of the HCuOs ([19] and many more). It is a membrane protein, but it does not contribute to the proton electrochemical gradient across the membrane [51, 80], meaning that it does not pump protons and it picks up the protons needed for both oxygen and NO reduction from the periplasm. This is intriguing, as both reactions catalyzed by NOR are highly exergonic (for NO to $N_2O$, $E^{0'}$ = 1.2 V [12] and for $O_2$ to $H_2O$ $E^{0'}$= 0.8 V). C*c*O is known to use the energy from the reaction with oxygen to both selectively use only protons from the cytosolic side of the membrane and to actively pump protons across the membrane [104, 105].

In figure 7, three different HCuOs are compared, concerning their ability to pump protons, to reduce NO and $O_2$ and from which side of the membrane protons for chemistry are taken.

In conclusion, sequence similarity and NO-reducing capability seem related and coupled to proton uptake from the periplasm, whereas proton pumping seems connected to oxygen reduction, but NOR diverges by not pumping protons with oxygen as substrate.

## 1.6 Protons are special

The coupling of proton transfer to electron transfer in proteins is fundamental for energy conservation and there are several examples of proton pumps, the light-driven proton pump bacteriorhodopsin (bR) [106, 107] and miscellaneous gated proton channels [108-110] are found in nature.

[7]With a twinkle in the eye, Castresana and Saraste pointed out the pentapeptide "GAMLA" (old in Swedish) in the *P. stutzeri* NorB, as a support, amongst all others, for the primordial character of this subunit [19].

### 1.6.1 Proton pathways in proteins

The composition of proton pathways has a general pattern with polar, acidic and basic residues lining the path and coordinating water molecules [111]. This is seen in the D-pathway in the catalytic subunit (SUI) of C*c*O from *R. sphaeroides*, which starts with the conserved D132 (hence the name D-pathway) and continues through the protein via three asparagines, a tyrosine, three serines and end at a glutamate (the E286) near the active site. The polar/acidic residues coordinate an array of water molecules leading to the highly conserved E286 [112], where the protons are believed to be transferred along the ordered water molecules by the so-called Grotthus mechanism [111, 113, 114].

In principle, this mechanism means that one proton enters on one side of a "water-row" resulting in the organization of the waters in such fashion that another proton is expelled on the other side of the "row" due to electrostatic interactions or the driving force from high p$K_a$ reaction intermediates. After the ejection of the proton, the water molecules rearrange themselves to the starting position, ready to transfer another proton [111].

### 1.6.2 Glutamates in proteins and proton pathways

Glutamates are found "everywhere" it seems. Glutamate is a neuronal transmitter substance and a taste enhancer that can cause the Chinese restaurant syndrome [115]. Turning back to the bacterial world we have to consider smaller systems and smaller "syndromes". If proton pathways can be called a syndrome, it is a common one, and if someone asked; are glutamates common parts of this "syndrome" then *yes* is the answer.

In the end of the D- pathway of CcO a crucial glutamate is found (the E286, *R. sphaeroides* numbering), which is believed to be a branching point, where protons are either pumped or directed to the active site [116]. In voltage-gated proton channels, glutamates are found to have a crucial function as well. For example in a $Cl^-/H^+$ exchanger the ion selectivity is proposed to be determined by two conserved glutamates [117], where one of them has also been proposed to function as a gate for the $Cl^-$ pathway [118]

The cytochrome $bc_1$ complex has also been shown to have a crucial glutamate that facilitates proton transfer through a rotational mechanism [119] and let us not forget the ATP synthase. This enzyme has a conserved glutamate (rarely an aspartate) in the ion binding site of the c-ring [120].

It has been shown that several glutamates are involved in proton transfer in the light-driven proton pump bacteriorhodopsin, holding roles of coordinating protonated water clusters [121]. In the photosynthetic reaction centre several glutamates are involved in coordinating structured water molecules [122], where one of the glutamates has the important role of delivering protons to the reduced quinone [123].

Even the green fluorescent protein (GFP) has a proton pathway, beginning with a glutamate-lysine cluster and extending into the interior of the protein where another glutamate sits near the active site [124]. Interestingly, in the same article it is proposed that the GFP might be a portable proton pump (!).

If so, nature is certainly ingenious.

Finally, Rutz and co-workers could demonstrate that in *Escherichia coli* negatively charged residues can play an active and direct role as topogenic determinants; they found that the negatively charged residue has a distance-window from the membrane of about 6 residues [125].

#### 1.6.2.1 Glutamates in the NorB

In the *P. denitrificans* NorB sequence 17 glutamates are found and 11 of them are more or less strictly conserved (the E74, E75, E80, E122, E125, E198, E202, E222, E225 and E267) among all NorB sequences presented in [12, 77, 78, 80, 126].

In this thesis, five of these glutamates, the E122, E125, E198, E202 and E267, which are *strictly* conserved in the cNORs and more or less "strictly" conserved in the qNORs will be considered. (One of them, the E125 is an aspartate in some of the qNORs, see paper II). The importance of these five residues for the activity, but not for the assembly of the enzyme has earlier been shown by aspartate-, alanine- and glutamine substitutions [27, 127].

The position of E122 and E125 at the periplasmic surface in a model ~25 Å from the active site, makes them both possible candidates for being involved in proton transfer. Interestingly, the equivalent of these surface glutamates are conserved in $cbb_3$ oxidase. When the corresponding residue to the E122 in the $cbb_3$ from *Vibrio cholerae* or *R. sphaeroides* was substituted with either glutamine or glycine the activity decreased to less than 1 % of wild type activity. When the same substitutions were done on the corresponding residue to the E125, the activity decreased to 47 % in *V. cholerae* and 11 % in *R. sphaeroides* [128]. This underlines the importance of these residues and also points to the relationship between $cbb_3$ and NOR, where the $cbb_3$ oxidase might use the area around the two glutamates as both an exit and entry point for protons.

The E198, E202 and E267 residues are found in the middle of the membrane (near the binuclear site), which is energetically costly and therefore implies special functions for them. No corresponding residues are found in the traditional A1-C*c*Os or the $cbb_3$ oxidases [78].

E198 is part of the strictly conserved core sequence HLWVEG (where the histidine is the invariant H194) and could therefore have the same function as E286 (*R. sphaeroides* numbering) in cytochrome $aa_3$ oxidase subunit I, which is part of the core sequence HP**E**VYI and delivers protons to the active site [129, 130]. Hendriks *et al.* [86] suggested that the glutamate could

be a proton storage site to the reaction due to its predicted proximity to the active site and Thorndycroft *et al.* [127] proposed the E198 to be part of the proton pathway from the periplasm.

E198 has also been suggested to be a ligand to the non-heme iron, $Fe_B$ [27, 62], due to its position and since only three other obvious ligands are present (the invariant histidines H194, H245 and H246, that per definition should be $Fe_B$ ligands).

The roles of these five conserved glutamates will be further dealt with in the "Results and discussion" section.

# 2 METHODOLOGY

In this part, I will describe, very briefly, the main methods that are of local design and not well known.

Different variants of UV-vis spectroscopy have been used, since the HCuOs have very characteristic metal containing co-factors[8], most of which are easily detectable in this region of the spectrum. The c-type NORs have typical spectra in the UV-vis region (figure 5), which is due to the absorption properties of the co-factors heme *c*, heme *b* and heme $b_3$ (figure 8 ), whereas the non-heme iron, $Fe_B$, is not detectable with our methods, but can be investigated with e.g. Electron Paramagnetic Resonance (EPR) spectroscopy, see for example in [27, 64].

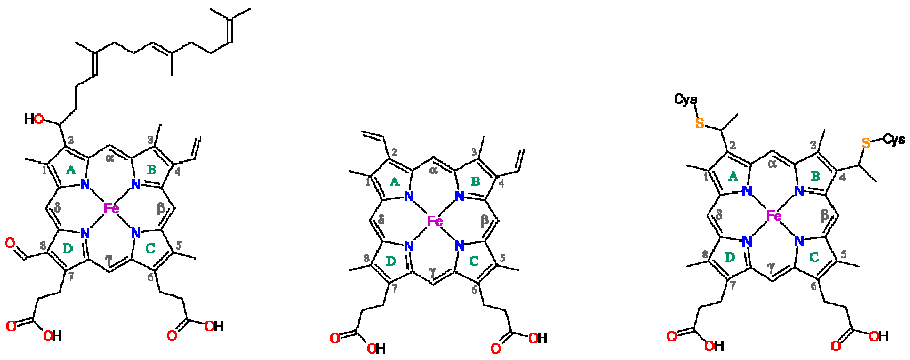


**Figure 8**. **Three examples of heme.** Heme *a* (found in cytochrome *c* oxidase), *b* and *c* that are found in e.g. cNOR from *P. denitrificans*, see introduction.
Modified from http://metallo.scripps.edu/promise/HAEMMAIN.html#haem

## 2.1 Flow-flash and stopped-flow

**The flow-flash method** has been used in order to follow the single-turnover reaction between the fully reduced NOR and $O_2$. The measurements were performed on a locally constructed setup described in [131]. In a few words,

[8] This moreover gives them a shiny bright color.

the fully reduced CO-bound NOR was mixed with an oxygenated buffer in a modified stopped-flow apparatus, see figure 9.

After a delay, a laser flash was applied, dissociating CO and allowing $O_2$ to bind and start the reaction, which is monitored in the Soret (400-500 nm) and alpha region (500-650 nm) in the range from microseconds to seconds.

For the pH-dependence measurements, the buffer concentration in the NOR solution was decreased to 10 mM (HEPES at pH 7.5) and mixed with different kinds of buffers depending on desired pH. For details, see Materials and Methods in paper I and III.

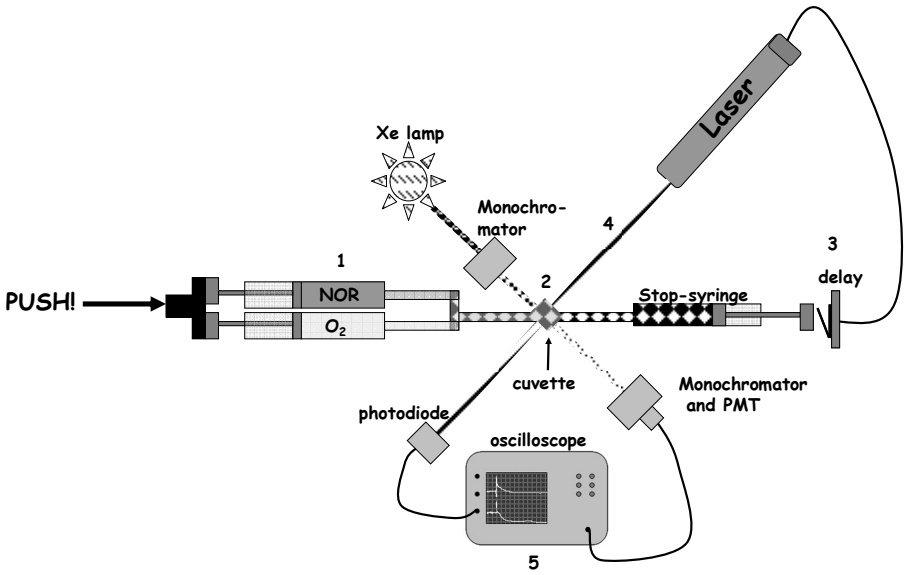


**Figure 9. The flow-flash set up.** NOR and the $O_2$ saturated buffer (substrate) (1) is mixed in a cuvette (2). The forward movement of the liquid in the system makes the stop-syringe hit the delay switch (3). After a pre-set delay time a short laser flash (4) is applied on the sample in the cuvette. The laser flash dissociates the CO bound to NOR ➔ the $O_2$ can bind and the reaction can start. The change in absorbance is followed time resolved by a spectrophotometer (Xe lamp, monochromator1 and 2, cuvette and PMT) and monitored on an oscilloscope (5). The picture is a modified version of the original made by Lina Salomonsson and is published with courtesy of Lina Salomonsson.

**The stopped-flow method** has been used in order to follow multiple turnovers. The technique works as the flow-flash technique, only that no laser flash is applied (no CO is bound to NOR), thereby the reaction starts at the time of mixing.

### 2.1.1 Proton uptake measurements

To be able to measure changes in pH during the flow-flash/stopped-flow reaction with $O_2$, the sample was made unbuffered and proton uptake was followed with the pH-sensitive dye phenol red. Phenol red has a broad absorbance peak at 560 nm in its unprotonated form, but during the single turnover experiments on the **solubilised enzyme**, the proton uptake was monitored at 570 nm in order to circumvent interference from the oxidized form of heme *b*, which also has a maximum at 560 nm. Proton uptake during *multiple turnovers* in the **vesicle reconstituted enzyme** (vNOR) was monitored at 559 nm since cytochrome *c* (which was used as the reductant) has an isobestic point at this wavelength. During a *single turnover* in the vNOR it was found that the background signal with buffer was better (less noise) at 568 nm, therefore data was collected at this wavelength (Reimann *et al.* unpublished).

For each experiment, data was also collected *with* buffer, which was subtracted from traces obtained *without* buffer, to make sure that the oxidation of heme *b* did not interfere with the proton uptake-signal. During each proton uptake experiment, the exhaust was collected and the buffering capacity was determined in order to verify that the signals were the result of an increase in pH and to determine how many protons were taken up per NOR. For further details, see Materials and Methods in paper I and II.

## 2.2 Measurement of electrical charge translocation

In this method, NOR reconstituted into liposomes according to the Bio-Beads technique described in [132-134] was used.

The voltage changes across the NOR oriented in the membranes was measured in a setup (figure 10) built by Håkan Lepp as described in [134]. The setup is based on the method originally developed by Drachev *et al.* [135] and in essence the same as the one used by Verkhovsky, Wikström and colleagues described in [136].

In short, the measuring cell (figure 10) consists of two compartments divided by a teflon film, covered by a lipid monolayer. The enzyme solution containing fully reduced NOR with CO bound reconstituted into vesicles (SUV = small unilamellar vesicle) was added to one of the compartments. Binding to the teflon-lipid surface was then initiated by addition of $CaCl_2$. After incubation the solutions in both chambers were exchanged for buffer, the measuring cell was put in an airtight chamber. The atmosphere was exchanged for nitrogen and then CO.

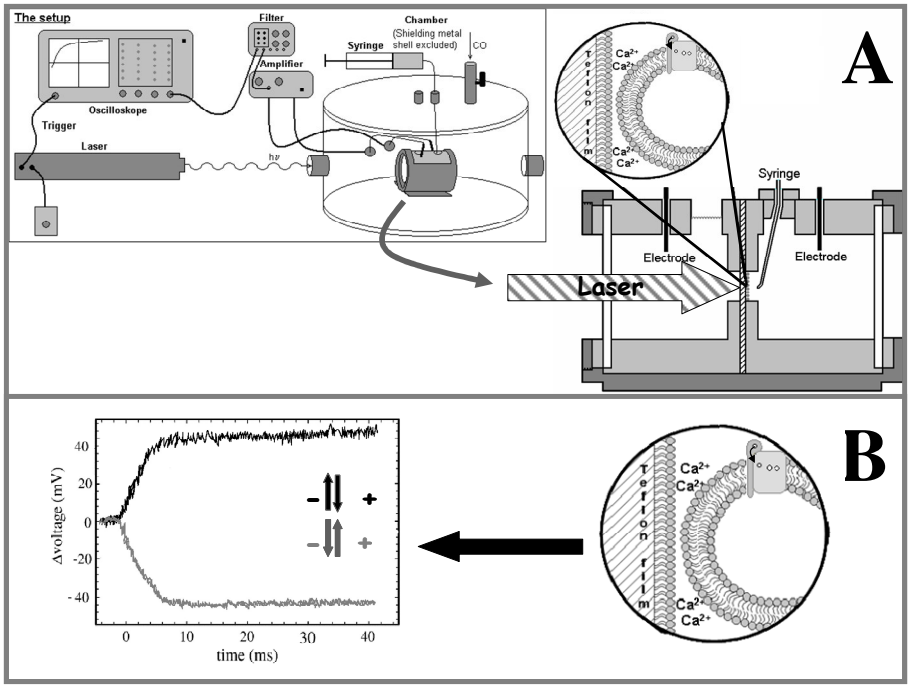


**Figure 10. The electrometric set-up.** *Panel A:* Seen to the left is a picture of the whole set-up. To the right is a zoom of the measuring cell with a further zoom of the teflon-lipid surface where a vesicle containing NOR is visible. *Panel B:* To the right is, as before, a zoom of the lipid covered teflon-lipid surface. Note the electron movement from heme *c* to heme *b* in NOR. This charge movement would give a trace with a negative slope, see to the left where imaginary results are presented. A proton moving from the outside of the vesicle to the interior would give a trace with a positive slope. This is a modified version of a picture originally made by Håkan Lepp (Stockholm University). Published with courtesy of Håkan Lepp.

The reaction is then initiated by injection of an oxygenated solution followed by a short laser flash that dissociates CO. Voltage changes associated with charge transfer perpendicular to the membrane were measured using Ag/AgCl electrodes inserted on each side of the teflon membrane.

In this setup the electrodes are placed in such fashion that *positive signals* represent either a positive charge moving from outside the membrane into the interior or a negative charge moving from the interior to the outside of the membrane. Similarly, a *negative signal* denotes a positive charge moving from the interior of the membrane to the outside or a negative charge moving from the outside to the interior (figure 10).

The amplitude of the signal is proportional to the distance that the charges move inside the membrane, the number of charges that move inside each enzyme and the total amount of reacting enzyme.

# 3 RESULTS AND DISCUSSION

## 3.1 The main topics of this chapter

In this chapter, I will present and discuss the most important results from my studies as reported in the articles and some of the conclusions that can be drawn from them. Each article is referred to with its Roman numeral (I-IV).

## 3.2 Improvement of NOR expression

The NOR from *P. denitrificans* contains heme *c* and in order to accomplish a complete NOR enzyme one has to consider that heme *c* is not expressed in *E. coli* unless the milieu is anaerobic and nitrate is present [137, 138]. To circumvent this matter Butland *et al.* [27] co-introduced the plasmid pEC86 [139, 140] into *E. coli* JM109 cells used for the recombinant expression of NOR. The pEC86 constitutively expresses the *E. coli* cytochrome *c* maturation genes (the *ccmABCDEFGH*) and should thereby increase the expression substantially.

Nonetheless, the expression was initially tediously low in my hands (~50% of the yield reported by Butland *et al.* (i.e. ~0.5 mg NOR/L broth) and the bacteria seemed long gone into the stationary phase at the time for harvest. In order to try to make the expression levels more gratifying the bacteria were grown in terrific broth (TB) [141]. With this modification the yield increased a little, but the breakthrough came after a power failure during one night, lasting for 5 hours. Unexpectedly, the power failure resulted in a yield of ~ 2.5 mg NOR/L broth, which is an increase of 5 times. Now, how could that be?

Due to the power failure the temperature and the aeration were decreased and based on the theory that cytochrome *c* is better expressed during anaerobic/reducing conditions the higher yield makes sense. The lowered temperature moreover moderated the bacterial growth, which should prevent the culture from reaching stationary phase and thereby could result in a more vivid protein-expressing culture. The cultivation procedure was successfully repeated and resulted in the procedure described in figure 11. This procedure is briefly described in paper III.

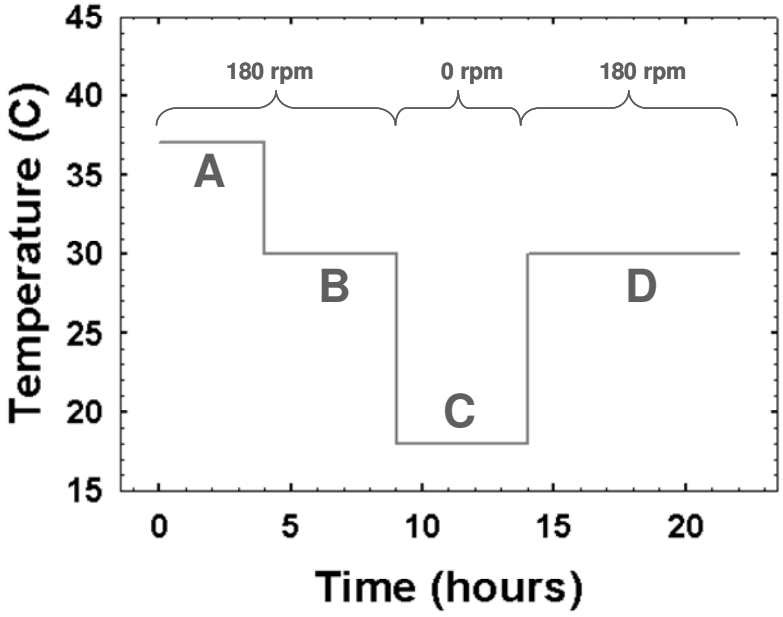


Figure 11. The optimized cultivation schedule. Bacterial growth and NOR expression were done in four steps; A: 0-4 hours, 180 rpm, 37˚C, B: (1 mM IPTG added) 4-9 hours, 180 rpm, 30˚C, C: 9-14 hours, 0 rpm, 18˚C, D: 14-22 hours, 180 rpm, 30˚C. Proceeding step A: a 100 ml LB over-night pre-culture from which inocula of 1 ml where taken. rpm (rounds per minute) stands for the intensity of shaking during the cultivation.

## 3.3 Why oxygen?

During my Ph.D.-studies I mainly worked with the side reaction performed by NOR, the reduction of oxygen to water, see scheme 3 below.
But what is the sense of using $O_2$ as the substrate during an investigation of an NO reductase?

NOR is an NO reductase but it can reduce $O_2$ as well and thus related to the classical oxygen reducers. This makes the $O_2$ reduction reaction interesting from a mechanistic and evolutionary point of view. For example, the $O_2$ –binding to heme $b_3$ is only ~5 times slower (time constant ~ 40 µs at 1 mM $O_2$ , see paper I) compared to the oxygen binding to the heme $a_3$ in the classical C*c*O from *R. sphaeroides* (time constant ~ 8 µs at 1 mM $O_2$) [142].

Moreover, studies of NOR mutants have shown that the $O_2$ and NO reduction activities are well correlated [27, 127] together with paper III and IV. In addition, the electrometric results are very similar, see paper II and [86] irrespective of substrate.

Finally, my main focus was to study proton uptake and for this purpose $O_2$ is better to work with, simply because $O_2$ does not have side reactions that automatically lower the pH in the (unbuffered) system I have used (and NO *is* toxic).

## 3.4 Paper I –The reaction between NOR and $O_2$

### 3.4.1 The product is water

It has long been known that some c-type NORs can reduce $O_2$ [27, 50, 51] and it has been assumed that the product of the reaction is water. In paper I, we established that this is indeed the case by determining how many electrons (from cyt. *c*) are used per oxygen molecule during turnover. If the product of the reaction was hydrogen peroxide ($H_2O_2$) it should consume two electrons per oxygen molecule (scheme 4). If the product of the reaction instead was $H_2O$ it should consume four electrons per oxygen molecule (scheme 3).

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad (4e^- / O_2)$$ Scheme 3.

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (2e^- / O_2)$$ Scheme 4.

Our results clearly showed a consumption of four electrons per oxygen molecule, which identifies water as product. This is, although not very surprising, certainly interesting if one keeps in mind to which superfamily the NORs belong to.

### 3.4.2 Oxygen binds to the high-spin heme $b_3$

Since the NOR from *P. denitrificans* was shown to consume 4 electrons per oxygen molecule, a fully reduced NOR (four cofactors loaded with one electron each) should be able to bind and fully reduce one oxygen molecule and leaving NOR completely oxidized.

During the reaction between the fully reduced NOR and $O_2$, three major kinetic phases were observed in the time range from 1 µs to ~2 s in the visible region of the spectrum (figure 12). The first observed phase could be assigned to oxygen binding to the heme $b_3$ ($\tau \sim 40$ µs at 1 mM $O_2$).

The assignment was based on the observation that the rate of this phase was strictly dependent on oxygen concentration and occurred without any detectable electron transfer. Moreover, the kinetic difference spectrum (see

paper I) of the 40 μs-phase was very similar to the corresponding spectrum in myoglobin (as presented in [143]), which is an well studied oxygen binding protein with a *b*-type heme [143, 144].

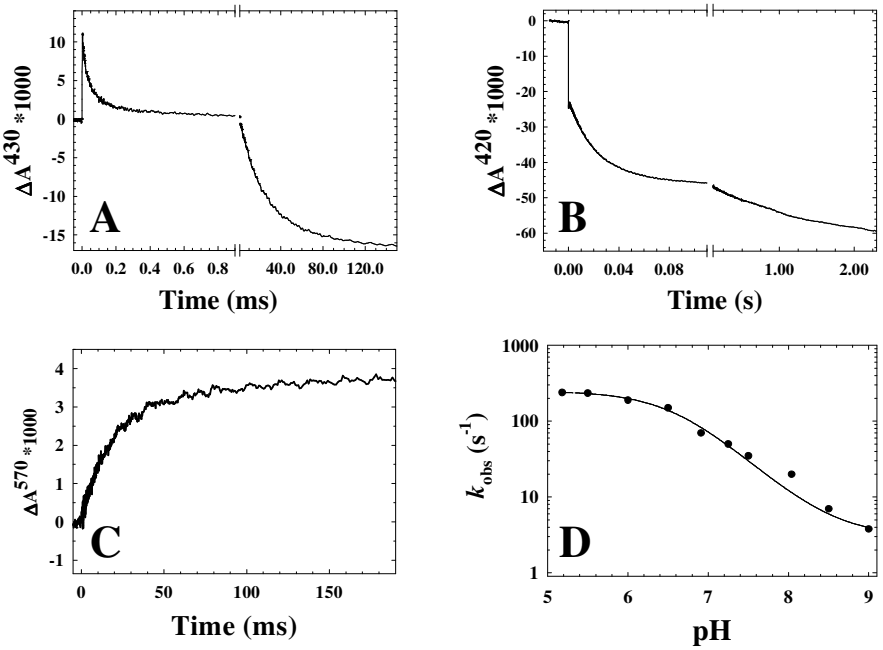


Figure 12. Main results in paper I. *Panel A:* Oxygen binding to the heme $b_3$ and heme oxidation on the ms time scale. *Panel B:* oxidation on the ms time scale and s time scale. *Panel C:* proton uptake from the bulk solution on the ms time-scale in NOR. *Panel D:* the observed rate constant versus pH in wild type solubilised NOR. For more details, see paper I.

### 3.4.3 Oxidation is coupled to proton uptake

After oxygen binding to the heme $b_3$ the second distinct kinetic phase occurs on the ms-time scale (figure 12).

In this phase, electron transfer from the low-spin heme *c* and *b* to the bound oxygen occurs with a $\tau \sim 25$ ms (pH 7.5) and was shown to be strongly dependent on pH (figure 12).

The phase shows a saturating rate constant at low pH (~pH 5.5) and a decrease in rate towards increasing pH values (almost zero at ~pH 8.5). The pH-dependence of the rate constant was found to be well described by a model (see equation 5 and 6), taking into account a *two step* process. The observed rate constant ($k_{obs}$) is determined by the transfer rate ($k_H$) of a proton from an internal protonatable group (AH) to the binuclear site (step one), whereby AH is in rapid equilibrium with the bulk solution (step two). In this model, the observed rate constant, $k_{obs}$, is dependent on the *fraction* of the

internal group that is in the protonated form ($\alpha_{AH}$), which in turn is dependent on the bulk pH and the $pK_a$ of the internal protonatable group ($pK_{AH}$).

$$k_{obs}(pH) = \alpha_{AH}(pH)\, k_H \qquad \text{Equation 5.}$$

$$\alpha_{AH}(pH) = \frac{1}{1+10^{pH-pK_{AH}}} \qquad \text{Equation 6.}$$

When this model was applied to the experimental data (figure 12) a $k_H$ ($k_{max}$) of 250 $s^{-1}$ ($\tau$ = 4 ms) and a $pK_{AH}$ of ~6.6 could be determined.

Since the model suggests a rate limiting step defined by an internal group that is in equilibrium with the bulk solution we aimed to observe proton uptake of the NOR needed for the reaction with NO or $O_2$. Indeed, $H^+$ uptake in the ms-time range could be observed with a pH-sensitive dye (figure 12).

At pH 7.5 a time constant of ~25 ms was determined, which coincides with the oxidation of the low-spin hemes, see above (and paper I). About 1 $H^+$/reacting NOR is taken up during this oxidative phase (for calculations see paper I). This phase corresponds to the oxidation of ~ 40 % of the low-spin hemes *b* and *c*, thus the number of protons taken up per fully oxidized NOR gives 2.5$H^+$.

The NOR needs 4 $H^+$ during one turnover with oxygen, but in this work only the oxidative half of the reaction has been studied. If the number of protons that are taken up during the oxidative phase (during the here called ms- and s phases) can be determined (as x), it automatically gives the number of protons taken up during the reductive part (as 4-x). If the calculation of 2.5 $H^+$/fully oxidized NOR holds, then 1.5 $H^+$ are taken up during the reduction of the enzyme, whereby one proton presumably is needed for the release of the µ-oxo bridge between $b_3$ and $Fe_B$ [70, 73, 145].

In back-flow experiments, Hendriks *et al.* [86] showed that the equilibration of electrons between heme *c* and *b* occurs with an observed rate constant $k_{obs}$ = 3 x $10^4$ $s^{-1}$ (a time constant of ~ 33 µs) and that the equilibration between heme *b* and $b_3$ was even more rapid. Thus electron transfers between the heme groups are <u>not</u> limiting steps of the 25 ms phase. However, in addition to this phase being limited by proton transfer, there could be a limit from the fraction reduced binuclear site, that is determined by the midpoint potentials of the cofactors (for example heme $b_3$ has a midpoint potential of 30-60 mV [27, 70], which is low for an enzyme that is supposed to reduce its substrate). With NO bound to the binuclear site, the midpoint potential might be different than with $O_2$ bound. The NO-binuclear site complex might have a higher potential, thus the probability of having electrons at the binuclear site is higher, which in turn would increase the rate constants for $H^+$-coupled

electron transfer. In the reduction of NO, the rates for the ms phase *is* increased (time constants of 2 ms and 10 ms at pH 8.0) [86], which would point to an increase in potential of heme $b_3$ .upon binding of NO.

The time constant with $O_2$ is in the same time range and it corresponds well with the observed NO turnover activity (30-80 $s^{-1}$ [79, 86] and others) around this pH. The proton transfer from the group with a p$K_a$ = 6.6 to the active site could thus be the limiting step in the reaction with NO as well since the NO turnover shows a strong pH-dependence in the same pH-range where this residue titrates (pH = 6-8 [79]). However, the overall pH optimum for NO turnover is 5 [54, 55] and thus other titratable residues are also important.

The ms phase represents a distinct step in the catalytic cycle and it is limited by the rate of proton transfer. Therefore, *if* the proton transfer pathway were affected in some way, then the rate constant of this phase would also be affected. Mutations in the $H^+$ pathway would either slow down the rate or abolish this phase such that this reaction represents an excellent 'handle' for studying such effects in mutant NORs (see below).

### 3.4.4 The completion – oxidation on the time scale of seconds

As mentioned above, the 25 ms phase does not result in full oxidation of the low-spin hemes *b* and *c*. A third distinct oxidative phase was observed (figure 12) with a time constant $\tau$ = 1-2 s ($k$ = 0.5-1$s^{-1}$).

This phase involved further oxidation of the low-spin hemes (as judged by the kinetic difference spectrum) and was interpreted to possibly represent the final step in the reaction yielding the fully oxidized enzyme. At this stage $H_2O$ could form at the catalytic site, and possibly the μ-oxo bridge that has been observed between the $Fe_B$ and the heme $b_3$ in the "resting" enzyme [73, 79] could be reformed.

The enzyme oxidizes, but to exactly evaluate what happens during this phase is difficult. A population of NORs still have CO bound after the laser flash (due to less than 100% photolysis efficiency, see Material and Methods in paper I) and their slow oxidation interferes with the s-time scale.

## 3.5 Paper II - Protons are taken from the periplasm!

Bacterial NORs are membrane proteins and members of the heme-copper oxidases (HCuOs) superfamily of which most members pump protons across the membrane and the reaction with both NO and $O_2$ is highly exergonic.

In the NOR case, however, it was concluded that the catalyzed reaction was non-electrogenic [86, 146, 147], i.e. not contributing to the proton electrochemical gradient, in spite of all the energy available and the enzyme being located in the membrane.

With these facts in hand, it was proposed that the protons should be taken from the periplasm, although strange it seemed [28, 86].

Well, are they?

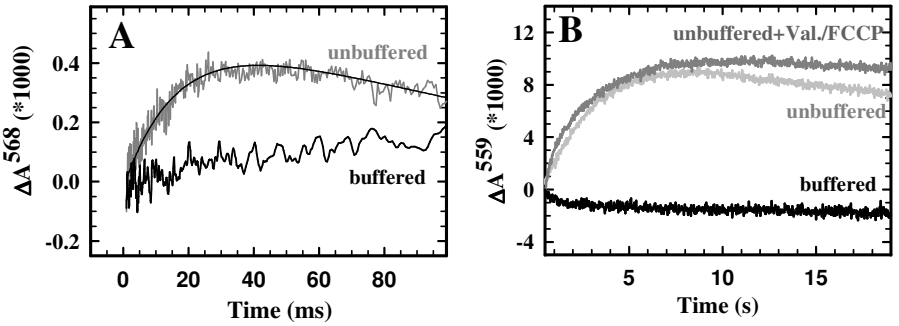


**Figure 13. NOR takes up protons from the periplasmic side of the membrane.** *Panel A:* proton uptake from outside a NOR-containing vesicle during a single turnover in an unbuffered solution is seen (grey trace) and the same experiment, but in the presence of buffer. *Panel B:* proton uptake during multiple turnovers in an unbuffered solution (*light grey trace*), proton uptake during multiple turnovers in an unbuffered solution but in the presence of valinomycin ($K^+$ ionophore) and FCCP (a protonophore) in order to dissipate any electrochemical proton gradient formed (*dark gray trace*) and finally the signal in the presence of buffer (*black trace*). For details see paper II.

In figure 13, data on NOR reconstituted into lipid vesicles (vNOR) with a pH-sensitive dye added to the outside are presented. The results demonstrate proton uptake from the outside of the vesicle membrane (corresponding to the periplasm in the bacterium). *In panel A* proton uptake (by the fully reduced NOR with CO bound) during a single turnover in an unbuffered solution is seen (grey trace) together with the control trace (proton uptake in a buffered solution) in order to exclude that the signal arise from anything else but proton uptake. *In panel B* three traces of proton uptake during multiple turnovers are presented; *i)* in an unbuffered solution (light grey trace), *ii)* in the presence of the protonophore FCCP and the ionophore valinomycin in

order to dissipate any formation of an electrochemical proton gradient (dark grey trace) and *iii)* in the presence of buffer (pH: 7.6) in order to exclude that the absorbance changes were due to something other than proton uptake from the outside of the vNOR. In parallel with these measurements, oxidation of cyt. *c* was followed at 550 nm (paper II), thereby demonstrating the coupling between proton uptake and oxidation of heme *c* (they display the same kinetics).

Furthermore, the "respiratory control ratios" (RCR) (see Materials and Methods in paper II) were = 1 for both $O_2$ and NO (also shown in [86]) demonstrating that no electrochemical gradient is formed concomitantly with turnover.

Electrometric flow flash were also done and showed a net build-up of a positive potential (time constant $\tau$ ~ 5 ms at pH 7.5, similar to the vNOR time constant $\tau$ ~15 ms for the proton-coupled electron transfer). In our set-up this corresponds either to a positive charge moving from the periplasmic side into the interior of the enzyme, *or* a negative charge moving towards the periplasm. The latter alternative is, however, unlikely since the electrons should move from heme *c* and *b* towards the binuclear site buried in the membrane. Electron movements from heme *b* to heme $b_3$ are expected to be electrically silent, since that movement would be in parallel to the membrane.

The net build-up is positive it should represent proton transfer from the periplasmic side, either from an internal site or from the surface of the protein. Because the net signal is positive, more protons than electrons are moving, or the protons are move a longer distance than the electrons.

In the results with NO (see paper II) a small negative phase is seen before the major positive signal. This is also described in [86] where the authors interpreted this as a movement of a charged amino acid simultaneously with the 2-electrons oxidation of the active site during the first turnover of NO to $N_2O$. With $O_2$ as substrate this negative dip is replaced by a lag-phase,in agreement with our optical data, showing that no oxidation occurred before the ms-phase.

The rate constant for relaxation of the built-up potential across the vesicle membrane (2-5 $s^{-1}$) made it impossible to perform a reliable determination of any charge movements during the $\tau$ ~1-2 s phase.

We also observed an accelerated time constant of the ms phase ($\tau$ ~15 ms) in the vNOR compared to solubilised NOR ($\tau$ ~25 ms). This effect could be a result of the influence of the membrane, where the protein is under lateral pressure by the lipids [148] and where proton transfer rates could be accelerated due to the rapid transfer along the membrane ([149-151]), which locally creates a lower pH due to attraction of protons by the negatively charged lipid head groups.

Regardless of whether NO or $O_2$ is used as substrate, protons are taken from the outside (periplasm) and the internal proton-transfer reactions in NOR are similar demonstrating that $O_2$ is a good substrate model for investigating proton-transfer reactions in NOR. Moreover, it is likely that the actual transfer of protons is rate-limiting for the reactions on the ms time scale with both NO and $O_2$.

So, the results in paper II demonstrated that, yes, cNOR from *P. denitrificans* takes the protons for the reaction (with both oxygen and NO as substrate) from the periplasmic side of the membrane.

### 3.5.1 NOR is not a proton pump. Why?

It is assumed that a protein situated in a membrane and catalyzing an energetically favorable reaction has to use this energy to do something. However, could not the location be a remainder of evolution or for organizational reasons?

Maybe the NO reduction mechanism evolved in order to rapidly remove the poisonous NO rather than conserving energy (since the steady state concentration of NO in the bacterium is kept in the nM range [12]).

If NOR would have to be an efficient proton pump, it would work near equilibrium, which means that NO clearance would be slowed. However, in my view the bacterial NORs are not very rapid. The turnover number is around 50 $s^{-1}$ compared to 1100 $s^{-1}$ in the fungal enzymes [60, 61]. On the other hand, the enzymes *producing* NO, nitrite reductase (NIR), has a turnover number of about 6 $s^{-1}$ [152, 153], thus the "low" turnover in NOR should not create any problem for the bacterium. Furthermore, *if* the bacterium has a cytochrome *c* nitrite reductase (which is a soluble protein), like in *E. coli*, which has, in addition to its NIR activity, an NO ➔ $N_2O$ turnover rate of ~ 840 NO $s^{-1}$ [154], it will be fine.

Basic chemistry could offer an explanation to why NOR does not pump protons. Blomberg *et al.* suggested [155] that the p$K_a$ values of the intermediates formed at the catalytic site during NO reduction might not be high enough to drive pumping. The C*c*Os, on the other hand, have very high p$K_a$ values for the $O_2$ reaction intermediates [156] making them favorable for protonation, which can be used to run pumping.

Since NORs do not contribute to the conservation of energy, one can ask; why are the bacterial NORs situated in the membrane? Pereira *et al.* [78] suggested, based on sequence alignments, that NORs most possibly evolved from the HCuOs (and not the other way around as suggested by Saraste *et al.* [19]). This suggests that the location in the membrane could be an evolutionary rest.

Considering how toxic both NO and nitrite are a "complex" anchored in the membrane could be envisaged. This complex, where all denitrification enzymes are put together would effectively shuttle NO and $NO_2^-$ without

wasting them and nitrate reductase (NAR), NOR and the $NO_3^-/NO_2^-$ antiporter could anchor the assembly.

The bacterial NORs are rather slow with either substrate and besides getting rid of NO they have no further function (they do no good) and yet, the cell-system works just fine.

NOR may not be of 'perfect' design; it does not pump protons across the membrane contributing to the conservation of energy *and* detoxifying NO, but it works.

## 3.6 Paper II - A proton pathway in the c-type nitric oxide reductase

Being convinced that the protons are taken from the periplasm we aimed to pinpoint the proton route into the binuclear site. For this purpose a 3D-model was constructed of the catalytic subunit, the NorB, based on the homology to the known structures of other HCuOs (figure 14). Moreover, multiple alignments of 12 NorB- and 7 NorZ sequences (catalytic subunit of qNORs) were done, (see paper II plus supplementary data). Residues found to be (strictly) conserved or very similar in all sequences *and* likely to participate in proton transfer in a path stretching from the periplasm into the binuclear site were identified by the alignement.

Based on the results a proton pathway was suggested (figure 14).

The pathway starts from around E122 and E125 (which are approximately 25 Å from $Fe_B$) and stretches through NorB via (maybe R121) D185, T243, S264, E198, E267 and end at the active site. The strictly conserved E202, see below, was not suggested to be part of the proton pathway.

In this section I will examine the proposed pathway and compare the residues to their potential counterparts in *cbb*$_3$ oxidases and (the classical) cytochrome *c* oxidases (in the A1 group [78], A1-C*c*O) in order to examine their relationship concerning proton pathways leading to the periplasm/intermembrane space. The results are presented in table 1.

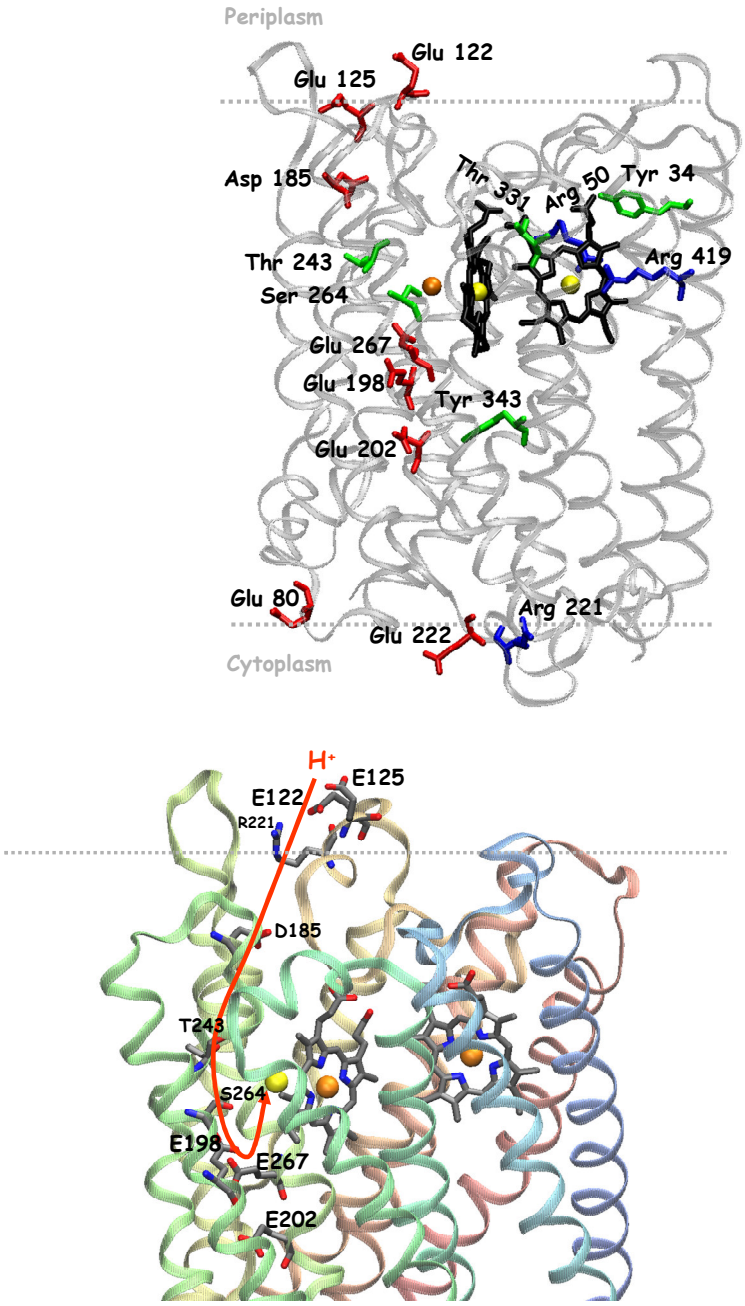


**Figure 14. The model of *P. denitrificans* NorB and the proposed proton pathway**. *Upper panet:* Polar/protonatable residues that are more or less strictly conserved are highlighted. The acidic residues are in red, basic in blue and hydroxyls in green. *Lower panel*: A zoom in to the suggested proton transfer pathway, which is indicated by an arrow. Both pictures show $Fe_B$ (yellow sphere), heme $b_3$ and heme $b$ (the iron spheres are orange).

Glutamate at position 122 (*E122*) is strictly conserved throughout all sequences aligned in our work, whereas glutamate at position 125 (*E125*) is an aspartate in five of the seven NorZs. Nevertheless, this emphasizes the need for an acidic residue at this position. The equivalents of these surface glutamates are conserved in the *cbb*$_3$ enzyme and have been shown to be important for activity [128]. A residue equivalent to E122 is not found in the A1-C*c*Os [77, 78], see table 1. In the E125 case a small polar residue (T or S) holds this position or is located one residue up or down in the sequence.

The distance from these surface glutamates to the next "strictly" conserved residue, the D185, is ~15 Å, but if the close by semi-conserved R121 is included together with a water molecule the distance decreases to ~9Å.

Arginine at position 121 (*R121*) is conserved in all NorBs aligned in paper II, but is a tyrosine in the NorZ sequence. This indicates the need for a hydrophilic residue with a high side chain p$K_a$ (~12.5 for arginine and ~ 10 for tyrosine) that e.g. has the ability to hydrogen bond a coordinated water molecule. The equivalent residue in the *cbb*$_3$ enzyme is either an arginine or a lysine, again a hydrophilic residue with a very high p$K_a$ (~10.5). In the A1-C*c*Os no obvious counterpart is found [77, 78].

Aspartate at position 185 (*D185*) is conserved in all NorBs, but is a methionine or an alanine in the NorZs, however, in the NorZ position equivalent to position 186 in NorB (where a lysine or a glutamine is found) one finds either an aspartate or a glutamate. This points towards the need for an acidic residue in this area in both proteins. In the *cbb*$_3$ oxidase sequence presented in [77, 78] an aspartate is found in all except for one alanine. This points to the same function of this aspartate in both the *cbb*$_3$ enzyme and the NORs. In the A1-C*c*O sequence no obvious equivalent amino acid is found [77, 78].

Threonine at position 243 (*T243*) is approximately 6.5 Å from $Fe_B$ and 13 Å from the D185 and conserved in all but one (where it is a leucine) of our aligned sequences, which underlines the requirement for a polar residue at this position in the NORs. In *cbb*$_3$ , no equivalent residue is found at this location, but ~ one helical-turn proceeding this position is a conserved tyrosine found and one helical-turn further on two of the histidines that are present in all HCuOs are found ( and in the *cbb*$_3$ oxidase case they coordinate $Cu_B$ [77, 78]. None of the A1-C*c*Os have a threonine at the position equal to T243, but they have in ~ 50 % of the cases a serine and sometimes a threonine is found ~ one helical-turn proceeding this position, see table 1 and [77, 78].

**Table 1. A comparison between the proposed proton pathway residues in NOR and the equivalent residues in *cbb*$_3$ oxidase and A1 C*c*Os.**

| NOR residue | *cbb*$_3$ residue | C*c*O residue |
|---|---|---|
| R121 | K or R | Y or P |
| E122 | E | P |
| E125 | E | S/T or A/V, but in the A/V case a T or S is found one amino acid up or down |
| D185 | D (A in one) | Y, W, N, S, A, but the amino acids around is almost always a Q or an R |
| T243 | A and a Y one helical-turn proceeding the A. | W, G, P or V, but sometimes an S one helical-turn proceeding the W, G, P or V. |
| S264 | S | T |
| E198 | G | Y[9] |
| E267 | L | I or L |
| **Outside pathway** | | |
| E202 | T | F, I or L |

[9]This is tyrosine 288 that is cross-linked to histidine 284 (*R. sphaeroides* numbering).

Serine at position 264 (*S264*) is ~7.5 Å from T243 and ~6.4 Å from the $Fe_B$ and is strictly conserved in all our aligned sequences, which indicates the necessity for a small polar residue at this position. This residue is also conserved in all the *cbb*$_3$ oxidases aligned in [77, 78], whereas the A1-C*c*Os has a threonine at this position. In all three cases a small polar residue seems to be of importance.

Glutamate at position 198 (*E198*) is especially interesting, since it is part of a signature sequence (HLWV**E**G, see introduction) [77, 126] and strictly conserved in both NorB and NorZ. Other residues in the suggested pathway, are closer to the active site (for example the S264), judged by the model, but we included this residue in the pathway together with the E267, since their importance for turnover activity has been demonstrated [27, 127]. The equivalent core sequence in the *cbb*$_3$ oxidase (HNAVGF) does not possess the conserved glutamate [77, 126], but a polar residue (asparagine), whereas the A1-C*c*Os core sequence (HP**E**VYI) does have a glutamate, but not at the same position [77, 78]. Instead a tyrosine (Y288, *R. sphaeroides* numbering) is found, which has been shown to be covalently-linked to the $Cu_B$ ligand

H284 [157-159]. The glutamate in A1-C*c*O (E286) is the immediate proton donor to the active site [129, 130].

The glutamate in position 267 (*E267*) is conserved in all NOR sequences aligned in paper II. Both *cbb*$_3$ oxidases and C*c*Os lack a glutamate in the equal position. This could be explained by the architecture of their binuclear site. They have a copper, not a non-heme iron, and the coordination of the $Cu_B$ is fulfilled (by three histidines), whereas in the NORs more ligands are needed for the non-heme iron (three histidines and three additional ligands that are unknown at present). Glutamate 267 might thus play a role in the coordination of $Fe_B$.

All in all, the sequence alignments show that the NORs and the *cbb*$_3$ oxidases show high similarities along their proposed $H^+$ pathways. This could indicate that the two classes of enzymes use the same path, but in the *cbb*$_3$ the path might have a dual function (exit point for pumped protons during the reduction of $O_2$ and entry point during the reduction of NO, Huang *et al.*, unpublished). In the A1-C*c*Os [77, 78], on the other hand, the residues aligning our suggested pathway do not match as clearly, which suggests that these use another pathway for proton pumping into the periplasm in the bacterium/intermembrane space in the mitochondria. Moreover, it should be noted that many of the suggested proton pathway residues in the NorB model are too far away from each other in order to make any hydrogen bonds. Consequently, either more water molecules are needed (more than those that were fitted in the model in paper II), or more protonatable groups or conformational changes in order to have a complete proton transfer pathway.

The proposal of the proton pathway leads to further experimental investigation of the roles of the five conserved glutamates found in the cNORs (the E122, E125, E198, E202 and E267, *P. denitrificans* numbering), of which four had earlier been found to be necessary for turnover [27, 127].

## 3.7 Paper III and IV - The roles of five conserved glutamates

Now, "what" might this interesting group with a p$K_a$ of 6.6 that we found in the reaction between NOR and $O_2$ be (see above and paper I)?

In the *P. denitrificans* NorB sequence 17 glutamates are found (figure 15) of which five are facing the periplasm (E42, E119, E122, E125 and not visible is E401) and nine are located on the cytoplasmic side of the membrane (E75, E76, E78, E80, E222, E225 and E443 and not visible are E459 and E462). Eleven of these glutamates are more or less strictly conserved in all known NorB proteins (the E74, E75, E77 E80, E122, E125, E198, E202, E222, E225 and E267). Here I will only cover five of these (E122, E125,

E198, E202 and E267) which are strictly conserved in cNORs and have earlier been studied by Butland *et al.* [27] and Thorndycroft *et al.* [127]. Furthermore, it is worth mentioning that E80 (sometimes a D) aligns with D132 (*R. sphaeroides* numbering) in C*c*O, which is at the entrance of the D-channel in this enzyme and faces the cytoplasm. This residue is not conserved among the $cbb_3$ oxidases, which presumably do not possess a D-pathway [128].

E122 and E125 are presumed to be located on the surface of the enzyme facing the periplasm (figure 15) and E198, E202 and E267 are believed to be positioned within the membrane, which is energetically expensive and therefore implies special functions. E198 is at a distance of ~ 9 Å from the $Fe_B$ and part of the conserved core sequence as described above. E267 is located ~ 8 Å from $Fe_B$ and supposed to be very close to the H194 (~3 Å in the model). E267 is also close to S264 (~ 4 Å) and the distance in between E267 and E198 is only ~ 4.8 Å. Judged by these distances, from E198 and E267

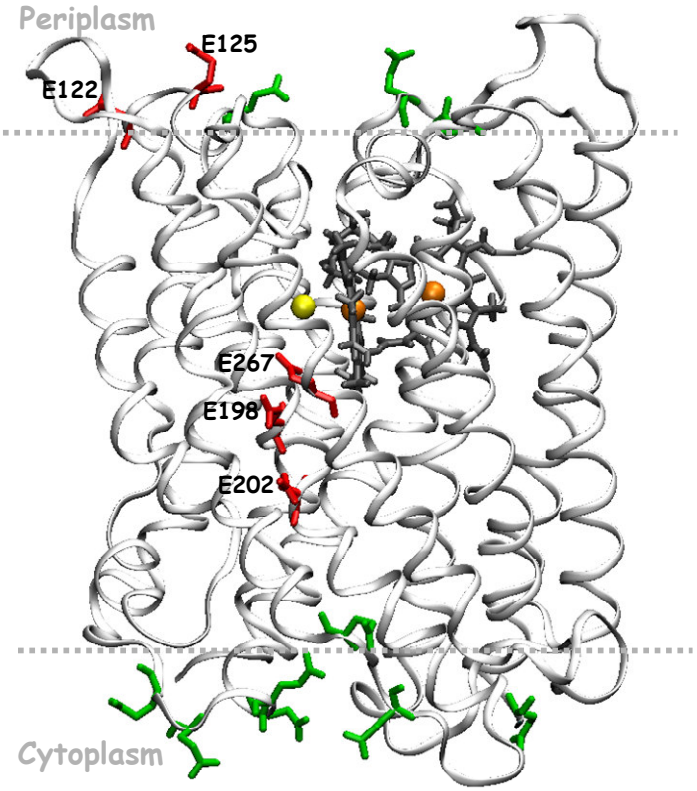


**Figure 15. The model of NorB with glutamates pointed out.** The glutamates labeled red are the ones discussed in this thesis and $Fe_B$, heme $b_3$ and heme *b* are shown as spheres, where $Fe_B$ is yellow .

to both $Fe_B$ and other protonatable residues in the area, any of them is a possible candidate either for ligating $Fe_B$ *or* being part of the proton pathway.

E202 is the diverging glutamate of the five in the sense that it is positioned in the middle of the membrane, with a distance of > 10 Å from either $Fe_B$, E267, H194 *and* all other conserved protonatable residues proposed for the proton pathway, except E198 to which it has a distance of ~4.5 Å. Moreover, E202 can be replaced by an alanine retaining wild type activity ([27] and below).

None of these five glutamates is found in the corresponding positions in the more classical HCuOs, such as the A1 HCuOs from for example *Bos taurus, P. denitrificans* or *R. sphaeroides*.

In the case of E125, E198 and E202, Butland et al [27]showed, by alanine substitutions, that E125 and E198 are necessary for the turnover activity of theNOR, but not for assembly, and that the E202A mutation did not abolish the turnover activity, but lowered it to around 40% of wild type. Thorndycroft *et al.* [127] showed that alanine substitutions of E122 and E267 also abolish turnover activity. Moreover, when E122 and E125 were substituted either to an aspartate, glutamine or asparagine in the, the mutant enzymes where more or less "dead", with the notable exception of E122D, which retained ~80% of wild type activity in whole cells measurements [127].

From these data, Thorndycroft suggested that E122 and E125 form the entrance of a proton conducting "E-pathway" leading from the periplasm to the catalytic site. Let us see what further clues we gain from the results of paper III.

### 3.7.1 Paper III - The proton entrance hall – the E122 and E125

In paper III, the role of the two surface glutamates (E122 and E125) wasinvestigated by site-directed mutagenesis. The fully reduced E122A/D/Q and E125A/Q variants in the reaction with $O_2$ was studied by time-resolved optical spectroscopy (figure 16).

The spectroscopic characterization (see supplementary data of paper III) of the oxidized E122-variants displayed wild type spectra and CO recombination studies (not shown) in all E122-variants also displayed wild type rates. In figure 16, oxygen binding ($\tau$ ~40 μs, at 1 mM $O_2$) to heme $b_3$ is, moreover, observed in all E122-variants. These results imply that the binuclear site is intact.

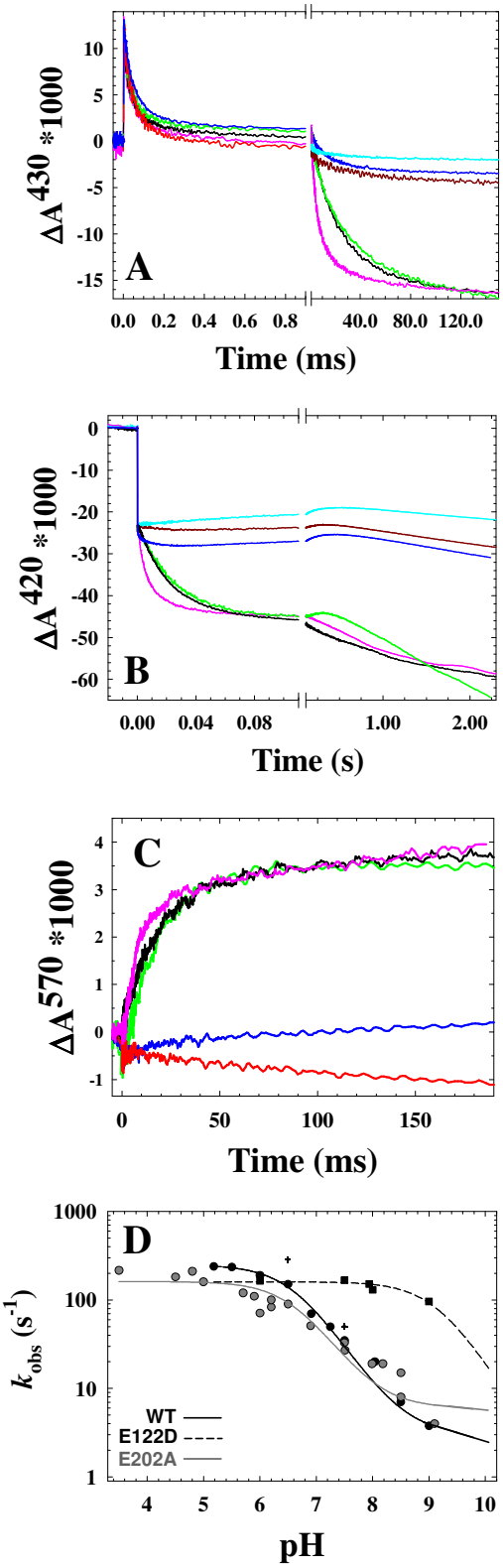


**Figure 16. Main results on the different glutamate variants studied in paper III and IV.** Traces are labeled as follow; **WT**, **E122Q/E198A/E125A/E125Q (one trace)**, **E122D**, **E202A**, **E267A** and **E122A**.
*Panel A:* Oxygen binding to the heme $b_3$ and heme oxidation on the ms time scale. None of the variant NORs showed any significant alteration in binding rates or amplitudes during $O_2$ –binding.
*Panel B:* Heme oxidation on the ms time scale and s time scale.
*Panel C:* Results from proton uptake experiments on wild type, E122D, E202A, E267A and E198A. NOR variants.
*Panel D:* The observed rate constant versus pH. N.B. that the p$K_a$ for this phase in the E122D-variant is shifted upwards more than 2.5 pH units compared to wild type NOR (from ~ 6.6 to ~ 9.1). Seen as two cross around pH 6.5 (τ~3.5 ms) and 7.5 (τ~20 ms), are the residual oxidation of the E267A NOR variant. For clarity reasons, not all variants are represented by a unique trace due to their similarities. Wild type data are from paper I. For more details, see paper III and IV.

Since E122 and E125 are predicted to be far from the active site these results are not surprising. However, the results for $\tau$ = 25 ms phase deviated significantly from wild type, where all, but E122), showed severe inhibition of this phase.

Because the ms-phase is strongly dependent on pH and coupled to proton uptake, the interpretation of the inhibitory effect of mutating these residues is that the proton pathway is blocked. If the E122 and E125 are forming the entrance to the pathway they would be necessary for proton uptake to the active site.

The results of the E122D corroborate this scenario. The E122D is the only variant, in paper III, that keep the ms phase (figure 16) and takes up protons, but the p$K_a$ of the ms-phase is dramatically increased by ~2.5 pH units (from ~ 6.6 to ~ 9.1, figure 16).

The instant thought is that E122 is the group with a p$K_a$ of ~ 6.6 which is raised to ~9.1 on the substitution to an aspartate, but we find this unlikely. As judge by the model, E122 is located at the surface of the protein and either of the carboxylates (on the glutamate or the aspartate) is likely in contact with waters and therefore such a large p$K_a$ shift is hard to imagine. Instead, the results imply that E122 defines the milieu around the residue with the p$K_a$ of 6.6, with which it possibly interacts, directly or via one (or more) water molecule(s). In the E122D enzyme this interaction might be lost due to shortened side chain and the p$K_a$ ~ 6.6 group has either ended up in a more hydrophobic environment or the role as the titratable group is taken over by another residue with a higher p$K_a$.

An example where a similar scenario is observed is in bacteriorhodopsin (bR) from *Halobacterium salinarium*. In the proton exit path of bR a Zundel ion ($H_5O_2^+$) is coordinated by a group of residues (either polar or acidic). The water cluster (figure 17) with its proton is believed to represent the "proton release site" in which the proton is stabilized by two deprotonated glutamates (the E194 and E204, *H. salinarium* numbering). The two glutamates are located close to the extracellular surface and the substitution of E194 by an aspartate resulted in a significant increase of the observed p$K_a$ of the proton release group [121]. The substitution to a glutamine of either of the glutamates resulted in no proton release. In the E194D bR variant, the proton in the water cluster can not hydrogen bond to the aspartate due to a too long distance leading to protonation of E204. In the E194D enzyme, E204 becomes the new, high p$K_a$, proton release group.

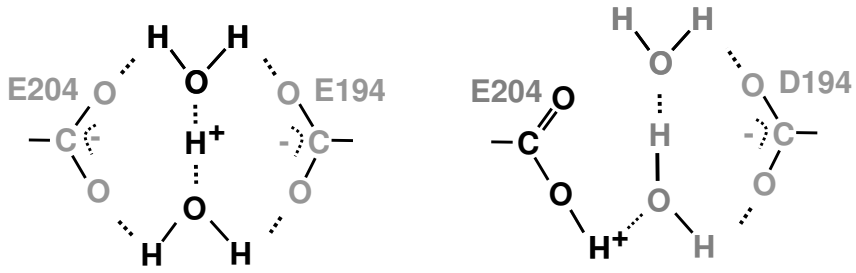


**Figure 17.** Schematic drawing of the proton release group for bR wild type and the aspartate substitution of E194 (*H. salinarium* numbering). Modified from [121].

We believe that NOR could have a similar arrangement. The results in paper III strongly support that E122 and E125 (which both are crucial for steady-state activity [27, 127]) sit at the aperture of the proton pathway and possibly coordinate a protonated water cluster, which could be the group with a p$K_a$ of 6.6. When the E122 is substituted by an aspartate it can no longer coordinate the protonated water cluster, which in turn leads to protonation of E125. The E125 variants are severely inhibited in the ms phase when substituted by an alanine or glutamine, therefore this glutamate also most likely takes part in proton uptake. E125 is located, as judged by the model, a little bit further into the membrane, which could explain the acquired high p$K_a$.

To our surprise, the E125D variant was not stable outside the membrane (Flock, Thorndycroft *et al.*, unpublished), which implies that this glutamate might have a dual function in NOR. It could be part of the proton pathway entry point *and* have a structural function. For example, it has been shown that glutamates are involved in the orientation of membrane proteins [125].

Finally, an interesting idea; Garczarek et al [160] have also shown that bR has *another* water cluster which participate in proton transfer, further into the protein coordinated by two aspartates. What if NOR has a protonated water cluster near the binuclear site, which is coordinated by E198 and E267.

Having established that the proton transfer pathway in NOR comprises E122 and possibly E125 the next task was to investigate the conserved glutamates situated in the membranous part.

### 3.7.2 Paper IV - E198 and E267 are components in the proton pathway

The optical spectra for the E198A and E267A variants were shown to be similar to the wild type (data not shown), as well as the oxygen binding to heme $b_3$ (figure 16) The CO recombination, however, was slightly altered, indicating that the milieu around the binuclear site is mildly affected. Why CO binding in these variants is affected, when $O_2$ – and also NO binding in E198A (Lachmann *et al.* unpublished) occurs as in wild type, is not known.

In the reaction between the fully reduced NOR variants and $O_2$, the 25 ms-phase in both E198A and E267A displays a severe and specific inhibition and no proton uptake is observed (figure 16). A small oxidation, only at 430 nm, in the E198A variant was sometimes seen (10% of the wild type amplitude and with a time constant of ~20 ms, data not shown). This oxidation was pH-*in*dependent and therefore interpreted to be a process other than the "ordinary" 25 ms-phase. It could be an ~1 electron transfer to the bound oxygen, since heme $b_3$ that has a low midpoint potential (40-60 mV [27, 70]), not likely would stay in the reduced state. In the E198A mutant the midpoint potential of heme $b_3$ is even lower (2 mV [27]). E198 is a good candidate for shuttling protons into the heme $b_3$- $Fe_B$ site, in part because it is relatively close to it (~9Å) and also since it is within the conserved core sequence near the H194 (~4 Å in the model), see above).

The E267A mutant did show a small pH-dependent heme oxidation, ~10% of wild type amplitude (figure 16), which is intriguing since no protons are taken up during this phase. Only a small drift, although with a positive slope, was recorded a couple of times during the proton uptake measurements. That *could* be interpreted as a *very* slow uptake (figure 16).

The results for both variants strongly suggest that they are part of the proton pathway according to our model, not in the immediate vicinity of the $Fe_B$. Both E198 and E267 are very close to the H194 (with distances of ~4 and 3 Å, respectively). Thereby they could affect the immediate environment (as seen by the mildly affected CO recombination) of this ligand or actually be the residues that directly give protons to the active site. *If* the E198 and E267 would be ligands of $Fe_B$, a more striking effect of an alanine substitution would be expected.

Thus, we propose that E198 and E267 are, if not directly delivering protons into the reaction, at least providing protons indirectly via, e.g. structured waters.

### 3.7.3 Paper IV – The role of E202

The E202A variant binds oxygen in a wild type manner and proton uptake from the bulk clearly remains coupled to the τ~25 ms phase (figure 16). Therefore this residue is not suggested to be part of the proton pathway.

The slightly lowered $k_{max}$ (figure 16) and the rate-reduction in the last phase could be due to secondary effects on for example the E198, which is ~4.5 Å from E202 in the model. The slightly altered CO recombination rates and amplitudes are intriguing, since these results indicate that E202 might be closer to the binuclear site than anticipated from the model.

E202 is strictly conserved in all NOR sequences aligned in paper II and is approximately one helical turn down from E198 in the sequence (a possible position to function as a $Fe_B$ ligand), but has a distance of about 14 Å to $Fe_B$ and about 10 Å to H194, as judged by the model. No counterpart are found to E202 in the *cbb*$_3$ or A1 C*c*Os; in the *cbb*$_3$ oxidases the corresponding position is held by a threonine and the A1-C*c*Os in most cases a leucine. A deep membrane buried location of a glutamate that is not participating in proton transfer and having a too long distance from the $Fe_B$ to be a ligand, is puzzling since this construction is energetically unfavorable.

So far we can not propose any good explanation to its function and why it is strictly conserved.

### 3.7.4 Future perspectives on the proton pathway in NOR

The future steps in the investigation of the proton pathway from the "wrong" side involve to further pinpoint the other participants. This is work already in progress by the Pia Ädelroth group.

Recent preliminary results, with oxygen as substrate, in the D185V mutant (Lachmann *et al.* unpublished results) support the suggestion that this residue is part of the proton pathway. Initial experiments with the S264A mutant corroborate the importance of this residue (Lachmann *et al.* unpublished results). Additional substitutions of the other suggested proton pathway participants are under way and interesting results are awaited.

## 3.8 Paper I-IV - Summary of science

In this thesis it has been concluded that the c-type NOR from *P. denitrificans* binds oxygen to heme *b*$_3$ and that the product of oxygen reduction is water (paper I). Moreover, we could demonstrate that the oxidation of the enzyme is coupled to proton uptake from the bulk solution and limited by proton transfer from an internal protonatable group with the p$K_a$ ~6.6 (paper I). During the catalysis we have shown that c-type NOR takes protons from the

periplasm during both $O_2$ and NO reduction (in paper II) and from these results a proton pathway leading from the periplasm to the active site (based on a model presented in paper II) was proposed.

In order to scrutinize our proposal we investigated the five strictly conserved glutamates situated in the catalytic subunit (NorB) (paper III and IV). In paper III our investigations established that the E122 (predicted to face the periplasm) and possibly the E125 define the protonatable group with the p$K_a$ =6.6 found in paper I. In paper IV, we concluded that two of the other conserved glutamates, E198 and E267 (predicted to be in the membranous part of the enzyme and close to the active site), are part of the proton pathway. Judged by the location of E198 and E267, both are candidates for delivering protons to the active site. The fifth conserved glutamate, E202, was concluded not to be part of the proton pathway (paper IV).

All in all; four of the five strictly conserved glutamates in the catalytic subunit participate in the proton pathway from the periplasm.

## 3.9 Finally and the end

The purpose of my PhD project aimed to elucidate the mechanism and energetics of NO- and $O_2$ -reduction by bacterial nitric oxide reductases (NOR).

Now, at the end, I can say that I have unraveled some of the puzzling mysteries of the NORs mini-machinery, although there is, certainly, material left for others to dig into[9].

[9] "Many shall run to and fro, and knowledge shall be increased." Daniel 12:4

# 4 POPULÄRVETENSKAPLIG SAMMANFATTNING

Till alla er som undrat vad jag håller på med, egentligen.

## 4.1 Andning utan syre

Visste du att du är beroende av forntida ”bakterier” för att kunna andas?!

Syret du andas tas upp av de röda blodkropparna i lungorna och transporteras sedan i blodet ut i kroppen.
Kroppen är uppbyggda av celler och i cellerna finns små bönformade ”lådor” som kallas för mitokondrier och de är våra kraftverk.

Mitokondrierna var för länge, länge sedan bakterier som våra celler började samarbeta med och cellen ger mitokondrien en bra miljö att leva i. Mitokondrien, i sin tur, ger cellen energi som den kan använda och behövs det mycket energi blir mitokondrierna flera.

Det är till mitokondrierna syret åker och här ovandlas det till vatten av en minimaskin som heter cytokrom *c* oxidas (C*c*O). C*c*O fungerar som en katalysator, dvs den underlättar omvandlingen av syre till vatten, och den är dessutom en pump. Vad pumpar C*c*O för något?

C*c*O pumpar något som kallas för protoner (det sura i citronen) från en våning till en våning upp, så att det blir en stor skillnad mellan de olika våningarna. Det här är ett sätt att lagra energi på, eftersom protonerna vill forsa tillbaka, men hindras av golvet/taket mellan våningarna. Man kan säga att C*c*O laddar våra batterier, eftersom den här uppdämda energin används av en annan minimaskin (ATP-syntas) som med hjälp av forsen tillverkar ATP som våra celler kan använda som energikälla. Tänk dig ett vattenkraftverk, precis så fungerar det inne i din kropp!

*Avhandlingen* handlar om en släkting till C*c*O som heter NO reduktas (NOR). NOR är också en katalysator och finns i bakterier som är släkt med mitokondrier, men de här bakterierna använder sig av NOR för att ”andas” utan syre.

NOR katalyserar omvandlingen av giftig kvävemonoxid (NO) till lustgas ($N_2O$) och kan dessutom katalysera omvandlingen av syre till vatten, men NOR pumpar ingenting! Jämfört med C*c*O är NOR mycket trög vid

katalysen av syre till vatten, men C*c*O å andra sidan kan inte omvandla NO till lustgas. Det här gör oss forskare förbryllade eftersom de två katalysatorerna har ungefär samma konstruktion.

I avhandlingen har jag försökt förstår hur NOR gör vid omvandlingen av syre till vatten och för att det ska ske behövs protoner. Hur gör NOR för att få in protonerna till platsen där katalysen sker?

Det visade sig att NOR har en liten protontunnel från, vad vi forskare tycker, ”fel” sida av ”golvet/taket” och att den innehåller byggstenar som heter glutamat (finns bl.a. i Aromat).

Avhandlingen gör så att vi förstår mer om hur proton-tunnlar i celler ser ut och fungerar och jag har dessutom jämfört designen av NORs proton-tunnel med hur liknande tunnlar ser ut i C*c*O och i en annan minimaskin (*cbb*$_3$ oxidas), som är mer lik NOR .

Även om C*c*O i dina celler är byggd på ungefär samma sätt som NOR i bakterierna så har det hänt en massa saker sedan celler och bakterier började samarbeta för miljontals år sedan. C*c*O och NOR använder förmodligen inte samma protontunnlar, medan NOR och *cbb*$_3$ verkar delvis göra det.

# 5 ACKNOWLEDGEMENTS

Det är många viktiga personer som har passerat under resans gång, som har gjort det lättare, roligare och inspirerande. Många har bidragit, för en avhanling är ju inget man knåpar ihop själv på en kafferast.....

**Pia Ädelroth**, min "handpekare" och "halvgeneral", tack för att du jämt är redo att avbryta vad du än håller på med (jag har varit bortskämd!). Tack för din naturliga talang vad gäller projekt och för att du är den där magiska personen som med ett lugn omedelbart ser vilken knapp som inte är på, som klappar lasern och den blir glad och sedan ger mig en frukt, som skriver artiklar med grym elegans, som inte kan skumma text, som litar på mitt omdöme när det gäller vardagslivet, som varit så generös med att låta mig gå på konferenser, som alltid är en utmaning vid diskussioner och "gräl" och tack för att du är en sån lojal och ambitiös handledare med goda tryffelrecept.

**Peter Brzezinski**, min andre handledare, tack för att du är en sån entusiastisk forskare. Jag vet ingen som kan bli så ivrig när man visar hopplösa, fula, och allmänt brusiga spår! Dessutom, tack för att du puttade mig in i Pia's armar, det är ju lite din "förtjänst" också!

**Elzbieta Glaser**,tack för att du varit en sån engagerad utvärderare och för en utvecklande tid i jämställdhetsgruppen.

Vår förre Prefekt och nu Dekan, **Stefan Nordlund**, tack för din ambition och omtanke när det gäller oss doktorander, för att du "årrrdnar"[10] och vill förbättra. Tack för att du granskat exjobb, forskarplan och avhandling med en sån noggrannhet och för att du dessutom tar dig tid att diskutera vissa mer filosofiska stycken. Tack också för att du är den du är, nämligen den man alltid kan gå till när det blir "för mycket", den som har bananer och näsdukar vid behov, som har glimten i ögat och viljan att diskutera. Dessutom, tack för en lärorik tid i institutionsstyrelsen och för insikten om att en lunch-diet innehållande filmjölk + frukt kommer man långt på.

**Inger Carlberg**, tack för att du är så positiv, för att du alltid lyssnar på alla mina ideér kring undervisningen och för att du är så tålmodig när jag, ibland väldigt ofta, poppat in full med något som jag m-å-s-t-e diskutera!

**Agneta Norén**, tack för en intressant tid med alla tonåringar!

[10] Ett Flock-klan uttryck.

**Ing-Marie Nilsson,** tack för ett gott samarbete med våra krävande studenter.

**Ach soo mein wunderbara kollegor Joachim Reimann undt Peter Lachmann,** danke shöön for your bracing faces, kicking jokes, bad German jokes ;-), ambition, smartness and big hearts! More Dublin and Ekerö!

**The heme-copper oxidase symbiosis group**, former and present members, Jag vill särskilt tacka **Håkan Lepp** en smart, rolig musikfantast och ”ryggpolare” som väldigt tålmodigt varit min idépopparvägg, **Ida Namslauer** den f.d. bakterie-damen, nu laser-bejb, **Emelie Svahn** vår live-lady , **Linda Öjemyr** sporty-bruden, **Rosa Lönneborg** vår politiska gren , **Gustav** “dude” **Nordlund**, **Ann-Louise Johansson** vår nya ordnung-lady. **Yafei Huang** our former super-talented postdoc, good luck in Uppsala! Our postdoc **Laila Singh,** for being cheerful and supporting.

**Christoph von Ballmos**, our brand new postdoc with a new point of view, a twinkle in the eye and a strong driving force ;-).

Tack, **Gisela Brändén**, **Magnus Brändén**, **Lina Salomonsson**, **Gwen Gilderson**, **Andreas Namslauer** and **Kristina Faxén** som var de stora coola snubbarna och förebilderna när jag gjorde mitt exjobb.

I like to put in an extra *thank you* to **Linda, Joachim, Peter L and Christoph** for putting in such energy in finding that many “moreover” and “the” and “root noodles” in my thesis and for making it shorter and comprehensible.

**Dr. H. Gyllberg**, tack för den mycket givande tiden i doktorandrådet och för att du är en så smart, drivande och omtänksam bissnissbejb!

Ett stort tack till alla ni som har och nu gör allt det där administrativa i vår vardag smidigt och lätt; **Ann Nielsen**, för dina läskiga e-mail och för att du räknat om min tid….några gånger, **Anki Holmström**, leg. Blankett-drottning, Ann och Anki för alla fester ni fixat och grejat med , **Charlotta Andersson** vår nya administratör och “vem har beställt”-drottning, **Bogos Adali** allas vår glada och dansande “pratar alla språk i världen” allfixare, **Peter Nyberg**, vår glada vips fixadatorn mannen, **Eddie Egerfjäll** vår förre detta storskämtare, borrare och allfixare, **Bengt Eurenius** vår talangfulla tekniker och revolutionär, **Torbjörn Astlind** vår kaktusfantast och dator-räddare (tack för min räddade hårddisk!).

**Pam Miss Piff;** du grymma dam, tack för stunderna i skrivstugan, vovverundan och de spanska diskussionerna kring de nu klassiska företeelserna ”Good enough” och ”Dr. Schlock”, mer kommer! Ser fram emot tejp och köttbullar i november! ;-)

**Jan Berglin**, stort tack för äran att få våldföra mig på din konst och dessutom publicera det hemska resultatet i min avhandling (bild 18)! Tack!

**Mina föräldrar, Jan och Mona Abelsson**, tack för er generositet, stöd och tålamod. *Tack mamma*, för att du är så omtänksam och alltid har de dina i tankarna och tack för att jag fick sitta i finrummet när jag pluggade till StoraTentan. Att plugga blir så lätt när någon kommer smygande med kaffe och smörgåsar! *Tack pappa*, för att du alltid är intresserad av hur det går, för att du är så nyfiken på hur det går till vid universitetet och för att du är en utmaning under diskussionspassen. Jag har genuin träning tack vare dig.

**Mina svärföräldrar, Åke och Britta Flock**, tack för många och inspirerande diskussioner kring "ett trevligt lamm" och för att ni sådde det vetenskapliga fröet tidigt i vår vänskap.

Slutligen, min **kära och älskade familj;**

**Erik och Johan Flock,** mina älskade, glada, knasiga, stöttande, mysiga, muntra, talangfulla, smarta, omtänksamma, schysta, coola barn! Tack för konstverket och för att ni har peppat mig!

**Per Flock, min stora kärlek;** du roliga, smarta, snygga, XX, stöttande, uppmuntrande, roliga, omtänksamma, pedagogiska, coola och underbara man! Du förklarade tålmodigt 1 mol för mig och jag dängde boken i huvudet på dig, men se vad som hände! Jag ska inte skriva att jag inte förtjänar dig, du blir så mörk i synen då, men du är helt enkelt för bra för att vara sann! ☺

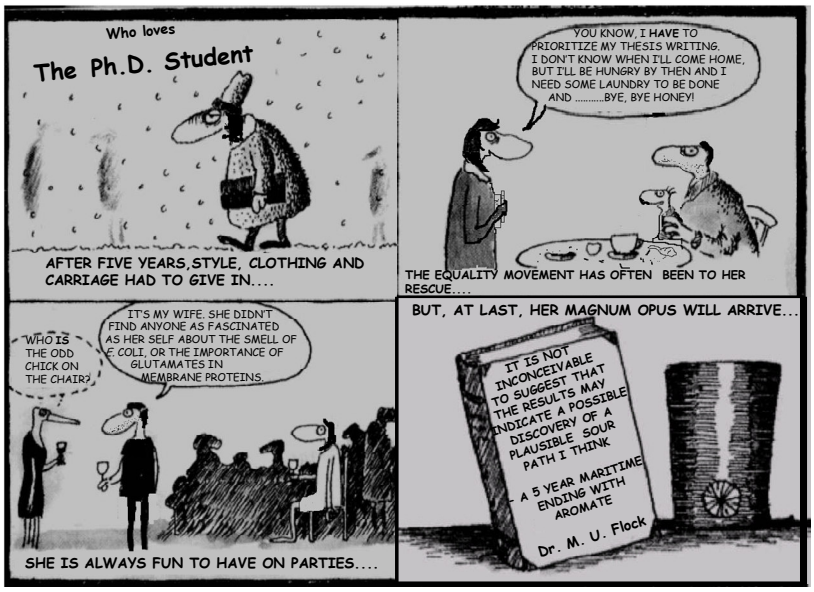


**Figure 18.** "**Who loves the Ph.D. student**" is an ugly modification of the original Vem älskar doktoranden?" by Jan Berglin [161] and published with courtesy of Jan Berglin.

# 6 REFERENCES


1. Nelson, D.L. and M.M. Cox, *Lehninger Principles of biochemistry*. 2005, New York*: W. H. Freeman and Company*.
2. Williams, D.R. *Earth Fact Sheet*. NASA Earth Fact Sheet Last Updated: 19 April 2007 [cited; Available from: http://nssdc.gsfc.nasa.gov/planetary/factsheet/earthfact.html.
3. Deacon, J. *The Microbial World: The Nitrogen cycle and Nitrogen fixation*. Institute of Cell and Molecular Biology, The University of Edinburgh [cited; Available from: http://www.biology.ed.ac.uk/research/groups/jdeacon/microbes/nitrogen.htm.
4. MacLean, A.M., T.M. Finan, and M.J. Sadowsky, Genomes of the Symbiotic Nitrogen-Fixing Bacteria of Legumes. *Plant Physiol.*, 2007. **144**: p. 615-622.
5. Duce, R.A., J. LaRoche, K. Altieri, K.R. Arrigo, A.R. Baker, D.G. Capone, S. Cornell, F. Dentener, J. Galloway, R.S. Ganeshram, R.J. Geider, T. Jickells, M.M. Kuypers, R. Langlois, P.S. Liss, S.M. Liu, J.J. Middelburg, C.M. Moore, S. Nickovic, A. Oschlies, T. Pedersen, J. Prospero, R. Schlitzer, S. Seitzinger, L.L. Sorensen, M. Uematsu, O. Ulloa, M. Voss, B. Ward, and L. Zamora, Impacts of Atmospheric Anthropogenic Nitrogen on the Open Ocean. *Science*, 2008. **320**: p. 893-897.
6. Garrocho-Villegas, V., S.K. Gopalasubramaniam, and R. Arredondo-Peter, Plant hemoglobins: What we know six decades after their discovery. *Gene*, 2007. **398**: p. 78-85.
7. Peters, J.W. and R.K. Szilagyi, Exploring new frontiers of nitrogenase structure and mechanism. *Curr Opin Chem Biol*, 2006. **10**: p. 101-108.
8. Sittig, M., Fertilizer Industry: Processes, Pollution Control and Energy Conservation. *Noyes Data Corp., N.J.*, 1979.
9. Navarro-González, R., C.P. McKay, and D.N. Mvondo, A possible nitrogen crisis for Archaean life due to reduced nitrogen fixation by lightning. *Nature* 2001. **412**: p. 61-64.
10. Arp, D.J., L.A. Sayavedra-Soto, and N.G. Hommes, Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch Microbiol*, 2002. **178**: p. 250-255.
11. Klotz Martin, S.L., Nitrifyers genomics and evolution of the nitrogen cycle. *FEMS Microbiol. Lett.*, 2008. **178**: p. 146-156.

12. Zumft, W.G., Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.*, 1997. **61**: p. 533-616.
13. Houghton, J.T., M.G.M. Filho, B.A. Callander, N. Harris, A. Kattenberg, and K. Maskell, Climate Change 1994 – radiative forcing of climate change and an evaluation of the IPCC IS92 emission scenarios. *IPCC (Intergovernmental Panel on Climate Change) (1995) Cambridge University Press, Cambridge.*, 1995.
14. Russell, R., *Layers of Earth's Atmosphere*. 2005, Windows to the Universe.
15. Crutzen, P.J., Atmospheric chemical processes of the oxides of nitrogen, including nitrous oxide. In: Denitrification, Nitrification and Atmospheric Nitrous Oxide *Delwiche, C.C., Ed., Wiley and Sons, New York.*, 1981: p. 17–44.
16. Nixon, S.W., Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia*, 1995. **41**: p. 199-219.
17. de Vries, S., M.J.F. Strampraad, S. Lu, P. Moenne-Loccoz, and I. Schroder, Purification and characterization of the MQH(2): NO oxidoreductase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*. *J. Biol. Chem.*, 2003. **278**: p. 35861-35868.
18. Zumft, W.G., Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J. Inorg. Biochem.*, 2005. **99**: p. 194-215.
19. Saraste, M. and J. Castresana, Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.*, 1994. **341**: p. 1-4.
20. Bru, D., A. Sarr, and L. Philippot, Relative Abundances of Proteobacterial Membrane-Bound and Periplasmic Nitrate Reductases in Selected Environments. *Appl. Environ. Microbiol.*, 2007. **73**: p. 5971-5974.
21. Bertero, M.G., R.A. Rothery, M. Palak, C. Hou, D. Lim, F. Blasco, J.H. Weiner, and N.C.J. Strynadka, Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. *Nature Struct. Biol.* , 2003. **10** p. 681-687.
22. Godden, J., S. Turley, D. Teller, E. Adman, M. Liu, W. Payne, and J. LeGall, The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. *Science*, 1991. **253**: p. 438 - 442.
23. Kuznetsova, S., G. Zauner, T.J. Aartsma, H. Engelkamp, N. Hatzakis, A.E. Rowan, R.J.M. Nolte, P.C.M. Christianen, and G.W. Canters, The enzyme mechanism of nitrite reductase studied at single-molecule level. *Proc Natl Acad Sci U S A*, 2008. **105**: p. 3250 - 3255.
24. Suzuki, S., K. Kataoka, and K. Yamaguchi, Metal Coordination and Mechanism of Multicopper Nitrite Reductase. *Acc. Chem. Res.*, 2000. **33**: p. 728 -735.
25. Wijma, H.J., L.J.C. Jeuken, M.P. Verbeet, F.A. Armstrong, and G.W. Canters, A Random-sequential Mechanism for Nitrite Binding and Active Site Reduction in Copper-containing Nitrite Reductase. *J. Biol. Chem.*, 2006. **24**: p. 16340 - 16346.

26. Prudencio, M., R. Eady, and G. Sawers, Catalytic and spectroscopic analysis of blue copper-containing nitrite reductase mutants altered in the environment of the type 2 copper centre :implications for substrate interaction. *Biochem. J.*, 2001. **259**: p. 259-266.
27. Butland, G., S. Spiro, N.J. Watmough, and D.J. Richardson, Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme. *J. Bacteriol.*, 2001. **183**: p. 189-199.
28. Zumft, W.G., Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J. Inorg. Biochem.*, 2004. **99**: p. 194-215.
29. Flock, U., N.J. Watmough, and P. Ädelroth, Electron/Proton Coupling in Bacterial Nitric Oxide Reductase during Reduction of Oxygen. *Biochemistry*, 2005. **44**: p. 10711-10719.
30. Flock, U., F.H. Thorndycroft, A.D. Matorin, D.J. Richardson, N.J. Watmough, and P. Ädelroth, Defining the Proton Entry Point in the Bacterial Respiratory Nitric-oxide Reductase. *J. Biol. Chem.*, 2008. **283**: p. 3839-3845.
31. Paraskevopoulos, K., S.V. Antonyuk, R.G. Sawers, R.R. Eady, and S.S. Hasnain, Insight into Catalysis of Nitrous Oxide Reductase from High-resolution Structures of Resting and Inhibitor-bound Enzyme from *Achromobacter cycloclastes*. *J. Mol. Biol.* , 2006. **362**: p. 55-65.
32. Tavares, P., A.S. Pereira, J.J.G. Moura, and I. Moura, Metalloenzymes of the denitrification pathway. *J. Inorg. Biochem.*, 2006. **100**: p. 2087-2100.
33. Koyu, F., J.M. Chan, J.A. Bollinger, M.L. Alvarez, and D.M. Dooley, Anaerobic purification, characterization and preliminary mechanistic study of recombinant nitrous oxide reductase from *Achromobacter cycloclastes*. *J. Inorg. Biochem.*, 2007. **101** p. 1836-1844.
34. Alonse, A., B. Etaniz, and M.D. Martinez, The determination of nitrate in cured meat products. A comparison of the HPLC UV/VIS and Cd/spectrophotometric methods. *Food Addit. Contam.*, 1992. **9**: p. 111-117.
35. Bruning-Fann, C.S. and J.B. Kaneene, The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Vet. Hum. Toxicol.*, 1993. **237**: p. 521-253
36. Duncan, C.D., H.; Johnston, P.; Green, S.; Brogan, R.; Leifert, and L.G. C.; Smith, M.; Benjamin, N. , Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat. Med.*, 1995. **1**: p. 546-551.
37. Dubois, M., F. Bailly, G. Mbemba, J.-F. Mouscadet, Z. Debyser, M. Witvrouw, and P. Cotelle, Reaction of Rosmarinic Acid with Nitrite Ions in Acidic Conditions: Discovery of Nitro- and Dinitrorosmarinic Acids as New Anti-HIV-1 Agents. *J. Med. Chem.*, 2008. **51**: p. 2575-2579.

38. Vazquez-Torres, A., T. Stevanin, J. Jones-Carson, M. Castor, R.C. Read, and F.C. Fang, Analysis of Nitric Oxide-Dependent Antimicrobial Actions in Macrophages and Mice. *Meth. Enzymol.*, 2008. **437**: p. 521-538
39. Iwase, K., K. Iyama, K. Akagi, S. Yano, K. Fukunaga, E. Miyamoto, M. Mori, and M. Takiguchi, Precise distribution of neuronal nitric oxide synthase mRNA in the rat brain revealed by non-radioisotopic in situ hybridization. *Mol. Brain Res.*, 1998. **53**: p. 1-12.
40. Bolan, E.A., K.N. Gracy, J. Chan, R.R. Trifiletti, and V.M. Pickel, Ultrastructural localization of nitrityrosine within the caudate-putamen nucleus and the globus pallidus of normal rat brain. *J. Neurosci.*, 2000. **20**: p. 4798-4808.
41. Snyder, S.H., Nitric Oxide: First in a New Class of Neurotransmitters? *Science*, 1992. **257**: p. 494-496.
42. Stewart, V.C. and S.J.R. Heales, Nitric oxide-induced mitochondrial dysfunction: Implications for neurodegeneration. *Free Radical Biology & Medicine*, 2003. **34**: p. 287-303.
43. Stuart-Smith, K., Demystified...Nitric oxide. *J Clin Pathol: Mol Pathol* 2002. **55**: p. 360–366.
44. Feelisch, M. and J.S. Stamler, Methods in nitric oxide research. *John Wiley & Sons, Ltd., Chichester, United Kingdom*, 1996.
45. Achike, F.I. and C.Y. Kwan, Nitric oxide, human diseases and the herbal products that affect the nitric oxide signalling pathway. *Clin Exp. Pharmacol. Physiol.* , 2003. **30**: p. 605-615.
46. Chu, C., S. Wu, F. Lee, S. Wang, F. Chang, H. Lin, C. Chan, and S. Lee, Splanchnic hyposensitivity to glypressin in a haemorrhage/transfused rat model of portal hypertension: role of nitric oxide and bradykinin. *Clin Sci.*, 2000. **99**: p. 475–482.
47. Merchant, K., H. Chen, T.C. Gonzalez, L.K. Keefer, and B.R. Shaw, Deamination of Single-Stranded DNA Cytosine Residues in Aerobic Nitric Oxide Solution at Micromolar Total NO Exposures. *Chem. Res. Toxicol.*, 1996. **9**: p. 891-896.
48. Nietzsche, F.W., *Also sprach Zarathustra – Ein Buch für Alle und Keinen*. 1883–1885.
49. Zafiriou, O.C. and Q.S. Hanley, Nitric Oxide and Nitrous Oxide Production and Cycling during Dissimilatory Nitrite Reduction by *Pseudomonas perfectomarina*. *J. Biol. Chem.*, 1989. **264**: p. 5694-5699.
50. Fujiwara, T. and Y. Fukumori, Cytochrome cb-type nitric oxide reductase with cytochrome c oxidase activity from *Paracoccus denitrificans* Atcc 35512. *J. Bacteriol.*, 1996. **178**: p. 1866-1871.
51. Sakurai, N. and T. Sakurai, Isolation and characterization of nitric oxide reductase from *Paracoccus halodenitrificans*. *Biochemistry*, 1997. **36**: p. 13809-13815.
52. Matsubara, T. and H. Iwasaki, Enzymatic Steps of Dissimilatory Nitrite Reduction in *Alcaligenes faecalis*. *J. Biochem*, 1971. **69**: p. 859-868.

53. Carr, G.J. and S.J. Ferguson, Nitric oxide formed by nitrite reductase of *Paracoccus denitrificans* is sufficiently stable to inhibit cytochrome oxidase activity and is reduced by its reductase under aerobic conditions. *Biochim. Biophys. Acta*, 1990. **1017**: p. 57-62.
54. Heiss, B., K. Frunzke, and 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.*, 1989. **171**: p. 3288-3297.
55. Dermastia, M., T. Turk, and T.C. Hollocher, Nitric oxide reductase. Purification from *Paracoccus denitrificans* with use of a single column and some characteristics. *J. Biol. Chem.*, 1991. **266**: p. 10899-10905.
56. Girsch, P. and S. deVries, Purification and initial kinetic and spectroscopic characterization of NO reductase from *Paracoccus denitrificans*. *Biochim. Biophys. Acta*, 1997. **1318**: p. 202-216.
57. Tsukamoto, K., S. Nakamura, and K. Shimizu, SAM1 semiempirical calculations on the catalytic cycle of nitric oxide reductase from *Fusarium oxysporum*. *J. Mol. Struc. Theochem*, 2003. **624**: p. 309-322.
58. Oshima, R., S. Fushinobu, F. Su, L. Zhang, N. Takaya, and H. Shoun, Structural Evidence for Direct Hydride Transfer from NADH to Cytochrome P450nor. *J. Mol. Biol.* , 2004. **342**: p. 207-217.
59. Park, S., H. Shimizu, S. Adachi, A. Nakagawa, I. Tanaka, K. Nakahara, H. Shoun, E. Obayashi, H. Nakamura, T. Iizuka, and Y. Shiro, Crystal structure of nitric oxide reductase from denitrifying fungus *Fusarium oxysporum*. *Nat. Struct. Biol.*, 1997. **10**: p. 827-832.
60. Shiro, Y., M. Fuji, T. Iizuka, S.-i. Adachi, K. Tsukamoto, K. Nakahara, and H. Shoun, Spectroscopic and kinetic studies on reaction of cytochrome P450nor with nitric oxide. *J. Biol. Chem.*, 1995. **270**: p. 1617-1623.
61. Shiro, Y., Reaction mechanism of nitric oxide reductase and its relation to molecular structure. *RIKEN Review*, 1999. **24**: p. 56-58.
62. Hendriks, J., A. Warne, U. Gohlke, T. Haltia, C. Ludovici, M. Lübben, and M. Saraste, The active site of the bacterial nitric oxide reductase is a dinuclear iron center. *Biochemistry*, 1998. **37**: p. 13102-13109.
63. Hendriks, J., A. Oubrie, J. Castresana, A. Urbani, S. Gemeinhardt, and M. Saraste, Nitric oxide reductases in bacteria. *Biochim. Biophys. Acta*, 2000. **1459**: p. 266-273.
64. Field, S., F. Thorndycroft, A. Matorin, D. Richardson, and N. Watmough, The respiratory nitric oxide reductase (NorBC) from *Paracoccus denitrificans*. *Methods Enzymol*, 2008. **437**: p. 79-101.
65. Vollack, K.-U. and a.W.G. Zumft, Nitric Oxide Signaling and Transcriptional Control of Denitrification Genes in *Pseudomonas stutzeri*. *J. Bacteriol.*, 2001. **183**: p. 2516-2526.
66. Braun, C., and W. G. Zumft, Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of

*Pseudomonas stutzeri* at nitric oxide. *J. Biol. Chem.*, 1991. **266**: p. 22785-22788.
67. de Boer, A.P.N., J.v.D. Oost, W.N.M. Reijnders, H.V. Westerhow, A.H. Stouthamer, and R.J.M.v. Spanning, Mutational analysis of the nor gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *Eur. J. Biochem.*, 1996. **242**: p. 592-600.
68. Kastrau, D.H.W., B. Heiss, P. M. H. Kroneck, and 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.*, 1994. **222**: p. 293-303.
69. Kastrau, D.H., B. Heiss, P.M. Kroneck, and 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*, 1994. **222**: p. 293-303.
70. Grönberg, K.L., M.D. Roldan, L. Prior, G. Butland, M.R. Cheesman, D.J. Richardson, S. Spiro, A.J. Thomson, and N.J. Watmough, A low-redox potential heme in the dinuclear center of bacterial nitric oxide reductase: implications for the evolution of energy-conserving heme-copper oxidases. *Biochemistry*, 1999. **38**: p. 13780-13786.
71. Berks, B.C., S.J. Ferguson, J.W. Moir, and D.J. Richardson, Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta*, 1995. **1232**: p. 97-173.
72. Kannt, A., H. Michel, M.R. Cheesman, A.J. Thomson, A.B. Dreusch, H. Körner, and W.G. Zumft, *The electron transfer centers of nitric oxide reductase: Homology with the heme-copper oxidase family in Biological electron-transfer chains: genetics, composition and mode of operation*, ed. G.W. Canters and E. Vijgenboom. 1998, Dordrecht*: Kluwer Academic Publishers, , The Netherlands,*. 279-291.
73. Moënne-Loccoz, P., O.M.H. Richter, H.W. Huang, I.M. Wasser, R.A. Ghiladi, K.D. Karlin, and S. de Vries, Nitric oxide reductase from *Paracoccus denitrificans* contains an oxo-bridged heme/non-heme diiron center. *J. Am. Chem. Soc.*, 2000. **122**: p. 9344-9345.
74. Berthold, D., N. Voevodskaya, P. Stenmark, A. Gräslund, and P. Nordlund, EPR Studies of the Mitochondrial Alternative Oxidase - Evidence for a diiron carboxylate center. *J. Biol. Chem.*, 2002. **277**: p. 43608–43614.
75. DiMatteo, A., F.M. Scandurra, F. Testa, E. Forte, P. Sarti, M. Brunori, and A. Giuffré, The $O_2$-scavenging Flavodiiron Protein in the Human Parasite *Giardia intestinalis*. *J. Biol. Chem.*, 2008. **283**: p. 4061-4068.
76. Siedow, J.N. and A.L. Umbach, Plant Mitochondrial Electron Transfer and Molecular Biology. *The Plant Cell*, 1995. **7**: p. 821-831.

77. Hendriks, J., U. Gohlke, and M. Saraste, From NO to OO: Nitric oxide and dioxygen in bacterial respiration. *J. Bioenerg. Biomembr.*, 1998. **30**: p. 15-24.
78. Pereira, M.M., M. Santana, and M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases. *Biochim. Biophys. Acta*, 2001. **1505**: p. 185-208.
79. Field, S.J., L. Prior, M.D. Roldan, M.R. Cheesman, A.J. Thomson, S. Spiro, J.N. Butt, N.J. Watmough, and D.J. Richardson, Spectral properties of bacterial nitric-oxide reductase: resolution of pH-dependent forms of the active site heme b3. *J. Biol. Chem.*, 2002. **277**: p. 20146-20150.
80. Reimann, J., U. Flock, H. Lepp, A. Honigmann, and P. Ädelroth, A pathway for protons in nitric oxide reductase from *Paracoccus denitrificans*. *Biochim. Biophys. Acta*, 2007. **1767**: p. 362-373.
81. Qian, J., D.A. Mills, L. Geren, K. Wang, C.W. Hoganson, B. Schmidt, C. Hiser, G.T. Babcock, B. Durham, F. Millett, and S. Ferguson-Miller, Role of the conserved arginine pair in proton and electron transfer in cytochrome *c* oxidase. *Biochemistry*, 2004. **43**: p. 5748-56.
82. Kapetanaki, S.M., S. Field, R. Hughes, N. Watmough, U. Liebl, and M. Vos, Ultrafast ligand binding dynamics in the active site of native bacterial nitric oxide reductase. *Biochim. Biophys. Acta*, 2008. **1777**: p. 919-924
83. Kumita, H., K. Matsuura, T. Hino, S. Takahashi, H. Hori, Y. Fukumori, I. Morishima, and 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.*, 2004. **279**: p. 55247-55254.
84. Grönberg, K.L., N.J. Watmough, A.J. Thomson, D.J. Richardson, and S.J. Field, Redox-dependent open and closed forms of the active site of the bacterial respiratory nitric-oxide reductase revealed by cyanide binding studies. *J. Biol. Chem.*, 2004. **279**: p. 17120-17125.
85. Blomberg, L.M., M.R. Blomberg, and P.E. Siegbahn, Reduction of nitric oxide in bacterial nitric oxide reductase--a theoretical model study. *Biochim. Biophys. Acta*, 2006. **1757**: p. 240-252.
86. Hendriks, J.H., A. Jasaitis, M. Saraste, and M.I. Verkhovsky, Proton and electron pathways in the bacterial nitric oxide reductase. *Biochemistry*, 2002. **41**: p. 2331-2340.
87. Pinakoulaki, E., S. Stavrakis, A. Urbani, and C. Varotsis, Resonance Raman detection of a ferrous five-coordinate nitrosylheme $b_3$ complex in cytochrome $cbb_3$ oxidase from *Pseudomonas stutzeri*. *J Am Chem Soc*, 2002. **124**: p. 9378-9.
88. Stavrakis, S., E. Pinakoulaki, A. Urbani, and C. Varotsis, Fourier transform infrared evidence for a ferric six-coordinate nitrosylheme $b_3$ complex of cytochrome $cbb_3$ oxidase from *Pseudomonas stutzeri* at ambient temperature. *J. Phys. Chem.*, 2002. **106**: p. 12860-12862.

89. Philippot, L., Denitrification in pathogenoc bacteria: for better or worse? *Trends Microbiol.*, 2005. **13**: p. 191-192.
90. Seib, K.L., H.-J. Wu, S.P. Kidd, M.A. Apicella, M.P. Jennings, and A.G. McEwan, Defenses against Oxidative Stress in *Neisseria gonorrhoeae*: a System Tailored for a Challenging Environment. *Microbiol. Mol. Biol. Rev.*, 2006. **70**: p. 344-361.
91. Rock, J.D., M.R. Mahnane, M.F. Anjum, J.G. Shaw, R.C. Read, and J.W. Moir, The pathogen *Neisseria meningitidis* requires oxygen, but supplements growth by denitrification. Nitrite, nitric oxide and oxygen control respiratory flux at genetic and metabolic levels. *Mol Microbiol.* , 2005. **58**: p. 800-809.
92. Rock, J.D., M.J. Thomson, R.C. Read, and J.W.B. Moir, Regulation of denitrification genes in *Nesseria meningitis* by nitric oxide and the respressor NsrR. *J. Bact.*, 2007. **189**: p. 1138-114.
93. van der Oost, J., A.P. de Boer, J.W. de Gier, W.G. Zumft, A.H. Stouthamer, and 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.*, 1994. **121**: p. 1-9.
94. Wikström, M., Cytochrome c oxidase: 25 years of the elusive proton pump. *Biochim. Biophys. Acta*, 2004. **1655**: p. 241-247.
95. Wikström, M.K., Proton pump coupled to cytochrome c oxidase in mitochondria. *Nature*, 1977. **266**: p. 271-273.
96. Wikström, M., K. Krab, and M. Saraste. Cytochrome Oxidase: A synthesis. 1981, London*: Academic Press*.
97. Brzezinski, P. and P. Ädelroth, Design principles of proton-pumping haem-copper oxidases. *Curr. Opin. Struct. Biol.*, 2006. **16**: p. 465-472.
98. Brzezinski, P., Redox-driven membrane-bound proton pumps. *Trends Biochem. Sci.*, 2004. **29**: p. 380-387.
99. Ferguson-Miller, S. and G.T. Babcock, Heme-copper terminal oxidases. *Chemical Reviews*, 1996. **96**: p. 2889-2907.
100. Forte, E., A. Urbani, M. Saraste, P. Sarti, M. Brunori, and A. Giuffrè, The cytochrome $cbb_3$ from *Pseudomonas stutzeri* displays nitric oxide reductase activity. *Eur. J. Biochem.*, 2001. **268**: p. 6486-6491.
101. Torres, J., V. Darley-Usmart, and M.T. Wilson, Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. *Biochem. J.*, 1995. **312**: p. 169-173.
102. Benamar, A., H. Rolletschek, L. Borisjuk, M.-H. Avelange-Macherel, G. Curien, H.A. Mostefai, R. Andriantsitohaina, and D. Macherel, Nitrite–nitric oxide control of mitochondrial respiration at the frontier of anoxia. *Biochim. Biophys. Acta*, 2008. **Article in press - Uncorrected proof**.
103. Ghafourifar, P. and C. Richter, Mitochondrial Nitric Oxide Synthase Regulates Mitochondrial Matrix pH. *Biol. Chem.*, 1999. **380**: p. 1025–1028.

104. Wikström, M., Pumping of protons from the mitochondrial matrix by cytochrome oxidase. *Nature*, 1984. **308**: p. 558-560.
105. Wikström, M., Identification of the electron transfers in cytochrome oxidase that are coupled to proton-pumping. *Nature*, 1989. **338**: p. 776-778.
106. Lanyi, J.K., Proton transfer in the bacteriorhodopsin photocycle. *Biochim. Biophys. Acta*, 2006. **1757**: p. 1012-1018.
107. Neutze, R., E. Pebay-Peyroula, K. Edman, A. Royant, J. Navarro, and E.M. Landau, Bacteriorhodopsin: a high-resolution structural view of vectorial proton transport. *Biochim. Biophys. Acta*, 2002. **1565**: p. 144-167.
108. DeCoursey, T.E. and V.V. Cherny, Common themes and problems of bioenergetics and voltage-gated proton channels. *Biochim. Biophys. Acta*, 2000. **1458**: p. 104-119.
109. DeCoursey, T., Voltage-Gated Proton Channels and Other Proton Transfer Pathways. *Physiol. Rev.*, 2003. **83**: p. 475-579.
110. DeCoursey, T., Voltage-gated proton channels. *Cell. Mol. Life Sci.*, 2008: p. 1-20.
111. Wraight, C.A., Chance and design-Proton transfer in water, channels and bioenergetic proteins. *Biochim. Biophys. Acta*, 2006. **1757**: p. 886-912.
112. Konstantinov, A.A., S. Siletsky, D. Mitchell, A. Kaulen, and R.B. Gennis, The roles of the two proton input channels in cytochrome *c* oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer. *Proc. Natl. Acad. Sci. USA*, 1997. **94**: p. 9085-90.
113. Cukierman, S., Et tu, Grotthuss! and other unfinished stories. *Biochim. Biophys. Acta*, 2006. **1757**: p. 876-885.
114. Grotthuss, C.J.T., Mémoire sur la décomposition de l'eau et des corps qu'elle tient en dissolution à l'aide de l'électricité galvanique. *Ann. Chim.*, 1806. **58**: p. 54-74.
115. Tarasoff L. , K.M.F., Monosodium L-glutamate: a double-blind study and review. *Food Chem Toxicol.*, 1995. **33**: p. 69-78.
116. Pomès, R., G. Hummer, and M. Wikström, Structure and dynamics of a proton shuttle in cytochrome c oxidase. *Biochim. Biophys. Acta*, 1998. **1365**: p. 255-260.
117. Accardi, A., M. Walden, W. Nguitragool, H. Jayaram, C. Williams, and C. Miller, Separate Ion Pathways in a Cl-/H+ Exchanger. *J. Gen. Physiol.*, 2005. **126**: p. 563-570.
118. Miller, C., ClC chloride channels viewed through a transporter lens. *Nature*, 2006. **440**: p. 484-489.
119. Crofts, A.R., S. Lhee, S.B. Crofts, J. Cheng, and S. Rose, Proton pumping in the $bc_1$ complex: A new gating mechanism that prevents short circuits. *Biochim. Biophys. Acta*, 2006. **1757**: p. 1019-1034.
120. Meier, T., P. Polzer, K. Diederichs, W. Welte, and P. Dimroth, Structure of the Rotor Ring of F-Type $Na^+$-ATPase from *Ilyobacter tartaricus*. *Science*, 2005. **308**: p. 659-662.

121. Garczarek, F., L.S. Brown, J.K. Lanyi, and K. Gerwert, Proton binding within a membrane protein by a protonated water cluster. *Proc. Natl. Acad. Sci. USA.*, 2005. **102**: p. 3633-3638.
122. Hermes, S., J.M. Stachnik, D. Onidas, A. Remy, E. Hofmann, and K. Gerwert, Proton Uptake in the Reaction Center Mutant L210DN from *Rhodobacter sphaeroides* via Protonated Water Molecules. *Biochemistry*, 2006. **45**: p. 13741-13749.
123. Ädelroth, P., M.L. Paddock, L.B. Sagle, G. Feher, and M.Y. Okamura, Identification of the proton pathway in bacterial reaction centers: Both protons associated with reduction of $Q_B$ to $Q_BH_2$ share a common entry point. *Proc. Natl. Acad. Sci. USA*, 2000. **97**: p. 13086-13091.
124. Agmon, N., Proton Pathways in Green Fluorescence Protein. *Biophys. J.*, 2005 **88**: p. 2452–2461.
125. Rutz, C., W. Rosenthal, and R. Schülein, A Single Negatively Charged Residue Affects the Orientation of a Membrane Protein in the Inner Membrane of *Escherichia coli* Only When It Is Located Adjacent to a Transmembrane Domain. *J. Biol. Chem.*, 1999. **274**: p. 33757–33763.
126. Watmough, N.J., G. Butland, M.R. Cheesman, J.W. Moir, D.J. Richardson, and S. Spiro, Nitric oxide in bacteria: synthesis and consumption. *Biochim. Biophys. Acta*, 1999. **1411**: p. 456-474.
127. Thorndycroft, F.H., G. Butland, D.J. Richardson, and 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.*, 2007. **401**: p. 111-119.
128. Hemp, J., H. Han, J.H. Roh, S. Kaplan, T.J. Martinez, and R.B. Gennis, Comparative Genomics and Site-Directed Mutagenesis Support the Existence of Only One Input Channel for Protons in the C-Family (cbb(3) Oxidase) of Heme-Copper Oxygen Reductases. *Biochemistry*, 2007. **46**: p. 9963-9972.
129. Ädelroth, P., M.S. Ek, D.M. Mitchell, R.B. Gennis, and P. Brzezinski, Glutamate 286 in cytochrome $aa_3$ from *Rhodobacter sphaeroides* is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen. *Biochemistry*, 1997. **36**: p. 13824-13829.
130. Ädelroth, P., M. Karpefors, G. Gilderson, F.L. Tomson, R.B. Gennis, and P. Brzezinski, Proton transfer from glutamate 286 determines the transition rates between oxygen intermediates in cytochrome c oxidase. *Biochim. Biophys. Acta*, 2000. **1459**: p. 533-539.
131. Brändén, M., H. Sigurdson, A. Namslauer, R.B. Gennis, P. Ädelroth, and P. Brzezinski, On the role of the K-proton transfer pathway in cytochrome c oxidase. *Proc. Natl. Acad. Sci. USA*, 2001. **98**: p. 5013-5018.
132. Jasaitis, A., M.I. Verkhovsky, J.E. Morgan, M.L. Verkhovskaya, and M. Wikström, Assignment and charge translocation stoichiometries

of the major electrogenic phases in the reaction of cytochrome c oxidase with dioxygen. *Biochemistry*, 1999. **38**: p. 2697-2706.
133. Rigaud, J.L., B. Pitard, and D. Levy, Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta*, 1995. **1231**: p. 223-246.
134. Lepp, H., E. Svahn, K. Faxén, and P. Brzezinski, Charge transfer in the K-pathway linked to electron transfer to the catalytic site in cytochrome c oxidase. *Biochemistry*, 2008. **47**: p. 4929-4935.
135. Drachev, L.A., A.A. Jasaitis, A.D. Kaulen, A.A. Kondrashin, E.A. Liberman, I.B. Nemecek, S.A. Ostroumov, A. Semenov, and V.P. Skulachev, Direct measurement of electric current generation by cytochrome oxidase, H+-ATPase and bacteriorhodopsin. *Nature*, 1974. **249**: p. 321-324.
136. Wikström, M., J.E. Morgan, and M.I. Verkhovsky, Proton and electrical charge translocation by cytochrome-c oxidase. *Biochim. Biophys. Acta*, 1997. **1318**: p. 299-306.
137. Thöny-Meyer, L., F. Fischer, P. Kunzler, D. Ritz, and H. Hennecke, *Escherichia coli* genes required for cytochrome *c* maturation. *J. Bacteriol.*, 1995. **177**: p. 4321-4326.
138. Choe, M. and W.S. Reznikoj, Identification of the regulatory sequence of anaerobically expressed locus aeg-46.5. *J. Bacteriol.*, 1993. **175**: p. 1165-1172.
139. Reincke, B., L. Thony-Meyer, C. Dannehl, A. Odenwald, M. Aidim, H. Witt, and a.B.L. H. Ruterjans, Heterologous expression of soluble fragments of cytochrome $c_{552}$ acting as electron donor to the *Paracoccus denitrificans* cytochrome *c* oxidase. *Biochim. Biophys. Acta*, 1999. **1411**: p. 114–120.
140. Arslan Engin, H.S., Rachel Zufferey, Peter Kuönzler, and Linda Thoöny-Meyer1, Overproduction of the Bradyrhizobium japonicum c-Type Cytochrome Subunits of the cbb3 Oxidase in Escherichia coli. *Biochem Biophys Res Commun*, 1998. **251**: p. 744-747.
141. Tartof, K.D. and C.A. Hobbs, Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus*, 1987. **9**: p. 12.
142. Verkhovsky, M.I., J.E. Morgan, A. Puustinen, and M. Wikström, Kinetic trapping of oxygen in cell respiration. *Nature*, 1996. **380**: p. 268-270.
143. Verkhovsky, M.I., J.E. Morgan, and M. Wikström, Oxygen binding and activation: early steps in the reaction of oxygen with cytochrome c oxidase. *Biochemistry*, 1994. **33**: p. 3079-3086.
144. Wilson, M.T. and B.J. Reeder, Oxygen-binding haem proteins. *Experim. Physiol.*, 2008. **93**: p. 128-132.
145. Cheesman, M.R., W.G. Zumft, and A.J. Thomson, The MCD and EPR of the heme centers of nitric oxide reductase from *Pseudomonas stutzeri*: evidence that the enzyme is structurally related to the heme-copper oxidases. *Biochemistry*, 1998. **37**: p. 3994-4000.
146. Shapleigh, J.P. and W.J. Payne, Nitric oxide-dependent proton translocation in various denitrifiers. *J. Bacteriol.*, 1985. **163**: p. 837-840.

147. Bell, L.C., D.J. Richardson, and S.J. Ferguson, Identification of nitric oxide reductase activity in *Rhodobacter capsulatus*: the electron transport pathway can either use or bypass both cytochrome $c_2$ and the cytochrome $bc_1$ complex. *J. Gen. Microbiol.*, 1992. **138**: p. 437-443.
148. Mouritsen, O.G., *Life - As a matter of fat. The emerging science of lipidomics*. The frontiers collection, ed. M.D. Daniela Dragoman, Avshalom C. Elitzur, Mark P. Silverman, Jack Tuszunski and H. Dieter Zeh. 2005*: Springer-Verlag Berlin Heidelberg*.
149. Georgievskii, Y., E.S. Medvedev, and A.A. Stuchebrukhov, Proton Transport via the Membrane Surface. *Biophys. J.*, 2002. **82**: p. 2833-2846.
150. Alexiev, U., R. Mollaaghababa, P. Scherrer, H.G. Khorana, and M.P. Heyn, Rapid long-range proton diffusion along the surface of the purple membrane and delayed proton transfer into the bulk. *Proc Natl Acad Sci U S A*, 1995. **92**: p. 372-376.
151. Serowy, S., S.M. Saparov, Y.N. Antonenko, W. Kozlovsky, V. Hagen, and P. Pohl, Structural Proton Diffusion along Lipid Bilayers. *Biophys. J.*, 2003. **82**: p. 1031-1037.
152. Arese, M., W.G. Zumft, and F. Cutruzzola, Expression of a fully functional $cd_1$ nitrite reductase from *Pseudomonas aeruginosa* in *Pseudomonas stutzeri*. *Protein Expr Purif*, 2003. **27**: p. 42-48.
153. Laratta, W.P., M.J. Nanaszko, and J.P. Shapleigh, Electron transfer to nitrite reductase of *Rhodobacter sphaeroides* 2.4.3: examination of cytochromes $c_2$ and $c_Y$. *Microbiology*, 2006. **152**: p. 1479-1488.
154. Wonderen, J.H.v., B.n.d. Burlat, D.J. Richardson, M.R. Cheesman, and J.N. Butt, The Nitric Oxide Reductase Activity of Cytochrome *c* Nitrite Reductase from *Escherichia coli*. *J. Biol. Chem.*, 2008. **283**: p. 9587-9594.
155. Blomberg, L.M., M.R. Blomberg, and P.E. Siegbahn, A theoretical study on nitric oxide reductase activity in a ba(3)-type heme-copper oxidase. *Biochim. Biophys. Acta*, 2006. **1757**: p. 31-46.
156. Namslauer, A., A. Aagaard, A. Katsonouri, and P. Brzezinski, Intramolecular proton-transfer reactions in a membrane-bound proton pump: the effect of pH on the peroxy to ferryl transition in cytochrome c oxidase. *Biochemistry*, 2003. **42**: p. 1488-1498.
157. Michel, H., The mechanism of proton pumping by cytochrome c oxidase. *Proc. Natl. Acad. Sci. USA*, 1998. **95**: p. 12819-24.
158. Blomberg, M.R., P.E. Siegbahn, G.T. Babcock, and M. Wikström, O-O bond splitting mechanism in cytochrome oxidase. *J. Inorg. Biochem.*, 2000. **80**: p. 261-269.
159. Ostermeier, C., A. Harrenga, U. Ermler, and H. Michel, Structure at 2.7 A resolution of the *Paracoccus denitrificans* two- subunit cytochrome *c* oxidase complexed with an antibody FV fragment. *Proc. Natl. Acad. Sci. USA*, 1997. **94**: p. 10547-53.

160. Garczarek, F. and K. Gerwert, Functional waters in intraprotein proton transfer monitored by FTIR difference spectroscopy. *Nature*, 2006. **439**: p. 109-112.
161. Berglin, J., Samlade serier. *42 Förlag*, 1992.