REVIEW

Folia Microbiol. 45 (3), 197–203 (2000) http://www.biomed.cas.cz/mbu/folia/

From No-confidence to Nitric Oxide Acknowledgement: A Story of Bacterial Nitric-Oxide Reductase

M. KOUTNÝ

Department of Environmental Technology, Faculty of Technology Zlín, Brno University of Technology, 762 72 Zlín, Czechia

e-mail mkoutny@zlin.vutbr.cz

Received 23 May 2000

ABSTRACT. The review briefly summarizes current knowledge of the bacterial nitric-oxide reductase (NOR). This membrane enzyme consists of two subunits, the smaller one contains hæm C and the larger one two hæms B and nonhæm iron. The protein sequence and structure of metal centres demonstrate the relationship of NOR to the family of terminal oxidases. The binuclear Fe–Fe reaction centre, consisting of antiferromagnetically coupled hæm B and nonhæm iron, is analogous to Fe–Cu centre of terminal oxidases. The data on the structure and function of NOR and terminal oxidases suggest that all these enzymes are closely evolutionally related. The catalytic properties are determined most of all by the relatively high toxicity of nitric oxide as a substrate and the resulting strong need to maintain its concentration at nanomolar levels. A kinetic model of the action of the enzyme comprises substrate inhibition. NOR does not conserve the free energy of nitric oxide reduction because it does not work as a proton pump and, moreover, the protons coming into the reaction are taken from periplasm, *i.e.* they do not cross the membrane.

Abbreviations

CCCP	3-chlorophenylhydrazono-malononitrile ('carbonyl cyanide 3-chlorophenylhydrazone')
COX	aa_3-cytochrome-*c* oxidase
NIR	nitrite reductase
NOR	nitric-oxide reductase
PMS	*N*-methylphenazonium methanesulfonate ('phenazine methosulfate')
TMPD	*N,N,N',N'*-tetramethyl-1,4-benzendiamine

CONTENS

1 INTRODUCTION

During the last fifteen years nitric oxide has been a molecule of particular interest for animal and human physiologists. Simultaneously, bacterial physiologists have found it relevant to their field and have been trying to confirm its appearance and the pathways of its production and consumption, although the first reports on the NO occurrence in bacterial cells have been met with scepticism.

2 NITRIC OXIDE: DANGEROUS AGENT

Nitric oxide, "the ultra low molar mass hormone" and at the same time one of the simplest and highly reactive nitrogen species (Sigler *et al.* 1999), is a small hydrophobic molecule which can easily permeate through biological membranes, and which contains an unpaired electron. Despite its high reactivity, it has a considerably long half-life in biological systems. Higher NO concentrations are toxic for living cells. This toxicity results from its reactivity with metalloproteins containing transition metals and with biological thiols and amines. The main sites of its toxic action are hæm Fe, nonhæm Fe and Cu containing enzymes (Packer 1996; Zumft 1997). NO is also mutagenic to bacterial DNA, obviously because of its deaminating and nitrosating activity (Wink *et al.* 1991). Because of this, there is a strong need for the denitrifying cell to maintain the concentration of NO produced by nitrite reductase at a very low level (nmol/L). Mutations leading to a lack of NOR activity and causing an abnormal increase of the steady-state NO concentration are lethal (Braun and Zumft 1991).

However, NO non-producing bacteria can also come in to contact with NO coming from their environment and can possess defense systems for its detoxification. Soil bacteria of the genus *Pseudomonas*,

closely related to *P. fluorescens*, can oxidize NO to nitrite (Koschorreck *et al.* 1996). Bacterial pathogens in animal bodies have to face nitric oxide produced by activated macrophages.

3 BRIEF HISTORY OF NITRIC OXIDE DISCOVERY IN BACTERIAL DENITRIFIERS

Originally, there was a deep distrust of NO existence as an obligatory intermediate. Before its role in animal organisms was known, some researches took the presence of NO in living cell to be pure nonsense. Experiments with isotopically labelled N-oxoanions have been interpreted as evidence for a direct reduction of NO_2^- to N_2O by a single enzyme – nitrite reductase (St. John and Hollocher 1977; Garber and Hollocher 1981). However, during subsequent years observations demonstrating role of NO as a free intermediate and NOR as a separate enzyme have accumulated.

Firstly it has been shown that all purified nitrite reductases with physiological electron donors reduce nitrite to nitric oxide and not to nitrous oxide (*e.g.*, LeGall *et al.* 1979; Liu *et al.* 1986). An experiment has proved that denitrifying bacteria can use NO as a terminal electron acceptor (Garber *et al.* 1982; Shapleigh and Payne 1985, 1986). NO reductase activity has been shown to be associated with the cytoplasmic membrane (Grant and Hochstein 1984). It has also been shown that, in the presence of the well-known uncoupler CCCP, nitrite is transformed to a potent inhibitor of terminal oxidases. Furthermore, the inhibitor has been identified as NO released due to the blocking of NOR activity by CCCP (Kučera *et al.* 1987). At appropriate CCCP concentrations, damped oscillations of NO concentration can even be observed (Kučera 1992). Cells lacking active NIR after insertion of *Tn*5 transposon are still been able to reduce NO (Zumft *et al.* 1988). Although the steady-state concentration of NO in bacterial cell appears to be very low, a possibility has been found to trap the NO produced during denitrification by the added high-affinity scavenger deoxyhemoglobin (Goretski and Hollocher 1988), or mammalian cytochrome *c* (Kučera *et al.* 1987). Formation of the NO–hemoprotein complex has been confirmed by its characteristic spectral properties. Progress in analytical methodology has also made possible the direct detection of free NO by mass spectrometry (Zafiriou *et al.* 1989). Definitive evidence has been provided by the purification and characterization of NOR from *Pseudomonas stutzeri* (Heiss *et al.* 1989; Kastrau *et al.* 1994).

4 HOW TO OBTAIN NITRIC-OXIDE REDUCTASE

Bacterial NOR has hitherto been isolated from *Pseudomonas stutzeri* (Heiss *et al.* 1989), several strains of *Paracoccus denitrificans* (Carr and Ferguson 1990; Dermastia *et al.* 1991; Fujiwara and Fukumori 1996), *Paracoccus halodenitrificans* (Sakurai and Sakurai 1997) and *Achromobacter cycloclastes* (Jones and Hollocher 1993). It is expressed under microaerobic or anaerobic conditions.

The enzyme is membrane-bound, so the first step of purification must be the isolation of the membrane fraction and its solubilization. Suitable detergents are lauryl maltoside (Girsch and de Vries 1997), octyl glucoside (Dermastia *et al.* 1991) and sucrose monocaprate (Fujivara and Fukumori 1996). On the other hand, nonionic detergents as Triton X-100 and Brij-35 have not proved to be suitable (Carr and Ferguson 1990), although in one case Triton X-100 has been also successfully used in the purification of the enzyme from *P. stutzeri* (Heiss *et al.* 1989). A substantial drop of activity is always observed after the addition of the detergent, caused probably by delipidation of the enzyme. Complete delipidation after treatment with a more aggressive detergent such as Triton X-100 leads to an almost complete loss of activity. An addition of phospholipids or some detergents, *e.g.*, octyl thioglucopyranoside, can then restore a significant part of the activity (Kastrau *et al.* 1994).

In the following purification step, a combination of ion-exchange chromatography and gel filtration (Heiss *et al.* 1989), immobilized-metal-ion affinity chromatography (Hendriks *et al.* 1998), or chromatography on hydroxyapatite (Fujiwara and Fukumori 1996) have been found to be applicable. NOR has been eluted at about 0.4 mol/L NaCl from the frequently used anion-exchanger Q-Sepharose under a NaCl gradient (Hendriks *et al.* 1998). The high purity of enzyme is necessary for reliable VIS and Molecular Circular Dichroism data and is critical for the reliability of EPR data (Hendriks *et al.* 1998; Sakurai 1998).

5 POLYPEPTIDE COMPOSITION; HÆM AND METAL CONTENT

Pure NOR consists of two polypeptides with molar mass 17 and 38 kDa (Heiss *et al.* 1989). The smaller subunit carries covalently bound hæm C and the larger one contains hæm B. Analysis of the hæm con-

tent by the pyridin hæmachrome method has shown that hæms B and C are present at a ratio of 2.1 ± 0.1. In both the detergent solution and the native cytoplasmic membrane, the enzyme probably occurs as a dimer of two NOR-BC monomers. Four iron atoms per NOR-BC monomer have been found (Hendriks *et al.* 1998). There are no indications of copper presence in the enzyme (Fujiwara and Fukumori 1996; Hendriks *et al.* 1998). Exceptions to the above pattern are enzymes recently isolated from *Alcaligenes eutrophus* H16 (Cramm *et al.* 1997) and *Synechocystis* sp. PCC6803 (Kaneko *et al.* 1996), where the N-terminal extension of the NOR-B sequence containing two more membrane-spanning helices provides a functional analogue for the NOR-C subunit.

6 SPECTRAL PROPERTIES

Visible spectra. The spectra of the reduced enzyme exhibit a maximum at 551 nm suggesting the presence of a low spin hæm C and a shoulder at 558 nm suggesting a low spin hæm B. A broad absorption peak at around 590 nm appears in the spectra of the oxidized enzyme and is disappearing during its reduction. Because of the similarity with the spectrum of the b_0 quinol oxidase, the signal (Moody and Rich 1994) has been taken to be high spin hæm B. The CO-difference spectrum of the reduced enzyme also shows evidence of the occurrence of the high spin hæm B (Girsch and de Vries 1996). The electronic spectra of NOR show considerable similarities with the spectra of *cb*-type cytochrome-*c* oxidases (Garcia-Horsman *et al.* 1994; Gray *et al.* 1984).

EPR spectra. The oxidized enzyme displays the signals of the low spin hæm C and the low spin hæm B. The signals are consistent with the assumed coordination of the hæms, *i.e.* His–Met in the case of the hæm C and His–His in the case of the hæm B (Girsch and de Vries 1996; Hendriks *et al.* 1998). The absence of any signal originating from high spin hæm B indicates magnetic coupling between the hæm and another paramagnet in its close vicinity (Cheesman *et al.* 1998). The most probable candidate is a nonhæm iron ion. After reduction of the enzyme, both low spin signals disappear and, after the addition of NO to reduced NOR solution, new strong signals emerge documenting the disturbance of magnetic coupling between the two iron centers. The signal at $g = 4$ has been assigned to the Fe(II)–NO complex of nonhæm iron (Hendriks *et al.* 1998). The previously observed signal at $g = 2.009$, which has been interpreted as manifestation of nonhæm iron, is most likely due to contamination.

7 STRUCTURE MODEL

The NOR-B subunit is a highly hydrophobic membrane protein. After analysis of its sequence, 12 transmembrane helices have been predicted (Zumft *et al.* 1994). A comparison of the primary structures of NOR-B and subunit I of cytochrome-*c* oxidase from several organisms has displayed the sequence homology of the proteins. This enables us to use structural information obtained from the X-ray analysis of bacterial aa_3-cytochrome-*c* oxidase to derive a model representing the arrangement of the transmembrane helices in the cytoplasmic membrane. The resulting model shows a compact protein, where the transmembrane helices are arranged in three semicircles surrounding metal prosthetic centres (Zumft and Körner 1997). The NOR-B subunit contains low spin hæm B and high spin hæm B. The presence of a further metal center has for some time been the subject of speculation. Now we have sufficient evidences to confirm nonhæm iron as the additional prosthetic group (Hendriks *et al.* 1998). Low spin hæm B is probably coordinated by His^{60} and His^{349}, which are analogues of conserved hæm coordinating histidines in COX I. High spin hæm B and nonhæm iron are located in close proximity to each other and form a binuclear reaction centre of NO-reductase analogous to the a_3Cu_B center of COX. EPR data, predicting the existence of antiferromagnetic coupling between high spin hæm B and another paramagnet in its close neighbourhood, provide strong support for this concept (Girsch and de Vries 1996; Cheesman *et al.* 1998). The data are also consistent with an assumed oxo or hydroxo bridge ligand between the two iron atoms (Cheesman *et al.* 1998). Some other ligands are again analogues of conserved histidines noted in COX, the others are still unknown (Zumft and Körner 1997).

The NOR-C subunit consists of a globular periplasmic domain with attached hæm C and a transmembrane helix anchoring the whole polypeptide in the cytoplasmic membrane (Zumft and Körner 1997).

8 CATALYTIC PROPERTIES OF NITRIC-OXIDE REDUCTASE

NOR catalyses the reductive condensation of two NO molecules to form N_2O (cf. overall reaction):

$$2\ NO + 2\ H^+ + 2e^- \rightarrow N_2O + H_2O$$

In the particular case of *P. denitrificans* cytochrome c_{550} (the physiological donor of electrons) appears to be the soluble periplasmic cytochrome *c*. However, its deletion does not cause a loss of activity, which suggests the possibility of an alternative electron supply, for example from the periplasmic blue-copper protein pseudoazurin (Ferguson 1994) also identified as an alternative electron donor in the case of nitrite reductase (Koutný *et al.* 1999). Because the isolated membrane fraction alone has a high succinate–nitric oxide oxidoreductase activity, we can deduce the existence of longitudinal electron flow along the cytoplasmic membrane, possibly mediated by a membrane-associated cytochrome *c* (*unpublished results*). The physiological activity has not yet been reconstituted *in vitro* (Goretski and Hollocher 1988).

Activity assay can make use of various artificial electron donors, PMS (Miyata 1971), TMPD–horse cytochrome *c* (Goretski and Hollocher 1988), or TMPD alone (Kučera 1992) being the most popular ones. The probable site of electron entrance into the enzyme is the hæm C in the NOR-C subunit.

After adding ascorbate to the solution of the oxidized enzyme, the fast reduction of hæm C and low spin hæm B is followed by the slower reduction of high spin B (Sakurai *et al.* 1998). From these observations, the electron pathway in the enzyme can be deduced as going from hæm C to low spin hæm B and then to the binuclear reaction centre consisting of high spin hæm B and a nonhæm iron ion (Fig. 1).

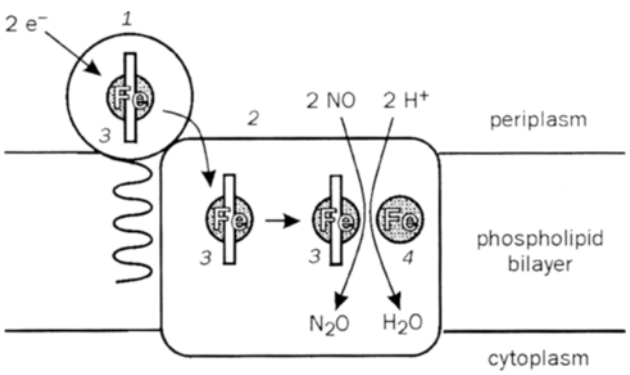

Fig. 1. Model of the structure and action of bacterial nitric-oxide reductase; the enzyme consists of two subunits, the smaller one (*1*) being a membrane anchored *c*-type cytochrome while the larger (*2*) one is an integral membrane protein; the positions of the hæms (*3*) and the nonhæm Fe (*4*) are schematically shown; the *arrows* indicate the flow of electrons and the reactants to the binuclear reaction centre of the enzyme.

The mechanism of NO reduction and N–N bond formation is still not completely clear. Spectral data have not provided convincing evidence confirming the presence of the assumed intermediates in the reaction centre. A more detailed kinetic study is also lacking. The two most discussed concepts suggest that (*i*) the primary product of the enzyme is a nitroxyl anion (NO^-) which dimerizes to form hyponitrite in an instant nonenzymic reaction; the hyponitrite is subsequently protonated and after the cleavage of water yields N_2O (Hendriks *et al.* 1998); or (*ii*) an N–N bond is established precisely in the reaction centre of the enzyme (Girsch and de Vries 1996). The arrangement and binding of the two NO molecules in the binuclear reaction centre also needs to be better understood (Zumf 1997). The above possible reaction mechanisms lends relevance also to our observation that hyponitrite does not affect NO reduction (*unpublished results*).

A typical feature of NO reductase kinetics is substrate inhibition (Kučera 1992). After addition of NO to the enzyme solution (100 μmol/L) we can observe an initially slow but gradually accelerating substrate consumption resulting in a sigmoidal shape of the [NO] time trace. The probable reason of the inhibition is the binding of NO to hæm. A kinetic model of NOR action comprising substrate inhibition has been established (Girsch and de Vries 1996) and afterwards critically reviewed and revised (Koutný and Kučera 1999). The main features of the model can be summarized as follows: (*i*) the inhibition is acompetitive; (*ii*) an inactive complex is formed in the reaction of NO with the oxidized enzyme, and some spectroscopic

data show that NO really binds to the oxidized form of the enzyme (Fujiwara and Fukumori 1996); (*iii*) the extend of inhibition depends on the redox state of the enzyme which is influenced by the rate of electron supply. The latter finding might explain why some authors have observed a linear decrease of NO concentration with time (Carr *et al.* 1989 and 1990) while others have taken the sigmoidal shape of the curve as suggesting substrate inhibition (Goretski and Hollocher 1990; Dermastia *et al.* 1991; Kučera 1992). The discrepancy can results from differences in the electron donor systems used by the authors. The above described kinetic model has provided an inhibition constant K_i = 3 μmol/L (Koutný and Kučera 1999). The substrate inhibition is obviously one of the causes of the observed oscillations of NO concentration (Kučera 1992).

Although NO can freely diffuse through biological membranes, its concentration in the interior of the membrane is elevated compared to the layer of polar phospholipid heads or surrounding aqueous phase (Subczynski 1996). This could be the reason why the binuclear reaction centre of NOR is localised inside the lipidic bilayer of the cytoplasmic membrane (Zumft 1997).

9 GENES FOR NITRIC OXIDE REDUCTION

The genes are located inside the *nor* cluster adjacent to the *nir* cluster which contains genes necessary for reduction of nitrite. Two structural genes, *norC* and *norB*, are followed by four other ORFs with less known function. The *norE* gene has been found to display sequence homology with subunit III of COX. Despite the relationship of the two enzymes, the product of the gene has not yet been purified as a component of NOR. Knockout of the gene causes a decrease in NO reductase activity (de Boer *et al.* 1996).

The expression of the *nor* genes is under the control of the transcription factor NNR (van Spanning *et al.* 1997). The signal, or one of the signals, for NNR activation could be NO itself (van Spanning *et al.* 1999).

10 EVOLUTIONARY RELATIONSHIP OF NO A O_2 REDUCTIONS

After searching protein sequence databases, about 20 % homology has been found between NorB and the main catalytic subunit of aa_3-cytochome-*c* oxidase (Zumf *et al.* 1994). Such a level of uniformity is not considered to be sufficient for confirmation of the relationship between the enzymes. However, further studies have provided evidence in favour of the relationship. The membrane spanning helix predictive algorithm has located twelve such segments in the NOR-B sequence in accordance with the known situation in COX I. Moreover, conserved histidine residues coordinating hæm A and the a_3Cu_B centre have their counterparts in the NOR-B sequence (van der Oost *et al.* 1994; Saraste and Castresana 1994; Zumft and Körner 1997). A remarkable analogy has been found between the structure and function of metal centers of the enzymes. The binuclear centre comprising the antiferromagnetically coupled hæm iron of hæm B and the non-hæm iron ion appears to be closely related to the a_3Cu_B centre of COX. The most notable difference is the presence of Fe instead of Cu. How the specificity of proper metal insertion is achieved, when both enzymes are present in one cell, continues to remain unclear. An analogue of the other hæm B in NOR is the hæm A in COX (van der Oost *et al.* 1994).

The cytochrome-*c* oxidase of aa_3 type is a member of a larger enzyme family of terminal oxidases which exhibit a high level of sequential and structural homology. The relationship of NOR to these enzymes along with some later conceptions of young earth's atmosphere (Kasting 1990) has inspired a theory placing an ancestral NOR at the beginning of evolution leading to all terminal oxidases.

11 ENERGY CONSERVATION

The high positive midpoint potential for the NO/N_2O couple (1.18 V) gives the cell a theoretical opportunity to use the free energy from NO reduction for proton pumping across the cytoplasmic membrane similarly to cytochrome-*c* oxidases (Hendriks *et al.* 1997). However, the actual experimental results clearly show that NOR neither works as a proton pump nor actually produces a membrane potential because protons for reduction are taken from the periplasm (Shapleigh and Payne 1985; Carr *et al.* 1989; Bell *et al.* 1992).

The detoxification of nitric oxide produced by periplasmic nitrite reductase appears to be the primary and essential function of NOR. During evolution, any mutations leading to better energy conservation but negatively affecting the above mentioned basic role could be abandoned. Progenies of ancestral NOR,

terminal oxidases, metabolizing relatively harmless oxygen, then could fully direct their evolution to maximizing the energy gain.

REFERENCES

BELL L.C., RICHARDSON D.J., FERGUSON S.J.: 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.* **138**, 437–443 (1992).

DE BOER A.P., VAN DER OOST J., REIJNDERS W.N., WESTERHOFF H.V., STOUTHAMER A.H., VAN SPANNING R.J.: Mutational analysis of the nor gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *Eur.J.Biochem.* **242**, 592–600 (1996).

BRAUN C., ZUMFT W.G.: Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of *Pseudomonas stutzeri* at nitric oxide. *J.Biol.Chem.* **266**, 22785–22788 (1991).

CARR G.J., FERGUSON S.J.: The nitric oxide reductase of *Paracoccus denitrificans*. *Biochem.J.* **269**, 423–429 (1990).

CARR G.J., PAGE M.D., FERGUSON, S.J.: The energy-conserving nitric-oxide-reductase system in *Paracoccus denitrificans*. Distinction from the nitrite reductase that catalyses synthesis of nitric oxide and evidence from trapping experiments for nitric oxide as a free intermediate during denitrification. *Eur.J.Biochem.* **179**, 683–692 (1989).

CHEESMAN M.R., ZUMFT W.G.: 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* **37**, 3994–4000 (1998).

CRAMM R., SIDDIQUI R.A., FRIEDRICH B.: Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. *J.Bacteriol.* **179**, 6769–6777 (1997).

DERMASTIA M., TURK T., HOLLOCHER T.C.: Nitric oxide reductase. Purification from *Paracoccus denitrificans* with use of a single column and some characteristics. *J.Biol.Chem.* **266**, 10899–10905 (1991).

FERGUSON S.J.: Denitrification and its control. *Antonie Van Leeuwenhoek* **66**, 89–110 (1994).

FUJIWARA T., FUKUMORI Y.: Cytochrome *cb*-type nitric oxide reductase with cytochrome *c* oxidase activity from *Paracoccus denitrificans* ATCC 35512. *J.Bacteriol.* **178**, 1866–1871 (1996).

GARBER E.A., CASTIGNETTI D., HOLLOCHER T.C.: Proton translocation and proline uptake associated with reduction of nitric oxide by denitrifying *Paracoccus denitrificans*. *Biochem.Biophys.Res.Commun.* **107**, 1504–1507 (1982).

GARBER E.A., HOLLOCHER T.C.: ^{15}N tracer studies on the role of NO in denitrification. *J.Biol.Chem.* **256**, 5459–5465 (1981).

GARCIA-HORSMAN J.A., BERRY E., SHAPLEIGH J.P., ALBEN J.O., GENNIS R.B.: A novel cytochrome-*c* oxidase from *Rhodobacter sphæroides* that lacks CuA. *Biochemistry* **33**, 3113–3119 (1994).

GIRSCH P., DE VRIES S.: Purification and initial kinetic and spectroscopic characterisation of NO reductase from *Paracoccus denitrificans*. *Biochim.Biophys.Acta* **1318**, 202–216 (1997)

GORETSKI J., HOLLOCHER T.C.: The kinetic and isotopic competence of nitric oxide as an intermediate in denitrification. *J.Biol.Chem.* **265**, 889–895 (1990).

GORETSKI J., HOLLOCHER T.C.: Trapping of nitric oxide produced during denitrification by extracellular hemoglobin. *J.Biol.Chem.* **263**, 2316–2323 (1988).

GRANT M.A., HOCHSTEIN L.I.: A dissimilatory nitrite reductase in *Paracoccus halodenitrificans*. *Arch.Microbiol.* **137**, 79–84 (1984).

GRAY K.A., GROOMS M., MYLLYKALLIO H., MOOMAW C., SLAUGHTER C., DALDAL F.: *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome-*c* oxidase without a CuA center. *Biochemistry* **33**, 3120–3127 (1994)

HEISS B., FRUNZKE K., ZUMFT W.G.: Formation of the N–N bond from nitric oxide by a membrane-bound cytochrome *bc* complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri*. *J.Bacteriol.* **171**, 3288–3297 (1989).

HENDRIKS J., WARNE A., GOHLKE U., HALTIA T., LUDOVICI C., LUBBEN M., SARASTE M.: The active site of the bacterial nitric oxide reductase is a dinuclear iron center. *Biochemistry* **37**, 13102–13109 (1998).

JONES A.M., HOLLOCHER T.C.: Nitric oxide reductase of *Achromobacter cycloclastes*. *Biochim.Biophys.Acta* **1144**, 359–366 (1993).

KANEKO T., SATO S., KOTANI H., TANAKA A., ASAMIZU E., NAKAMURA Y., MIYAJIMA N., HIROSAWA M., SUGIURA M., SASAMOTO S., KIMURA T., HOSOUCHI T., MATSUNO A., MURAKI A., NAKAZAKI N., NARUO K., OKUMURA S., SHIMPO S., TAKEUCHI C., WADA T., WATANABE A., YAMADA M., YASUDA M., TABATA S.: Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109–136 (1996).

KASTING F.J.: Bolide impacts and the oxidation state of carbon in the earth's early atmosphere. *Origins Life Evol.Biosphere* **20**, 199–231 (1990).

KASTRAU D.H., HEISS B., KRONECK P.M., ZUMFT W.G.: Nitric oxide reductase from *Pseudomonas stutzeri*, a novel cytochrome *bc* complex. Phospholipid requirement, electron paramagnetic resonance and redox properties. *Eur.J.Biochem.* **222**, 293–303 (1994).

KOSCHORRECK M., MOORE E., CONRAD R.: Oxidation of nitric oxide by a new heterotrophic *Pseudomonas* sp. *Arch.Microbiol.* **166**, 23–31 (1996).

KOUTNÝ M., KUČERA I., TESAŘÍK R., TURÁNEK J., VAN SPANNING R.J.: Pseudoazurin mediates periplasmic electron flow in a mutant strain of *Paracoccus denitrificans* lacking cytochrome c_{550}. *FEBS Lett.* **448**, 157–159 (1999).

KOUTNÝ M., KUČERA I.: Kinetic analysis of substrate inhibition in nitric oxide reductase of *Paracoccus denitrificans*. *Biochem.Biophys.Res.Commun.* **262**, 562–564 (1999).

KUČERA I., KOZÁK L., DADÁK V.: Aerobic dissimilatory reduction of nitrite by cells of *Paracoccus denitrificans*. *Biochem.Biophys.Acta* **894**, 120–126 (1987).

KUČERA I., LAMPARDOVA L., DADÁK V.: Control of respiration rate in non-growing cells of *Paracoccus denitrificans*. *Biochem.J.* **246**, 779–782 (1987).

KUČERA I.: Oscillations of nitric oxide concentration in the perturbed denitrification pathway of *Paracoccus denitrificans*. *Biochem.J.* **286**, 111–116 (1992).

LEGALL J., PAYNE W.J., MORGAN T.V., DER VARTANIAN D.V.: On the purification of nitrite reductase from *Thiobacillus denitrificans* and its reaction with nitrite under reducing conditions. *Biochem.Biophys.Res.Commun.* **87**, 355–362 (1979).

LIU M.-Y., LIU M.-C., PAYNE W.J., LEGALL J.: Properties and electron transfer specificity of copper proteins from the denitrifier *Achromobacter cycloclastes*. *J.Bacteriol.* **166**, 604–608 (1986).

MIYATA M.: Studies on denitrification. XIV. The electron donating system in the reduction of nitric oxide and nitrate. *J.Biochem. (Tokyo)* **70**, 205–213 (1971).

MOODY A.J., RICH P.R.: The reaction of hydrogen peroxide with pulsed cytochrome b_0 from *Escherichia coli*. *Eur.J.Biochem.* **226**, 731–737 (1994).

VAN DER OOST J., DE BOER A.P., DE GIER J.W., ZUMFT W.G., STOUTHAMER A.H., VAN SPANNING R.J.: The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. *FEMS Microbiol.Lett.* **121**, 1–9 (1994).

PACKER L.: *Nitric Oxide. B. Physiological and Pathological Processes. Meth.Enzymol.* **269** (1996).

SAKURAI N., SAKURAI T.: Isolation and characterization of nitric oxide reductase from *Paracoccus halodenitrificans*. *Biochemistry* **36**, 13809–13815 (1997).

SAKURAI T., SAKURAI N., MATSUMOTO H., HIROTA S., YAMAUCHI O.: Roles of four iron centers in *Paracoccus halodenitrificans* nitric-oxide reductase. *Biochem.Biophys.Res.Commun.* **251**, 248–251 (1998).

SARASTE M., CASTRESANA J.: Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.* **341**, 1–4 (1994).

SHAPLEIGH J.P., PAYNE W.J.: Nitric oxide-dependent proton translocation in various denitrifiers. *J.Bacteriol.* **163**, 837–840 (1985).

SIGLER K., CHALOUPKA J., BROZMANOVÁ J., STADLER N., HÖFER M.: Oxidative stress in microorganisms. I. Microbial *vs.* higher cells – damage and defenses in relation to cell aging and death. *Folia Microbiol.* **44**, 587–624 (1999).

VAN SPANNING R.J., DE BOER A.P., REIJNDERS W.N., WESTERHOFF H.V., STOUTHAMER A.H., VAN DER OOST J.: FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol.Microbiol.* **23**, 893–907 (1997).

VAN SPANNING R.J., HOUBEN E., REIJNDERS W.N., SPIRO S., WESTERHOFF H.V., SAUNDERS N.: Nitric oxide is a signal for NNR-mediated transcription activation in *Paracoccus denitrificans*. *J.Bacteriol.* **181**, 4129–4132 (1999).

ST. JOHN R.T., HOLLOCHER T.C.: Nitrogen-15 tracer studies on the pathway of denitrification in *Pseudomonas aeruginosa*. *J.Biol. Chem.* **252**, 212–218 (1977).

SUBCZYNSKI W.K., LOMNICKA M., HYDE, J.S.: Permeability of nitric oxide through lipid bilayer membranes. *Free Radic.Res.* **24**, 343–349 (1996).

WINK D.A., KASPRZAK K.S., MARAGOS C.M., ELESPURU R.K., MISRA M., DUNAMS T.M., CEBULA T.A., KOCH W.H., ANDREWS A.W., ALLEN J.S.: DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254**, 1001–1003 (1991).

ZAFIRIOU O.C., HANLEY Q.S., SNYDER G.: Nitric oxide and nitrous oxide production and cycling during dissimilatory nitrite reduction by *Pseudomonas perfectomarina*. *J.Biol.Chem.* **264**, 5694–5699 (1989).

ZUMFT W.G., BRAUN C., CUYPERS H.: Nitric oxide reductase from *Pseudomonas stutzeri*. Primary structure and gene organization of a novel bacterial cytochrome *bc* complex. *Eur.J.Biochem.* **219**, 481–90 (1994).

ZUMFT W.G., DOHLER K., KÖRNER H., LOCHELT S., VIEBROCK A., FRUNZKE K.: Defects in cytochrome cd_1-dependent nitrite respiration of transposon *Tn5*-induced mutants from *Pseudomonas stutzeri*. *Arch.Microbiol.* **149**, 492–498 (1988).

ZUMFT W.G., KÖRNER H.: Enzyme diversity and mosaic gene organization in denitrification. *Antonie Van Leeuwenhoek* **71**, 43–58 (1997).

ZUMFT W.G.: Cell biology and molecular basis of denitrification. *Microbiol.Mol.Biol.Rev.* **61**, 533–616 (1997).