Characterization of NosL, a novel Cu(I) protein of the nitrous oxide reductase gene cluster
by Michele Ann McGuirl

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University

Abstract:
Metal chaperone structure and function is an emerging area of research in the field of bioinorganic chemistry. The assembly of the catalytic copper center in nitrous oxide reductase requires the assistance of auxiliary proteins. The isolation, and characterization of these ancillary proteins has not been previously reported. This investigation describes the cloning, recombinant expression, purification, and characterization of NosL from Achromobacter cycloclastes. NosL is an auxiliary protein of the nos (nitrous oxide reductase) gene cluster. Recombinant NosL has a high affinity Cu(I) binding site. Studies to elucidate the function of NosL suggest that it is not the electron-transfer partner to nitrous oxide reductase. In vivo investigations of the phenotype associated with nosL minus strains indicate that NosL is not strictly required for the production of active N2OR, under the laboratory conditions employed in the study.

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APPROVAL

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Michele Ann McGuirl

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

David M. Dooley — (Signature) — Date 9/29/99

Approved for the Department of Chemistry and Biochemistry

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Date 9/30/99

This thesis is dedicated to Erica Smith. May she, too, follow her dreams under God's guidance.

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ABSTRACT

Metal chaperone structure and function is an emerging area of research in the field of bioinorganic chemistry. The assembly of the catalytic copper center in nitrous oxide reductase requires the assistance of auxiliary proteins. The isolation, and characterization of these ancillary proteins has not been previously reported. This investigation describes the cloning, recombinant expression, purification, and characterization of NosL from *Achromobacter cycloclastes*. NosL is an auxiliary protein of the *nos* (nitrous oxide reductase) gene cluster. Recombinant NosL has a high affinity Cu(I) binding site. Studies to elucidate the function of NosL suggest that it is not the electron-transfer partner to nitrous oxide reductase. *In vivo* investigations of the phenotype associated with *nosL* minus strains indicate that NosL is not strictly required for the production of active N_2OR, under the laboratory conditions employed in the study.

INTRODUCTION

Overview of Denitrification

Denitrification is a key step of the global nitrogen cycle (Figure 1), providing the pathway by which fixed nitrogen is returned to the atmosphere (1,2). In the nitrogen cycle, atmospheric nitrogen is reduced to ammonia by a select group of bacteria. The ammonia is then assimilated by plants, animals and microbes. When returned to soil or water by the degradation of waste or decaying matter, released ammonia is either re-assimilated or converted to nitrate or nitrite through the nitrification pathway. Nitrate thus formed experiences one of two fates. First, nitrate may be reduced back to ammonia via the assimilatory reduction pathway. This reaction occurs in plants, fungi, and microbes. Alternatively, a subfamily of bacteria known as denitrifiers may convert nitrate to nitrogen gas via the denitrification pathway and thus complete the nitrogen

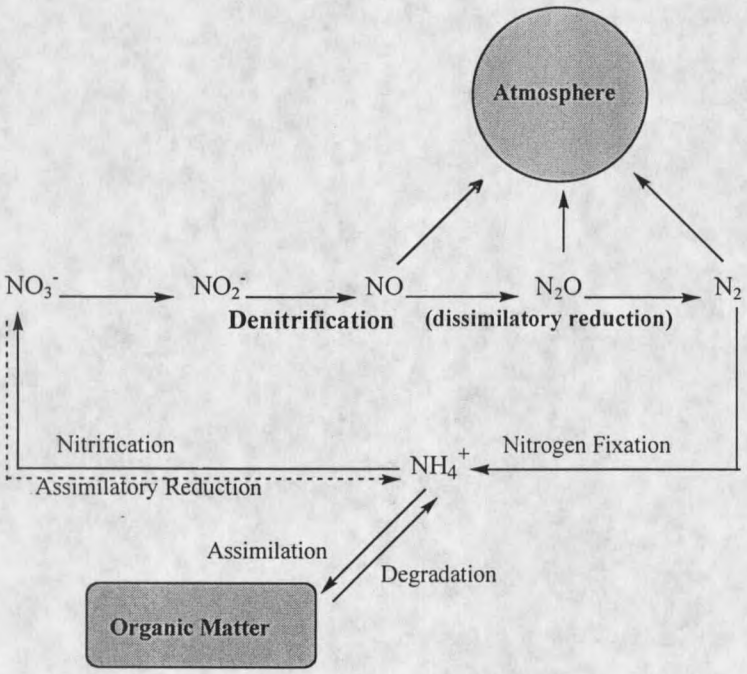

Figure 1. The global nitrogen cycle.

cycle. Recently several fungi have also been shown to reduce nitrate to nitrous oxide, but lack the enzyme to complete the final step and produce N_2 (3,4).

Denitrification provides an alternative respiratory pathway for denitrifying bacteria under oxygen-limited conditions, with N-oxides acting as terminal electron acceptors in lieu of O_2. Although denitrification has traditionally been considered an anaerobic process, several bacteria have been shown to be capable of aerobic denitrification. In either case, nitrate is reduced to dinitrogen by the action of four discrete metalloenzymes as shown below in Equation 1:

$$NO_3^- \xrightarrow[\text{nitrate reductase}]{} NO_2^- \xrightarrow[\text{nitrite reductase}]{} NO \xrightarrow[\text{nitric oxide reductase}]{} N_2O \xrightarrow[\text{nitrous oxide reductase}]{} N_2 \quad \text{(Eq. 1)}$$

The electrons for the reaction are provided by small, soluble electron transfer proteins, which are in turn supplied by a membrane-bound bc_1 complex (5). In the bc_1 complex, electron transfer is coupled to H^+ pumping, which drives ATP synthesis (Figure 2).

Denitrification has been associated with several detrimental environmental effects. For example, denitrifying bacteria efficiently utilize nitrate that is applied as fertilizer to crops (1,6,7). Depending on soil conditions, up to 40% of the fertilizer may be lost to denitrifiers, which results in millions of dollars in wasted resources annually in the United States alone. Moreover, in fungi and a few bacteria, the denitrification pathway ends prematurely with the production of the greenhouse gas nitrous oxide (2,4,8). N_2O has a remarkably stable half-life of about 150 years (7,8). Studies show that N_2O reaches the stratosphere, where it is oxidized to NO by reaction with singlet oxygen, which is produced

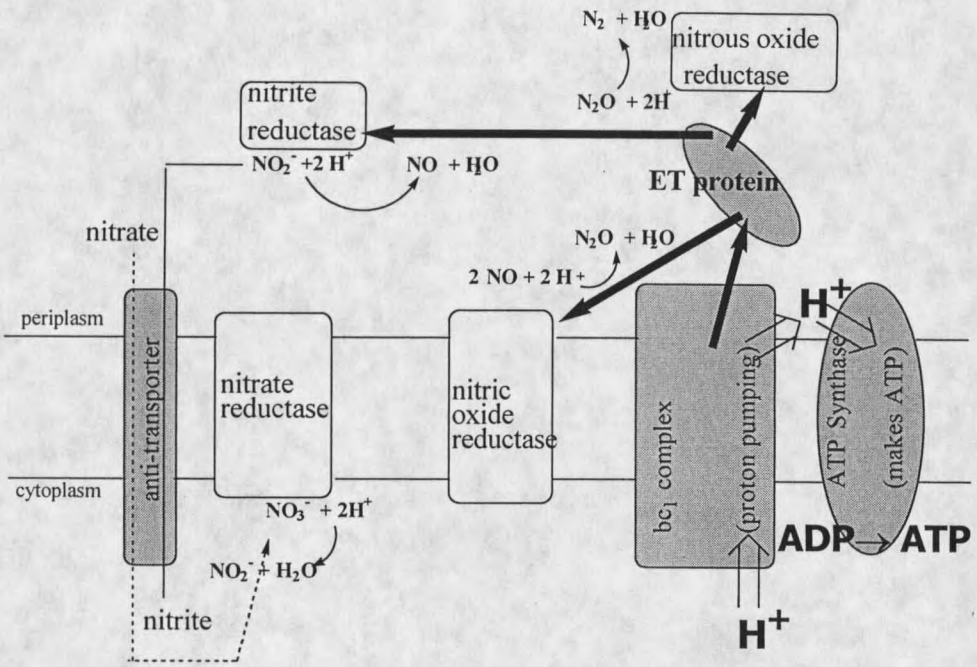

Figure 2. The Denitrification Pathway. ➡ represents electron transfers between proteins. ⇨ represents the flow of protons used to drive ATP synthesis. For simplicity, only the membrane-bound form of nitrate reductase is shown.

by the photochemical decomposition of O_3 (9). NO continues reacting with O_3, further depleting the earth's protective ozone layer.

While some aspects of denitrification are environmentally harmful, others are beneficial and have begun to be exploited commercially. For example, denitrifiers have been successfully used in bioremediation to remove nitrates from water supplies (10-12). It is perhaps ironic that the same organisms that mandate the use of high levels of fertilizer also serve to purify the groundwater that, as a result, becomes contaminated. Another area of environmental concern for which denitrification may provide answers is N_2O pollution. In addition to the N_2O produced from biological sources (1,8), significant amounts of N_2O are

released as a waste product during industrial nylon production (13) and biomass burning (14). Studying the biological mechanism of N_2O reduction to N_2 may aid the design of an inexpensive biomimetic catalyst. In addition to these anthropogenic applications, denitrification in marine sediments plays an important role in regulating the N-oxide flux in seawater, accounting for the removal of over 50% of N-oxides from the marine environment (15,16).

Key Enzymes of Denitrification

In the first step of denitrification, the enzyme nitrate reductase (NaR) catalyzes the 1-electron reduction of nitrate to nitrite (1,2,5). Both periplasmic and membrane-bound forms of NaR have been identified, and the role of the periplasmic NaR has long been debated (2,5). It now appears that the periplasmic enzyme plays different roles in different organisms, serving as the primary NaR during anaerobic denitrification in some bacteria (17,18), while in others it functions only during the aerobic process (19).

In addition to being the first enzyme of denitrification, NaR is also found in the assimilatory reduction pathway. In the latter, product nitrite is enzymatically converted to ammonia. Regardless of the pathway in which they function, NaRs typically contain the cofactor molybdopterin guanine dinucleotide (MGD), and an iron-sulfur electron-transfer center, in addition to heme centers (2,5). The structural homology among NaRs is not surprising, given that the enzymes catalyze the same reaction. Recently molybdenum-free membrane-bound and periplasmic NaRs have been isolated from the vanadate-reducing

bacteria *Pseudomonas isachenkovii*, but structural information on these variants is limited (20).

In the second step of denitrification, nitrite is reduced to nitric oxide by the enzyme nitrite reductase (NiR) (2). Two types of NiRs have been characterized from denitrifiers: a copper enzyme (CuNiR) (21-24) and a heme-containing protein (cd_1NiR) (25-30). While denitrifiers contain either CuNiR or cd_1NiR, but not both, it has been shown that CuNiR can be functionally produced in a cd_1NiR$^-$ mutant strain (31). Both types of NiR have been extensively characterized and bear little resemblance to each other, or to the assimilatory NiR, which reduces nitrite to ammonia.

Once nitric oxide is released from NiR it is further reduced to nitrous oxide by the enzyme nitric oxide reductase (NOR). NOR, which is found in the cytoplasmic membrane, contains one type *c* and two type *b* cytochromes, in addition to a non-heme iron (32-37). Several structures for the active site of NOR have been proposed, including species in which the non-heme iron is linked by a μ-oxo or hydroxy bridge to one of the *b* hemes (37). Two possible mechanisms for NO reduction have been suggested. In the first, a single NO is bound and reduced to the nitroxyl anion (NO^-), which is then non-enzymatically converted to N_2O (38). Alternatively, two molecules of NO could bind to the dinuclear center, setting up the formation of the N-N bond (37). Additional biophysical characterization of NOR is needed to fully understand the structure/function relationship of the metal sites in this intriguing enzyme.

Nitrous oxide reductase (N_2OR) is the terminal enzyme of denitrification and reduces N_2O by two electrons to N_2. Although N_2ORs are typically soluble, periplasmic enzymes (39-44), at least one may be membrane-associated (45). To date, all N_2ORs have been found to be homodimers that contain two distinct dinuclear copper centers per subunit. The electron transfer copper site, called Cu_A, is analogous to the Cu_A site in cytochrome *c* oxidase (46-52), and the catalytic copper site, called Cu_{Cat}, is where N_2O is thought to bind and be reduced (53-55).

It is noteworthy that the terminal oxidases of aerobic respiration, *e.g.*, cytochrome oxidase and quinol oxidase, have structural components that closely resemble elements of NOR and N_2OR (2, 56-59). Additionally, denitrification genes have been identified in several halophilic and hyperthermophilic members of the archaea kingdom (2,60-62). Thus, the study of the denitrification genes and their related proteins may provide clues to the evolution of enzymes involved in bioenergetic processes.

Regulation of Denitrification

The regulation of denitrification genes has been extensively studied in *Pseudomonas stutzeri, Paracoccus denitrificans, Pseudomonas aeruginosa,* and *Rhodobacter sphaeroides* (63–77). Regulation responds to both the oxygen tension and the type of N-oxide present. While in principle two transcriptional regulators (one for O_2, one for N-oxides) could globally control the biosynthesis of all denitrifying enzymes, the data indicate that a far more complex situation exists. For example, in *P. stutzeri* both nitrate and nitrite induce the

transcription of all four denitrification enzymes, yet different transcriptional factors are used for each enzyme (63,64). Moreover, the response to exogenous signals differs among the denitrifiers studied, which hampers the efforts to identify and characterize regulatory proteins involved in denitrification. As previously mentioned, nitrite induces the production on NiR and NOR in *P. stutzeri*, while in *P. aeruginosa* and *R. sphaeroides* NO is the regulatory signal for the production of these enzymes (66,68,69). Despite this difference, regulation of NiR and NOR is tightly coupled in all organisms examined, perhaps to prevent the accumulation of NO, which may be toxic to cells.

The expression of denitrification proteins in response to oxygen is controlled by FNR-like proteins (2,65,67,70-77). FNR, the fumarate and nitrate reduction regulator protein discovered in *E. coli*, is related to CRP, the cyclic AMP receptor protein (78). Unlike CRP, FNR contains an N-terminal Cys-ligated iron-sulfur moiety that acts as an O_2 sensor. FNR recognizes a palindromic TTGAT-N_4-ATCAA sequence and both positively and negatively regulates transcription. FNR analogs from several denitrifiers have been identified and shown to be directly involved in denitrification: these include ANR, DNR, and NNR, as well as FnrA, FnrD, and FnrP. Some of these proteins possess the N-terminal Cys cluster indicative of the O_2-sensing Fe-S cluster, while others more closely resemble the FixK regulator in that they lack a Cys cluster. Evidence suggests that denitrification uses a cascade of multiple O_2-responsive regulatory proteins within the same organism.

Structure of Nitrous Oxide Reductase

The structural gene of N_2OR, *nosZ*, has been sequenced entirely for seven different denitrifiers (59,79-83) and partially for an additional 44 organisms (mostly uncultured marine bacteria) (84-87). Comparison of these translated amino acid sequences (Appendix A) reveals a C-terminal Cu_A binding motif that is similar to the Cu_A region of cytochrome *c* oxidase (59,79,87). In the latter enzyme, the two coppers of the dinuclear Cu_A center are bridged by two Cys residues, forming a near-coplanar geometry. Two His, one Met, and the peptide carbonyl oxygen of Glu provide the other ligands to the dinuclear site (51,52). In N_2OR, Glu is replace by Trp in all known sequences except that from *Ralstonia eutropha*, where the corresponding amino acid is His.

In addition to the amino acids that are assigned as Cu_A ligands, there are several conserved amino acids (9 His, 3 Met) that are frequently coordinated to copper, and are therefore candidates for Cu_{Cat} ligands (59,79,80). Despite the lack of a strictly conserved Cys (other than the Cys associated with the Cu_A center), numerous spectroscopic studies have long been interpreted as evidence for a Cu-Cys thiolate bond in the Cu_{Cat} site of *P. stutzeri.* (53,54,88,89). Sequence analysis suggests that a Cys residue found in a variable position (within a 5-residue stretch) near the Cu_{Cat} domain might provide the thiolate bond (59). Alternatively, Farrar *et al* have proposed that all spectroscopic signatures previously thought to arise from a Cu_{Cat}-thiolate bond are in fact associated with a structural variant of the Cu_A