Purification and characterization of gene products of the nitrous oxide reductase gene cluster
by Shannon Michelle Henery

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

Abstract:
Denitrification is a key pathway in the global nitrogen cycle. It comprises a series of reductions from nitrate through nitrite, nitric oxide, nitrous oxide and finally to dinitrogen gas. This dissimilatory pathway is carried out by soil, marine, and freshwater organisms, removing nitrogen from the biosphere and returning it to the atmosphere. Understanding the steps of the pathway on a molecular level could have a substantial impact on agriculture, control of air pollution, as well as on removal of nitrate pollution from water.

The focus of this research was on the final reduction of nitrous oxide (N2O) to dinitrogen gas by the enzyme nitrous oxide reductase (N2OR). This work involved purification and characterization of N2OR as well as some of its accessory proteins, which are required for production of active enzyme containing all of the necessary copper. N2OR was purified in several different forms, each containing different amounts of copper, different levels of activity, and showing different spectroscopic characteristics. N2OR is a homodimer containing two Cu sites per monomer. These are the binuclear, CuA electron transfer site and the novel, tetranuclear, sulfide-bridged CuZ catalytic site.

The genes coding for the accessory proteins are organized into three separate transcriptional units in a cluster with the nosZ (N2OR) gene. These units are nosR, nosDFYL, and nosX. The accessory protein NosX, which is speculated to be involved in either assisting regulation of N2OR synthesis or loading of the CuA site in N2OR, was purified and shown to be a flavoprotein containing FAD as the cofactor. Another auxilliary protein, NosD, was purified with two other proteins (one being N2OR) tightly bound to it. This work begins the elucidation of the roles of these accessory proteins in N2OR processing.

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APPROVAL

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Shannon Michelle Henery

This dissertation has been read by each member of the dissertation 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.

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ABSTRACT

Denitrification is a key pathway in the global nitrogen cycle. It comprises a series of reductions from nitrate through nitrite, nitric oxide, nitrous oxide and finally to dinitrogen gas. This dissimilatory pathway is carried out by soil, marine, and freshwater organisms, removing nitrogen from the biosphere and returning it to the atmosphere. Understanding the steps of the pathway on a molecular level could have a substantial impact on agriculture, control of air pollution, as well as on removal of nitrate pollution from water.

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INTRODUCTION

The Global Nitrogen Cycle and Denitrification

The global nitrogen cycle comprises a variety of redox reactions. Most of these reactions are linked to electron transport pathways in the cytoplasmic membrane and subsequent ATP sysnthesis in bacteria. The exception to this is nitrogen fixation, which requires ATP (1). In soil, nitrogen is typically found as nitrate under aerobic conditions and as ammonia or ammonium under anaerobic conditions (2).

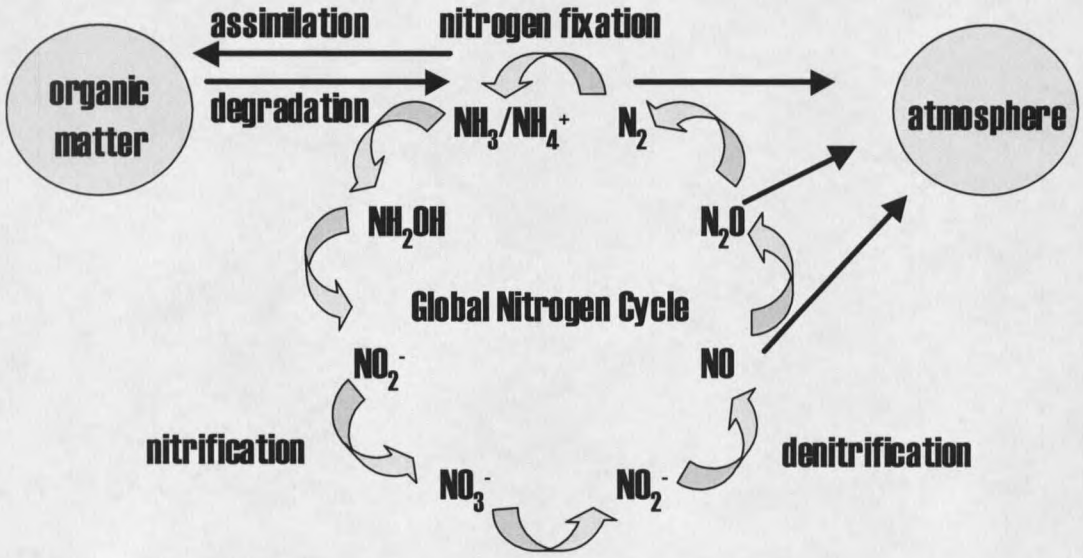

Figure 1. A simplified overview of the global nitrogen cycle.

Denitrification is a very important pathway in the global nitrogen cycle. It is the process responsible for removal of fixed nitrogen from the biosphere, sending it back into

the atmosphere in the form of N_2. Many bacteria carry out denitrification as a form of facultative anaerobic respiration in anaerobic and semi-anaerobic conditions where N-oxides are available, with the N-oxides replacing oxygen as terminal electron acceptors (3;4).

Understanding the denitrification pathway will prove invaluable for many reasons. Nitrate applied to the soil for agriculture pollutes drinking water when it leaches into aquifers, rivers, or other ground water. In addition, 5-10% of nitrogen applied to the soil as fertilizer is lost to the atmosphere in the form of NO (5-7). N_2O, the final intermediate in the denitrification pathway, is 310 times more effective as a greenhouse gas than carbon dioxide, and its alarming 0.25% per year increase in our atmosphere (8) originates from anthropogenic sources such as release from soil organisms reducing nitrates applied in agriculture, biomass burning, and nylon manufacture (2;9-11). Approximately 5% of the greenhouse effect due to anthropogenic sources is a result of atmospheric N_2O (12). In addition to being a potent greenhouse gas, N_2O has a half life of 120 years in the atmosphere. Finally, N_2O is the primary source of NO_x gases in the stratosphere. Oxidation of N_2O in the stratosphere to NO causes destruction of ozone (3;12;13). With N_2O at its highest level in the past 45,000 years (15), its continuing increase could contribute substantially to ozone depletion.

The Metalloenzymes of Denitrification

The denitrification pathway consists of a series of reductions from nitrate to nitrite, nitrite to nitric oxide, nitric oxide to nitrous oxide, and finally to dinitrogen. These steps are each catalyzed by a specific oxidoreductase shown in scheme 1 (4).

$$\underset{\text{nitrate reductase}}{NO_3^-} \rightarrow \underset{\text{nitrite reductase}}{NO_2^-} \rightarrow \underset{\text{nitric oxide reductase}}{NO} \rightarrow \underset{\text{nitrous oxide reductase}}{N_2O} \rightarrow N_2$$

Scheme 1. The denitrification metalloproteins and their reductions.

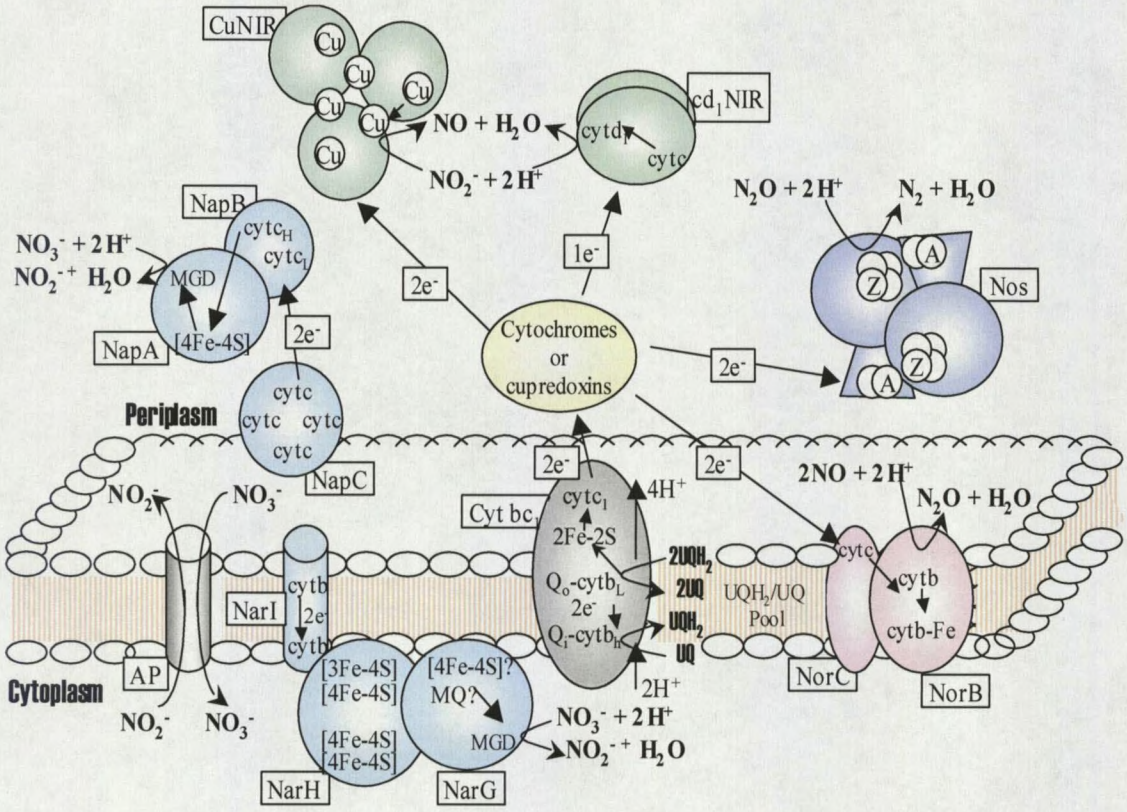

Figure 2. Orientation of the metalloenzymes of denitrification.

Each of these reductases are metalloproteins, and extensive work is being carried out to reveal the structures and mechanisms of them in order to better understand this very important pathway. X-ray structures are available for the proteins involved in each step (16-21) with the exception of nitric oxide reductase, which is an integral membrane protein.

Nitrate reductase.

Nitrate reductase can be found in cytoplasmic (Nas), periplasmic (Nap) and membrane bound (Nar) forms. Nas is the assimilatory nitrate reductase and will not be discussed further here. All of the nitrate reductases contain molybdopterin cofactors in their active sites (17;18). Nar catalyzes the following reduction (UQ=ubiquinone; UQH_2=menahydroquinone):

$$NO_3^- + UQH_2 \rightarrow NO_2^- + UQ + H_2O \qquad (19)$$

Most of the Nap enzymes have multiple subunits, with one subunit (NapA) containing a [4Fe-4S] center and a molybdenum coordinated by two molybdopterin guanine dinucleotide moieties. The Nar enzyme is composed of three subunits. The Nar subunits α and β are located in the cytoplasm at the membrane surface and contain a Mo center coordinated by a *bis*-molybdopterin guanine dinucleotide (MGD) cofactor (in the α component) along with FeS centers, while the γ subunit is forms a transmembrane α-helical structure containing two heme groups.

These heme groups donate electrons to the FeS centers located in the β and possibly the α subunit (2). EPR and mutagenesis experiments indicate that each of the

two hemes are probably coordinated by two axial His ligands (20). These transmembrane oriented hemes are predicted to be common in bacterial electron transport chains(21).

Nitrite reductase.

The structural gene for nitrite reductase, NirS, is encoded in a gene cluster comprising genes for its transcriptional regulation, cofactor assembly/insertion (27). The reductase is found in three forms. One form is a multiheme cytochrome c nitrite reductase which reduces nitrite to ammonia. The other two forms, which are found in denitrifying bacteria, are the dimeric cytochrome cd_1 enzyme and a trimeric, Cu containing enzyme (22). The two dissimilatory nitrite reductases catalyze the following reduction (with c = cytochrome and Cu = blue-copper pseudoazurin):

$$NO_2^- + c^{2+}/Cu^+ + 2H^+ \rightarrow NO + H_2O + c^{3+}/Cu^{2+} \qquad (19)$$

A structure for the multiheme, cytochrome *c* nitrite reductase has been determined (23). This structure shows it to be a homodimer with each monomer containing five tightly packed hemes. The orientation of the hemes has been found to be conserved in a number of multiheme proteins exhibiting different functions and structures. The active site heme is surrounded by a channel lined with positively charged residues which stabilize the anionic substrate and store protons used in reducing the nitrite to ammonia (22).

The cytochrome cd_1 nitrite reductase is a homodimeric protein that contains a *c*-type heme as well as a d_1-type heme in each monomer (2;22). Crystal structures have shown that the d_1-type heme Fe is the site at which the nitrite is bound (18;22). The *c*-