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
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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.

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 auxiliary 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.
PURIFICATION AND CHARACTERIZATION OF GENE PRODUCTS OF THE 
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by

Shannon Michelle Henery

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APPROVAL

of a dissertation submitted by

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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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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 synthesis 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).

\[
\begin{align*}
\text{NO}_3^- &\rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2
\end{align*}
\]

\text{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):

$$\text{NO}_3^- + \text{UQH}_2 \rightarrow \text{NO}_2^- + \text{UQ} + \text{H}_2\text{O} \quad \text{(19)}$$

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 $\alpha$ and $\beta$ 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 $\alpha$ component) along with FeS centers, while the $\gamma$ subunit is forms a transmembrane $\alpha$-helical structure containing two heme groups.

These heme groups donate electrons to the FeS centers located in the $\beta$ and possibly the $\alpha$ 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 cd₁ 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):  

\[
\text{NO}_2^- + \text{c}^{2+}/\text{Cu}^+ + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O} + \text{c}^{3+}/\text{Cu}^{2+}
\]  

(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 cd₁ nitrite reductase is a homodimeric protein that contains a c-type heme as well as a d₁-type heme in each monomer (2;22). Crystal structures have shown that the d₁-type heme Fe is the site at which the nitrite is bound (18;22). The c-
type heme transfers an electron to the $d_1$ heme. It is interesting to note that cytochromes $cd_1$ from different organisms show different residues ligated to the heme centers and therefore different ligand switching during catalysis. The crystal structure of the oxidized form from *Thiosphaera pantotropha* shows His-His coordination of the $c$-type heme and His-Tyr coordination of the $d_1$ heme (24). It was correctly hypothesized from this crystal structure that the Tyr ligand would be lost upon reduction of the enzyme, making the $d_1$ heme pentacoordinate for nitrite binding. Crystal structures of the reduced form showed this change in $d_1$ coordination (18). Ligand switching was also observed for the $c$ heme upon reduction, going from His-His coordination to His-Met coordination. In contrast the oxidized form of $cd_1$ nitrite reductase from *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* has His-Met coordination at the $c$ heme (25). While the $d_1$ heme in the enzyme from *P. aeruginosa* is also coordinated by a Tyr, it differs from the *T. pantotropha* enzyme in the fact that the ligand comes from the other monomer for *P. aeruginosa* (26).

![Figure 3. Proposed mechanism for cytochrome cd1 nitrite reductases (33).](image-url)
The mechanism of nitrite reduction at the heme $d_1$ center, shown in figure 3, has been fairly well understood for several years now. Studies of synthetic heme $Fe^{2+}-NO^+$ compounds (34;35) indicated that a ferrous heme $d_1-NO^+$ intermediate would be very unstable (although it has been observed by FTIR), and that an electron transfer producing ferric heme $d_1$ and NO is likely to take place (33).

The intermediate ferrous heme $d_1$-NO resulting from a one electron reduction of heme $d_1$-NO$^+$ is unlikely to be physiologically relevant as the half life of NO bound to ferrous heme is measured in days (36). This slow dissociation is also reflected in the strong inhibitory effect of NO on its own formation (33). Thus, formation of the $Fe^{3+}$-NO species via an internal electron transfer appears to be the most likely path for $cd_1$ nitrite reductases to follow.

The Cu containing, homotrimeric nitrite reductases are divided into two categories, green and blue, and both contain two different Cu centers. These are a type 1 center and a catalytic, type 2 Cu site. The type 1 center has a distorted trigonal planar geometry with 2His and a Cys being the planar ligands and the Met being a weak axial ligand. The geometry of this center is likely to be responsible for the color differences in the blue and green forms of the enzyme, showing His-Cu-Met angles of 115° and 132° respectively (27). Additionally the Cu-S(Met) distance in the green type I center of the structure from $A. cycloclastes$ is somewhat shorter than that observed in the typical type I Cu center, but resonance Raman exciting into the 485 nm band of the green form also showed that this center exhibited ligand to metal charge transfer from Cys (33). The Cu in the type 2 center is ligated by two His from one monomer and a third His from another
monomer. A fourth ligand is found to be water in the structure from *A. cycloclastes* and a chloride in the structure from *A. xylosoxidans*. This exogenous ligand has been shown to be replaced by the substrate in structures from both organisms (28;29). The oxidized type 2 center has been proposed (see figure 4) to bind nitrite by both of its oxygen atoms based on structural data. A different crystallographic study performed on the oxidized enzyme from another organism indicates that a Cu-N bond may form after an isomerization (30). Because Cu(I) is found to coordinate N in a model complex, and O-coordination is expected for Cu(II), until it is known whether the type II center is reduced before or after binding of NO₂⁻, neither orientation can be ruled out. Thus the mechanistic details remain to be worked out.

Figure 4. Catalytic mechanism for copper-containing nitrite reductases proposed by Boulanger and Murphy (41).
Nitric Oxide Reductase.

Nitric oxide reductase is a membrane bound, heterodimeric enzyme that catalyzes the following reaction:

\[
2\text{NO} + 2e^{2+} + 2H^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} + 2c^{3+}
\] (19)

The subunits are typically 17 kDa and 53 kDa. The smaller subunit contains a heme c while the larger subunit contains two hemes b and non-heme iron (19;22;31). The amino acid sequence of the larger heme b containing subunit predicts 12 helices spanning the membrane. NO reductase is predicted to have membrane topology very similar to that of cytochrome c oxidase (CcO), and it contains the six conserved His residues found to bind the prosthetic groups in CcO (9). A high spin b heme and non-heme iron appear to make up a novel, dinuclear catalytic center (29;31-35). This model of the catalytic center is supported by the fact that the non-heme iron and the high spin heme b are EPR silent, implying possible antiferromagnetic coupling. Resonance Raman indicates that, in addition to the low spin, hexacoordinate hemes b and c, a high spin pentacoordinate heme b, presumed to be the catalytic site, is also present. Upon addition of CO (making the catalytic heme b hexacoordinate), the vibration at 218 cm\(^{-1}\) due to pentacoordinate, ferrous heme disappears. The current model for the catalytic site based on this and other data consists of the binding of two molecules of NO to the two iron atoms in the catalytic site, followed by breakage of the His-iron bond (of the high spin heme b iron center). This maintains the pentacoordinate state seen by resonance Raman. Upon reduction of NO to N\(_2\)O the oxidized iron becomes oxo bridged. Reduction and
protonation of the active site then returns it to a diferrous state with heme b once again ligated to His (35).

Nitrous Oxide Reductase (N2OR).

Nitrous oxide reductase is a periplasmic homodimer with a molecular weight of ~67 kDa per monomer and catalyzes the following reduction (with c = a cytochrome or pseudoazurin):

\[ \text{N}_2\text{O} + 2c^{2+} + 2H^+ \rightarrow \text{N}_2 + \text{H}_2\text{O} + 2c^{3+} \]  \hspace{1cm} (19)

Each monomer contains a maximum of 6 Cu atoms arranged in a binuclear Cu_A site and a novel, tetranuclear, sulfide-bridged Cu_Z site. A schematic of the Cu_A center can be seen in figure 7 below. Cu_A is responsible for electron transfer (as in cytochrome c oxidase) however the identity of the physiological electron donor to this center remains to be determined. Inhibition studies on the cytochrome bc_1 complex in Rhodobacter species showed it to be partially responsible for electron transport to nitrous oxide reductase, but an as yet unidentified alternate path was also found to be present (47). A homolog of cytochrome c_2 (an electron acceptor in the path from cytochrome bc_1), cytochrome c_550, has been proposed to be involved in transport of electrons to nitrous oxide reductase in P. denitrificans (48). The Cu_Z center is postulated to be the catalytic site. Cu_Z is the first known biological copper sulfide cluster (see figure 11).

The first steps toward identification of the N2OR enzyme were carried out by Matsubara and Mori in 1968 (36), in which inhibition of N_2O reduction was observed in the presence of metal ligating compounds. Copper was identified as the specific metal required for N_2O reduction in Alcaligenes in 1980 (37) and in Pseudomonas stutzeri.
(formerly *Pseudomonas perfectomarinus*) in 1982 (38). In 1982 the first purification of \( N_2 \)OR from *Pseudomonas stutzeri* was reported by Zumft and Matsubara (39).

With the exception of the membrane bound forms of \( N_2 \)OR from *Flexibacter canadensis* (40) and *Thiobacillus denitrificans* (41), \( N_2 \)OR has typically been found to be a soluble, periplasmic protein (52). In addition to the presence of a signal peptide in the sequence for the *nosZ* structural gene (42), preliminary cell fractionation work carried out by Zumft and Minagawa in 1988 suggested a periplasmic location for \( N_2 \)OR (56;44). Komer and Mayer demonstrated its periplasmic location more conclusively in *P. stutzeri* in 1992, using Immunogold labelling of ultrathin sections of the cells at low temperature, as well as formation of spheroplasts and two-dimensional electrophoresis (45). The Immunogold label was found to be in the periphery of the cell even in mutants deficient in Cu chromophore assembly. This study as well as studies carried out by Zumft, et al. (46) led to the proposal that \( N_2 \)OR was translocated through the cytoplasmic membrane in an inactive, apo-form with subsequent insertion of the Cu chromophore taking place in the periplasm.

**The *nos* genes and gene products.**

Insertion of the Cu chromophore was found to require the presence of several genes in an approximately 8 kbp region (46). Transposon Tn-5 mutagenesis of *Pseudomonas stutzeri* produced a mutant that synthesized an inactive apo-\( N_2 \)OR in levels comparable to the wild-type organism (47). By SDS-PAGE and crossed immunoelectrophoresis it was shown that \( N_2 \)OR was present in some of the mutant
strains, but accumulation of N₂O during growth on nitrate indicated that the enzyme was inactive. Purification of the inactive N₂OR revealed a dimeric protein, but lacking Cu. In 1990 Zumft et al. mapped the Tn5 insertions known to cause production of apo-N₂OR to a region immediately downstream of the structural gene for N₂OR (46). Sequencing of the 3.2 kbp region showed it to contain at least three genes in one transcriptional unit, nosDFY (see Figure 5).

![Figure 5. Organization of the nitrous oxide reductase (nos) gene cluster. The four transcriptional units are shown separated by vertical lines.](image)

Tn5 mutations were mapped to each of the three genes, and in each of the three different mutants the N₂OR isolated contained no more than 2 Cu atoms/140 kDa. N₂OR from these mutant strains also exhibited no activity even when cultures were grown in the presence of excess (200 μM) Cu(II) (46). Nos cluster Tn5 mutants in this study were detected by inability to grow on N₂O. Because no mutations were mapped to the region downstream of nosY, it is likely that loss of nosL (sequenced in later studies) is not detrimental to formation of the chromophore under the conditions used in these experiments. The nosF and nosY genes were found to overlap with the genes upstream from them, implying possible "transcriptional readthrough from the nosD promoter and a
closely controlled stoichiometric formation of the gene products (46)." The nosRZDFY gene cluster was also located in the nod megaplasmid of Sinorhizobium meliloti (formerly Rhizobium meliloti) in 1996 by Chan et al. using Tn5 mutagenesis and Southern hybridization (48).

The nosD gene product was predicted to be a hydrophilic protein with a molecular weight of ~46 kDa and has a signal sequence, indicating a periplasmic location. Its periplasmic location was also confirmed by a comparison using 2D gel electrophoresis on the periplasmic fractions from both wild type and nosD Tn5 mutant cultures. A spot at pl 5.7 and 45 kDa was missing in the mutant (46). Attempts to purify NosD from a heterologous expression system failed. Although the annotation for each nosD gene sequence published predicts a periplasmic Cu binding protein, alignment of the eight NosD sequences published to date (see Appendix B) reveals only one conserved His, 2 conserved Met, and 2 conserved Tyr. There are no strictly conserved Cys residues. To date there is still no conclusive evidence that NosD binds Cu.

The amino acid sequence of the NosF protein showed the two nucleotide binding motifs found to be conserved in a family of nucleotide-binding proteins (62). The nosY gene product is a hydrophobic protein and has a predicted molecular weight of ~28 kDa. Hydropathy plot analysis of the amino acid sequence of NosY showed six predicted transmembrane helices (46). The orientation of the gene products of the nos cluster in a bacterial cell is shown in Figure 6.

In addition to the DFY genes of the nos cluster, nosA was also found to be important for Cu chromophore formation in N2OR in certain strains of P. stutzeri. NosA
is a Cu-binding protein that forms a channel through the outer membrane, and can cause production of apo-N₂OR when mutated (46). Expression of NosA occurs under anaerobic (and perhaps semi-anaerobic) conditions, and is repressed by exogenous Cu concentrations of 20 μM and higher. In 1991 Ingraham et al. cloned and sequenced nosA finding the predicted amino acid sequence to be homologous to part of BtuB, an outer membrane vitamin B₁₂ receptor in *E. coli* (49). The presence of a short, highly conserved sequence of amino acids homologous to IutA, FepA, FhuA, and BtuB (all *E. coli* TonB-dependent outer membrane proteins) may, with more detailed characterization of these proteins, eventually give clues to the role of NosA in Cu loading of N₂OR because these proteins, with the exception of BtuB, are all involved in metal transport and are also induced by limiting their substrates (49).

![Figure 6. Orientation of the nos gene products in gram negative bacterial cells.](image)

In the 1985 Tn5 mutagenesis studies carried out by Zumft et al., the regulatory component of the *nos* gene cluster was also identified and characterized. NosR is predicted to span the membrane seven times and contain the 2[4Fe-4S] clusters found in
some bacterial ferredoxins (48;50). Its molecular weight is predicted to be 81,872 kDa. A helix-turn-helix motif found in the central hydrophilic domain of NosR indicates that it may be a positive regulatory factor (51). The c-terminal “CX₃CP-helix-helix-CX₃CP-FeS-FeS” domain is similar to the membrane proteins NapH and MauN, which are believed to have electron transfer functions and interact with the periplasmic components NapG and MauM respectively (66).

The **nosL** gene in the **nos** gene cluster of *P. stutzeri* was identified in 1996 by Zumft et al., and was proposed to code for a protein disulfide isomerase based on the presence of an N-terminal CHVC thiol/disulfide pair characteristic of these proteins (67). The **nosL** and **nosX** genes were located in *Sinorhizobium meliloti* by Chan et al. in 1997 (52). The predicted primary structures of NosL from *S. meliloti* and *A. cycloclastes* both lacked the residues that had led to the proposal of a possible disulfide isomerase function for the NosL gene product. The **nosL** gene product was predicted to be anchored by a lipid moiety to the outer membrane via a thioether bond to its N-terminal Cys (69).

In work published by McGuirl et al. NosL has been heterologously expressed in *E. coli* (in a form with the N-terminal Cys replaced by Met-Asp to eliminate the problematic lipid anchor), allowing purification of a monomeric protein with a MW of 18,540 which binds one Cu(I) under anaerobic conditions and forms an apo, dimeric form under aerobic conditions (69;66). Reaction of NosL with reagents such as pyridine disulfide (PDS) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) indicated free Cys (not metal or disulfide bound) only in the monomeric apo-protein, but not in the Cu(I) containing monomer or the dimeric apo-protein even under denaturing conditions (69).
EXAFS data reported for NosL best fit a first shell of Cu-(O,N)S₂, although a four coordinate site including at least two sulfur atoms cannot be ruled out (69). Attempts to determine the function of NosL have been unsuccessful to date. While a role in electron transfer to N₂OR has been essentially ruled out via visible absorption and EPR studies, the facts that the nosL gene is found in the same transcriptional unit as three genes (nosDFY) necessary for Cu₂ formation, and that the protein is able to easily release the copper ion upon oxidation help support the hypothesis that it could function as a metallochaperone for formation of the catalytic site of N₂OR (69). Unfortunately, neither Tn5 nor gentamicin resistance cassette interruption of the nosL gene have any apparent phenotype under the conditions studied to date (69), with the exception of a slight decrease in the growth rate of P. stutzeri upon insertion of a gentamicin resistance cassette into the gene (67). While BLAST searches have failed to reveal homologs to NosL, the signal sequence of NosL shows similarity to that of an outer membrane lipoprotein e (P4) from Haemophilus influenzae (67;70). The e (P4) protein has been found to be an unusual phosphomonoesterase required for heme transport into H. influenzae (70). This protein requires the presence of divalent copper for its catalytic activity. The significance, if any, of the small similarity between e (P4) and NosL is still unclear.

Tn5 insertion into the region corresponding to the newly discovered NosX gene in S. meliloti resulted in a Nos⁻ phenotype (52). This gene was found to reside in a fourth and final complementation group of its own, and was predicted to be a periplasmic protein based on the presence of a signal sequence. The homology of NosX to NirX (and
NosR to NirI) could indicate that NosX is involved in transcriptional regulation with NosR, as was found for the NirIX system in *P. denitrificans* (70). This potential link between NosR and NosX was also previously proposed by Chan et al. (52) based on "anomalous" behavior of strains carrying Tn5 mutations in both genes during a complementation study. Adding to the list of possible functions for NosX, Spanning et al. published mutagenesis results in 2000 that indicated possible involvement of NosX (and/or its homolog NirX) in formation of the Cu$_A$ center of N$_2$OR (71). To date no further evidence has been reported that corroborates this proposed function.

In summary, the *nos* gene cluster typically comprises four separate transcriptional units, each necessary for the production of fully functional N$_2$OR. The gene products of the *nos* cluster have been pursued in the Dooley lab, resulting in purification and at least partial characterization of N$_2$OR, NosD, NosL, and NosX, with future purifications of NosF and NosY very close at hand.

**Spectroscopic characterization of N$_2$OR.**

Although it was known in 1990 that genes in the *nos* cluster were necessary for formation of the Cu chromophore in N$_2$OR, the true nature of the Cu site(s) would not be understood for another decade. As early as 1982 Zumft and Matsubara (53) may have seen different forms of N$_2$OR during isoelectric focusing, although only in the form of a "pink-to-violet band (often double band, but otherwise no discernible properties)." This aerobically isolated enzyme was pink in color, showed a copper content of ~8 Cu atoms/120,000 MW, and would later be called form II. In 1985 Zumft, Coyle, and
Frunzke demonstrated that anaerobically isolated N\textsubscript{2}OR from \textit{P. stutzeri} was spectroscopically distinct from and exhibited higher activity than the enzyme previously isolated aerobically (54). The previously isolated pink, aerobic form, form II, (see figure 5a below) showed a specific activity of 0.9-2.1 \textmu mol N\textsubscript{2}O/min-mg protein (55), while the specific activity of the anaerobically isolated purple form, form I, was 3-5 fold higher with a maximum of 6.9 \textmu mol N\textsubscript{2}O/min-mg protein (43;53-55). The visible absorption spectrum of the high activity purple form showed maxima at 540 and 780 nm, with the 480 and 620 nm maxima observed in the pink form being present only as shoulders (see figure 7b).

Upon dithionite reduction, both forms exhibited a single maximum at 640 nm, resulting in an inactive blue form, form III. Copper chromophores reduced by dithionite had been found to exhibit virtually no absorption in the visible region, making the the
strong ~640 nm absorption in N$_2$OR unusual and indicating the possible presence of Cu that could not be reduced even in the presence of an excess of dithionite.

It was proposed that the "reduced" catalytic Cu site was "stabilized by thiol or disulfide sulfur with substantial spin density delocalized onto sulfur" (56). Oxidation by ferricyanide under aerobic conditions reverted both blue forms (originating from purple and pink) to the pink form, as determined by the activity and visible spectrum (55). Reduction of the purple form I to form III was found to be fully reversible under anaerobic conditions using ferricyanide, hydrogen peroxide, and nitric oxide as oxidants (56).

Titrations with dithionite were carried out, revealing two phases in the reduction process. The first phase took place in less than 30 seconds and consisted of a decrease in the 540 and 640 nm absorbance by 50% (56). The second phase was slower, taking minutes rather than seconds, and was identified by the appearance of the 650 nm peak. An isosbestic point was present at 625 nm. These titrations were also carried out at various pH values, showing a decrease in the intensity of the 650 nm absorption band at higher pH (56).

Both spectrophotometric and gas chromatographic assays were run on the pink and purple forms of N$_2$OR (55). The spectrophotometric assay used photochemically reduced benzyl viologen as the electron donor. Inhibition of the oxidation of benzyl viologen was observed at low enzyme concentrations. The gas chromatographic assay also showed inhibition after an initially rapid rate. This assay was carried out using hydrogenase and a headspace of H$_2$ gas. It was speculated that the reducing conditions of
this reaction led to the inhibition as the blue form of N$_2$OR, obtained from reduction via dithionite, ascorbate, or ferrocyanide, had been found to be inactive (55). Reactivation was achieved by addition of ferricyanide, leading to the conclusion that active enzyme must require an intermediate redox state that could not be attained with the reductants used. It is interesting to note that when inactive, apoprotein was produced by anaerobic dialysis in cyanide under reducing conditions, over 90% of the Cu could be reconstituted, but the visible absorption spectrum revealed a new species, form IV.

The reconstituted N$_2$OR showed absorption maxima at 480 nm, 530 nm, and 800 nm, but the 620 nm band found in pink N$_2$OR was no longer present (55). This new form did not regain activity, and could not be reduced to the blue form III with an excess of dithionite. The nearly apo form, form V, obtained in the Tn5 mutagenesis studies discussed above was found to be a catalytically inactive, pink form. Loss of the pink color was observed upon addition of 0.5e$^\ddagger$/Cu, but no blue form was produced and the protein remained inactive (56). Ability to be reduced to form III appears to be necessary for high N$_2$OR activity.

In 1988 a multifrequency EPR study carried out by Kroneck et al. revealed several unusual signals including a seven line hyperfine pattern indicative of a mixed valence, S=1/2 [Cu(1.5)-Cu(1.5)] center observed in both the anaerobic and aerobically isolated forms (forms I and II respectively) (55). The unpaired electron appeared to be delocalized between the two equivalent Cu nuclei, giving rise to hyperfine splitting in the $g\parallel$ with $A\parallel=38.3$ G and ($g\parallel=2.18$) (57). This narrow splitting, being half that expected from a typical blue Cu center, results from the spin density of the unpaired electron being
highly delocalized over the two Cu ions. These $g_\parallel$ hyperfine signals were determined to account for 60-70% of the total Cu present, with the remainder being EPR silent. This seven line hyperfine, in addition to the visible absorption maximum at ~800 nm showed that N$_2$OR was likely to contain a Cu$_A$ site similar to beef heart cytochrome c oxidase (see Figure 8). Furthermore Dooley et al. had published resonance Raman data in 1987 that suggested a Cu(II)S$_2$(cys)$_2$N(his) site as the source of the 540 nm absorption maximum from N$_2$OR (58).

Resonance Raman data from 633 nm excitation showed modes in the low activity form (form II) that were somewhat similar to type I (blue) copper sites, and were not found in data obtained using 514.5 nm excitation. Some modes observed in the 514.5 nm data were also missing from the 633 nm data, indicating that different Cu chromophores were likely to be responsible for the 540 and 630 nm absorption bands (58). While the possibility remained that the 540 nm chromophore was simply being converted to a 630 nm chromophore upon changes in geometry or coordination number, it was also apparent

Figure 8. The Cu$_A$ binuclear center as determined from the crystal structure of N$_2$OR from *Pseudomonas nautica.*
that the 630 nm chromophore of the low activity form was essentially identical to the blue species obtained upon reduction by dithionite (58).

In the multifrequency EPR work discussed above, hyperfine splitting with $A_\perp = 28$ G was observed in the $g_\perp$ region ($g_\perp = 2.03$) of spectra obtained from the purple, high-activity form (55). In addition a "broad, feature-less signal" found in the region containing the seven line signal seemed to account for approximately 30% of the total Cu content, and was present in both forms I and II. Upon reduction of form I to the blue form (form III) this signal could be seen more clearly and was found to represent between 10% and 30% of the total Cu (55). This broad signal is not present in $N_2OR$ V, and is therefore clearly not originating from the CuA center.

Pulsed EPR studies published in 1989 (59) showed probable His ligation to the EPR-detectable Cu ions. ESE studies in the same publication resulted in the conclusion that multiple distinct imidazole liganded Cu(II) sites were present. Depending on pH of the sample as well as other unknown factors, 50% to 80% of the Cu was found to be EPR nondetectable (56;59). Approximately 47% of the total Cu was EPR silent in form V. EXAFS data published the same year (60) helped to confirm the presence of a CuA-type site in both the oxidized and reduced forms. Cu-(S, Cl) interactions at 2.3 and ~2.6 Å as well as Cu-(N, O) interaction at 2.0 Å were necessary for a fit to the data. These distances are nearly the same as those observed in cytochrome $c$ oxidase. It was determined that ~8S were required per 8 Cu ions at 2.3 Å. Similar EXAFS results were reported for the enzyme from *Pseudomonas aeruginosa* in 1991 (80).
XAS Cu K-edge data taken on form I showed no difference in the edges at pH 9.8 and 7.5, leading to the conclusion that the 10 fold increase in activity at high pH was probably not due to ligation of Cu or oxidation state differences. Comparison of the MCD spectra of cytochrome c oxidase and N2OR confirmed that N2OR contained a CuA-type site (60). Finally, the conserved sequence Gly-Xaa-Xaa-Xaa-Xaa-Cys-Ser-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-XXaa-Cys-Xaa-Xaa-His was found to be present in several cytochrome c oxidase subunits II and N2OR, providing the potential ligands seen by resonance Raman, EPR, and EXAFS (79;81).

In CD spectra of N2OR published by SooHoo and Hollocher no strong positive bands were present and therefore no correlation to spectra obtained for canonical type I centers. The only strong bands were negative and located between 540 and 580 nm for N2OR from P. aeruginosa (82). Because the reported amounts of paramagnetic Cu in the Cu centers of N2OR varied so widely, saturation magnetization experiments at 0.2, 1.375, 2.75, and 5.5 T were used by Dooley et al in order to obtain a more accurate estimate (83). It was concluded from this work that 25% of the average Cu content was paramagnetic, implying that the remaining Cu was either Cu(I) and/or antiferromagnetically coupled Cu(II). CD/MCD characterization by Dooley et al showed spectra which indicated several low energy transitions likely to arise from Cu(II)(S)2(N)2 or Cu(II)(S) centers (84). EXAFS data reported in the same paper revealed a decrease in the number of (N,O) scatterers for N2OR V when compared to that from form I, indicating that histidine ligated Cu was missing. Furthermore, the presence of near
infrared bands in the CD spectra of forms I and II, but not in form V indicated that Cu(II) sites other than CuA must also be present (84).

With at least 50% of the total Cu in N2OR being EPR silent, either much of the Cu was reduced and inaccessible to oxidants, or antiferromagnetic coupling was preventing its detection. Interestingly, Farrar et al had observed as early as 1991 that ascorbate reduction brought the enzyme from an S = 1/2 state showing the seven line hyperfine by EPR, through an EPR “silent” state (very little signal), and finally (with PMS as a mediator) into another S = 1/2 state which could be reduced no further (85). From these data they were able to draw the conclusion that there were two different types of centers present, with the mid-reduction, EPR silent state being a result of one center being fully reduced Cu(I)-Cu(I) and the other being most likely antiferromagnetically coupled Cu(II)-Cu(II). Based on MCD charge transfer transitions, they also predicted that both centers were likely to have Cys copper coordination (85). It was clear that the measured Cu content when combined with the various signals seen by EPR, resonance Raman, EXAFS, MCD, and UV-Visible spectroscopy, excluded the N2OR Cu sites from being cannonical type 1 (blue), type 2 (square planar), or type 3 (binuclear Cu(II)) centers, and the true nature of the chromophore(s) remained a mystery for more than a decade after these studies were published.

Until 1998 the widely accepted model for the Cu sites in N2OR was that of two binuclear centers, CuA and CuZ. CuA was thought to be the site responsible for electron transfer to the catalytic site CuZ based on it's similarity to the CuA site of cytochrome c oxidase. CuA was understood to exist in an oxidized [Cu(I)-Cu(II)] state and a reduced
[Cu(I)-Cu(I)] state, as seen by the disappearance of its spectral features upon reduction of the enzyme. Virtually nothing was known about the nature of the CuZ center, and it was proposed to cycle between [Cu(II)-Cu(II)] and [Cu(I)-Cu(II)] (1).

In 1998 Farrar, Zumft, and Thomson proposed CuZ to be a variant of the CuA electron transfer center (1). This was based on the fact that thiolate coordination was indicated for both Cu centers in previous resonance Raman spectra (58) as well as MCD in current work, but lack of conserved cysteines beyond the two required for CuA made interpretation of the data otherwise difficult. The table on the following page is a summary of the forms of CuA/CuZ/CuZ* proposed in 1998:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>Z</th>
<th>Z*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Enzyme</td>
<td>CuII/CuI</td>
<td>CuII/CuI</td>
<td>CuII/CuI</td>
</tr>
<tr>
<td></td>
<td>S=1/2</td>
<td>S=0</td>
<td>S=1/2</td>
</tr>
<tr>
<td></td>
<td>A=480, 540, 800</td>
<td>A=540, 650</td>
<td>A=650</td>
</tr>
<tr>
<td>Semi-Reduced Enzyme</td>
<td>CuI/CuI</td>
<td>CuII/CuII</td>
<td>CuI/CuI</td>
</tr>
<tr>
<td></td>
<td>S=0</td>
<td>S=0</td>
<td>S=0</td>
</tr>
<tr>
<td></td>
<td>A=540, 650</td>
<td>A=540, 650</td>
<td></td>
</tr>
<tr>
<td>Reduced Enzyme</td>
<td>CuI/CuI</td>
<td>CuII/CuI</td>
<td>CuI/CuI</td>
</tr>
<tr>
<td></td>
<td>S=0</td>
<td>S=1/2</td>
<td>S=0</td>
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<tr>
<td></td>
<td></td>
<td>A=650</td>
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</tr>
</tbody>
</table>

Table 1. Proposed variations of the CuA site of N2OR from P. stutzeri (1).

This interpretation required that the CuA/CuZ center be capable of losing 2e\(^-\) to become [Cu(II)-Cu(II)], and that "CuZ" escape complete reduction in the presence of excess dithionite. The nature of the catalytic site of N2OR would then remain a mystery in this model, with "CuZ" simply being an alternate state of CuA. To further complicate the proposal of CuZ as a variant of CuA, a form called CuZ* was designated as a variant of CuZ.
In 2002 Rasmussen et al published spectroscopic characterizations of “anaerobic” (purified aerobically after storage of lysed cells in liquid N₂) and aerobic forms of N₂OR which had each been oxidized with ferricyanide, reduced with ascorbate, and further reduced using dithionite, with the hope of producing an explanation for the Cu₂⁺ phenomenon (87). The conclusion drawn is that Cu₂⁺ is a form of the Cu₂ center arising from exposure of the enzyme to O₂, still showing the same Cu content as the “anaerobic” form, but giving rise to distinct spectroscopic signals and increased activity. Unfortunately, the Cu contents reported in that publication did not support their conclusion, showing 8.4 Cu atoms/dimer in the aerobic form and 10.5 Cu atoms/dimer in the anaerobic form. Additionally, no activity numbers have ever been reported in which an oxygen exposed, Cu depleted form of N₂OR exhibited higher activity than a form isolated anaerobically with higher Cu content, and all activity numbers are excluded from this publication leaving it highly suspect. In addition, Alvarez et al recently reported Zₘᵥ⁺ (Cu₂⁺) as the low activity form of N₂OR from P. stutzeri (88). The data reported by Rasmussen et al clearly indicate (in contrast to their conclusions) that the Cu₂⁺ center is a Cu depleted version of Cu₂. This is in agreement with the results shown in the N₂OR chapter of this thesis in which Cu depleted Cu₂ was characterized and found to show distinct spectroscopic signals.

In 2000 two forms (purple and blue) of N₂OR were purified from Pseudomonas nautica 617 (61). These forms were chromatographically distinct and both were found to contain a surprising 6 Cu/monomer. The 6 Cu ions were determined crystallographically to be arranged in two centers, Cuₐ and Cuₗ. In the structural data resolved to 2.4 Å, Cuₐ
was found to be a binuclear site much like the Cu_A center in cytochrome c oxidase (see figure 8), giving rise to the typical 7-line hyperfine with \( g_x = g_y = 2.021, A_x = A_y = 0 \text{ G,} g_z = 2.178, \) and \( A_z = 40 \text{ G} \) in the purple form (see figure 7). Cu_A in the blue form appeared to be in the EPR silent, \( S=0, \) Cu(I)-Cu(I) state, while in the purple, oxidized form its 7-line pattern indicated the \( S=1/2, \) Cu(I)-Cu(II) state. Cu_Z was determined to be a novel, tetranuclear cluster responsible for the 640 nm absorption band and a 4-line EPR spectrum with \( g_x = 2.015, A_x = 15 \text{ G,} g_y = 2.071, A_y = 20 \text{ G,} g_z = 2.138, \) and \( A_z = 70 \text{ G.} \)

According to Prudencio et al. (61), Cu_Z remains in a reduced, \( S=1/2 \) state in both forms. Thus the only apparent difference between the purple and blue native forms of the enzyme is in the oxidation state of Cu_A. The purple form of N_2OR eluted at a lower ionic strength during anion exchange than the blue form, and exhibited a slightly higher level of activity than the blue form as well. The visible absorption spectra of the two forms differ in the fact that the purple form shows a much higher 540 nm absorption band and exhibits 480 and 800 nm absorption bands not seen in the blue form.

In the center EPR spectra below, simulations were generated using the following ratios of 7-line pattern (Cu_A) to 4-line pattern (Cu_Z): 0.45 Cu_A/0.55 Cu_Z for the purple form and 0.08 Cu_A/0.92 Cu_Z for the blue form. In the EPR spectra to the right in Figure 9 below, the oxidized form (A) appears to be identical to the purple form of the native enzyme, and is therefore a mixture of the 7-line Cu_A hyperfine and the 4-line Cu_Z hyperfine. This agrees with their result that Cu_Z oxidation state was redox inactive. In spectrum B of the reduced form from the same data set, only the 4-line Cu_Z hyperfine is seen, consistent with the fact that Cu_A is reduced and is therefore EPR silent. This
reduced form is again identical to the blue, native form. In spectrum C the difference spectrum of "purple minus blue" shows the expected 7-line $\text{Cu}_A$ hyperfine lacking the signals from $\text{Cu}_Z$ (61).

![Figure 9](image)

Figure 9. (left) UV-visible spectra of P. nautica N2OR. (A) Purple form; (B) Blue form; (C) dithionite-reduced enzyme. (center) EPR spectra of the as-purified (A) purple and (B) blue N2OR from P. nautica. (right) EPR spectra of the (A) oxidized and (B) reduced forms of N2OR from P. nautica as well as the (C) difference spectrum "A minus B" (89).

The crystal structure of N$_2$OR.

The crystal structure of N$_2$OR from P. nautica can be seen below, and was refined to 2.4 Å (62). The model of the tetranuclear $\text{Cu}_Z$ center showed coordination by 7 His and 3 OH or H$_2$O ligands, one being a central bridging OH$^-$. Each monomer consists of an N-terminal 7-blade $\beta$-propeller fold containing the $\text{Cu}_Z$ center and a c-terminal cupredoxin fold containing the $\text{Cu}_A$ center. The dimer exhibits domain sharing, with the $\text{Cu}_A$ site from one monomer interacting with the $\text{Cu}_Z$ site from the other monomer. A Met and a Phe residue from the cupredoxin domain are the only residues found in the solvent channel between the $\text{Cu}_A$ and $\text{Cu}_Z$ centers (62). Each monomer has a Cl$^-$ bound near the $\text{Cu}_Z$ center and 2 Ca$^{2+}$ ions located near the dimer interface (90;91).
The fact that spectroscopic data continued to indicate sulfur ligation even upon complete reduction of CuA to a spectroscopically silent form was puzzling in light of the OH$^-$-bridged model of CuZ based on the *P. nautica* structure. Sulfide analysis of native and mutant forms of N$_2$OR, as well as resonance Raman of the reduced form at 647 nm, revealed the presence of inorganic sulfide as the bridging ligand in the CuZ center (63). A more recent crystal structure of the enzyme from *Paracoccus denitrificans* solved to 1.6 Å, as well as spectroscopic data and chemical analysis has confirmed the structure of CuZ as a μ$_4$-sulfide bridged tetranuclear center (88;92-94). Using variable temperature, variable field MCD, Q band EPR, XAS, and density functional theory (DFT) calculations, Chen et al were recently able to show that the CuZ center in the reduced enzyme from *P. nautica* is in an $S = \frac{1}{2}$ state with 3Cu(I)/1 Cu(II) (94). The DFT
calculations resulted in a delocalized wave function with 42% of the spin density found on Cu1 (it being the oxidized Cu), 16% on Cu2, 8% on Cu3 and 3% on Cu4. The μ₄-bridging sulfur was also found to have σ bonds to Cu1 and Cu2 allowing it to hold 14% of the spin density, contributing to significant delocalization of the unpaired electron (94).

Figure 11. Structure of the sulfide-bridged, tetranuclear Cu₄ center of N₂OR.

Research Goals

The focus of this work was to purify and fully characterize N₂OR and the putative metallochaperone ABC transport system from _Achromobacter cycloclastes_. Purification of N₂OR was fine-tuned continuously over the course of three and a half years, eventually achieving resolution of multiple forms of the enzyme differing in copper content, activity level, and perhaps oxidation state of the copper ions.
In addition purification of the gene products of the *nosDFYL* operon was pursued in order to determine the substrate and function of this ABC transporter. A role in formation of the catalytic Cu$_Z$ center had been indicated for the transporter, but its strong resemblance to bacterial ABC importers and numerous results indicating that copper insertion takes place in the periplasmic space make the likelihood of Cu import by this system very slim. Purification and characterization of the gene product of the *nosX* transcriptional unit was also carried out, with the hope of assigning a possible function for the protein.
NITROUS OXIDE REDUCTASE (N₂OR)

Introduction

Nitrous oxide reductase (N₂OR) is a soluble, periplasmic, homodimeric protein comprising two distinct copper centers per monomer. Each monomer typically has a molecular weight around 67,000 Da and an average pI of 5.9 (66). The structural gene for N₂OR, nosZ, codes for an extraordinarily long signal sequence of ~50 amino acids, which includes a conserved twin-arginine motif for Sec-independent translocation to the periplasm. This motif is conserved in a number of periplasmic and membrane bound proteins requiring cofactor insertion or other processing (95). An R20D mutation in N₂OR from P. stutzeri resulted in cytoplasmic apo-protein, indicating that the twin-arginine motif of the signal sequence is indeed necessary in the biosynthetic pathway of N₂OR, and leading to the proposal that this specialized signal sequence provides translocational information involved in associating N₂OR with its Cu center assembly proteins (95).

Transcription of the nosZ gene is under the control of six different promoters in P. stutzeri (96). This provides for transcriptional control in response to varying environmental conditions and presence of regulatory signals. N₂OR is constitutively expressed at low levels under aerobic conditions, with derepression taking place in anaerobic conditions. The extent to which nosZ transcription is derepressed is higher in
the presence of NO$_3^-$ than N$_2$O (96). The promoter regions of several denitrification genes have been found to contain FNR boxes. *rpoN* consensus sequences have also been found in the promoter regions of several *nosZ* and *nosD* genes, indicating involvement of the $\sigma^{54}$ transcription factor (81).

To date, the only report of purification of N$_2$OR from *A. cycloclastes* is that by Hulse and Averill (64), in which the enzyme was reported to be a pink, high activity, monomeric form with a molecular weight of 72 kDa. The work reported in this thesis establishes the fact that, contrary to the earlier report, N$_2$OR from *A. cycloclastes* can be purified in dimeric blue, purple, pink, and intermediate forms with varying levels of activity.

Purification of N$_2$OR from *A. cycloclastes* was pursued in this work using a recombinant strain containing a multi-copy, broad host range plasmid containing the entire *A. cycloclastes nos* gene cluster. The recombinant strain was developed in order to increase protein expression to levels that would make possible further spectroscopic characterization and x-ray crystallographic work. Much remained to be elucidated regarding the nature of the active sites of the enzyme at the time that these purifications were begun. Even today, with two crystal structures and extensive spectroscopic characterizations, the existence of so many forms of the enzyme leaves questions to be answered.
Experimental Methods

Growth and Induction of recombinant

*Achromobacter cycloclastes* (Ac/pMLnos).

Overexpression of the gene products of the *nos* cluster was facilitated by subcloning of the entire gene cluster into the multi-copy, broad host range vector pML10 (98). The plasmid pML10 was first downsized 2 kbp by a SalI-SalI deletion. After ligation, this product was then linearized using the restriction enzyme Sse8387I. Conveniently, the *nos* cluster of *A. cycloclastes* is flanked by Sse8387I restriction sites, allowing ligation directly into the Sse8387I site of the expression vector. This vector could then be amplified in *E. coli*, and then electroporated into *A. cycloclastes* using the protocol discussed below. Purification work on N\(_2\)OR from *Achromobacter cycloclastes* began with growth of 10 mL LB Gm 50 (50 ppm Gentamicin) starter cultures in a shaking incubator or water bath at 30\(^\circ\) C and 240 rpm shaking for 12 hours. The starter cultures were then scaled up to 500 mL of LB Gm 50 in 1 L flasks to limit the amount of headspace over the cultures. The flasks were covered with foil, and were then grown in the shaker at 30\(^\circ\) and 240 rpm shaking for 12-18 hours. At an optical density (O.D.\(_{600}\)) \approx 1.0 the cultures were induced by adding 1 mL of 5 mM CuSO\(_4\)\(_\cdot\)5H\(_2\)O and 3.33 mL 1.5 M KNO\(_3\) to achieve final concentrations of 10 \(\mu\)M Cu and 10 mM NO\(_3^-\). The shaking rate was reduced to 120 rpm for induction, creating oxygen limited conditions. The induction was carried out for 24 hours. Cultures were then harvested by centrifugation for 10 minutes at 7,000 g for osmotic shock.
Osmotic Shock Protocols.

The following osmotic shock protocols were used in an attempt to isolate the periplasmic fraction containing N\textsubscript{2}OR:

**Protocol 1**

1. Centrifuge induced cultures for 10 minutes at 7,000 g and room temperature. Discard supernatant and weigh pellets.

2. Resuspend pellets in 30 mM TrisCl pH 8.0 containing 20% sucrose using a ratio of 80 mL/g cells. Add EDTA to 1 mM and stir at room temperature for 5-10 minutes.

3. Centrifuge at 10,000 g for 10 minutes at room temperature. Remove supernatant.

4. Resuspend pellet in the same volume of ice cold MgSO\textsubscript{4}. Stir on ice for 10 minutes.

5. Centrifuge at 10,000 g for 10 minutes. Supernatant should be periplasmic fraction.

**Protocol 2 (based on pET Manual, 7th Ed., procedure #2, pp. 50)**

1. Resuspend cell pellets in 20 mM TrisCl pH 8.0 containing 20% sucrose and 2.5 mM EDTA to 5.0 OD\textsubscript{500} units/mL, and incubate on ice for 10 minutes.

2. Centrifuge at 15,000 g for 30 seconds.

3. Resuspend in the same volume of ice cold 20 mM TrisCl, pH 8.0 containing 2.5 mM EDTA. Incubate on ice for 10 minutes.

4. Centrifuge at 15,000 g for 10 minutes. Supernatant should be periplasmic fraction.

**Protocol 3**

1. Pellet 750 mL cultures (centrifuging at 7,000 g for 10 minutes) and discard supernatant.

2. Resuspend the Pellet in 188 mL AP buffer (30 mM TrisCl, 20% sucrose (w/v), pH 8.0).

3. Centrifuge again to pellet cells.

4. Resuspend pellet in 9.375 mL AP buffer.

5. Transfer to centrifuge tubes, and add 469 µL lysozyme solution (10 mg lysozyme in 1 mL AP buffer).

6. Incubate on ice 20 minutes.
7. Centrifuge to pellet (at high speed for 4 minutes at 4°C). Transfer supernatant (periplasmic fraction) to a fresh, chilled container.

**Protocol 4**

1. To approximately 10 g cell pellet add 10% v/v Triton x-100 (0.1 mL), 10 µL mercaptoethanol, and 2.5 mL glycerol.
2. Stir vigorously for 30 minutes.
3. Add 30 mL extraction buffer (20 mM KPO₄, pH 7.0 and 1 mM EDTA) containing 0.2 mg/mL lysozyme and 10 µg/mL DNase. Add this rapidly and stir for 30 minutes.
4. Add sigma protease inhibiting cocktail and centrifuge at 14,500 g for 10 minutes.


1. Resuspend cells in 0.4 culture volume (80 mL/g) of 30 mM TrisCl, pH 8.0, and 20% sucrose. Add EDTA to 1 mM and incubate 5-10 minutes at room temperature with stirring.
2. Centrifuge at 10,000 g for 10 minutes at 4°C.
3. Resuspend pellet in the same volume of ice cold 5 mM MgSO₄. Stir 10 minutes on ice.
4. Centrifuge at 10,000 g for 10 minutes at 4°C again. The supernatant should be the periplasmic fraction.

**Protocol 6**

1. Centrifuge cells at 7,000 g for 10 minutes. Then resuspend in 30 mL total volume (per ~15 g cell paste) of 100 mM TrisCl, 500 mM Sucrose, 2 mg/mL polymyxin B sulfate, pH 8.0.
2. Incubate 30 minutes on ice.
3. Pellet cells at 14,000 g. The supernatant should be the periplasmic fraction.

**N₂OR Purification.**

In finding that *A. cycloclastes* is resistant to osmotic shock, subsequent preliminary N₂OR purification attempts began with sonication of the cell paste in low ionic strength buffer (20 mM TrisCl, pH 8.0). The cell suspension was sonicated in 50 mL aliquots with a w/v ratio of 1/10 at a power level of 7 and 50% duty cycle. The
temperature of the solution was kept at 15°C or below. After sonication, the suspension was then centrifuged at 7,000 g for 10 minutes at 4°C. To the supernatant an equal volume of DEAE Sepharose was added and the slurry was stirred slowly for a half hour. The batch bound DEAE was then loaded into a fast flow column and washed with 20 mM TrisCl, pH 8.0 until the A$_{280}$ was low and unchanging, indicating that unbound protein had all been removed. The bound protein was eluted using 0.5 M NaCl in the same buffer mentioned above, and then dialyzed vs. 20 mM TrisCl, pH 8.0 in order to remove the NaCl. Next the protein was batch bound to 8 mL cytochrome c agarose (4% cross-linked beads) pre-equilibrated with 20 mM TrisCl, pH 8.0 in the same manner as with DEAE. Again the batch bound material was loaded into a column (this time an FPLC HR-10 column from Pharmacia) and washed with the Tris buffer until no unbound protein appeared to be washing off any longer. The elution was again carried out using 0.5 M NaCl. The following steps resulted in excellent resolution of N$_2$OR from a large peak of contaminants on the cyt c agarose column: gradient to 0.1 M NaCl over 5 column volumes, 2 column volumes at 0.1 M NaCl, gradient to 0.25 M NaCl over 5 column volumes, ramp to 0.5 M NaCl over 1 column volume, hold at 0.5 M NaCl for ~6 column volumes. At this point the protein was better than 95% pure as seen by SDS-PAGE. S200 gel filtration using a 100 mL bed volume column at a flow rate of 9 mL per hour was used for the final purification step and resulted in protein that was nearly 100% pure when analyzed by SDS-PAGE (see Figure 13).

Other column materials used in later purifications include hydroxylapatite, MonoQ, ultrogel, and biogel. The most recent purification method, used to obtain
resolution of multiple forms of N$_2$OR, begins with harvest of the cells from ~4 L of culture grown to O.D$_{600}$ of 1.0-1.3 with 240 rpm shaking at 30°C and induced for 24 hours by reducing the shaking rate to 120 rpm, adding Cu$_2$SO$_4\cdot$5H$_2$O to a final concentration of 0.1 mM, and adding KNO$_3$ to a final concentration of 0.01 M. Harvest of the cells is carried out by centrifugation at 7,000 g at 4°C for 10 minutes. The cell paste is then frozen at -80°C. Thawed cells are then resuspended in 20 mM TrisCl, pH 8.0 in a w/v ratio of 1/2.5 and run through a French press at least twice at 12,500 psi. Alternately the cells have also been broken by sonication on ice in 50 mL aliquots at a power level of 7 and a 50% duty cycle, although this procedure requires approximately 10 x 3 minute rounds of sonication with 2 minute cooling times, therefore becoming very long and tedious for large amounts of cell paste. The crude extract is then centrifuged at 30,000 g for 20 minutes, and remaining cell debris is removed from the supernatant by ultracentrifugation for 1 hour at 45,000 rpm in a Ti50 rotor. The cleared lysate is then injected directly onto a 200 mL DEAE Fast Flow Sepharose column pre-equilibrated with 20 mM TrisCl, pH 8.0. The column is washed with 10 bed volumes of the Tris buffer, and elution is carried out using a linear gradient maker containing 1 L 20 mM TrisCl, pH 8.0 and 1 L of the same buffer containing 0.5 M NaCl. Fractions of approximately 8-10 mL are collected. Fractions appearing purple or pink are then analyzed by SDS-PAGE to determine purity of the N$_2$OR and the cleanest fractions are pooled and concentrated to a final volume of no more than 10 mL in a 150 mL Amicon concentrator using a YM30 or YM50 membrane.
The concentrated sample is then dialyzed vs. 2 x 1 L of 20 mM MES, pH 6.5, and then injected onto a MonoQ HR-10 column (Pharmacia). The following gradient is used to resolve peaks of N2OR differing by Cu content, visible spectrum, EPR signals, and activity (see Figure 12): ramp from no salt in 20 mM MES, pH 6.5 to 0.075 M NaCl, hold at 0.075 M NaCl for 2 column volumes, ramp to 0.15 M NaCl, hold at 0.15 M NaCl for 2 column volumes, ramp to 0.4 M NaCl, hold at 0.4 M NaCl for 10 column volumes, ramp to 1 M NaCl, and hold at 1 M NaCl for 3 column volumes. Fractions of 3 mL were collected throughout this method. Fractions within each peak were pooled based on level of purity as seen by SDS-PAGE and on closeness of the 620 nm to 530 nm ratios among the samples within a peak. Each form of N2OR resolved on the MonoQ column was then concentrated for ultrogel filtration. Activity assays were performed according to a final protocol modified by Jeannine Chan.

![Figure 12](image-url). MonoQ elution profile showing separation of three forms of N2OR from A. cycloclastes.
Determination of Protein Concentration.

An extinction coefficient for absorption at 280 nm was calculated using CD/MCD data obtained from a Jasco 710 Spectropolarimeter. The analysis was run twice on each of two separate samples of N2OR from A. cycloclastes and calculations were performed as those reported by Holmquist and Vallee (65). The resulting extinction coefficients were 93.4, 107.35, 105.6, and 105.6 mM⁻¹ cm⁻¹. The average of 104.9 mM⁻¹ cm⁻¹ was used for concentration determinations in all further work, based on sample absorption measured at 280 nm.

N2OR Specific Activity Assay (modified by Jeannine Chan). Solutions: (enough for about 30 assays)

50 mM Potassium phosphate buffer, pH 7.1
0.5801 g K₂HPO₄ (MW = 174.2)
0.2273 g KH₂PO₄ (MW = 136.1)
100 mL DI H₂O

Note: Using pK₂ = 6.8

Dissolve the K₂HPO₄ and KH₂PO₄ in the DI H₂O. Check and adjust pH as necessary using pH meter. Divide into 5 25-mL glass serum vials, and seal with gray butyl luckstoppers/Al seals. Bubble Ar gas through the solutions (at least 30-40 minutes before use).

5 mM Benzyl viologen
0.0205 g benzyl viologen (MW = 409.4)
10 mL 50 mM potassium phosphate, pH 7.1

Dissolve viologen in buffer in a 25-mL glass serum vial, and seal with a gray butyl stopper/Al seal. Bubble Ar gas through the solution (at least 30 minutes).

~100 mM Sodium dithionite
0.050 g Na₂S₂O₄ (estimate MW ~ 200; actual MW = 174)
2.5 mL 50 mM potassium phosphate, pH 7.1

Weigh out the dithionite and place in a 9-mL glass serum vial. Seal with a gray butyl stopper/Al seal. Thoroughly flush vial with Ar gas. Using a gas-tight syringe, add the 2.5 mL of Ar-flushed 50 mM potassium phosphate, pH 7.1 (minimal gas formation upon addition of buffer means the buffer was well-deoxygenated).

N₂O-saturated buffer

Place about 3 mL of 50 mM potassium phosphate buffer, pH 7.1, in a 9-mL glass serum vial. Use a red rubber stopper to seal (Note: stopper should fit tightly). Bubble N₂O gas through the solution either continuously throughout the assays or for ~15-20 minutes, leaving a constant overpressure of N₂O over the solution afterwards.
**20 mM Benzyl viologen**  
(if using batch activation)  
0.0164 g benzyl viologen (MW = 409.4)  
2 mL 50 mM potassium phosphate, pH 7.1  

Dissolve viologen in buffer in a 9-mL glass serum vial, and seal with a gray butyl stopper/Al seal. Bubble Ar gas through the solution (at least 30 minutes).

**~10 mM Sodium dithionite**  
(if using batch activation)  
200 μL ~100 mM Na$_2$S$_2$O$_4$  
2 mL 50 mM potassium phosphate, pH 7.1  

Place buffer in a 9-mL glass serum vial. Seal with a gray butyl stopper/Al seal. Thoroughly flush vial with Ar gas. Using a gas-tight syringe, add the ~100 mM Na$_2$S$_2$O$_4$.

### Activation of N$_2$OR:

**Method#1 – activation of individual assay samples**

Remove lids from 0.6-mL microcentrifuge tubes, place inside 9-mL glass serum vials, and seal with gray butyl stoppers/Al seals. Purge vials with Ar gas. Add ~200 μL of 100 mM Na$_2$S$_2$O$_4$ to the bottom of each vial (acts as a trap for O$_2$). Thaw proteins and transfer desired amounts into the Ar-purged vials. Flush Ar gas over the surfaces of the protein solutions, occasionally tapping the tubes and avoiding evaporation (typically only very small volumes of N$_2$OR are needed, hence the need to be particularly careful about evaporation). If dilution of protein solution is performed, use Ar-flushed 50 mM potassium phosphate buffer, pH 7.1, as the diluent prior to flushing Ar over the protein solutions. An ideal concentration of protein is 1 mg/mL.

Flush as many sealed, empty glass serum vials (5-mL and/or 9-mL) with Ar as the number of assays to be run. To each vial, add the following:

- 2.7 mL 50 mM potassium phosphate, pH 7.1
- 300 μL 5 mM benzyl viologen
- 3 μL ~100 mM Na$_2$S$_2$O$_4$
- x μL 1 mg/mL N$_2$OR (x = 2.5-10 μL)

Swirl to mix. Let solutions sit at room temperature for 2 - 3 hours for complete activation.

**Method#2 – batch activation of N$_2$OR**

Remove lids from 0.6-mL microcentrifuge tubes, place inside 9-mL glass serum vials, and seal with gray butyl stoppers/Al seals. Purge vials with Ar gas. Add ~200 μL of 100 mM Na$_2$S$_2$O$_4$ to the bottom of each vial (acts as a trap for O$_2$). To the bottoms of the 0.6-mL tubes inside the Ar-purged vials, add enough 50 mM potassium phosphate, pH 7.1, 20 mM benzyl viologen, and 10 mM Na$_2$S$_2$O$_4$ to end up with the following concentrations:

- 2 mM benzyl viologen
- 1 mM Na$_2$S$_2$O$_4$
- 1 mg/mL N$_2$OR

Ideally at least 5 μL of protein will be used (lower concentrations of N$_2$OR stock are therefore easier to use). The protein should be added last.

- e.g., for a 60 mg/mL N$_2$OR stock, add 235 μL 50 mM potassium phosphate, pH 7.1
- 30 μL 20 mM benzyl viologen
- 30 μL 10 mM Na$_2$S$_2$O$_4$
- 5 μL 60 mg/mL N$_2$OR

Either tap vial/tube or pump solution using a gas-tight syringe to insure solution is well mixed. Let solutions sit on bench for 2.5 - 3 hours for complete activation.

### The Assay:
Place micro stir bars into each of the two cuvets with screw tops. Place new rubber seals into the caps.

Turn on the H₂O circulator (needed for stirring). Set the temperature to 25 degrees Celsius.

HP UV-vis spectrometer in Room 18
Method = N2ORAY (in Shannon's folder)
Blank the spectrometer on 50 mM potassium phosphate, pH 7.1.

Method #1 – activation of individual assay samples
Using the 5-mL gas-tight syringe, transfer an activated assay sample to an Ar-purged cuvet. Add 1-2 μL additional 100 mM Na₂S₂O₄ so that the starting absorbance at 600 nm is approximately 1.0.

Method #2 – batch activation of N₂OR
To an Ar-purged cuvet, add

- 2.7 mL 50 mM potassium phosphate, pH 7.1
- 300 μL 5 mM benzyl viologen
- 3 μL ~100 mM Na₂S₂O₄ (should give starting A₆00ₐ₇~1.0)
- x μL 1mg/mL batch activated N₂OR (x = 2.5-10 μL)

Hit F7 to enter the time-based measurement mode. F7 again to start the run. Data is collected every 6 seconds. After about 60-120 seconds, should be able to assess any N₂OR-independent oxidation of the benzyl viologen (hopefully there is little to none).

Inject 80 μL N₂O-saturated buffer to begin N₂OR-dependent oxidation of the benzyl viologen. Spectrometer is programmed to collect data for 360 seconds.

Examine the initial linear part of the curve to determine rate of oxidation. The rate will need to be converted to minutes.

Be sure to run several assay blanks (3) before running protein assays. Use 5 μL of buffer in place of x μL of N₂OR.

Additional Assay Notes/Suggestions:

As outlined in the protocol, solutions are prepared in Wheaton glass serum vials with gray butyl stoppers and Al crimp seals. If these are not available, then solutions should at least be prepared in glass containers that can be sealed (e.g., with either screw tops with septa or rubber stoppers that make tight contact with the inner mouth of the containers).

As long as an overpressure of Ar is maintained in the vials, the solutions should theoretically not require continuous bubbling with Ar in order to stay de-oxygenated.

As a general rule of thumb, when removing solution/volume from a sealed vial, the volume should be replaced with Ar – i.e., keep an Ar line open into the vial to always maintain the same overpressure and prevent air from being pulled in.

A stock of 100-50 mM potassium phosphate buffer can be made ahead of time and stored so it doesn't need to be made fresh every time. The benzyl viologen solutions should also keep.

Dithionite solutions need to be made fresh every time.

If doing a Kₘ determination for N₂O or some other experiment where the [N₂O] needs to be known, then should use continuous bubbling so that the pressure of N₂O is theoretically the same as atmospheric pressure. But if just determining specific activities or an experiment where N₂O should be in excess, then continuous bubbling should not be necessary and an overpressure maintained instead.

Methyl viologen actually works better for batch activation of N₂OR, however it is a known carcinogen.
Activation method #2 is a little easier than Activation method #1—partly because there are fewer vials to deal with (and clean). Theoretically it should also give more consistent results between replicate samples, but it is not necessarily the case.

Sulfide Analysis (protocol from Jeannine Chan).

I. Broderick’s Modification of Beinert’s Basic Procedure: For more information see references (100-102).

   a) Add protein sample (approximately 6-9 nmol S\(^2^-\) will give an absorbance in the middle of the standard curve) to a siliconized 1.5 mL Eppendorf tube, keeping tube capped unless making an addition.
   b) Add water, pH 8, to bring sample volume to 300 µL.
   c) Simultaneously add 900 µL 1% Zn(OAc)\(_2\) and 45 µL 12% NaOH, immediately recap tube, and vortex. Process one tube at a time. (Theoretically, the OH\(^-\) denatures the protein and prevents loss as H\(_2\)S(g); the Zn\(^2+\) is to capture the S\(^2-\) as ZnS(s).)
   d) Let sample sit for 12-15 hours. (The time is rough for the incubation period, and one time, better values were actually attained when allowed to incubate for about 24 hours.)
   e) Add 225 µL 0.1% DMPD and 6 µL 23 mM FeCl\(_3\) under the surface of the sample, immediately recap tube, and vortex. Process one tube at a time.
   f) Centrifuge for 15 minutes to pellet any precipitated protein.
   g) Let sample sit at least 30 minutes (longer is fine) at 20-25 degrees Celsius.
   h) Read absorbance at 670 nm.

Solutions:

1. Water, pH 8 (very dilute NaOH)
2. 1% Zn(OAc)\(_2\) in DI H\(_2\)O
   - prepared just before use
3. 12% NaOH in DI H\(_2\)O
4. 0.1% \(N,N\)-dimethyl-\(p\)-phenylenediamine (DMPD) monochloride in 5 N HCl
5. 23 mM FeCl\(_3\) in 1.2 N HCl

Na\(_2\)S Stock Standard:

   a) Fill a 100-ml volumetric flask with 0.1-0.01 N NaOH, seal with a tightly-fitted rubber stopper, and purge the solution with Ar.
   b) Rinse a crystal of Na\(_2\)S 9H\(_2\)O (about the size of a small pea) with H\(_2\)O, pH 9; blot it dry with a KimWipe; and immediately weigh it.
   c) Immediately add the crystal to the Ar-purged NaOH; add a stir bar and mix.

Na\(_2\)S Working Standard:

Depending on the resulting concentration of the Na\(_2\)S stock standard (which depends on the size of the crystal used), the standard will likely need to be diluted (roughly 100 µM Na\(_2\)S is a convenient working standard concentration).

   a) Fill a 10-ml volumetric flask with 0.1-0.01 N NaOH, seal with a tightly-fitted rubber stopper, and purge with Ar.
   b) Remove x ml of NaOH solution
   c) Replace with x ml of Na\(_2\)S stock standard solution, and invert to mix. (e.g., for a 10-fold dilution, x = 1 ml).

Suggestions:

1. When rinsing the Na\(_2\)S 9H\(_2\)O, hold the crystal over a paper towel with forceps and drop the H\(_2\)O, pH 9 over the crystal. Be sure to work in a hood to avoid fumes.
2. Standard range = 0.75 – 15 nmol sulfide (15 nmol should give an absorbance around 0.25-0.3)
3. Once the working standard is made, it is not necessary to work anaerobically, though it should be used immediately, and it's probably wise to minimize exposure to oxygen.
4. It's generally thought the two main ways that $S^{2-}$ is lost are (1) oxidation and (2) loss as H$_2$S(g) upon acidification. The latter seems to be the bigger source of error and can be minimized by having as little head space in the sample tube as possible. (So, for instance, if 0.6ml Eppendorf tubes are used instead of 1.5ml tubes, the volumes used should be divided by 3; or if 2ml tubes are used, the volumes used should be multiplied by 4/3; etc.) To minimize oxidation, solutions could be flushed with Ar or the assay could be run in a glove box, but so far, this hasn't seemed necessary.

Beinert's Basic Procedure is virtually identical to Broderick's modified method, except mixing is accomplished using a stir bar (a micro stir bar will fit in 2ml Eppendorf tubes, though Beinert had small glass tubes with glass stoppers). In Beinert's procedure, the solutions are stirred very gently so as to not disturb the top and suck in air; also, all additions are made toward the bottom of the tube or at least under the surface of the solution. For all the extra effort, however, Broderick's modified method gives equally satisfactory results, if not a little better.

The following is a brief summary of an alternate protocol involving more effort for similar results (at least for N$_2$OR) for $S^{2-} + S^0$, (92).

II. Beinert's Procedure for $S^{2-}$ and $S^0$ in Stable Fe-S Proteins

a) Mix 5 µL protein, 13 µL 20 mM Hepes, pH 7.5, and 7 µL saturated CsCl solution.
b) Mix 200 µL saturated guanidine-HCl solution and 10 µL DTT-EDTA solution.
c) Place 600 µL water, pH 11, in a tube and add 4 µL DTT-EDTA solution. Mix.
d) Add 80 µL guanidine-HCl-DTT-EDTA solution from step (b) to the bottom of the tube.
e) Add 20 µL protein solution from step (a) to the bottom of the tube, under the guanidine-HCl-DTT-EDTA solution.
f) Add 80 µL guanidine-HCl-DTT-EDTA solution from step (b) to the bottom of the tube, under the protein solution. Deliver in spurts - protein solution should be layered between the two guanidine-HCl-DTT-EDTA layers.
g) Add 860 µL water, pH 12, on top of liquid column, being careful not to disturb the layers.
h) Cap tube and let sit for 2 hours at 18-20 degrees Celsius.
i) Add 300 µL DMPD to the bottom of the tube.
j) Add a stir bar to the tube and gently stir.
k) Just before the solution becomes homogeneous, add 10 µL 50 mM FeCl$_3$ under the surface of the solution, and increase the stirring rate.
l) Let sample sit at least 30 minutes at 23-25 degrees Celsius.
m) Read absorbance at 670 nm. $\varepsilon_{670\text{nm}} = 34.5$ mM$^{-1}$cm$^{-1}$ (at least according to Beinert).

(Solutions:
1. 20 mM Hepes, pH 7.5
2. Saturated CsCl (R.T.) in DI H$_2$O
3. Saturated guanidine-HCl (R.T.) in DI H$_2$O
4. 100 mM dithiothreitol (DTT) in 20 mM EDTA, pH 7.5)
5. Water, pH 11 (diluted NaOH) - can add phenolphthalein
6. Water, pH 12 (diluted KOH) - can add phenolphthalein
7. 0.1% N,N-dimethyl-p-phenylenediamine (DMPD) monochloride in 5 N HCl
8. 50 mM FeCl₃ in 1.2 N HCl

N-terminal sequencing of N₂OR.

A 500 µL sample of pure N₂OR containing ~450 pmoles of protein was dialyzed vs. 1 L doubly deionized H₂O containing 0.02% SDS for 3 hours. Initial dilution of 2 µL to the 500 µL volume for the dialysis was also carried out using the SDS solution. The sample was then dried in a speed-vac. N-terminal sequencing of the sample was carried out in the lab of Gerhard Munske in the Department of Biochemistry and Biophysics at Washington State University.

Western Blotting for N₂OR (Without Blocking).

1.) After transferring proteins from gel to Immobilon-P membrane, dry the membrane by soaking it in 100% methanol for 10 seconds. Place blot on filter paper and wait for the methanol to evaporate (~15 minutes). This step is crucial.

2.) Incubate blot for 1 hour with primary antibody diluted in blocking buffer containing 0.05% Tween-20 (the best dilution of the antibody "1651" has been 1:2000 (eg. 7.5 µL antibody into 15 mL buffer)). Blocking buffer = 1 % BSA in phosphate buffered saline (PBS)

3.) Wash the blot in PBS 2 times for 10 seconds (exceeding this time may reduce signal to noise).

4.) Incubate the blot for 30 minutes with the secondary antibody ( ) diluted in blocking buffer containing 0.05% Tween-20 (1 µL Sigma anti-rabbit IgG alkaline phosphatase into 30 mL buffer).

5.) Wash blot in PBS 2 times for 10 seconds.

6.) Add stain solution to blot and incubate until the signal reaches the desired contrast: Wash blot with de-ionized water to stop reaction.

   Stain solution:
   1 mL Tris buffer (1.0 M, pH 9.5)
   100 µL MgCl₂ (1.0 M)
   66 µL NBT (nitroblue tetrazolium, 50 mg/mL in 70% dimethylformamide)
   8.8 mL H₂O

7.) Air dry membrane and store in dark place.
Electroporation. Transformation of *A. cycloclastes* was carried out using the following protocol:

**Preparation of Bacteria**
1. Grow 50 mL culture of *A. cycloclastes* in LB with shaking at 30 degrees to an OD of 0.5.
2. Ice culture for 2 hours, then centrifuge at ~4°C to collect bacteria.
3. Wash bacteria once with 40 mL ice cold water.
4. Suspend in 40 mL ice cold water, hold on ice for 3 hours, and then centrifuge to collect bacteria.
5. Wash one time with 20 mL ice cold 10% glycerol.
6. Decant glycerol wash and resuspend bacteria in residual wash.
7. Keep on ice one hour.
8. Store at -80°C.

**Electroporation**
1. A Bio Rad MicroPulser set on the standard *E. coli* method was used
2. Mix 200-400 ng plasmid DNA (in 1 to 5 µL) with 50 µL prepared bacteria.
3. Immediately add mixture to iced, 0.1 cm sterile cuvette, and pulse with electroporator.
4. Add 0.5 mL SOC to cuvette, transfer to a sterile culture tube, and incubate at 30 degrees with shaking for 1 hour.

**Plating**
After the one hour incubation, plate 100-200 µL bacterial suspension on LB plate with appropriate antibiotic. Incubate plates at 30 degrees for up to three days.

**Determination of Copper Content by AA.**

Standards were always made immediately before taking measurements, and covered the range from 0.25-2.0 ppm. Ten mL standards of 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 ppm were made using doubly deionized water and 1,000 ppm Cu reference solution in a 10 mL volumetric flask. As a check for extraneous copper in the buffer containing the protein, the Cu content of the same buffer minus the protein was measured.
Results

Very little or no \( N_2 \text{OR} \) was seen in shockates, indicating that the standard \( E. \text{coli} \) and \( Sinorhizobium \text{meliloti} \) osmotic shock protocols were not efficient for isolation of periplasmic proteins from \( A. \text{cycloclastes} \). Some of the osmotic shock protocols attempted resulted in complete cell lysis, which again was not the goal in using these methods. Sonication became the method of choice until larger scale purifications made the use of French press much more practical. Once pure \( N_2 \text{OR} \) was obtained, n-terminal sequencing was used to ensure that the protein was processed (meaning that the n-terminal signal sequence was not present). The resulting sequence determined from the protein sample gave the first 13 amino acids as ATGAXGSVAPGKL. This sequence was found to be identical to the n-terminal sequence of mature \( N_2 \text{OR} \) with the exception of the X, which is D in the sequence obtained from the translated gene.

![Figure 13. SDS PAGE analysis of fractions of \( N_2 \text{OR} \) purified from \( A. \text{cycloclastes} \). MW markers: 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, and 14.4 kDa.](image)

The Cu content of the protein was found to be between 2.5 and 5 Cu per monomer for purifications prior to the resolution of multiple forms. A wide variety of visible
spectra had been observed from these initial purifications, all showing the 480 nm, 540 nm, and ~800 nm peaks known to arise from a CuA-like site, but with varying levels of the 620 nm absorption attributed to the CuZ site. Resolution of multiple forms eventually revealed that the previous purifications had possibly been mixtures of at least the three oxidized forms separated to date. Table 2 below shows the differences in Cu and S content as well as differences in specific activity among the three forms resolved during aerobic purification from *A. cycloclastes*.

<table>
<thead>
<tr>
<th>Form</th>
<th>Specific Activity $\mu$mol N$_2$O min$^{-1}$ mg$^{-1}$</th>
<th>Sulfide/monomer</th>
<th>Cu/monomer</th>
<th>Cu/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple</td>
<td>62</td>
<td>0.6</td>
<td>3.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Pinkle</td>
<td>31</td>
<td>0.6</td>
<td>2.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Pink</td>
<td>7</td>
<td>0.5</td>
<td>1.4</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Table 2. Specific Activities and Cu/S contents of the multiple forms of N$_2$OR from *A. cycloclastes*.*

The data in Figures 14 and 15 shown in pink come from a low Cu, low activity pink form. This form shows the typical CuA visible absorption bands around 480 nm, 540 nm, and 800 nm, and was found to elute from the MonoQ column at the highest ionic strength. This form eluted from the column as the third of the three N$_2$OR peaks resolved. The X-band EPR spectrum for the pink form (Figure 15) shows nitrogen hyperfine in the $g_\perp$ with $A_\perp = 14$ G. A signal with $A_\parallel = 180$ G, indicative of a type II center is also present. Finally, the seven line hyperfine signal of a CuA center is present
in the g∥. This form is the aerobic, low activity Form II discussed in the introductory chapter of this thesis.

\[\begin{align*}
0.35 - & 0.30 - 0.25 - 0.20 - 0.15 - 0.10 - 0.05 - 0.00 \\
400 & 500 & 600 & 700 & 800 & 900 & 1000
\end{align*}\]

**Figure 14.** Visible absorption spectrum of pink N₂OR from *A. cycloclastes*.

\[\begin{align*}
1000 & \quad 0 \quad -1000 \\
2400 & 2700 & 3000 & 3300 & 3600 & 3900
\end{align*}\]

**Figure 15.** X-band EPR spectrum of pink N₂OR from *A. cycloclastes*. 
The visible absorption spectrum of the second N₂OR peak resolved on the MonoQ column can be seen in Figure 16 below, shown in magenta. The 620 nm peak is noticeably larger than any observed in the visible absorption of the pink form. Because this form falls between the pink and purple forms in every characteristic (elution from MonoQ, Cu content, specific activity, 620 nm visible absorption intensity, and EPR hyperfine signal intensities), this form was named "pinkle." The EPR spectrum in Figure 17 shows the nitrogen hyperfine found in the $g_\parallel$ with $A_\parallel = 14$ G, the type II Cu signal with $A_\parallel = 180$ G, and the seven line hyperfine with $A_\parallel = 40$ G, but the intensities of the signals are less than those found in the pink form.

![Figure 16. Visible absorption spectrum of pinkle N₂OR from A. cycloclastes.](image)
The purple form of the enzyme elutes from the MonoQ column at the lowest ionic strength of the three forms, and as can be seen in Figures 18 and 19, the purple form of N$_2$OR from *A. cycloclastes* shows a stronger visible absorption band at 640 nm than that observed in the "pinkle" form. The EPR spectrum of the purple form of the enzyme shows more strongly the seven line hyperfine pattern in the g$_\parallel$ arising from the Cu$_A$ center, with the type II signal in the g$_\parallel$ region and the g$_\perp$ hyperfine no longer present. This form is the aerobically purified equivalent to Form I discussed in the introduction chapter of this thesis.
Figure 18. Visible absorption spectrum of purple N$_2$OR from *A. cycloclastes*.

Figure 19. X-band EPR spectrum of purple N$_2$OR from *A. cycloclastes*. 
Overlays of the EPR spectra from the three forms of N₂OR obtained during aerobic purification are shown below in Figures 20-22. It is apparent that the type II signals (blown up in Figure 22) and the hyperfine structure in the $g_\perp$ (blown up in Figure 21) are strongest in the pink, lowest activity form, somewhat weaker in the "pinkle," mid-level activity form, and absent in the purple, high activity form of the enzyme. A difference spectrum is shown in Figure 23, in which the EPR spectrum of the purple form is subtracted from that of the pink form in order to remove the CuA signals, allowing a better view of the signals likely to be produced by the Cu$_Z$ site.

Figure 20. Overlay of the EPR spectra of purple, pinkle, and pink forms of N₂OR from *A. cycloclastes.*
Figure 21. Overlay of the EPR spectra of purple, pinkie, and pink forms of N\textsubscript{2}OR from \textit{A. cycloclastes} (range narrowed to focus on the differences seen in the nitrogen hyperfine).

Figure 22. Overlay of the EPR spectra of purple, pinkle, and pink forms of N\textsubscript{2}OR from \textit{A. cycloclastes} (region narrowed to focus on the differences in the type II signal).
Figure 23. Pink minus purple EPR difference spectrum showing removal of the Cu$_A$ seven line hyperfine to more clearly view the N-hyperfine and the type II signal.

The blue form of N$_2$OR from *Achromobacter cycloclastes*, which is isolated from highly concentrated cell extract (ie. conditions of low oxygen tension) exhibited the typical visible absorption band at 640 nm, much like the reduced form for which the crystal structure was obtained from *P. nautica*. The Cu$_A$ center is fully reduced to Cu(I)-Cu(I) causing its characteristic visible absorption bands to no longer be present in the spectrum (see Figure 24) (90). The EPR spectrum of the blue form shows the typical “broad-featureless” $S = \frac{1}{2}$ signal with possibly the poorly resolved hyperfine of spacing $A \approx 70$G seen previously (89).
Figure 24. Visible absorption spectrum of the blue form of *A. cycloclastes* N$_2$OR (purified from *S. meliloti*).

Figure 25. X band EPR of the blue form of *A. cycloclastes* N2OR (purified from *S. meliloti*).
Discussion

Just as Zumft and others had seen in early purifications of N$_2$OR, multiple forms of N$_2$OR can be isolated. While most early work isolated and distinguished the forms based on aerobicity, this may not be the only factor determining the activity and Cu content of the enzyme. As early as 1989 Jin et al separated "cathodic" and "anodic" forms of the purple enzyme using preparative isoelectric focusing (78). The cathodic form (pI = 4.97) exhibited higher activity than the anodic (pI = 5.06) form. In the recent work reported here it is found that N$_2$OR can be initially isolated in a high activity blue form when purified in highly concentrated cell extract (ie. extract with low oxygen tension). This initial step is carried out under aerobic conditions, therefore allowing the blue form to convert gradually to the "purple," "pinkle," and "pink" forms overnight with further exposure to oxygen. Storage of the blue form under argon appears to possibly prolong its lifetime, but does not seem preserve it indefinitely. Work to better resolve the various forms of N$_2$OR from *A. cycloclastes* was carried out with the hope of facilitating successful crystal growth. It now appears that homogeneity of redox state is crucial for formation of crystals (103).

It is unclear whether the multiple forms obtained in this work are a result of oxidation, degradation, or a combination of these factors. Observation of lower Cu content in aerobically purified N$_2$OR is not unprecedented. Initial purification of N$_2$OR from *Paracoccus denitrificans* resulted in apo-protein, but this was later revealed to be an artifact of the aerobic purification conditions (3). Additionally all reported Cu levels remained between 6 and 8 Cu per dimer until the structure from *P. nautica* revealed a
total of 12 Cu per dimer in 2000 (89;90). Loss of Cu from the Cu2 center, and
subsequent loss of the 640 nm absorption band, has been observed by Haltia et al in X-
ray crystal structures upon exposing the crystals to CN− or H2O2 (91). Perhaps the
prevalence of oxidized, Cu depleted forms of N2OR could indicate oxygen sensitivity
which acts to regulate the enzymatic activity when shifting to aerobic conditions.

An alternative explanation for the multiple forms of N2OR purified in this work
may be that the blue form is a fully reduced mixture of the forms that are revealed by
subsequent oxidation, and that these forms have been caught in different stages of
processing of the Cu2 site. CuA visible absorption bands are seen in all three forms after
the blue form has disappeared, while the 620 nm absorption arising from Cu2 appears to
be most pronounced in the purple form, slightly less intense in the pickle form, and much
lower (if not nonexistant) in the pink form. Resonance Raman spectra obtained by
Elizabeth Wilkinson also confirmed the fact that the three oxidized forms (purple, pickle,
and pink) of N2OR from A. cycloclastes showed the expected characteristically CuA
stretches.

EPR of each oxidized form shows the seven-line hyperfine in the g∥ with A∥ = 40
G, typical of a CuA site. The g⊥ region of the EPR in each form differs in the amount or
presence of nitrogen hyperfine with A⊥ = 14 G. In the purple form this hyperfine is
absent due to a more fully loaded Cu2 site containing mostly EPR silent Cu(I) and/or
greater antiferromagnetic coupling, making the site EPR silent. In addition, a type II Cu
signal is present with A∥ = 180 G in the pink form and to a lesser extent in the pickle
form (see Figures 15, 17, 20, and 22). This type II signal has now recently been reported
by Haltia et al in the aforementioned X-ray crystal studies involving Cu depleted CuZ after exposure to H2O2 (91). The purple form exhibits virtually no type II signal. These data agree with the EPR susceptibility work reported by Jin et al (78) in which an inverse correlation was observed between number of EPR detectable Cu sites and enzyme activity. In addition, variable temperature EPR studies on the purple, pinkie, and pink forms of the enzyme showed the type II signal to appear only at higher temperatures (~70 K), while the CuA seven line hyperfine was observed to be strongest at the lower temperatures down to 4 K. The temperature dependence of the signals indicates that the type II and CuA hyperfine signals arise from distinct copper centers. More specifically, the type II signal appears to come from the CuZ center, as the seven line hyperfine is well established as resulting from the CuA site.

The blue, reduced form of the enzyme shows the typical 640 nm absorption band, and EPR of the blue form shows the “broad featureless signal” (with a possible poorly resolved four line pattern showing $A_\parallel \approx 70$ G) described previously (89;91). Because the CuA center in this form is fully reduced to a Cu(I)-Cu(I) EPR silent state, the signal must be the result of one Cu in the CuZ center remaining in the cupric state or a pair of Cu atoms remaining in a coupled, mixed valence state. It has been proposed that the Cl ion found near Cu1 in the tetranuclear cluster is likely to be responsible for stabilizing an oxidized state (91). Spectroscopic studies by Chen et al indicate that the CuZ center in dithionite reduced N2OR exhibits a spin of $\frac{1}{2}$ with 3Cu(I) and a Cu(II), with the spin being delocalized over Cu1 and Cu2 via the bridging sulfide (94).
Contrary to the previous report by Hulse and Averill (97), native gel analyses run by both Jeanine Chan and myself indicate that N\textsubscript{2}OR isolated from \textit{A. cycloclastes} is dimeric. The band typically runs at 93 kDa, implying that the shape of the dimer is far enough from globular to run as a somewhat smaller protein would, but too large to be monomeric. X-ray crystal structures published to date have indicated a distance of 40 Å between Cu\textsubscript{A} and Cu\textsubscript{Z} within the same monomer, ruling out intramonomer electron transfer between the sites. A more feasible intermonomer Cu\textsubscript{A} to Cu\textsubscript{Z} electron transfer distance of 10 Å has been determined (91). Spectroscopic analyses and sequence homologies show N\textsubscript{2}OR from \textit{A. cycloclastes} to be similar to the enzymes for which crystal structures have been obtained, indicating that it will most likely involve the same domain sharing interactions required for activity.

Conclusions

It is important to note that the only form of N\textsubscript{2}OR previously reported to be purified from \textit{A. cycloclastes} is the pink, low activity form (although it was claimed to be a high activity form, numbers from other sources were reported inaccurately in the publication by Hulse and Averill, indicating that the activity numbers reported therein are not to be trusted). The work in this thesis is the first report of purification of multiple higher activity forms of N\textsubscript{2}OR from \textit{A. cycloclastes}. It is also the first report of more than two forms from a single purification from any of the organisms used to date. The multiple forms characterized in this thesis may offer an explanation for the Cu\textsubscript{Z}\textsuperscript{*} form.
previously discussed. It appears that the spectroscopically distinct forms of native enzyme reported to date may simply be a result of differing Cu content in the Cu$_Z$ center. Thus the “pinkle” form reported here could be a Cu$_Z$ form with the pink then being a new Cu$_Z^{**}$ form or vice-versa. Much remains to be learned about the various forms of N$_2$OR. The physiological relevance of the forms remains to be elucidated. Are the forms degradative states exhibiting Cu loss or are the forms representative of various levels of processing by the accessory Nos proteins, only becoming detectable upon oxidation? These questions were raised by the results reported here, but could not be answered definitively within the scope of this work.

Acknowledgements

All EPR spectra and the interpretations presented here are a result of work performed with the help of Elizabeth Wilkinson. Her contributions included (but were by no means limited to) providing the necessary information and insight to begin interpreting the nature of the multiple forms of N$_2$OR. The protocols for the activity and sulfide assays were modified and greatly improved by Jeannine Chan. The final 280 nm extinction coefficient numbers for N$_2$OR, based on CD/MCD analysis, were obtained by Doreen Brown.
THE NOS DFYL ABC "TRANSPORTER"

Introduction

For nearly two decades it has been known that the DFYL operon plays a role in CuZ formation. However, the nature of this role remains unclear. In addition to the finding that Tn5 insertions in these genes resulted in a CuA-only form V, a mutation in the signal sequence of N2OR was found to result in cytoplasmic, apo-protein (66). Cu could be inserted into the CuA site in vitro, implying that at least part of the protein was properly folded, and that the insertion of CuZ probably required the proximity of the periplasmic components. Features of the primary structures of the nosDFYL gene products indicate that they are likely to be members of the family of ATP-binding cassette (ABC) transporters (104). This is one of the largest families of proteins and comprises three main types of systems; importers, exporters, and systems not involved in transport (67). Classes of ABC systems lacking a transport function have been linked to cellular processes and their regulation. For example, ABC systems involved in DNA repair, translation, and regulation of gene expression have been discovered (68). Nearly 5% of the E. coli genome codes for ABC transport genes, resulting in a total of approximately 70 different transporters, which are probably necessary for the wide variety of environments to which the organism adapts. Typically ABC transporters are substrate specific and have been found for a wide variety of molecules including charged
molecules, hydrophobic molecules, organic ions, sugars, amino acids, proteins, and complex polysaccharides (69).

ABC transporters usually comprise a minimum of four domains. These are the two transmembrane domains (TMD) and two nucleotide (ATP) binding domains (NBD). The two TMDs each typically have six membrane-spanning α-helices. Together the TMDs make a channel through the membrane for transport of the substrate, and are found to contain substrate binding sites (108). Typically the ABC systems not involved in transport do not have TMDs (68). The two NBDs are found in the cytoplasm, and have a characteristic 215 amino acid domain which is involved in ATP-binding. The NBDs are both required and appear to alternate in hydrolyzing ATP rather than carrying out hydrolysis simultaneously. In addition to the four domains discussed thus far, a periplasmic binding protein (PBP) is also often present. The job of the PBP is to bind substrate with high affinity and specificity as well as confer directionality to the transporter (69). To date, 100% of the systems characterized that include a PBP have been found to be importers, while those lacking PBPs tend to be exporters (69). In Gram positive bacteria an equivalent component to the PBP can be found in the form of a lipoprotein anchored to the surface of the cell. Another component has been found to be necessary in some ABC transporters. This is an outer membrane protein which can either bring the substrate into the periplasm or, in the case of protein exporters, help transport the protein through the periplasm and outer membrane.

In September of 2001 the first high-resolution structure was reported for a complete ABC transporter (108). It confirmed that the pathway through which the
substrate crosses the membrane is composed of six predicted membrane spanning \( \alpha \)-helices of each TMD in the dimer (109). Each monomer in the homodimeric structure is composed of a TMD, a NBD, and an intracellular TMD loop that appears to bridge the TMD and NBD (108;109). This intracellular TMD bridge is likely to transduce signals between the NBD and the TMD.

In the case of the Nos ABC transporter, as discussed in the Introduction chapter of this work, exactly what is being transported is unclear. Although the system appears to be an importer due to the presence of periplasmic binding proteins, it also seems unlikely that it would import Cu into the cytoplasm for subsequent Cu\( \text{Z} \) formation in the periplasm. Purification of the components of the Nos system was pursued in this work with the hope of defining the function/mechanism of this ABC transporter.

**Experimental Methods**

**Molecular biology for n-terminal NosD tagging with Strep-tag II.**

NosD was n-terminally Strep tagged by John Bollinger using the plasmid pZpD (pUC18 containing the nos gene cluster with the nos\( Z \) gene removed). This construct was then Sse8387I (an isoschizomer of SbfI) restriction digested to remove the nos cluster from pUC. pMLA, a multi-copy; broad host range plasmid used successfully for expression of recombinant enzymes in *Achromobacter cycloclastes*, was also digested by Sse8387I, and subsequently treated with calf intestine alkaline phosphatase (CIP). The
fragments were then purified by 1% agarose gel electrophoresis (AGE). Bands of the appropriate size (~10,000 bp for pMLA and ~7,500 bp for the ZpD insert) were cut from the gel and extracted using the Bio Rad Prep-a-gene kit. Ligation of the ZpD insert into pMLA was carried out overnight at 16°C using T4 DNA ligase from Takara. The ligated plasmid was heat shocked into *E. coli* Top10F' according to the protocol suggested by Invitrogen. Transformants found to be positive for the *nos* cluster insert were then grown in 2 mL culture for mini-prep plasmid purification using an Eppendorf mini-prep kit. Six plasmid mini-preps were carried out and the purified plasmid was digested with Sse8387I restriction enzyme in order to confirm the presence of the insert. All six plasmid preps were found to contain the insert and two samples were then electroporated into electrocompetent *A. cycloclastes* according to the method discussed in Chapter 2 of this work.

**Preliminary growth and purification of n-terminally Strep tagged NosD.**

Two of the transformants were chosen for initial 250 mL culture growth with wild type *A. cycloclastes* grown as a control culture. These cultures were induced after they had reached an optical density (O.D.600) near 1.0. Induction consisted of addition of CuSO₄·5H₂O to obtain a concentration of 10 μM, addition of KNO₃ to obtain a concentration of 10 mM, and slowing of the shaking rate to 120 rpm from 240 rpm for 24 hours. The cells were harvested by centrifugation at 7,000 g for 10 minutes, resuspended in 30 mL of media and frozen overnight at -80°C. Upon thawing, the cells were again centrifuged at 7,000 g for 10 minutes. The pellets were each treated with 10 mL
Bacterial Protein Extraction Reagent (BPER). The lysate was centrifuged at 30,000 g for 20 minutes. Each supernatant was run through a 1 mL Streptactin sepharose column in separate purifications. The column was washed with 100 mM TrisCl, pH 8.0 (buffer W) containing 1 M NaCl after loading extract. These washes were collected in 1.5 mL fractions. Elution was carried out using buffer W with 2.5 mM desthiobiotin. Six 0.5 mL elution fractions were collected and concentrated to less than 50 µL each, using YM10 microcon concentrators.

Molecular biology for c-terminal NosD tagging with Strep-tag II.

The following oligos (Life Technologies, GIBCO BRL) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the c-terminus of the nosD gene were used in an inverse PCR reaction for insertion of Strep-tag II using inverse PCR:

5’CCAGCTGGCGCGGCCCTCGATGTGAGCCTGGAGATTGTCAGCG3’ priming from the antisense strand and
5’AGCCATCCGCAGTTCGAGAAGTAAAGCGTTTTCCAATCGGTGGAAG3’ priming from the sense strand. The inverse PCR reactions for tagging were carried out using Pfx polymerase in 20 µL volumes containing concentrations of 0.5, 1, 2, and 4 mM MgSO₄ with 2 µL Pfx enhancer added to each reaction. After an initial melting step of 3 minutes at 94° C, 30 cycles comprising a 1 minute melting step at 94° C, a 1 minute annealing step at 64° C, and a 12 minute extension at 68° were run. DpnI digestion was carried out for 20 minutes at 37° C after PCR to reduce template contamination. pZpD plasmid (see
table of plasmids in appendix A) was used as the template. The products from the reactions were then purified by 1% agarose gel electrophoresis and extracted using a BioRad "Prep-a-gene" kit. The purified product was then ligated and transformed into *E. coli* Top10F' by heat shock. Colonies were screened by PCR using a primer that spans the ligation point in the Strep tag. Positive colonies were then grown in 2 mL culture for plasmid mini-prep purification using an Eppendorf kit. The plasmid samples were then sequenced using the ABI Prism Big dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The two plasmid samples (called pZpDSt) found to contain the correct and complete tag were then subject to Sse8387I restriction digest for 2 hours at 37°C. pMLA plasmid was also digested by Sse8387I and treated with calf intestine alkaline phosphatase (CIP) for the last 30 minutes of the digest. An attempt to purify the fragments by 1% AGE revealed that both vectors containing the tag had an approximately 2,000 bp deletion in the *nos* cluster. There was no obvious explanation for the mysterious deletions, and their locations were unknown. In order to use the correctly sequenced tags, the small region immediately surrounding the tag would have to be removed by restriction digestion and placed into the original template. A BsrGI restriction site was located ~700 bp upstream of the 3' end of *nosD* and a KpnI restriction site was located ~370 bp downstream from the 3' of *nosD*. Unfortunately, the KpnI site was not unique and would result in three fragments when digesting the original vector for insertion of the tagged *nosD* 1,070 bp fragment.

The KpnI site was removed from the original pUC18 plasmid by KpnI digest followed by polishing of the sticky ends by T4 DNA polymerase. The pUC18 fragment
was then purified by 1% AGE, extracted from the gel, and religated overnight. The "KpnI minus" (KpnI) pUC18 was then KpnI digested again to linearize any plasmid that still contained the KpnI site, and the mixture of linear and circular plasmid was used for transformation into *E. coli* Top10F', taking advantage of the fact that linear DNA would not be taken up as readily as the circular DNA lacking the KpnI site. Transformants containing the KpnI pUC18 were then grown in 2 mL culture for mini-prep plasmid purification using a Qiaprep kit.

*Sse8387I* digests of KpnI pUC18, pZpD, and pcluster were carried out, and the fragments were gel purified by 1% AGE. The bands containing the ZpD portion of the *nos* cluster and the full *nos* cluster were each combined with 1/3 of the KpnI pUC18 band and then extracted from the gel for ligation overnight. KpnI pZpD and KpnI pCluster were then heat shocked into *E. coli* Top10F'. PCR screening of the transformants allowed selection for plasmid containing the *nos* cluster. Mini-prep plasmid purification was then carried out on the PCR positive constructs. The plasmids were digested with Sse8387I and analyzed by 1% AGE to confirm the presence of the *nos* cluster insert. The KpnI pCluster and pZpD were then BsrGI/KpnI double digested and only two fragments resulted from each. One of the two fragments was the untagged 1,070 bp region around the 3' end of *nosD*, which was discarded and replaced by the same fragment containing the tag.

A BsrGI/KpnI double restriction digest was carried out on the pZpDSt plasmid containing the mystery 2,000 bp deletion as well as a previous construct (pJQDStII) in which NosD had been successfully tagged on its c-terminal end but was not produced in
detectable quantities (possibly due to the fact that it contained only the DFYL operon under its own promoter). The 1,070 bp band was removed from the 1% agarose gel for each digested plasmid.

There were four ligation reactions as follows: pZpD based tag insert + pZpD, pZpD based tag insert + pCluster, pJQ based tag insert + pZpD, pJQ based tag insert + pCluster. These ligations were then transformed into *E. coli* Top10F′, and transformants were screened for the Strep tag by PCR. PCR positive transformants were grown in 2 mL culture for mini-prep plasmid purification. Purified pZpDSt and pClusterDSt were then Sse8387I restriction digested and the large band (nos cluster) extracted for insertion into Sse8387I digested pMLΔ. After ligation, pMLZpDSt and pMLClusterDSt were heat shocked into *E. coli* Top10F′. The transformants were screened by PCR as before to detect the presence of the tagged *nosD*, ensuring that recircularized pMLΔ was not selected for subsequent mini-prep purification. The purified plasmid was then Sse8387I digested to confirm the presence of the insert. Midi-prep scale plasmid purifications were used for electroporation into *A. cycloclastes*.

Culture growth and purification of c-terminally Step-tag II tagged NosD.

*A. cycloclastes* containing the plasmid pMLZpDSt was grown in Luria Bertani media containing 50 ppm gentamicin at an initial shaking rate of 240 rpm, a temperature of 30° C, and in volumes of 50 mL for starter cultures. When the starter cultures have grown to an O.D.₆₀₀ of at least 1.0, they are then transferred into 2 L batches of media in 2.8-3.0 L flasks. The shaking rate is held at 240 rpm until an O.D.₆₀₀ of 1.0-1.4 is
reached. At O.D. > 1.0 shaking is slowed to 120 rpm to create semi-anaerobic conditions. The induction also includes addition of 4 mL of 0.05 M CuSO4·5H2O and 20 mL of 1 M KNO3 to obtain final concentrations of 100 µM Cu2+ and 10 mM NO3⁻ respectively. After 24 hours of induction the cultures are harvested by centrifugation at 7,000 g for 10 minutes at 4° C. The pellets are then resuspended in a minimal volume (no more than 3x cell paste volume) of 100 mM TrisCl pH 8.0 and frozen at -80° C. After being thawed, the cells are lysed by either sonication or french press. Initial centrifugation at 30,000 g for 20 minutes is followed by ultracentrifugation at 45,000 rpm for one hour. The cell extract is loaded onto a 5 mL Streptactin sepharose column (Sigma-Genosys) equilibrated in 100 mM TrisCl pH 8.0 (buffer W). The column is then washed in buffer W containing 1 M NaCl. Elution of the tagged protein is carried out by running 2.5 mL aliquots of buffer W containing 2.5 mM desthiobiotin through the column.

**Molecular biology for c-terminal tagging of NosD with His₆.**

The following oligos (MWG Biotech) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the c-terminus of the nosD gene were used in an inverse PCR reaction for insertion of Strep-tag II using inverse PCR:

5'(CAC)₆TAAGCGTTTCCAATCGGTGGAAG3' priming from the antisense strand and 5’GCTGACAATCTCCAGGCTCACATCGAGGGCCGCGCCAGC3’ priming from the sense strand. The inverse PCR reactions for tagging were carried out using Pfx polymerase in 20 µL volumes containing concentrations of 0.5, 1, 2, and 4 mM MgSO4
with 2 μL Pfx enhancer added to each reaction. After an initial melting step of 3 minutes at 94° C, 30 cycles comprising a 1 minute melting step at 94° C, a 1 minute annealing step at 64° C, and a 12 minute extension at 68° C were run. Template for the reaction was a derivative of pCluster plasmid which had been downsized to 5.3 kbp by HindIII/KpnI double digest, with insertion of the fragment comprising the 3’ end of nosZ, nosD, and the 5’ end of nosF into double digested pUC18 vector (see table of plasmids in appendix A). The products from the inverse PCR were then purified by 1% agarose gel electrophoresis and extracted using a BioRad "Freeze and Squeeze" kit. The purified product was then ligated and transformed into E. coli Top10F’ by heat shock. Colonies were screened by PCR using a primer that spans the ligation point in the His tag. Positive colonies were then grown in 2 mL culture for plasmid mini-prep purification using a BioRad mini-prep kit. The plasmid samples were then sequenced using the ABI Prism Big dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). A BsrGI restriction site located 700 bp upstream of the 3’ end of nosD, and a KpnI restriction site located 370 bp downstream from the 3’ of nosD, were used to lift a fragment composed of the His tagged end of nosD out of pDF, for insertion into KpnI minus pCluster (which had been created previously for insertion of the Strep tagged version of this fragment). After transformation into E. coli Top10F’ by heat shock, colonies were screened for presence of the His tag. PCR positive colonies were then grown in 2 mL culture and mini-prep plasmid purifications carried out for subsequent Sse8387I digest. pCluster containing His6 tagged was then subject to Sse8387I digest in
order to remove the cluster from the pUC18 vector for insertion into the final Sse8387I linearized pMLΔ vector.

**NosD mutagenesis work.**

Attempts to mutate the potential Cu ligands in the c-terminal Strep-tag II construct of NosD were carried out using the Quick Change mutagenesis kit from Stratagene. The oligomers designed and ordered for NosD mutagenesis are as follows (changed nucleotides shown in bold):

- **M207S**
  - Down: 5'GCCGTGCACTTCTCGTACACCCGCAACACC3'
  - Up: 5'GGTGTTCGCGGTGTACGAGAAGTGACGGGC3'

- **M228S**
  - Down: 5'GGGCTTTGCGATCTCGTTCTCCAACCGCGC3'
  - Up: 5'GCGCGGGTTGGAGAACGAGATCGCAAGTGGCAAGCC3'

- **H295A**
  - Down: 5'GCCGCATTGGCATCTCCTTACCACGCGCTGTCG3'
  - Up: 5'CGAACCGGCGGTGAAGGCTATGCCAATGCAGCC3'

The primers for PCR screening of potential mutants were as follows:

- **M207S** 5'GCTTTGCCGTGCACTTCTC3'
- **M228S** 5'CATCTGGGCTTTGCGATCTC
- **H295A** 5'CTGCGGCATTGGCATAGCC3'

**Molecular biology for c-terminal NosF tagging with Strep-tag II.**

The following oligos (Life Technologies, GIBCO BRL) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the c-terminus of the nosF gene, were used in an inverse PCR reaction for insertion of Strep-tag II:

5'CCAGGCGCTGCGGCCCTCGATTTGCCCGTCCCTCCGGCTGAA3' priming from the antisense strand and
5'AGCCATCCGCAGTTGACAGGATGAGCCGCATCCTTGCCACCG3' priming from the sense strand. The tagging inverse PCR reaction involved copying the entire plasmid, and was found to be more successful on very small templates. Therefore the original pCluster plasmid (see Appendix A) was KpnI digested, and the ~5,800 bp fragment containing the c-terminus of nosF, the nosYLX genes, and pUC was religated for use as the template. PCR was run with an initial melting step of 3 minutes at 94° C, 30 cycles comprising a 1 minute melting step at 94° C, a 30 second annealing step at 65° C, and a 6 minute extension at 68° C. DpnI digestion was carried out for 20 minutes at 37° C after PCR to reduce template contamination. The products were then ligated, transformed into E. coli TopI O'F', and transformants were screened for presence of the Strep tag as for the c-terminal NosD tagging. The tagged region of each PCR positive plasmid sample was then sequenced. Plasmid containing the correct sequence was then KpnI restriction digested again to open if for ligation to the nosRZDF\textsubscript{partial} half of pCluster, which had been removed to produce smaller template for PCR. In addition, pZpD was KpnI digested, and the fragment corresponding to the nosRZDF\textsubscript{partial} half (but missing nosZ) was also ligated to the tagged nosF fragment. This resulted in two constructs, one containing tagged nosF in the complete gene cluster and one with the DFYL operon placed in front of the nosZ promoter. Both of these constructs were then screened for proper orientation of the ligation by Sse8387I digest.

For each construct (pZpD and pCluster based) three ligation products could be found after growth in E. coli TopI O'F' on ampicillin plates; the correctly ligated complete plasmid, the backward ligation of the two fragments, and reclosure of the small pUC
containing fragment. In order to simplify the Sse8387I digest results, the transformants were all resuspended together in 1xTE buffer, a plasmid mini-prep was carried out with subsequent XhoI digest for linearization and purification by 1% AGE. Only the larger band containing the two complete plasmid possibilities was re-transformed in *E. coli* Top10F'. This eliminated the many colonies that would otherwise be screened and found to contain only the religated, small fragment. Religation of the two larger XhoI linearized plasmids comprised by the large band, transformation, mini-prep purification, and subsequent Sse8387I/1% AGE analysis revealed some samples containing the fragment of the proper length to be the complete *nos* gene cluster. This band was extracted from the gel and ligated into Sse8387I digested, CIP treated pMLΔ.

Transformants resulting in positive PCR results from screening for Strep-tag II were then grown in 50 mL culture for plasmid midi-prep purification. The pMLClusterFSt plasmid was electroporated into *A. cycloclastes* for homologous expression. No pZpD based candidates were found during the XhoI analysis, thus the pCluster based construct was carried forward.

Purification of NosF.

Purification of NosF was attempted using the same 5 mL Streptactin sepharose column as for purification of NosD. After loading extract onto the column, the column was washed thoroughly with 100 mM TrisCl, pH 8.0, containing 1 M NaCl. Elution was carried out in 2.5 mL aliquots of 2.5 mM desthiobiotin in 100 mM TrisCl, pH 8.0. A second, albeit unsuccessful, attempt included use of a cibacron blue column with a total
volume of 5 mL. Purification using this column will be attempted again as a second step, when larger quantities of NosF are visible by SDS PAGE, allowing easier detection of NosF in wash vs. eluent fractions.

Molecular biology for c-terminal NosY tagging with Strep-tag II.

The following oligos (Life Technologies, GIBCO BRL) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the c-terminus of the nosD gene were used in an inverse PCR reaction for insertion of Strep-tag II using inverse PCR: 5'AGCCATCCGCAGGAGAAGTGAGGACCCGACTTCGCTTCG3' priming from the sense strand and 5'CCAGCTGGCGCGGCCCTCGATTGGGGTCACCTTTCGGAAGGCT3' priming from the antisense strand. The entire tagging protocol for NosY was identical to that for NosF (discussed previously). For the case of NosY, only the pZpD based construct was found during XhoI digest analysis, and therefore only pMLZpDYSt could be carried forward.

Purification of NosY.

Purification of NosY was attempted using 11 g of cell paste that had been grown and harvested in the same manner as for NosF purification. After thawing, the cell paste was resuspended in 100 mL of 100 mM TrisCl pH 8.0 and sonicated using the usual protocol from NosD and NosF purification. The crude extract was centrifuged at 30,000 g for 20 minutes and then ultracentrifuged for one hour at 45,000 rpm in a Ti50 rotor to
remove any remaining membrane material. The pellets from both centrifugations were resuspended in 100 mM TrisCl, pH 8.0 containing 1% LDAO. The pellets were then sonicated ~20 minutes to ensure full solubilization. The samples were again ultracentrifuged for one hour at 45,000 rpm in the Ti50 rotor to remove any remaining unsoluble material. The Streptactin sepharose column was equilibrated in the same LDAO containing buffer discussed above, and the solubilized membrane fraction was loaded onto the column by gravity flow. The column was then washed with 100 mM TrisCl, pH 8.0, 1% LDAO. The same buffer containing 2.5 mM desthiobiotin was used for elution of Strep tagged NosY from the column. A microdialyzer (10,000 MWCO) was used to exchange the LDAO with 0.5% SDS in order to possibly allow analysis of the protein by SDS PAGE.

Results

n-terminally Strep tagged NosD purification.

Attempts to purify n-terminally Strep tagged NosD by affinity chromatography using a Streptactin sepharose column were unsuccessful. The protein appeared to be present in the wash fractions, not in the eluent, as determined by SDS PAGE and western blotting for the Strep-tag II using streptavidin alkaline phosphatase conjugate (SAAP) for detection.
c-terminally Strep tagged NosD purification.

As can be seen in Figure 26, the eluent fractions from purification attempts on c-terminally tagged NosD contained contaminants that could not be removed by washing the column even with 1M NaCl in 100 mM TrisCl pH 8.0. The band seen at 67 kDa was confirmed to be N₂OR by western blotting using the protocol discussed in the previous N₂OR chapter of this thesis.

![Diagram](image)

**Figure 26.** Eluent fractions from affinity purification of NosD using Streptactin sepharose (1 M NaCl wash). MW markers: 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, and 14.4 kDa.

The band immediately below N₂OR, running at ~55 kDa, was n-terminally sequenced from a blot onto a PVDF membrane. The resulting sequence of MRAILEQ matched most closely with that of a putative propionyl-CoA carboxylase β-chain protein (MRAVLEQ) using a BLAST search. This contaminant could be removed by additional washing with 5 mM CuSO₄·5H₂O (see Figure 27). The contaminants at ~23 kDa and 14 kDa were also blotted onto a PVDF membrane, and the n-termini sequenced. The 23 kDa band gave a sequence of ADKKPGID(q?)AL(i?)X(d?)L which BLAST results
suggested to be biotin carboxyl carrier protein (BCCP) from *Sinorhizobium meliloti*. The 14 kDa band gave a sequence of APAAESARASKN which differed by two residues from a putative transketolase from *Aeropyrum pernix*. Western blotting for the Strep tag indicated that the 46 kDa band was NosD, and N-terminal sequencing confirmed this by matching exactly to the predicted sequence of AERAVMPGAGSL. A visible absorption spectrum was obtained on the mixture and the 480 nm and 540 nm absorption bands due to CuA in N2OR were apparent (see Figure 28). A 410 heme Soret band is also present in the visible spectrum. It has not been determined which protein in the mixture contains a heme center.

**Figure 27.** SDS PAGE of Strep-tag II NosD/N2OR eluent fractions from Cu wash. MW markers: 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, and 14.4 kDa.

**Figure 28.** Visible absorption of NosD/N2OR
Figure 29. NCBI BLAST result showing presence of one to two CASH domains in each NosD sequence.

**BLAST results for NosD.**

A BLAST search against the Conserved Domain Database was carried out using the predicted amino acid sequence of NosD from *A. cycloclastes* (http://www.ncbi.nlm.nih.gov/BLAST). A domain with homology to NosD was
identified from several S-layer type proteins from the pectate lyase superfamily. This
domain, called CASH (for carbohydrate binding proteins and sugar hydrolases), is found
in over 1000 proteins from all of the three kingdoms of life, and consists of a right
handed β helix structure (110). Sequences of NosD from other organisms also appeared
to contain at least one CASH domain, with many containing two (see Figure 29).

The amino acid sequence of NosD was threaded onto the structure of Pectin
Methylesterase from carrot (111) (PDB ID Igq8), resulting in the structural model for the
NosD CASH domain as shown below (Figure 30). Of the 442 amino acids which make
up the NosD primary structure, 266 were aligned sufficiently. The remaining 176 amino
acids were excluded from the models shown here. A surface model of the CASH domain
was created, and the electrostatic potential was mapped to this surface using the Swiss

**Figure 30.** Ribbon diagram of sideview (left) and end-on view (right) of the model of the NosD
CASH domain based on the structure of Pectin Methylesterase from carrot (111) (PDB ID Igq8).
Discussion

Attempts to purify NosD using the Strep-tag II affinity purification system via n-terminal tagging proved unsuccessful. All bands of the correct size seen in SDS PAGE appeared in the wash fractions. No protein was found in eluent fractions. The most logical explanation for detection of tagged protein, but only in wash fractions, is that the n-terminus may be buried during protein folding. SDS PAGE for western blot detection of the tag denatures the protein, exposing the tag.

Purification of c-terminally Strep tagged NosD resulted in elution of multiple proteins as can be seen in Figure 24. Because the affinity column can bind biotinylated proteins, it was expected that one or more of the contaminants could be binding to the column rather than interacting with NosD in any way. As shown in the figure, n-terminal sequencing confirmed that at least one of these proteins was indeed a biotinylated contaminant. Addition of Strep-tagged Xa protease to the eluent and a second run
through the Streptactin sepharose column should remove any biotinylated proteins in future purification attempts. The protease and biotinylated proteins will remain in the column while the NosD, having had its strep tag removed at the Xa site, will wash through.

Western blot for Strep-tag II and n-terminal sequence results confirmed that the 46 kDa band in SDS PAGE was indeed NosD. Western blot for N₂OR and the presence of its visible absorption bands helped confirm that the 67 kDa band was N₂OR. Because a solution containing 1 M NaCl and 5 mM Cu could not remove the N₂OR from the NosD bound to the affinity column, it became clear that the interaction was fairly tight. Perhaps more interesting is the fact that the N₂OR exhibits Cu visible absorption bands. This indicates that the N₂OR in the NosD/N₂OR complex does contain some Cu and is not bound in only an apo-form.

Native gel electrophoresis on the least contaminated preparation of NosD/Z revealed a band at ~93 kDa, (which tested positive for N₂OR by Western blot), a band at ~150 kDa (which also tested positive for N₂OR by Western blot), and a band at ~190 kDa, which did not appear to contain N₂OR. As discussed previously in the N₂OR chapter of this thesis, the 93 kDa band has been determined to be the N₂OR dimer based upon native gel analysis of pure, dimeric N₂OR and Western blotting. The band found at ~150 kDa was also shown to contain N₂OR by Western blotting, and is the correct size to be the “93 kDa” dimer of N₂OR in complex with one monomer of 46 kDa NosD. The ~190 kDa band remains to be explained, but is possibly a multimer of the remaining contaminant protein.
Annotations of the NosD gene have typically listed it as a putative periplasmic Cu binding protein, but alignment of the available NosD translated sequences reveals that the only conserved potential ligands to Cu are one His, two Met and two Tyr (see Appendix B). BLAST results for NosD indicate the presence of a CASH domain in the protein, but the implications of this are unclear at the moment. CASH domains are found in S-layer proteins, and are involved in binding carbohydrates. Exactly how this functionality would fit into the role of CuZ formation in N2OR is unclear, and much remains to be elucidated about the NosD/Z complex. Clearly the electrostatic potential calculations mapped to the surface for the CASH domain of NosD, based on a published structure of the domain, show dramatic patches of negatively charged residues wrapping around a large portion of the domain, with the surrounding positively charged patches well isolated by neutral residues. This dramatic sequestering of charged residues is likely to play an important role in interactions with N2OR.

While initial purification attempts on NosF and NosY were not entirely successful, potential bands of each protein were faintly visible by Coumassie blue stained SDS PAGE gels. Western blotting of these faint bands indicated that they may possibly be the desired product in each case (ie. positives were faint, but present).

Conclusions

This is the first report of purification of NosD. Although the protein was not purified to homogeneity, it was purified in complex with N2OR, a protein that had only
been speculated to interact with it in the past. Prior to this work no evidence had been obtained for a direct interaction of NosD with N_2OR. The complex showed the visible absorption bands characteristic of Cu_A in N_2OR, therefore demonstrating that the N_2OR was not bound in only apo-form, but did contain some Cu. It remains to be determined how much Cu is actually present in N_2OR in this complex, and if any N_2O reducing activity will be detected. Ideally, separation of the complex would answer the question of whether or not NosD actually is a Cu binding protein as has been the annotation from the sequence data reported to date.

In addition to purification work on NosD, NosF and NosY were also successfully tagged using the Strep-tag II system for affinity purification. Further work toward purification of these proteins should prove invaluable in reconstitution of the Nos system and in ascertaining its exact role in Cu_2 formation.
PURIFICATION AND CHARACTERIZATION OF THE FLAVOPROTEIN NOSX FROM ACHROMOBACTER CYCLOCLASTES

Introduction

The nosX gene was discovered in *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) in 1997, and was determined by Tn5 insertion to be necessary for N₂O reduction (70). While nosX genes have been identified in multiple organisms (see Appendix C), the sequence downstream of nosL in *P. stutzeri* does not appear to be nosX. The possibility remains that the nosX gene could reside elsewhere in the *P. stutzeri* genome. The translated *S. meliloti* gene appeared to contain a leader sequence of 31 residues as well as a putative transmembrane helix in the C-terminal domain, leading Chan et al. to predict a peripheral membrane protein. Others have predicted a periplasmic location for the NosX protein based on the presence of a double arginine motif in the N-terminus, which may indicate involvement of the TAT translocon in export to the periplasm (71).

NosX shows homology only to ApbE thiamine biosynthesis lipoprotein precursor (55% similar, 34% identity), NirX of *P. denitrificans* (64% similarity, 45% identity), and RnfF of *Rhodobacter capsulatus* (51% similarity, 26% identity). In this work, the nosX gene product was purified to homogeneity using the Strep-tagII affinity purification system, and characterized for the first time. NosX was found to be a soluble, periplasmic flavoprotein, with FAD (flavin adenine dinucleotide) as the cofactor.
Flavoproteins catalyze a wide variety of reactions including O₂ activation, aromatic hydroxylation, dehydrogenation, and one or two electron transfer. In addition flavoproteins with functions other than electron transfer functions are surfacing. One such example is gene transcription regulation involving NifL. The cofactors that distinguish flavoproteins from others are FAD and FMN, which can be bound noncovalently or less frequently in a covalent linkage to the 6 or 8 position in the isoalloxazine ring structure (see Figure 32) (112). Flavin cofactors result in strong visible absorption maxima which can be measured under varying conditions to probe for information about the environment in the flavin binding pocket of the protein, the
ionization state of the flavin, substrate binding, and kinetic parameters. The reduction path of a flavin depends greatly on the protein in which it is bound, and can consist of either a two electron reduction directly to the hydroquinone form or a one electron reduction to the semiquinone form. The semiquinone form comprises two possible radical species which can be distinguished spectroscopically, one being protonated (neutral or blue semiquinone), and the other monodeprotonated (anionic or red semiquinone) (112). The neutral/blue semiquinone typically shows an absorbance maximum at 580-620 nm while the anionic/red species exhibits a maximum absorbance at 380 nm and a sharp peak at 400 nm. The presence and lifetime of these species is highly dependent on the function of the flavoprotein as well as the environment in which the protein is found (e.g. pH, presence of substrate or inhibitors). Reduction to the hydroquinone form causes bleaching of the flavin chromophore and subsequent disappearance of the absorption maxima.

Experimental Methods

Molecular Biology for c-terminal tagging of NosX with Strep-tag II.

The nosX gene was tagged on its 3' end (the C-terminus of the gene product) using the Strep-tag II eight amino acid tag (-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys-) from Sigma-Genosys by the following protocol. The 9486 bp nos gene cluster from Achromobacter cycloclastes (ATCC 21921 IAM1013) was inserted into the Sse8387I restriction site of pUC18. The resulting plasmid, called pCluster, was then restriction
digested using KpnI restriction enzyme to give two fragments of 6,569 bp and 5,829 bp containing RZDFn-terminus and Fc-terminus YLXpUC respectively (see Figure 33). The fragment containing pUC18, the N-terminal portion of nosF, nosY, nosL, and nosX was religated and transformed into *E. coli* Top10F'. The 5,829 bp pFYLX plasmid was used as template in an inverse PCR reaction using primers (Life Technologies, GIBCO BRL) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the C-terminus of the nosX gene. The following oligomers were designed using the codon preferences of *A. cycloclastes*;

5'CCAGCTGGCGCGGCCCTCGATAACGGAGGCGGCTTCCAGGCG3' priming from the sense strand and

3'AGCCATCCGCGAGCTCGAGAAGTAAACGGCGACCAAGGAACGT5' priming from the antisense strand. The inverse PCR reactions for tagging were carried out using Pfx polymerase in 20 μL volumes containing concentrations of 0.5, 1, 2, and 4 mM MgSO4 with 2 μL Pfx enhancer added to each reaction. DpnI digestion was carried out for 20 minutes at 37° C after PCR to reduce template contamination. The products from the reactions were then purified by 1% agarose gel electrophoresis and subsequent extraction using a BioRad "Prep-a-gene" kit. The purified product was then ligated and transformed into *E. coli* Top10F' by heat shock. The oligos listed above each contain a portion of the Strep-tag II, causing the site of ligation of the PCR product to fall in the tag. PCR screen of *E. coli* Top10F' transformants using a primer designed to span the site of ligation in the tag allowed detection of successfully tagged constructs. PCR positive transformants were then grown for plasmid minipreps. The plasmid samples
were then sequenced using the ABI Prism Big dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). pFYLX containing the correct insertion was then restriction digested by KpnI and ligated back into the KpnI site of the 6,569 bp fragment from the original KpnI digestion of pCluster. Three products that would result in Ampicillin resistant *E. coli* Top10F’ were possible from this ligation. All transformants on the LB Ap60 plates were resuspended in 2.5 mL 1xLB per plate. Minipreps were carried out on the multi-transformant mixtures, and the plasmid samples were run on 1% agarose gel electrophoresis (AGE) after linearization by XhoI restriction digest. The smallest ligation product, the reclosure of pFYLX, could easily be removed by AGE. The remaining AGE bands of the mixture consisted of two 12kbp products ligated in the correct or reversed orientation. This mixture was extracted from the agarose gel and re-ligated for transformation into *E. coli* Top10F’. Plasmid minipreps from individual colonies were subject to Sse8387I restriction digest which would result in two fragments for both possible constructs. The fragments for the correctly oriented ligation would differ from those of the reversed insertion by at least 400 bp, and when run beside pCluster Sse digest as a standard could be easily discerned in 1% AGE. The intact nos cluster fragment from the correctly oriented product was then extracted from the gel and ligated into the Sse8387I site in the multi-copy, broad host range plasmid pMLΔ (98a). A midiprep of the resulting plasmid (pMLClustXSt) containing the entire nos gene cluster including the c-terminally tagged nosX gene yielded sufficient quantities for electroporation into *A. cycloclastes*. 
Figure 33. Tagging of $nosX$ using inverse PCR for insertion of Strep-tag II.
Molecular biology for c-terminal tagging of NosX with His$_6$.

The following oligos (MWG Biotech) designed to insert an Xa protease site, an Ala-Ser linker, and the Strep-tag II sequence at the c-terminus of the nosD gene were used in an inverse PCR reaction for insertion of Strep-tag II using inverse PCR:

'5GCTGGCGCGCCCCTCGATAACGGAGGCTTCCAGGC3' priming from the antisense strand and

5'CACCACCACCACCACCACCACCACCTAAGGTGGGCGACCCACAAGGAACG3'

priming from the sense strand. The inverse PCR reactions for tagging were carried out using Pfx polymerase in 20 μL volumes containing concentrations of 0.5, 1, 2, and 4 mM MgSO$_4$ with 2 μL Pfx enhancer added to each reaction. After an initial melting step of 3 minutes at 94° C, 30 cycles comprising a 1 minute melting step at 94°, a 1 minute annealing step at 64° C, and a 6 minute extension at 68° were run. Template for the reaction was a derivative of pCluster plasmid which had been downsized to 5.7 kbp by KpnI digest (see table of plasmids in appendix A). The products from the inverse PCR were then purified by 1% agarose gel electrophoresis and extracted using a BioRad "Freeze and Squeeze" kit. The purified product was then ligated and transformed into E. coli Top10F' by heat shock. Colonies were screened by PCR using a primer that spans the ligation point in the His tag. Positive colonies were then grown in 2 mL culture for plasmid mini-prep purification using a BioRad mini-prep kit. The plasmid samples were then sequenced using the ABI Prism Big dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). A candidate containing the correct sequence was then KpnI digested and ligated with the remaining half of pCluster removed prior to
tagging. The new pCluster transformants containing the His tag were then Sse8387I digested and the 9.6 kbp fragment was extracted from a 1% agarose gel electrophoresis gel. This band was then ligated with Sse8387I digested pMLA plasmid, and transformed into E. coli Top10F'. Midipreps were carried out on transformants testing positive for the inserted tag by PCR screen. The DNA was then electroporated into *A. cycloclastes* as described in the N$_2$OR chapter of this thesis.

**Culture growth, induction, and NosX purification.**

Ac/pMLClustXSt is stored in 15% glycerol at -80° C, and is initially grown on Luria Bertani Agar plates containing 50 ppm gentamicin at 30° C for approximately 48 hours. Next 50 mL starter cultures are grown in Luria Bertani Media containing 50 ppm Gentamicin at 240 rpm and 30° C, with subsequent scale-up to 2L cultures in 3 L flasks (for minimal headspace) to an optical density (O.D.) of 1.0-1.3 at 600 nm. At O.D. > 1.0 shaking is slowed to 120 rpm to create semi-anaerobic conditions. This induction also includes addition of 4 mL of 0.05 M CuSO$_4$·5H$_2$O and 20 mL of 1 M KNO$_3$ to obtain final concentrations of 100 μM Cu$^{2+}$ and 10 mM NO$_3^-$ respectively. After 24 hours of induction the cultures are harvested by centrifugation at 7,000 g for 10 minutes at 4° C. The pellets are then resuspended in a minimal volume (no more than 3x cell paste volume) of 100 mM TrisCl pH 8.0 and frozen at -80° C. After being thawed, the cells are lysed by either sonication or french press. Initial centrifugation at 30,000 g for 20 minutes is followed by ultracentrifugation at 45,000 rpm for one hour in a Ti50 or Ti60 rotor.
For Strep-tag II tagged NosX, approximately 1 mg of avidin is added to the lysate and incubated for at least ½ hour before the cell extract is loaded onto a 5 mL Streptactin sepharose column (Sigma-Genosys) equilibrated in 100 mM TrisCl pH 8.0 (buffer W). The column is then washed in buffer W containing 1 M NaCl. Elution of the tagged protein is carried out by running 2.5 mL aliquots of buffer W containing 2.5 mM desthiobiotin through the column.

Purification of His tagged NosX involves running the lysate through a Cell Through Talon metal affinity column (Clontech) equilibrated in 50 mM NaPO₄ buffer containing 300 mM NaCl at pH 7.0. The column is then washed with no less than 10 bed volumes of the same buffer containing 20 mM imidazole. The His tagged protein is then eluted using 0.25 M imidazole in the equilibration buffer.

NosX analysis.

To estimate the level of purity achieved, 8-25% gradient gels were used for SDS-PAGE and native gel electrophoresis. All gels were run using a Pharmacia PhastSystem. An electroblot of NosX on an Immobilon-P PVDF membrane was sent to Midwest Analytical for N-terminal sequencing. Flame AA was run to determine Cu content of NosX. UV-Visible spectra were measured on a Hewlett Packard 8452A Diode-Array Spectrophotometer. Fluorescence excitation and emission spectra were collected. NosX was sent to the Research Analytical Laboratory at the University of Minnesota for Inductively Coupled Plasma (ICP) emission spectroscopy. The samples were dry ashed
in quartz crucibles for 12 hours at 485° C, then dissolved in 3 mL of 20% HCl, and finally
diluted to a final volume of 6 mL with H₂O.

**Fluorescence analysis.**

Fluorescence analysis was carried out using a Spex Fluorolog 211 instrument in
the laboratory of Pat Callis at Montana State University and on an ISS PCI
spectrofluorometer in the Keck Biophysics Facility at Northwestern University.

**Mutagenesis Work.**

Attempts to mutate the GG motif, thought to form the flavin binding pocket,
involved two different methods. The first method tried was the Quick Change
mutagenesis kit from Stratagene. The oligomers designed and ordered for NosX
mutagenesis are as follows (changed nucleotides shown in bold):

GG→ AG: Down 5'CACCTGGCTAGCGCCGGCCGCTTCGG3'
        Up 5'CGAAAGCGCCCGGCGCTAGCCAGGTG3'

GG→ AA: Down 5'CACCTGGCTAGCGCCGCCCGCTTCGATCCG3'
        Up 5'CGGATCGAAGCGGGCGGCGCTAGCCAGGTG3'

GG→ GA: Down 5'CACCTGGCTAGCGGCGCCCGCTTCGATC3'
        Up 5'GATCGAAGCGGGCGCCGCTAGCCAGGTG3'

The primers for PCR screening of potential mutants were as follows:

GG→ AG: 5'AGTCCACCTGGCTAGCGGC3'
GG→ AA: 5'ACCTGGCTAGCGGCCGGC3'
GG→ GA: 5'ACCTGGCTAGCGGCCGC3'
The second method attempted for mutagenesis involved use of a Pfx polymerase PCR reaction as for the strep tagging method. Although the mutagenesis attempts to date were unsuccessful, this work will be pursued further.

Results

Purification and Characterization of NosX.

NosX was purified to nearly 100% homogeneity as seen by SDS-PAGE (figure 34) and showed a molecular weight of 30-32 kDa. The predicted molecular weight based on sequence information is 29,181 Da (30,805 Da when the 8 amino acid (aa) Strep-tag II, a 2 aa linker and the 4 aa Factor Xa protease site are included in the calculation).

![Figure 34. SDS-PAGE analysis of NosX eluent fractions. The molecular weight markers on the right hand side of the gel are as follows (from top to bottom): 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, and 14.4 kDa.](image-url)
N-terminal sequencing resulted in a 12 amino acid sequence of NAATRFWTGQAL (translated sequence of NosX predicts NAATRLWTGQAL), confirming the band to be NosX. The protein was found to be yellow in color and showed visible absorption maxima at 370 nm and 460 nm, indicating the presence of a flavin cofactor (see Figure 35).

Figure 35. UV-Visible spectrum of NosX from *A. cycloclastes*.

As expected, the fluorescence excitation spectrum exhibited similar maxima to those seen in the visible absorption spectrum (see Figure 36). From the fluorescence excitation spectrum it is apparent that the largest quantum efficiency is found at 465 nm and is therefore the most appropriate excitation wavelength for fluorescence emission spectra of NosX.
Figure 36. Fluorescence excitation spectrum of NosX.

Figure 37. Fluorescence emission spectrum of NosX at a concentration of 0.37 mg/mL.
The emission spectrum of NosX can be seen in Figure 37 and shows a maximum at 525 nm, as is typical for flavoproteins. The flavin cofactor was removed from NosX by boiling the sample for 10 minutes and then centrifuging to remove the denatured protein. The visible absorption spectrum of the NosX cofactor was measured, and upon comparing the absorption maxima to those obtained from spectra taken of pure FAD and pure FMN (Sigma) samples, was found to more closely match that of FAD. See Figure 38 and the corresponding Table 3.

![Absorption Spectrum](image)

**Figure 38.** Spectroscopic determination of the FAD cofactor.

<table>
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</tr>
</tbody>
</table>

**Table 3.** Spectroscopic absorption data for pure FMN (green), pure FAD (blue), and NosX cofactor (black), with λ = nm.
In order to confirm the identity of the cofactor more definitively a fluorescence emission experiment was carried out. The intensity of the emission was found to increase 13 fold when the flavin cofactor was freed from the protein environment (see Figure 39). Addition of snake venom phosphodiesterase to the free flavin cofactor resulted in an additional 3.5 fold increase in intensity (see Figure 39, left). Finally, fluorescence emission of pure FMN was measured before and after addition of the phosphodiesterase as a control. No change was observed for PDE treatment of FMN (see Figure 39, right).

![Fluorescence emission spectra confirming the identity of the FAD cofactor.](image)

Fluorescence excitation and emission spectra were also taken upon addition of either NO$_2^-$ or NO$_3^-$ to a sample of NosX. As can be seen in Figures 40 and 41, addition of stoichiometric and excess amounts of NO$_3^-$ caused an increase in both the fluorescence excitation and emission intensities, while Figures 42 and 43 show no obvious change or trend for the addition of either stoichiometric or excess amounts of NO$_2^-$. 
Figure 40. Effect of NO$_3^-$ on NosX fluorescence excitation.

Figure 41. Effect of NO$_3^-$ on NosX fluorescence emission.
Figure 42. Effect of NO$_2^-$ on NosX fluorescence excitation.

Figure 43. Effect of NO$_2^-$ on NosX fluorescence emission.
Discussion

NosX was purified and characterized with the hope of eventually elucidating its structure and function. Oxidized flavin cofactors exhibit two characteristic visible absorption peaks at \( \sim 360 \) nm and \( \sim 450 \) nm. The visible spectrum of NosX shows the presence of a flavin cofactor. The identity of that cofactor has been determined by two different methods. The first method, in which the visible absorption bands of the free cofactor, pure FAD, and pure FMN were compared, involved a very small shift in the \( \sim 450 \) nm absorption maximum. Given the amount of noise in the spectra compared and the small wavelength shift to be identified, the spectrophotometric method was used only for a preliminary determination of the cofactor. The more conclusive experiment involved addition of snake venom phosphodiesterase, which would remove the AMP moiety from an FAD cofactor, converting it to FMN (see Figures 32 and 39). FMN exhibits up to ten fold greater fluorescence emission intensity than FAD, due to the quenching caused by ring stacking involving the AMP moiety of FAD. Thus conversion of an FAD cofactor to FMN by the phosphodiesterase, would result in a dramatic increase in emission intensity.

SDS-PAGE analysis of purified NosX revealed a \( \sim 30-32 \) kDa band purified to nearly 100% homogeneity. The predicted molecular weight of 29,181 Da, calculated based on the translated gene, lacking the signal sequence, using Expasy (Expert Protein Analysis System, the proteomics server of the Swiss Institute of Bioinformatics), is slightly lower than that observed by SDS-PAGE. In the recent publication by Saunders et al. a molecular weight of 32.1 kDa was predicted (71). This value was determined
with the signal sequence included (Van Spanning, personal communication). Upon entering the gene sequence (minus the encoded n-terminal signal sequence) reported in the above publication (GenBank accession number AJ010260) into Expasy and SARS for comparison with the *A. cycloclastes* NosX prediction, molecular weights of 29,567 and 29,566 Da were obtained respectively. When the *A. cycloclastes* NosX molecular weight is calculated with the molecular weight of the Strep tag II, linker, Factor Xa protease cleavage site, and the FAD cofactor included, it matches more closely with the SDS PAGE observed molecular weight, resulting in a prediction of 31,606 Da.

The experiments involving addition of NO$_3^-$ and NO$_2^-$ resulted in interesting findings that may provide clues to a possible function for NosX. It is clear that NO$_3^-$ added in stoichiometric and excess amounts increases both the excitation and emission fluorescence intensities. It is unlikely that the FAD cofactor is simply being released from the quenching environment of the protein due to an increase in ionic strength, causing the increased fluorescence intensities, because the protein was already in a solution of 100 mM Tris pH 8.0 with 0.15 M NaCl. In addition, such effects on the fluorescence were not observed for the sample to which NO$_2^-$ was added. The FAD cofactor appears to be starting in an oxidized state (indicated by the visible absorption spectrum showing large peaks at 370 and 450 nm) which would imply that rather than an oxidation to increase fluorescence, perhaps a conformational change is triggered in NosX by NO$_3^-$ binding. Conformational change could reduce ring stacking that may take place between the FAD cofactor and certain residues (eg. Trp) in NosX. This would cause an increase in fluorescence intensity.
Conclusions

This work describes the first purification and characterization of the nosX gene product. NosX was found to contain FAD as a cofactor by two separate methods. Addition of nitrate, but not nitrite, increases the fluorescence intensity exhibited by NosX, but the details of this effect remain to be elucidated. It is known that N₂OR expression levels are much higher when the host organism is grown in the presence of NO₃⁻ than in the presence of N₂O, and that NO₂⁻, when added to the N₂OR activity assay, causes a first order turnover dependent inactivation of N₂OR (111). Perhaps a Nos protein, such as NosX, acts as a NO₃⁻ sensor in order to regulate NosZ transcription levels. This proposed transcriptional regulation involvement of NosX would, of course, require association with components such as NosR (an integral membrane component) and perhaps additional cytoplasmic components, as NosX is periplasmic.
RESEARCH SIGNIFICANCE AND FUTURE EXPERIMENTS

This work has focused on a complex system, required for the final step of denitrification. Prior to the purifications discussed above, the only form of *A. cycloclastes* N2OR reported in the literature was a pink, "high activity," monomeric form. It is clear from the current crystal structures reported that a monomer of N2OR should be completely inactive due to the large distance separating CuA and CuZ in each monomer. Only through domain sharing between the two monomers should the electron transfer and catalytic sites be found in close enough proximity for N2O reduction to take place.

Although it is known that the accessory proteins of the *nos* gene cluster are needed for assembly of the CuZ center, their roles have not been elucidated. Purification and characterization of these proteins is necessary in order to better understand the formation of the 4 Cu, sulfide bridged cluster. The work contained here includes the purification and partial characterization of two of the accessory proteins. In working to purify NosD, the first confirmation that NosD and N2OR interact directly was obtained. It was demonstrated that the N2OR in the NosD/N2OR complex did contain some Cu, at least in the CuA center. Crystallization trials will soon be carried out on this complex in hopes of obtaining a structure which could answer the question of how the CuZ site is assembled. Until a structure can be determined for the complex, analytical ultracentrifugation and native gel analysis should help to answer the question of how
many subunits of each protein are present. In addition, attempts to break the complex apart or express NosD in a host not containing an N$_2$OR gene will be very helpful in allowing determination of Cu content of NosD. Sulfide/sulfur analysis should also be carried out on NosD to determine if it is a periplasmic binding protein for sulfide.

NosX was purified and characterized in this research, revealing that it is an FAD containing flavoprotein. While this discovery does not reveal the exact role of NosX, the purification allows for future crystallographic work as well as various other experiments. Attempting various assays for flavoproteins exhibiting different functions may help to distinguish which family it may belong to and what its role in the nos system may be.

Two hybrid studies will be carried out soon, with the hope of determining which Nos proteins interact with NosX. Electrochemistry will be used to determine the redox potential of the flavin cofactor. Mutagenesis experiments designed to disrupt flavin binding or interaction of key residues with the flavin are planned, and effects of the NosX mutations on N$_2$OR expression levels and processing will be investigated.

In addition to the work completed on N$_2$OR, NosD, and NosX, the nosFY genes were also strep tagged for future purifications and characterization. Once NosY has been purified, attempts will begin for placing a NosY containing lipid bilayer on a surface plasmon resonance (SPR) chip. The four periplasmic components will then be washed across the chip, in order to determine which components interact directly with NosY. Many other SPR experiments involving washes of Cu, sulfate, sulfite, and sulfide in combination with periplasmic components could help in assigning a function for the ABC transporter. When all components of the ABC transporter have been purified work to
reconstitute the system in vesicles will begin. This will allow controlled additions of
Cu, SO$_4^{2-}$, SO$_3^-$, ATP, and any other components thought to be relevant for studying the
function of the transporter.
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Spectroscopic properties, bonding, and implications for the entatic/rack state. JACS 1996;7755-7768.

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APPENDIX A

TABLE OF PLASMIDS AND CONSTRUCTS
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>2.7 kbp</td>
<td>New England Biolabs (NEB)</td>
</tr>
<tr>
<td>pCluster</td>
<td>pUC18 with <em>nos</em> gene cluster from <em>A. cycloclastes</em> inserted into SbfI site</td>
<td>John Bollinger (JB), Dooley Lab</td>
</tr>
<tr>
<td>pZpD</td>
<td>pUC18 with <em>nos</em> gene cluster (with <em>nosZ</em> gene deleted, placing <em>nosD</em> against <em>nosZ</em> promoter) from <em>A. cycloclastes</em> inserted into SbfI site. DFYL operon placed immediately in front of Z promoter</td>
<td>JB</td>
</tr>
<tr>
<td>pDF</td>
<td>pUC18 containing HindIII/KpnI fragment of <em>nos</em> cluster comprising c-terminus of <em>nosZ</em>, <em>nosD</em>, and n-terminus of <em>nosF</em></td>
<td>Shannon Henery (SH), Rosenzweig Lab</td>
</tr>
<tr>
<td>pML10</td>
<td>multi-copy, broad host range plasmid containing gentamicin resistance gene</td>
<td>98a</td>
</tr>
<tr>
<td>pMLΔ</td>
<td>pML10 trimmed to SbfI sites</td>
<td>JB</td>
</tr>
<tr>
<td>pMLnos</td>
<td>pMLΔ with <em>nos</em> gene cluster from <em>A. cycloclastes</em> inserted into SbfI site</td>
<td>JB</td>
</tr>
<tr>
<td>pMLZpD</td>
<td>pMLΔ with with <em>nos</em> gene cluster (with <em>nosZ</em> gene deleted, placing <em>nosD</em> against <em>nosZ</em> promoter) from <em>A. cycloclastes</em> inserted into SbfI site. DFYL operon placed immediately in front of Z promoter</td>
<td>JB</td>
</tr>
<tr>
<td>pMLZpDSt</td>
<td>pMLΔ with <em>nos</em> cluster inserted (with <em>nosZ</em> gene deleted, placing <em>nosD</em> against <em>nosZ</em> promoter)</td>
<td>JB/SH</td>
</tr>
<tr>
<td>pMLClusterDSt</td>
<td>pMLΔ with <em>nos</em> cluster containing Strep-tag II on 3' end of <em>nosD</em> gene</td>
<td>SH</td>
</tr>
<tr>
<td>pMLDHis</td>
<td>pMLΔ with <em>nos</em> cluster containing His6 tag on 3' end of <em>nosD</em> gene</td>
<td>SH</td>
</tr>
<tr>
<td>pMLZpDYSt</td>
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<td>SH</td>
</tr>
<tr>
<td>pMLClusterFSt</td>
<td>pMLnos (above) with Strep-tag II inserted on 3' end of <em>nosF</em> gene</td>
<td>SH</td>
</tr>
<tr>
<td>pMLClustXSt</td>
<td>pMLnos (above) with Strep-tag II inserted on 3' end of <em>nosX</em> gene</td>
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</tr>
<tr>
<td>pMLXHis</td>
<td>pMLΔ with <em>nos</em> cluster containing His6 tag on 3' end of <em>nosX</em> gene</td>
<td>SH</td>
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APPENDIX B
LINEUP OF AVAILABLE NOSD SEQUENCES
Consensus

251 300
---R------M---N------G-A-M--S------N---

251 300

d.msf{Psd} SEQDQNYGIL MNYITYSTIT GNJVSDVQR

d.msf{Pfd} SEQDQNYGIL MNYITYSTLR DNVFSDV.R

d.msf{Pad} SIDENYGIL MNYITYSTLA GNKVEGVR.R

d.msf{Ac} SLGDRGSLML LNYANNAVDS GNVL

d.msf{Pdd} SLGDRGSLML LNYANNAVDT GNLI

d.msf{Rsd} SLRDRDHGVM LNYVNGAEBVA RNVL

d.msf{Smd} SDGDPDHGLL LNYANNNRTI GNIVGRRLQP ADRWLRKARS GHHVYKDEE

d.msf{Bjd} SDRRDPHGLL FNAYNYRDR RNRVTGGL.L...LTCPG GSEEGQDDLDER

Consensus S---D---G---NY---N---

301 350
---R------M---N------G-A-M--S------N---

301 350

d.msf{Psd} .....GTGQGS MIGGGEGKAL PTYNSLFTNI ENNHFEKSSL GTHLTAGS

d.msf{Pfd} .....GTGQGS MIGGGEGKAL PTYNSLFTRNI EGNHFERSAV GTHLTAGS

d.msf{Pad} .....STGQGT MIGGGEGKAL PTYNSLFTNRI EGNFAPDSAL GTHLTAGS

d.msf{Ac} .....RGTKQGL PTYNAHKNLV WGNRFSCGCI GTHLTAGS

d.msf{Pdd} .....RGTKQGL PTYNAHKNLI WDNRFQDCGSI GTHLTAGS

d.msf{Rsd} .....RGTJQGL PTYNAHRNVI DHRNFEGCST GTHLTAGS

d.msf{Smd} .....RQATGADR.. RLGPEKCV PTYANKNNRF RNIVGRLQP ADRENGA CAYFTAGS

d.msf{Bjd} .....GMPDTRKQG SRLSSPEKCV PTYNTHHKNRF RNWFIRCAI GYHTAGS

Consensus ---K----PIY---N---N---P---G-H-TAGS

351 400
---R------M---N------G-A-M--S------N---

351 400

d.msf{Psd} NRISGNAFVG NQQQKTVAS RTQWESVDAGR NNYWSLYGWL DRNNDGDGDI

d.msf{Pfd} NRISGNAFVG NQQQKTVAT RLQWESADGR NNYWSLYGWL DRNNDGDGDI

d.msf{Pad} NRISGNAFVG NQQQKTVAS RTQWESADGR NNYWSLYGWL DRNNDGDGDI

d.msf{Ac} NRISGNAFVG NQQQKTVAS RTQWESADGR NNYWSLYGWL DRNNDGDGDI

d.msf{Pdd} NRISGNAFVG NQQQKTVAS RTQWESADGR NNYWSLYGWL DRNNDGDGDI

d.msf{Rsd} NRTQKTVHGK RTLVESHEGR GNFSWPHGP DLNGDGADG

d.msf{Smd} NLIGSNSFIGN MRNOQKTVGT RHLNWSHEGR GNFSWPHGP DLNGDGADG

d.msf{Bjd} NEITGNAFVG NNRQKTVVGT RNLDWSSEGQ GNYWSNWPAF DLNGDGADG

Consensus N-----N-F--N-----Q---V-----W-----G----N---W-----S-----G---D---DG--D--

401 450
---R------M---N------G-A-M--S------N---

401 450

d.msf{Psd} AYEPNADVDR LLWLYQPQRL LMNSPSTIEVL RWQVAFPAV FIKSPGQDSHQ

d.msf{Pfd} AYEPNADVDR LLWLYQPQVL LMNSPSTIEVL RWQVAFPAV FIKSPGQDSHQ

d.msf{Pad} AYEPNADVDR LLWLYQPQRL LMNSPSTIEVL RWQVAFPAV FIKSPGQDSHQ

d.msf{Ac} FYRPNDLMQ ILWSQPAASL LTSGPAVIV KWSQRDFPPA LPGVGRDSAP

d.msf{Pdd} FYRPNDLMQ ILWSQPAASL LTSGPAVIV KWSQRDFPPA LPGVGRDSAP

d.msf{Rsd} FYRPNDLMQ ILWSQPAASL LTSGPAVIV KWSQRDFPPA LPGVGRDSAP

d.msf{Smd} FYRPNDLMQ ILWSQPAASL LTSGPAVIV KWSQRDFPPA LPGVGRDSAP

d.msf{Bjd} AYRPNDLVDR VLVTAPSAKLM LNSVQAVQL NWQAQAQPAP YPGVGIDSHP

Consensus ---PND--D--W--P-----L---P-----RW--Q--FP----GV--DS--P

451 490
---R------M---N------G-A-M--S------N---

451 490

d.msf{Psd} LMKLPTEKLL TEK.QEPS.

d.msf{Pfd} LMETPVQPS RNQAEQENAS.
| d.msf{Pad} | LMRMPAAEPR P............... ............... ............... |
| d.msf{Acd} | LMRPLTIPVP LEILAYEAEA AGRWTEGNYD DTDADNLQAH |
| d.msf{Pdd} | LMRPLTIPVP PEIEAYEAEA AGRWAKGNYD DIPDDLASH |
| d.msf{Rsd} | LMAPIDIPVP DDVARLEA.A VPPWPQG..E TSDATLACG |
| d.msf{Bjd} | LMVPAGRVAQ Q............... ............... ............... |
| Consensus | LIAPPFPSPG KEGGR............... ............... ............... |
APPENDIX C

LINEUP OF AVAILABLE NOSX SEQUENCES
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<tr>
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</tr>
<tr>
<td>nosx.msf(Pdx)</td>
<td>...MSLSRRR FLITITAVAA ...LLPAGLRA Q ...PG.RHW VQGALGARAS</td>
<td></td>
</tr>
<tr>
<td>nosx.msf(Smx)</td>
<td>...MMAA.AG LPLLLDLGRA EGAVAAVRWR ...GRALGAPAT</td>
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</tr>
<tr>
<td>nosx.msf(Bjx)</td>
<td>MAPANLTRRR MITIAAAATAG SAPISSGQGA ...SSTGAVRWR ...GSALGAVQS</td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>--------------- ...-A- -A-- --------------- -G-ALGA--</td>
<td></td>
</tr>
</tbody>
</table>

| 51 | 100 |
| nosx.msf(Acx) | IRIAHPI ...EAGTIVSRVM ABIDRLENIL SLYRANSALC RLNRGHLQD |
| nosx.msf(Pdx) | IRIIDHP ...EAGAIGHTAC ELIDRLENIL SLYRPSQALAA RLNRGVLGA |
| nosx.msf(Smx) | L.ILLLESAQA DAAGLIDRVRV AEVARLREVF SLYQRSALAA ELNRTGIAAA |
| nosx.msf(Bjx) | IDIFHPDRE. EAEIIRQDCL TEVRRLGQGF SLYRDSACIC ALNRSGLVIA |
| Consensus | ---I- ---A--- -E--RLE--- ...SLY---SA-- ...LNR-G--- |

| 101 | 150 |
| nosx.msf(Acx) | PPFELLLECLS LADAVHLSAG GPFDPQVQPL WTLAQ...SA ANGTRPGPAD |
| nosx.msf(Pdx) | PPFELLDCLS LAGAVRKSAG GFLPDQVQPL WSLWA...AA VNGRPPTPBE |
| nosx.msf(Smx) | PPPDLVNLLE ASRDFPQVQL WTLYABHFAA GDADPAGQPE |
| nosx.msf(Bjx) | PEPDMVALLK ASLQFSGLTD WQLYAHISSF ENPDPGGPPA |
| Consensus | P-----L- -------------G-FDP-VQPL W-L-A----- ---P-- |

| 151 | 200 |
| nosx.msf(Acx) | ...VDRVRVNS GWGKVKLGA AAITL.HPGMA TLNQGQGY VADRVAAMLE |
| nosx.msf(Pdx) | ...RRDAERT GWDRVRVLAAR RITAL.EPGMA TLNQGQGY VADRVAALBE |
| nosx.msf(Smx) | AAKRALKSRV GFDDKLEFNRD RCVFARPGMA TLNQGQGY ITDRVGLLK |
| nosx.msf(Bjx) | GKLAEALAKV GSSGIRVSEK LVALLRHGAA TLNQGQGY ATDRVVDVLR |
| Consensus | ------------- ...G- -------------T-TNGI-GQY --DR---L- |

| 201 | 250 |
| nosx.msf(Acx) | VEGLTIDILID TGFPRALGGR PGGGGWPHVL BT......G ERLPLRQRAV |
| nosx.msf(Pdx) | AEGLQDILID TGRALRGLG RGDGWDPVRL AE......G GAVGLRALL |
| nosx.msf(Smx) | DAGTANSLVS MGTERAIGSQ HDGRQYWVQGL ATREDASTPD SVHNLRLVRA |
| nosx.msf(Bjx) | ARGLSTTLVN MGTEIRALGAR ADGTIPLRVQL SDPDRAAGT ETIDLDRAV |
| Consensus | ---G----L- ---GE-RA-G--- --------------- ---L---RA- |

| 251 | 300 |
| nosx.msf(Acx) | ATSAPLGTTTF DQUARQGHLL DPLTQMPAPA NWRSVSFSAS SAAIADALAT |
| nosx.msf(Pdx) | ATSAPLGRTFS DEARGQGHHIL DPLSGARPARP VVRAWVSISAP GAGLADALST |
| nosx.msf(Smx) | ATSSPDQFRFF DDSRGPFQHIL DPLSG.RAPR LRRRVSVVAP TATADASFR |
| nosx.msf(Bjx) | ATTAGGFRFF DPAGRTHFLL DPTTRG.RSPA LRYTVSVIAP TATADASFR |
| Consensus | AT----G-F D----R-H-- DP---G----- -----R-VS-A-- A-A ADA--T |

| 301 | 350 |
| nosx.msf(Acx) | AACLLPDAG. ................. SIVAMNARF. DGVRLEAASV |
| nosx.msf(Pdx) | AACLLTDLQ. ................. EURALLDRF. DGTRLEAQR |
| nosx.msf(Smx) | AFSLGSSSAV .RIACEH. ....... SEL. ............ .TVDMIST. |
| nosx.msf(Bjx) | AFSLMPSATQI DJIAATRDFV AGSGLQNNWM EICAFNRGLR GVVAIRSSE |
| Consensus | A----L---- ----- ----F D-----R-H-- DP-----G----- -----R-VS-A-- A-A ADA--T |

| 351 | 370 |
| nosx.msf(Acx) | ................. ................. ................. |
| nosx.msf(Pdx) | QA. ................. ................. |
| nosx.msf(Smx) | SGAHE.RFG. ................. |
nosx.msf\{Bjx\}  SGIRNPRYG F PAHTLSKRAP
Consensus   ---------------