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# A Method of Determining the Relationship Between Copper ATPases and Nitrite Reductase

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**A Method of Determining the Relationship  
Between Copper ATPases and Nitrite Reductase**

A Major Qualifying Report

Submitted to the Faculty of

Worcester Polytechnic Institute

In Partial Fulfillment of the Requirements for the

Degree of Bachelor of Science

By

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Robert Le

Date: April 18, 2013

Approved:

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Biochemistry  
WPI Project Advisor

# Abstract

Copper containing nitrite reductase (NirK) is a homotrimeric protein in *Sinorhizobium meliloti* makes use of six copper ions in total, but the molecular mechanism by which it procures its copper ions is yet to be determined. The focus of this project was to investigate the role of certain copper ATPases in the metallation of nitrite reductase. The wild type strain was explored, and the Griess method of nitrite determination was used to determine nitrite reductase activity and understand the relationship between nitrite reductase and copper ATPases. The methods that have been developed during this project can then be further refined and used in the future to determine copper ATPase metallation targets in *S. meliloti*.

# Acknowledgements

I would like to thank Professor José Argüello for guiding me along the process of producing a major qualifying project and training me in the methods of biochemical research. I would also like to thank the students and postdoctoral fellows that I have met since coming to Professor Argüello's lab, especially Sarju Patel, who have been extremely welcoming, helpful, and have sacrificed a good bit of their time to help me out during the long data collection process of research.

I would also like to thank Professor Destin Heilman for his work as faculty advisor throughout all my years at WPI, as well as all of my very supportive friends and family who have stood by me during my college years.

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# 1. Introduction

## *1.1 Copper Enzymes and Homeostasis*

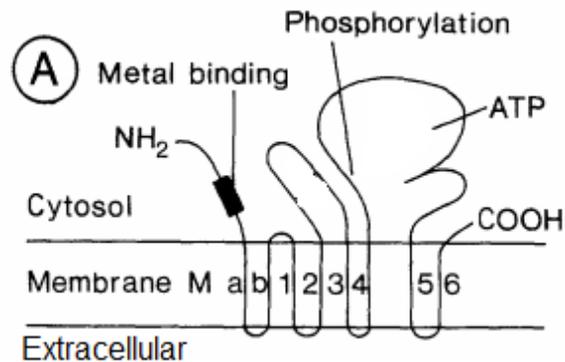
Copper is an essential component of cellular function. It is used in various functions such as respiration, nitrogen metabolism, and detoxification in proteins (1). In spite of the necessity of copper in roles throughout the cell, in large proportions, copper becomes extremely toxic to the cell (1, 2). Certain mechanisms have been acquired over time by various organisms to maintain homeostasis in the presence of excess copper. These methods include heavy metal transporters and chaperones that bind to heavy metals to direct them to other proteins that require their usage.

## *1.2 Copper ATPases*

The family of P-Type ATPases proteins constitutes a large number of different types of transmembrane transporters (3). These P-Type ATPases are transmembrane proteins that transfer ions such as copper across a membrane using the energy of ATP hydrolysis. P-Type ATPases are classified based on the ion that is transported. P-IA Type ATPases are simple bacterial ion pumps which transport  $K^+$ . P-IB Type ATPases are responsible for the removal of ions such as  $Cu^+$ ,  $Ag^+$ ,  $Zn^{2+}$ ,  $Cd^{2+}$ , or  $Pb^{2+}$ . P-II Type ATPases include a large array of transporters responsible for  $Ca^{2+}$ ,  $Na^+/K^+$ , or  $H^+/K^+$  ion pumping. P-III Type ATPases are  $H^+$  ion pumps found in plants and fungi used by these organisms to maintain pH within the cell. P-IV Type proteins are involved in lipid transport and maintaining the lipid bilayer. The role of P-V Type ATPases has yet to be elucidated (5).

P-IB Type ATPases are responsible for copper. As shown in Figure 1 (6), the structure of these enzymes presents two cytosolic loops, a shorter loop between transmembrane segments 3 and 4 and a longer loop which binds and hydrolyzes ATP. There is also a long strand of the N-

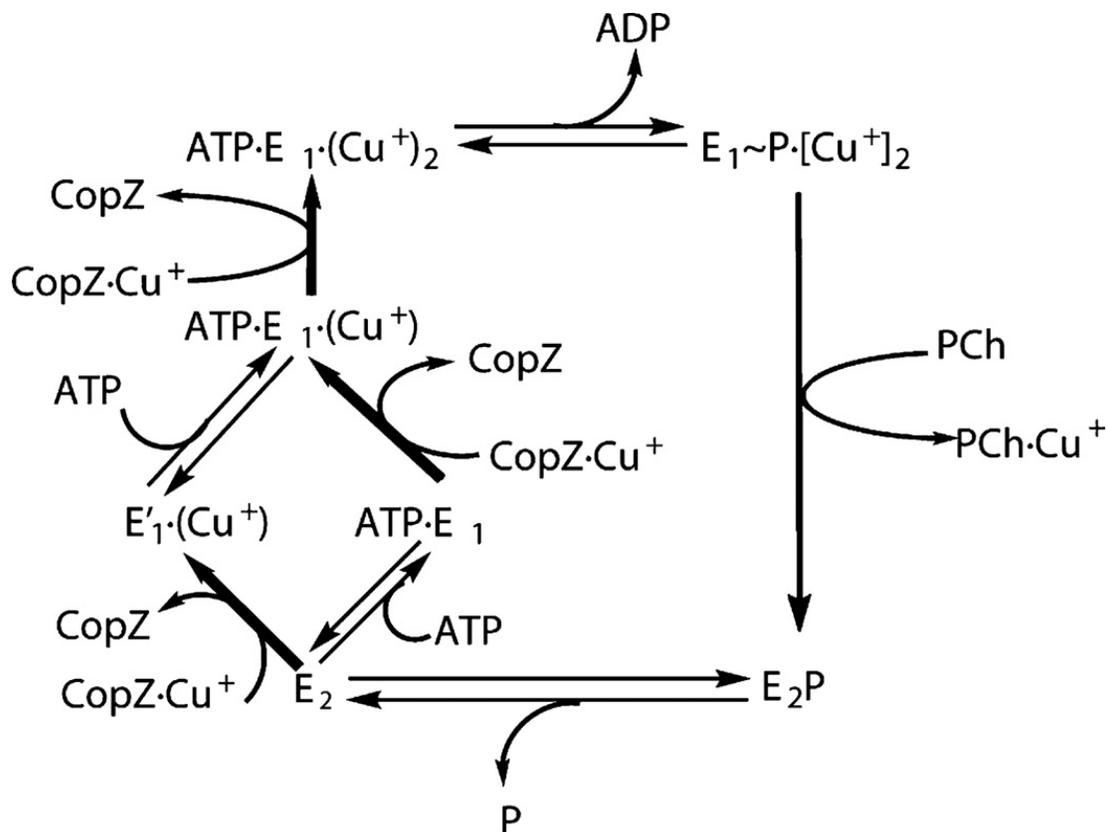
terminus of the protein in the cytoplasm as well as eight transmembrane segments. A metal binding site is located near the N-terminus of the ATPase, while phosphorylation takes place as marked.



**Figure 1. Membrane topology of bacterial P-1B Type ATPases.** Copper is bound where metal binding site is found for copper ATPases. Phosphorylation takes place as labelled in the larger of the two cytosolic loops (6).

Although there is a variety of P-Type, all utilize the same mechanism. For copper ATPases, they are unique in that copper does not move freely throughout the cell, and a step in the transport involves delivery of copper from a chaperone. In the cytosol, copper is bound to the chaperone protein CopZ, which allows for an efflux of cytosolic copper without allowing free ions to move through the cytoplasm (5).

Upon binding of the copper ion as brought by CopZ to the transmembrane metal binding domain, the site in Figure 1 marked with a P is phosphorylated (5). Upon phosphorylation, a conformational change takes place which results in the release of any bound ligands, and the enzyme changes from its E1 state to its E2 state as seen in Figure 2 (3). While the E1 state has a high affinity for metals, the E2 state prevents metal ions from returning to the cytosol. Upon dephosphorylation, the ATPase reverts back to its initial E1 state so that the cycle can restart (3).



**Figure 2. Catalytic Cycle of Metal Transporting ATPases.** E1, E2, E2P, and E1P are different conformations of the enzyme in this cycle with P indicating that the ATPase has been phosphorylated (5).

### 1.3 *Sinorhizobium meliloti*

*Sinorhizobium meliloti* is a gram-negative bacteria that undergoes symbiosis with certain legumes such as *Medicago sativa*, fixing nitrogen for the plants at the roots (7). Other legumes that it undergoes symbiosis with include *Medicago truncatula* and *Melilotus* spp (8, 9).

The symbiosis between various *Medicago* species and *S. meliloti* is done by the formation of root nodule. This is accomplished by the bacteria by differentiating into bacteroids which fix atmospheric nitrogen by use of nitrogenase (10). This differentiation process occurs when *S. meliloti* enters the plant cell. Nitrogen fixation and other pathways such as denitrification are

facilitated with the help of metalloenzymes such as ferredoxin, which donates electrons to proteins such as nitrogenase (11).

#### ***1.4 Copper ATPases in Sinorhizobium meliloti***

P-IB ATPases present in *S. meliloti* are of interest in Dr. Arguello's lab. These P-IB ATPases are ActP, FixI1, FixI2, Cut4, and Cut5. While ActP has been characterized previously in *Rhizobium leguminosarum* *bv.* *Viciae* as well as *S. meliloti*, the other putative transporters are currently being investigated and characterized for function (12). In the case of ActP, aptly named for its acid tolerance role in the cell, ActP is responsible for copper homeostasis in the case of low pH. Because of its role in low pH copper homeostasis, transcription is enhanced in such conditions, and the expression of ActP is controlled by the heavy metal response regulator HmrR.

In other studies, the *fix* cluster of proteins has been shown to be found upstream of cytochrome c oxidases. Deletion of *fixI* resulted in reduced oxidase activity, implying that the ATPase is in part responsible for cytochrome c oxidase metallation or are part of the coding region for the oxidase. One thing of note is that, in pathogenic as well as symbiotic bacteria, extra copies of these Cu<sup>+</sup> ATPases are frequently found (13). Although ActP and FixI have been investigated, it is not currently known what function Cut4 and Cut5 perform in *S. meliloti*.

#### ***1.5 Hypothesis: Copper ATPases have different targets, one of which is Nitrite Reductase***

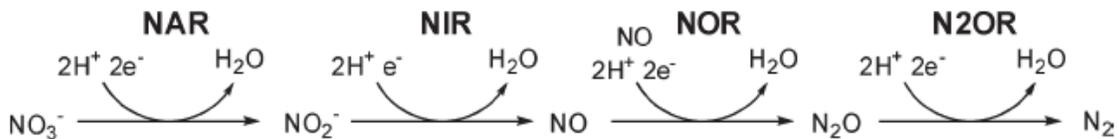
Based on the numerous copies of copper ATPases as well as their genetic environment in *S. meliloti*, it has been speculated that each protein is responsible for the metallation of a different target in the cell. One possible target that has been suggested is copper-containing nitrite reductase (NirK), a protein who relies on two copper ions for each of its three monomers. By mutating certain

copper transporting ATPases such as Cut4 or Cut5, the expected result may be a decrease in nitrite reduction activity in *S. meliloti*.

### 1.6 Nitrite Reductase

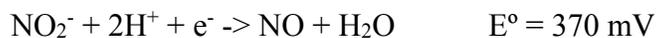
The denitrification pathway is an essential pathway necessary for nitrogen fixing bacteria of the *Rhizobia* order to allow them to survive under various O<sub>2</sub> concentrations, including the microaerobic conditions of the root nodule. In the case of limited oxygen, the *Rhizobia* bacteria change from aerobic respiration to denitrification so as to generate ATP (14).

The process begins with nitrate and ends with the production of dinitrogen, but there are multiple steps to the pathway as shown in Figure 3 (15). In addition, each step results in the production of one water molecule. Nitrate is reduced to nitrite by nitrate reductase. Nitrate in turn is reduced to nitric oxide by nitrite reductase. Nitric oxide is then converted to nitrous oxide by nitric oxide reductase, and the final step is reduction of nitrous oxide by nitrous oxide reductase to dinitrogen (15).



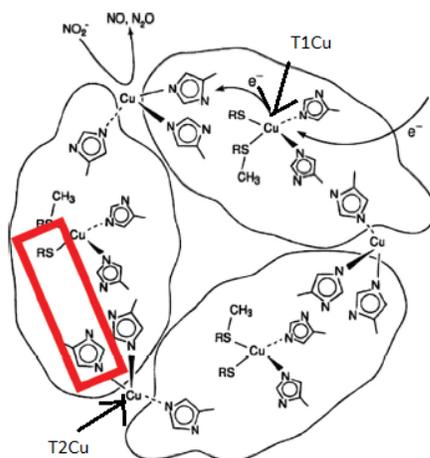
**Figure 3. Denitrification Scheme following the reduction of Nitrate to dinitrogen.** The enzymes used are Nitrate reductase (NAR), Nitrite Reductase (NIR), Nitric Oxide Reductase (NOR), and Nitrous Oxide Reductase (N2OR) (15).

Because of the role *S. meliloti* plays in fixing nitrogen in legumes, the ability to denitrify certain species of nitrogen in the cell is necessary under the microaerobic conditions of the root nodules. Thus, denitrification is a necessary element in *S. meliloti* symbiosis. One step in the denitrification pathway is the reduction of nitrite to nitric oxide as follows:



Copper containing Nitrite Reductase is a homotrimeric protein of 40kDa per monomer weight exported from the cytoplasm to the periplasm. Each monomer contains two copper ions: Type 1, a “blue” copper, and Type 2, a “normal” copper. The proposed mechanism of nitrite reduction involves the substrate binding to the Type 2 copper center with the Type 1 copper delivering the electrons required to reduce the substrate to nitric oxide (7).

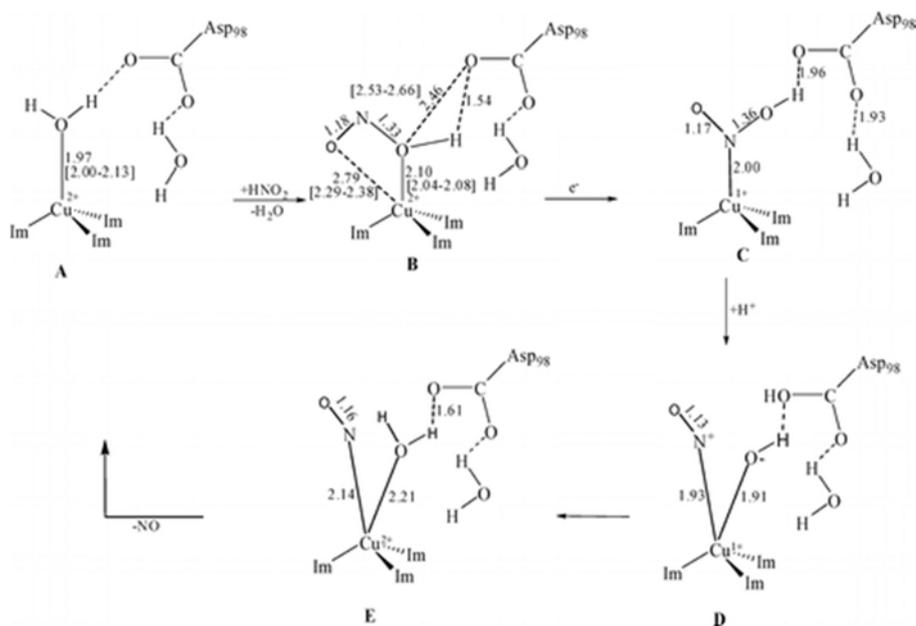
The copper centers of nitrite reductase are positioned so as to allow the electron transfer to move to the catalytic site. As seen in Figure 4 (15), the T1 center is positioned within one of the trimers of the protein, through which it obtains an electron by an electron donor. In the later reaction for the determination of activity, this donor is methyl viologen after it has an electron donated from a dithionite ion. The T2 center is located in between trimers, allowing for a position to interact with and bind to nitrite in order to reduce it to nitric oxide (15).



**Figure 4: Copper Nitrite Reductase homotrimer scheme with T1 and T2 Cu labelled.** Following an electron donation to the T1Cu site, the electron is transferred to the T2 site where the nitrite is reduced to nitric oxide. The red box labels the amino acids involved in electron transfer (15).

In particular, multiple kinetic parameters have been characterized from the wild type Rm2011 strain of *S. meliloti* previously by expressing NirK in *Escherichia coli*. It has been found that NirK in *S. meliloti* has a  $K_m = 0.55 \pm 0.06$  mM  $\text{NO}_2^-$ ,  $V_m = 89 \pm 3$  U  $\text{mg}^{-1}$  and  $k_{\text{cat}} = 178 \pm 2$   $\text{s}^{-1}$  (7). Others have expanded on NirK in other organisms such as *Nitrosomonas europaea* as well as the genes involved in the regulation of NirK (17).

As shown in Figure 5 (18), a proposed scheme shows the T2 copper center being surrounded by three histidines indicated by Im for the imidazole groups. While normally capturing a water molecule, the copper center binds to nitrite. Following binding of the oxygens to the copper coordinated with an aspartic acid on the side, the T2 center receives an electron donated by the T1 center, which allows the center to draw in and bind the nitrogen. While one oxygen is being held by the aspartic acid, the nitrogen is split from it, and the oxygen is converted to water while the resulting nitric oxide is released (18).



**Figure 5. Proposed mechanism for Nitrite Reductase.** The copper center mentioned in this figure is the T2 copper center. Nitrite is bound with the help of a flanking aspartic acid and split. The oxygen obtained is then used to form a water molecule (18).

Although nitrite reduction activity has been measured for both the cytoplasm as well as the periplasm, NirK has been found to be secreted into the periplasm (7). Sequence analysis has found that the *nirK* gene contains the signal sequence to be secreted unfolded into the periplasm via the sec pathway (19, 20). This would imply that the copper centers necessary for nitrite reduction are obtained in the periplasm where it is folded.

However, it has yet to be determined from where NirK obtains its copper. Because of the heavy involvement of copper in the process amongst a homotrimeric protein such as nitrite reductase, NirK presents an evident destination for copper following metallation by a copper ATPase. The focus of the project is to expand on methods to measure nitrite reduction activity in its original organism, *S. meliloti*, and to determine if NirK is a target for either the copper ATPase Cut4 or Cut5.

## 2. Materials and Methods

### 2.1 Strains used

*S. meliloti* strains were used in the experiments. Strains were grown in media containing 0.5% Tryptone, 0.3% Yeast Extract, and 3 mM CaCl<sub>2</sub> (TY medium). The wild type Rm2011 strain was grown with 200 µg/mL streptomycin.  $\Delta$ Cut4 cells, which lack the Cut4 copper ATPase, as well as  $\Delta$ Cut5 cells, which lack the Cut5 copper ATPase, were grown with 100 µg/mL Neomycin.

### 2.2 Periplasmic Fraction Extraction

Following growth in 5 mL cultures of TY media for 36-48 hours, 300 mL flasks of TY media were inoculated with the smaller 5 mL cultures. After incubating the cells for another day at 30°C, the OD of the culture was measured, and the cells were pelleted via centrifugation. The cells were then washed with 0.9% NaCl and pelleted once more. Cells were then incubated in 20% sucrose with 3 mM Tris/HCl (pH 8.0) at room temperature for ten minutes before being pelleted once more via room temperature centrifugation. Cells were then resuspended in icy milliQ water for 15 minutes before being pelleted, with the supernatant of this centrifugation being the periplasmic fraction. Both periplasmic and whole cell fractions were stored at -20°C in 20% glycerol.

### 2.3 Whole Cell Fraction Acquisition

During the NaCl wash of the periplasmic fraction extraction, 1 mL of the cell suspension was taken, pelleted, and resuspended in milliQ water. These cells were then sonicated for one minute and pelleted with the supernatant taken as the fraction of protein from the whole cell.

#### ***2.4 Protein Determination***

Protein was measured by Bradford method (21). Bradford reagent was prepared by filtering 0.01% Coomassie Blue, 5% ethanol, and 8.5% Phosphoric Acid through filter paper. 2  $\mu$ L of protein were added to culture tubes along with 1 mL of Bradford reagent and mixed. Protein concentration was measured using a spectrophotometer at 598 nm. Bovine Serum Albumin (BSA) was used as the standard of measurement for Bradford determination, and measurements are reported in mg/mL.

# 3. Results and Discussion

## 3.1 Periplasmic Protein Yield

It has been previously shown that nitrite reductase is exported from the cytoplasm to the periplasm in the cell. Because of this, attempts were made to extract the periplasmic fraction from *S. meliloti* cells. Following periplasmic extraction, the protein yield was measured for both fractions. The yield for periplasmic protein extracted on average was  $0.427 \pm 0.054$  mg per 300 mL culture over 7 extractions.

As previously discussed, periplasmic protein are limited in its yield, generally producing 300-400  $\mu$ g of protein for a 300 mL culture using TY media. One potential solution in the future is the possibility of optimizing the media. By using a richer medium, it may be possible to harvest more cells to use in the periplasmic extraction. Potential media that may allow for a higher yield of cells include 1.6% Tryptone, 1% Yeast Extract, and 3 mM CaCl<sub>2</sub> (2xYT medium with CaCl<sub>2</sub> substituted for NaCl) as well as 1% Tryptone, 0.5% Yeast Extract, and 3 mM CaCl<sub>2</sub> (LB medium with CaCl<sub>2</sub> substituted for NaCl).

Another consideration to factor in terms of growth is the capability of inducing NirK production within *S. meliloti*. Although there are methods presented in other papers, the growth of these cells requires anaerobic or micro-aerobic conditions which require specialized equipment to control. In addition to cell induction, the methods listed in these papers also require solutions prepared and used in assays to be kept in microaerobic conditions as well. Thus, finding a method of inducing NirK production would allow for a much easier determination of the relationship between NirK and the various copper ATPases being investigated.

### ***3.2 General Principal Determination of Nitrite Reductase Activity***

In investigating methods of determining the relationship between copper ATPases and nitrite reductase, the first step was to determine the reducing capability of the wild type as well as the mutants. One such method previously performed and adapted for this project was the use of the Griess Assay, a diazocoupling reaction that produces a pink color in the presence of nitrite detectable at 540 nm (22). In a previous study done by Ferroni et. al, this method was used for *E. coli* cells transformed to express nitrite reductase in order to characterize it (8). The method produced was one that determines nitrite reducing activity by observing leftover nitrite.

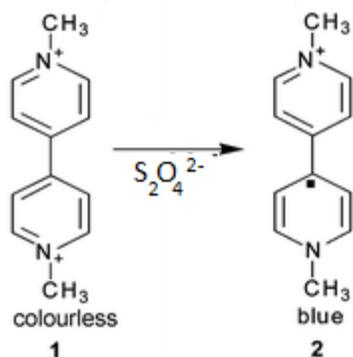
Another factor considered in this determination of nitrite reductase activity was the location of the protein extracted. Although it may have been possible to use protein extracted from the whole cell following a sonication, it was decided that the ratio of nitrite reductase to other proteins in a whole cell derived fraction would not be optimal for measurement. Although the amount of protein obtained from the entire cell was much larger than the periplasmic fraction, the concentration of nitrite reductase itself was unlikely to be much more significant. Thus, although the whole cell fraction was done, the main focus of this investigation was the periplasmic fraction. The use of cells themselves to reduce nitrite in solution was also looked into as a method to observe general activity. This method is discussed in section 3.4.

To determine nitrite reduction ability, the Griess method of nitrite determination was used. For periplasmic fractions and whole cell fractions, the fractions were used immediately following extraction. The protein fractions were incubated in eppendorf tubes with 30 mM sodium phosphate buffer (pH 7.0), 0.3 mM methyl viologen, 2.8 mM Sodium Nitrite, and 5 mM sodium dithionite in 0.4% NaHCO<sub>3</sub>, with the dithionite added last to begin the reaction for a total volume of 250 μL.

The choice of 2.8 mM nitrite concentration was made based on a previously determined  $k_m$  for the nitrite reductase of *S. meliloti* (8).

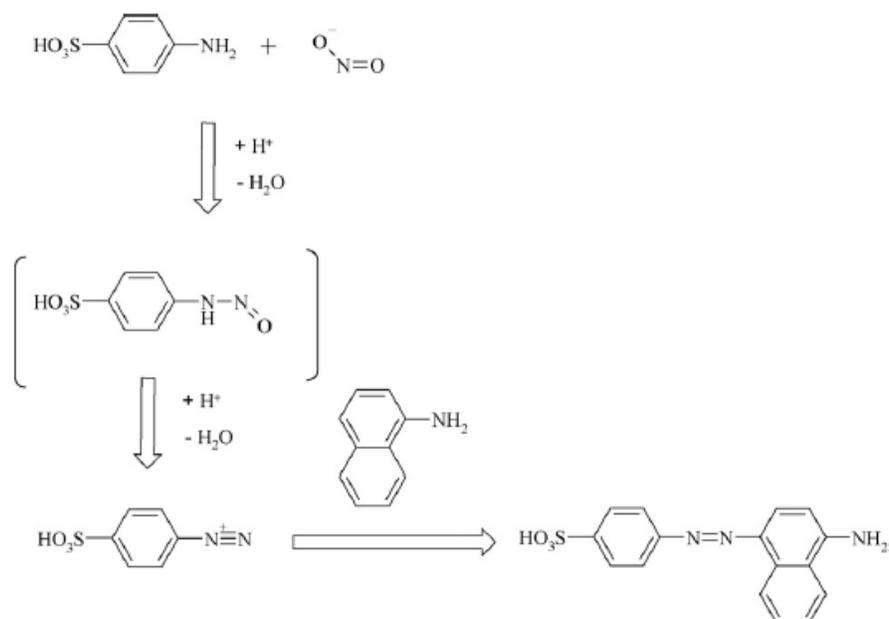
For the reaction, the methyl viologen was prepared and stored at  $-20^\circ\text{C}$  to prevent decomposition. While the sodium phosphate buffer was stored at  $4^\circ\text{C}$ , the sodium nitrite and sodium dithionite were prepared fresh. In particular, due to the volatile nature of the dithionite and its quick rate of decomposition in solution, the dithionite was prepared fresh before addition of the reaction tube.

Upon addition of the dithionite to the reaction, the mixture takes on a blue color. This is due to an electron donation chain started with the donation from dithionite to methyl viologen as seen in Figure 6 (23). The methyl viologen is then responsible for donation of an electron to the T1 copper center of the nitrite reductase so as to allow for the donation of an electron to the T2 copper center, which then proceeds to the catalytic reduction of nitrite to nitric oxide. The reaction was incubated at  $30^\circ\text{C}$  for fifteen minutes before being vortexed for ten seconds to end the reaction.



**Figure 6: Redox scheme of Methyl Viologen in the presence of dithionite.** Addition of sodium dithionite results in a donation of an electron to methyl viologen, which results in the formation of blue color. The methyl viologen is responsible for electron donation to the nitrite reductase during the assay, but the blue color only disappears upon vortexing the reaction so as to oxidize the sulfanilamide completely (23).

Following the reaction, a 1:50 dilution was done on the sample in order to bring it within the linear range of the Griess reaction. 250  $\mu\text{L}$  of 0.01% N-(1-Naphthyl)ethylenediamine dichloride (NED) and 250  $\mu\text{L}$  0.1% Sulfanilamide with 3M HCl were added to the mixtures, which caused a pink color to appear in the presence of nitrite. This pink color is the result of a coupling reaction depicted in the scheme shown in Figure 7 (22). For the original Griess reaction, in acidic solution, nitrite will react with sulfanilic acid to form a diazonium cation which then couples with naphthylamine and produces the pink color (22). The sulfanilic acid and naphthylamine has since then been substituted in modern experiments with sulfanilamide in acidic solution and naphthylethylenediamine, as the previous naphthylamine used in the coupling reaction has been found to be extremely carcinogenic. Following a ten minute incubation of the reaction, the result was then measured using a spectrophotometer at 540 nm using a glass cuvette.



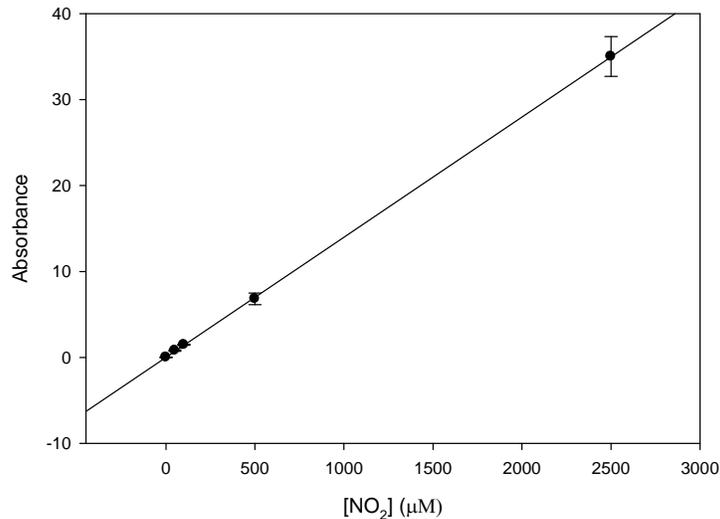
**Figure 7: Diazo-coupling Scheme of the original Griess reaction.** The addition of sulfanilic acid to nitrite results in the formation of the diazonium cation. Addition of  $\alpha$ -naphthylamine results in the final product, giving the product a pink hue. Since the original reaction was published, the reactants have later been substituted with sulfanilamide in acidic solution as well as naphthylethylenediamine to reduce the usage of more carcinogenic reactants in the Griess assay (22).

Following nitrite measurement, the detected nitrite was compared to a reaction with no protein added to the eppendorf tube. The difference in nitrite concentration between the reaction with no protein and the reaction containing sample protein was used to determine the amount of nitrite reduced by the periplasmic or whole cell protein fractions in  $\mu\text{M}$ . This concentration was then divided by the reaction time to determine activity. Results were then plotted using SigmaPlot and compared for general trends.

Using this method of determination for NirK activity, periplasmic and whole cell protein fractions were assayed for activity. Activity was measured against the amount of protein added to the reaction to observe nitrite reduction under increasing amounts of protein.

### ***3.3 Griess Assay Standard Curve***

To assess the linearity of the Griess reaction, a standard curve was plotted using the reaction volume listed previously. For the following experiments involving whole cells, in order to bring the system to saturation for NirK, the standard curve was measured for linearity up to a concentration of 2500  $\mu\text{M}$ . Figure 8 is the standard curve produced after 6 different experiments for different concentrations of nitrite from 0 to 2500  $\mu\text{M}$  nitrite. The values for the standards conform to a linear curve closely. Standards were prepared as described in the previous section without the addition of enzyme. For concentrations of nitrite higher than 100  $\mu\text{M}$ , the reaction volume was diluted to bring it at or below 100  $\mu\text{M}$ .



**Figure 8. Standard Curve of Griess Assay.** Using the diazocoupling method, nitrite was measured at concentrations to allow for measuring a saturated system for the nitrite reductase. Standard measurements were taken at 0 µM, 50 µM, 100 µM, 500 µM, and 2500 µM (N=6).

It can be seen from this figure that, although variation exists for the 500 µM and 2500 µM values, the overall graph can be seen as linear. Variance was caused from time to time due to the dilution process of a 1:50 dilution to bring high concentrations to below 100 µM, or the maximum concentration of the Griess reaction for which the reaction is linear. But after six experiments and six standard curves, the curves were overall very consistent from experiment to experiment.

### ***3.4 Whole Cell Determination of Nitrite Reductase Activity***

In addition to the protein measurement method described in the general principal determination of activity, an alternative method was investigated involving the use of whole cells as a way of measuring NirK activity. To assess nitrite reducing capabilities prior to their electrochemical assay of nitrate and nitrite reducing activities in a different *Rhizobium* species, Salome et. al performed a nitrite reducing assay that has been adapted in this project that normalizes the data according to protein measurements found within the cell (18).

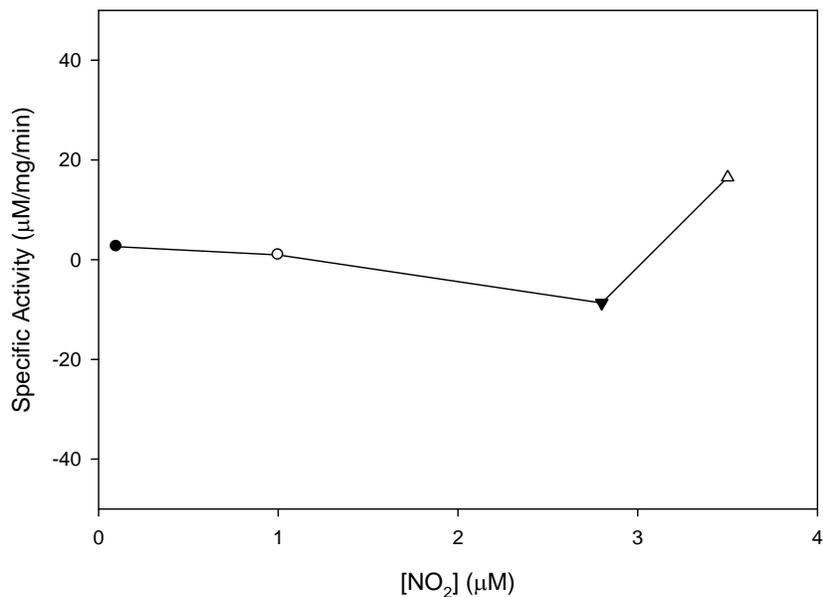
Following growth of whole cells, the OD of the cells was checked, and a 1 mL aliquot was sonicated to measure protein within the cell. An eppendorf tube was then prepared with 0.2 mL of cells and 2.5 mM sodium nitrite for a final volume of 0.4 mL. The cells were incubated at 30°C for two hours. Following incubation, the cells were centrifuged, and the supernatant was diluted and brought to a final volume of 250 µL in glass culture tubes. This dilution was then used in the diazo-coupling method involving Naphthyl ethylenediamine and Sulfanilamide used to measure nitrite mentioned previously.

By using the whole cell method, this assay provides an alternate method to measuring nitrite reducing activity within *S. meliloti*. One caveat of using the periplasmic fraction was the relatively low yield of protein that could be harvested from the cells. While whole cell fractions provided a higher yield than periplasmic protein, usage of whole cell fraction introduced the possibility that the amount of nitrite reductase found in the fraction was less in proportion to the periplasmic fraction. In particular, protein activity was measured against initial substrate concentration using this method as well as comparing different mutations of the copper ATPases versus the wild type *S. meliloti* strain.

The usage of periplasmic protein would be favored over whole cells due to the secretion of NirK to the periplasm because it allows for a more focused observation of NirK activity. However, the use of whole cells in saturated systems produced some indication that the mutants affect the ability of nitrite reduction. As a general method of determination, as was used in Salome et al, the whole cell assay works sufficiently to observe the ability of nitrite reduction for the cells.

### ***3.5 Activity vs. Nitrite Curve***

To characterize the capability of *S. meliloti* to reduce nitrite at various levels of nitrite saturation in a system, the whole cell assay was used at varying amounts of nitrite from 0.1 mM to 3.5 mM. This was done using 0. Figure 10 is a plot of one iteration of the experiment.



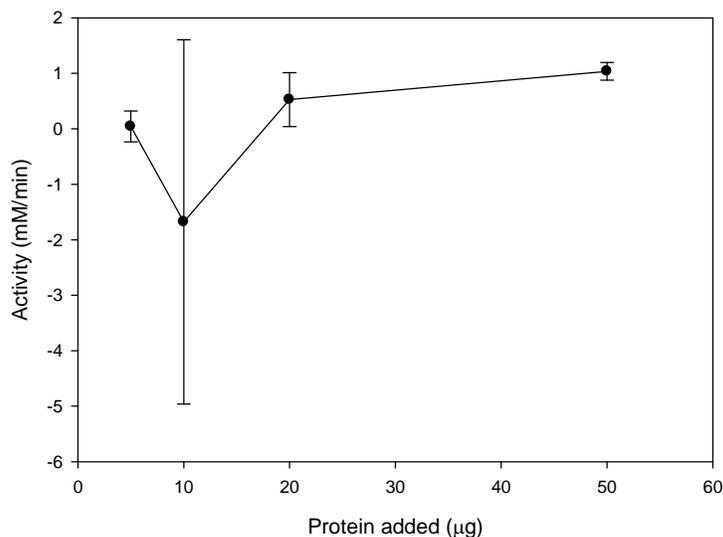
**Figure 10. Nitrite Reduction Substrate Curve.** Rm2011 *S. meliloti* wild type cells were incubated with varying concentrations of nitrite (0.1 mM, 1 mM, 2.8 mM, 3.5 mM) to observe nitrite reduction.

It was expected that the plot would follow a Michaelis-Menten trend in terms of activity. The point of 2.8 mM nitrite was selected because of the  $K_m$  listed previously by Ferroni et. al being 0.55 mM with saturation occurring approximately five times the  $K_m$ . However, while the data dips slightly at 1.0 mM, the activity dips further at 2.8 mM rather than increasing to saturation. Although measurements of whole cells at 2.5 mM (shown below) have produced viable data for the wild type, this occurrence at 2.8 mM nevertheless is brought up close to 20  $\mu\text{M}/\text{mg}/\text{min}$  after saturating the system with 3.5 mM of nitrite.

One possible explanation of the activity is that the cells have not been induced to produce NirK without the anaerobic environment under which denitrification takes place. The fit may also not fit a perfect Michaelis-Menten fit because the substrate saturation for the lower concentrations tested (0.1 mM and 1 mM) would not saturate the system or would eventually desaturate the system after enough nitrite has been reduced.

### 3.6 Activity vs. Periplasmic Protein Curve

To determine NirK activity of the wild type strain of *S. meliloti*, protein activity was measured against the amount of protein added using the periplasmic fraction of the wild type Rm2011 strain. This was done using the general principal determination of activity. Figure 9 shows the activity of the periplasmic fraction and its ability to reduce nitrite after a 15 min incubation from 5-50  $\mu\text{g}$  amounts of protein added.



**Figure 9. Wild-type (Rm2011) periplasmic protein curve.** Different concentrations (5, 10, 20, 50  $\mu\text{g}$ ) of periplasmic protein were measured for their ability to reduce 2.8  $\mu\text{M}$  sodium nitrite after N=3 trials.

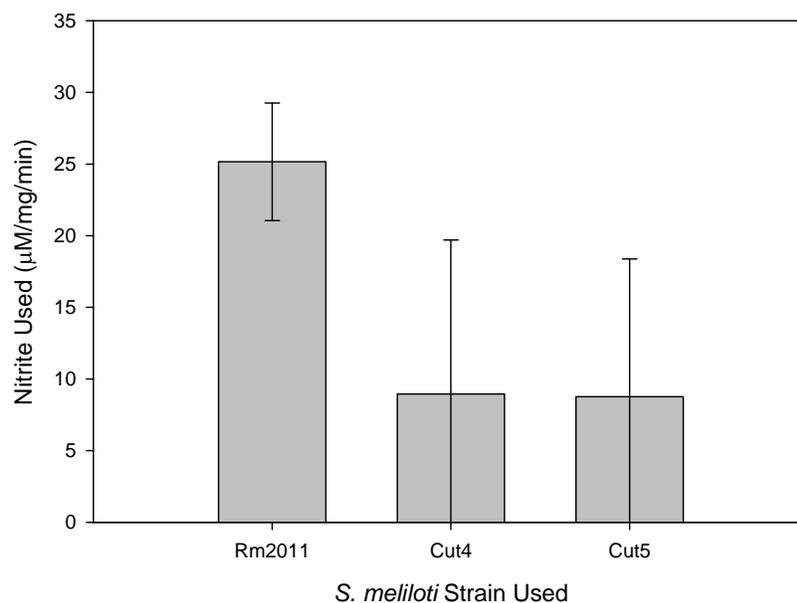
Although the iterations seem to suggest overall increasing nitrite reduction, there is an evident outlier in the data at 10  $\mu\text{g}$  of protein added. Barring that data point, however, the trend of the overall curve is one that increases, with close to zero mM of nitrite being processed per minute to one mM of nitrite per minute at 50  $\mu\text{g}$ .

This would suggest that if more periplasmic protein were obtained and added to the reaction, the nitrite reducing capability would increase to much more significant levels. Because of the limited amount of periplasmic protein yielded from each extraction, however, observing for significant amounts of nitrite reduction was limited until more cells could be extracted, and to observe activity, whole cells were used to observe the nitrite reductase activity within the cells.

### ***3.7 Mutant Whole Cell Activity Comparison***

Following an investigation into these methods, the effects of different mutations was observed for various mutants. Using the whole cell determination method previously described in section 3.4, the activity was measured for the wild type as well as the mutant strains in which certain copper ATPases are no longer expressed. The whole cell determination method was used in order to observe the general reduction capabilities of the cell, as the amount of periplasmic protein yielded was insufficient for measurement.

A total of three trials were done to observe the effects of missing two of the copper ATPases: Cut4 and Cut5. Figure 11 shows the results for the whole cell assay previously described at 2.5 mM using the Rm2011 wild type,  $\Delta\text{Cut4}$ , and  $\Delta\text{Cut5}$  mutant strains. Absorbance has been converted to activity in terms of micromolar nitrite reduced per minute per microgram of protein measured after a sonication of an aliquot of cells prior to performing the assays.



**Figure 11. Whole cell assay of three different strains of *S. meliloti*.** The wild type Rm2011,  $\Delta$ Cut4, and  $\Delta$ Cut5 mutant strains were all used in the whole cell assay to reduce nitrite at 2.5 mM. (N=3).

It can be seen that the wild type has a higher amount of activity in comparison to either mutant. However, the error bars after these three iterations are high. Standard deviation for whole cell  $\Delta$ Cut4 and  $\Delta$ Cut5 data large over three replicates. It is not known what causes these unusual readings, but the unusual readings have appeared over a series of experiments even though the standard curve done with each experiment has been fairly consistent.

Although the error bars are high, it is possible that these reactions are both lower than the wild type activity to some degree, but to determine which one has a greater amount of activity versus the other is something inconclusive based on the data at the moment. The lack of copper transporters appear to affect the ability for NirK to obtain the necessary copper centers required for nitrite reduction. Observations of the other copper ATPases found in *S. meliloti* (ActP, FixI1,

and FixI2), would contribute to understanding if NirK receives its copper from a single copper ATPase or if there is a target.

### **3.8 Conclusions**

The goal of this project was to determine the different targets of copper ATPases in *S. meliloti*. It was hypothesized that one of the targets of these ATPases is NirK. While the data is inconclusive about nitrite reductase being a target, methods have been developed to measure nitrite reduction activity in *S. meliloti*, and each can be critiqued for its successes and areas of improvement for further research.

One consideration that should be noted is the pathway that the denitrification is done. As previously discussed, the denitrification pathway is one method by which the cells synthesize ATP under oxygen limited conditions. One potential other method frequently mentioned in addition to incubating the cells with nitrite is growing the cells under anaerobic or microaerobic conditions to utilize the denitrification pathway. For this project, however, the equipment to grow the cells anaerobically was not available, and cells were limited to growth in a normal incubator under aerobic conditions.

As demonstrated with the standard curve, it has been shown that it is possible to get fairly consistent data points using the Griess assay. There are some dubious data points as well as the mutant data in the comparison of various mutants, but data collected for the wild type was for the protein curve and the mutant comparison was generally more reliable than the mutant data.

Obtaining more data points for protein activity versus initial substrate concentration would be one thing to continue as well as activity versus the amount of protein added to the reaction. Measuring protein activity with respect to time to observe nitrite reduction over different

incubation times would also be useful in characterizing NirK activity in *S. meliloti*. Other mutants may be tested to observe if the mutants used in this project are responsible or if mutations to any copper ATPase affect nitrite reduction capability.

## 4. References

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