COPPER-NITROSYL FORMATION BY NITRITE REDUCTASE

by

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B.Sc., The University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
August 2007

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Abstract

Copper-containing nitrite reductase (NiR) is part of the denitrification pathway employed by bacteria to generate energy from reducing equivalents during low oxygen availability. NiR is located in the periplasm and catalyzes the committed step of denitrification, the one electron reduction of nitrite to nitric oxide. The enzyme is a homotrimer with two spectroscopically distinct copper sites per monomer, classified as type 1 and type 2. The type 1 copper site is located near the surface of the protein and receives electrons from a physiological electron donor, such as pseudoazurin. The electrons are then passed onto the type 2 copper site, which is the catalytic center of NiR. The type 2 copper is located at the bottom of a 16 Å deep cavity at the interface between two adjacent subunits. Efficient electron transfer is ensured by the presence of a Cys-His bridge that connects the two copper sites.

A copper-nitrosyl intermediate is formed by NiR during catalysis; however, it has not been characterized previously. To address how NiR may interact with nitric oxide and to better understand the transition between substrate and product binding to the type 2 copper during catalysis, crystal structures and EPR spectra were analyzed of the substrate- and product-bound NiR. In the crystal structure of the substrate-bound NiR from Alcaligenes faecalis S-6 (AfNiR) extended to 1.4 Å resolution, the \( \text{NO}_2^- \) coordinates to the copper primarily by a single oxygen-atom (\( \text{Cu-O}_c \) distance of ~2 Å). In addition, the second oxygen and nitrogen atoms are situated ~2.3 Å from the metal, positioning nitrite almost face-on with respect to the copper. A copper-nitrosyl intermediate forms during the catalytic cycle of AfNiR. To examine a possible copper-NO interaction in AfNiR, crystals of the reduced enzyme were exposed to NO in an anaerobic environment. The x-ray crystal structure to 1.3 Å resolution reveals an NO molecule interacting with the catalytic center. The NO molecule coordinates in a side-on fashion such that both the N
and O atoms are equidistant from the copper (~2 Å). The oxidation states of the type 1 and type 2 copper atoms were monitored by EPR spectroscopy. Exposure of the reduced protein solution to NO results in a spectrum indicative of an oxidized type 2 copper site. Observation of NO bound to the catalytic center and measurement of the oxidation state of the metal gave rise to a formal description of the copper-nitrosyl in AfNiR as a Cu(II)-NO⁻. Comparison of the NO₂⁻-bound with the NO-bound crystal structure of AfNiR provides a plausible explanation of how coordination can change between copper-oxygen and copper-nitrogen during catalysis.

The copper-nitrosyl catalytic intermediate formed by AfNiR is formally described as Cu(I)-NO⁺, which is a system that has one less electron than the copper-nitrosyl proposed to be formed by the addition of exogenous NO to reduced AfNiR. Copper-nitrosyl complexes of oxidized wild-type and variant forms of AfNiR were formed by prolonged exposure of crystals to exogenous NO and the structures were determined to 1.8 Å or better resolution. Exposing oxidized wild-type crystals to NO results in the formation of nitrite bound to the type 2 copper. The type 1 copper site variant, H145A, prevents electron exchange with the type 2 site and the reverse reaction is disrupted. Instead, both oxidized and reduced H145A have NO bound side-on to the type 2 copper as observed previously with the reduced wild-type enzyme. Asp98 forms hydrogen bonds to both bound substrate and product. In the D98N variant of AfNiR, NO binding is partially disordered. EPR spectra of each AfNiR-NO complex under similar buffer conditions are indicative of stable copper-nitrosyls that are formally assigned as Cu(II)-NO⁻. Reaction schemes can account for the formation of a stable copper-nitrosyl when starting from either the oxidized or reduced H145A variant.

The ability of several small molecules to bind to the type 2 copper of AfNiR and inhibit the enzyme was examined by crystallography and enzyme kinetics. Inhibitors such as nitrate,
formate, acetate, nitrous oxide and azide are able to interact with the catalytic center. However, kinetic data showed that tested inhibitors weakly inhibit the enzyme ($K_i > 2$ mM). All inhibitors, except azide, bind ~0.15 Å further away from the copper than either the product or substrate. Azide binds with a Cu-N distance of ~2 Å and shows the strongest inhibition with a $K_{ic}$ of 2 mM. Mixed inhibition was observed for formate and acetate; however, kinetic data show that nitrate is an uncompetitive inhibitor with respect to nitrite. Through steric clashes and a requirement for a hydrogen bond with Asp98, the active site of NiR is able to favour nitrite binding and discriminate against other small molecules present in the periplasm.

Altogether the studies presented in this thesis indicate how AfNiR is specific for its substrates and how this specificity is fine tuned by the active site residues.
Acknowledgements

I would like to thank my supervisor Michael Murphy for his supervision over the years. I am grateful to my committee members, Drs. Tom Beatty, Lindsay Eltis and Lawrence McIntosh for their advice and support. I am especially thankful to Drs. Lindsay Eltis and A. Grant Mauk who generously opened their labs to me throughout my graduate career. I would like to thank to Dr. Federico Rosell who provided invaluable assistance during EPR spectroscopic data collection. I am grateful to all present and past members of the Murphy, Eltis and Mauk labs for their help and assistance. I also recognize Stanford Synchrotron Radiation Laboratory and support staff especially Dr. Tzanko Doukov for x-ray crystallography data collection.

Most importantly, I would like to thank my parents, Kalina and Ivan, and my brother Nikolay for their unconditional love and support.
Co-authorship statement

Scientific collaborations have provided valuable support for this thesis research. Below is a summary of the contributions of other scientists.

Chapter 3 was largely published in: Tocheva, E. I., Rosell, F. I., Mauk, A. G. and Murphy, M. E. P. 2004. Side-on copper-nitrosyl coordination by nitrite reductase. Science 304:867. The original hypothesis that NO might coordinate to type 2 Cu of NiR side-on was suggested by Dr. Peter Legzdins (Dept. of Chemistry, UBC). Introduction to working anaerobically in a glove box was provided by Drs. Lindsay Eltis, Fred Vaillancourt, Pascal Fortin and lab technician Cheryl Whitting. I did all the protein purifications, crystallization setups, data collection and processing. I also prepared all the EPR samples which were collected with the assistance of Dr. Fred Rosell in the lab of Dr. Grant Mauk. The initial draft of the manuscript and all figures were prepared by me. Drs. Fred Rosell, Grant Mauk and Michael Murphy edited the manuscript. Dr. Elinor T. Adman, William E. Antholine, Hans C. Freeman, Harry B. Gray, Peter Legzdins, and Tom G. Spiro helped with thoughtful comments on the manuscript and stimulating discussions.

Chapter 4 is a draft of a manuscript that will be submitted as: Tocheva, E. I., Rosell, F. I., Mauk, A. G. and M. E. P. Murphy. Copper-nitrosyls in wild-type and mutant forms of nitrite reductase. I performed all crystallographic experiments and prepared EPR samples for data collection by Dr. Fred Rosell. Schemes I-III were developed with the help of Dr. M. Murphy. I prepared all the figures and initial draft of the manuscript.

Chapter 5 is a draft of a manuscript that will be submitted as: Tocheva, E. I., Eltis, L. D. and M. E. P. Murphy. Inhibition of Alcaligenes faecalis nitrite reductase by substrate and
product analogues. I performed all the experiments and prepared all figures and initial draft. Dr. Lindsay Eltis helped with the design and trouble shooting of kinetic experiments.
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<td>$A$</td>
<td>Hyperfine coupling constant</td>
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<tr>
<td>Å</td>
<td>Ångstrom unit (1 Å = 0.1 nm)</td>
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<td>Amp'</td>
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<tr>
<td>BCA</td>
<td>2,2’-bicinchoninic acid</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>DFT</td>
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<td>DNR</td>
<td>Dissimilative Nitrate Respiration regulator</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotidetriphosphate</td>
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<td>ET</td>
<td>Electron Transfer</td>
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<tr>
<td>ETP</td>
<td>Electron Transfer Protein</td>
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<tr>
<td>EXAFS</td>
<td>Extended X-ray Absorption Fine Structure</td>
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<tr>
<td>$F_0, F_c$</td>
<td>Observed and calculated structure factors</td>
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<td>FNR</td>
<td>Fumarate and Nitrate Reductase regulator</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Phase Liquid Chromatography</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>G</td>
<td>Gauss (unit for field modulation)</td>
</tr>
<tr>
<td>$g$</td>
<td>$g$ factor (g tensor)</td>
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<td>H145A variant</td>
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<tr>
<td>H-bond</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan'</td>
<td>Kanamycin resistance</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Catalytic constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Competitive inhibition constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>$K_{iu}$</td>
<td>Uncompetitive inhibition constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria – Bertani broth</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>mT</td>
<td>Millitesla = 10 G</td>
</tr>
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NiR
Copper-containing nitrite reductase

cd/NiR
Heme-containing nitrite reductase

NO*
NO is a radical. The dot is mostly omitted in this thesis for clarity

NnrR
Nitrite and nitric oxide Reductase regulatory

OD<sub>600</sub>
Optical Density at 600 nm

O<sub>c</sub>
An oxygen atom that coordinates to type 2 copper with a bond length <2.1 Å

O<sub>nc</sub>
An oxygen atom that is considered non-coordinating to the type 2 copper (C-O<sub>nc</sub>) bond distance >2.1 Å

Paz
Pseudoazurin

PDB
Protein Data Bank

PMS
Peroxymonosulfate

<sub>R</sub>merge
Σ<sub>hkI</sub> Σ<sub>II(hkl)l</sub> / Σ<sub>hkI</sub> Σ<sub>II(hkl)l</sub>
r.m.s.
Root mean squared

<sub>R</sub>work
Σ |F<sub>obs</sub>1 - |F<sub>calc</sub>| / Σ |F<sub>obs</sub>1

SDS-PAGE
Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SOD
Superoxide dismutase

Tris
Tris(hydroxylmethyl)aminomethane

TPQ
Trihydroxylphenylalanine quinone

UV
Ultraviolet

Vis
Visible

YT-
Yeast Tryptone
1.1 Nitrogen and life

The growth of all organisms depends on the availability of mineral nutrients such as nitrogen, oxygen, phosphorus, potassium and sulfur. Nitrogen is required as an essential component of proteins, nucleic acids and other biologically relevant molecules. There is an abundant supply of nitrogen in the earth's atmosphere: nearly 80% in the form of N\textsubscript{2} gas. However, because of the triple bond between the two nitrogen atoms, N\textsubscript{2} is unavailable for use by most organisms (1). For atmospheric nitrogen to be utilized for growth, it must be converted to a form such as ammonia or nitrate that is more readily assimilated by living organisms (1).

1.2 The global nitrogen cycle

The nitrogen cycle is the continuous series of natural processes by which nitrogen passes from the air to soil or aquatic environments, to plants, and ultimately to sustain all animal life. It then returns back to the environment through decay or denitrification (Figure 1-1) (2). Four major processes comprise the global nitrogen cycle – nitrogen fixation, nitrification, nitrate assimilation, and denitrification.
Figure 1-1 Main processes in the global nitrogen cycle. Nitrogen fixation, nitrification, nitrate assimilation, anammox (anaerobic ammonium oxidation) and denitrification are the main processes that comprise the global nitrogen cycle. Chemical formulas of the intermediate species are shown. Figure is adapted from (3).
1.2.1 Nitrification

Autotrophic and heterotrophic microorganisms use nitrification, an aerobic process, to oxidize ammonia to nitrate via the formation of hydroxylamine and nitrite intermediates (4). Nitrifying bacteria are capable of converting the most reduced form of nitrogen, ammonia, to the most oxidised form, nitrate. Autotrophic organisms are the only ones to use this process to derive energy where the oxidation of ammonia to nitrite and nitrate involves the donation of electrons to oxygen. The first step, the oxidation of ammonia to hydroxylamine is catalyzed by the enzyme ammonia monoxygenase with oxygen serving as an exogenous electron acceptor (4). In the second step, hydroxylamine is oxidized to nitrite by the heme-containing soluble hydroxylamine oxidase. Ultimately, nitrite is oxidized to nitrate by an enzyme called nitrite oxidase to complete the nitrification pathway. *Nitrosomonas* spp. transform ammonia into nitrite, which is then converted to nitrate by *Nitrobacter* spp.

1.2.2 Nitrate assimilation

Enzymes involved in the synthesis of nitrogen-containing biological molecules, assimilatory nitrate and nitrite reductases, do not conserve energy. This process, referred to as nitrate assimilation, is the reverse of nitrification and reduces nitrate to nitrite and subsequently nitrite to ammonia (4). In nitrite assimilation, the catalytic subunit of the cytochrome c nitrite reductase catalyzes nitrite reduction to ammonia without liberating intermediates.

1.2.3 Nitrogen fixation

Nitrogen fixation, an essential biological step, is the conversion of atmospheric nitrogen to ammonia. The overall reaction is summarized with the following equation:

\[ \text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- \rightarrow 2 \text{NH}_3 + \text{H}_2 \]

Equation 1
The key enzyme performing this reaction, nitrogenase, is found in free living aerobes and anaerobes as well as in symbiotic bacteria. Nitrogenase is a complex enzyme consisting of two separate proteins, dinitrogenase and dinitrogenase reductase (2).

Microorganisms are capable of reducing N₂ to NH₃ and thus have a central role in providing more complex organisms with a useful form of nitrogen. Bacteria able to convert atmospheric nitrogen into reduced nitrogen compounds span a wide range of environments. Such bacteria include obligate anaerobes (e.g. *Clostridium pasteurianum*), facultative anaerobes (e.g. *Klebsiella* sp.), photosynthetic bacteria (e.g. *Rhodobacter* sp.), many cyanobacteria, obligate aerobes (e.g. *Azotobacter* sp.) and some methanogens (3).

### 1.2.4 Denitrification

Denitrification is the major mechanism that reduces nitrogen oxide species such as nitrate and nitrite to dinitrogen gas (Figure 1-1 and Figure 1-2). Denitrification balances nitrogen fixation by releasing nitrogen gas back into the atmosphere, thereby completing the nitrogen cycle. The overall equation for the whole denitrification process is:

$$2 \text{NO}_3^- + 10 \text{e}^- + 12 \text{H}^+ \rightarrow \text{N}_2 + 6 \text{H}_2\text{O} \quad \text{Equation 2}$$

A vast number of bacteria but also some archaea are able to denitrify. In addition, a few fungi have also been shown to possess components of the denitrification pathway (3). The bacterial denitrification pathway and its enzymes will be discussed in greater detail in section 1.3.

### 1.2.5 Anammox

Recently, an additional process called anammox (anaerobic ammonium oxidation) has been described (5). This process involves the direct conversion of ammonium to dinitrogen gas and can be summarized by the following overall reaction:

$$\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \quad \text{Equation 3}$$
Anammox is an important process in ocean environments and waste water treatment plants (5) and in addition to denitrification is responsible for the removal of fixed nitrogen to a gaseous form of nitrogen.

Figure 1-2 Lewis dot and resonance structure representation of nitrogen oxide species during denitrification. For simplicity only one of the resonance structures will be used in this thesis.
1.2.6 Human intervention

Early in the 20th century, a German scientist named Fritz Haber discovered how to convert atmospheric nitrogen to ammonia by fixing nitrogen chemically at high temperatures and pressures. The so-called Haber-Bosch process creates synthetic fertilizers that can be added directly to the soil (6). The Haber-Bosch process is helping feed the growing world population; however, the use of excessive fertilizers has serious health, agricultural and environmental hazards.

Not all of the nitrogen fertilizer applied to agricultural fields remains in the soil. Some is washed off by rain or irrigation water, where it leaches and accumulates into surface or ground water. In ground water that is used as a drinking water source, excess nitrogen in the form of nitrite can lead to cancer in humans and respiratory distress in infants (7). The environmental impacts are also severe. High levels of fixed nitrogen in water can result in uncontrolled algal growth and eventually deplete aquatic sources of oxygen and nutrients (7). Up to 20% of the applied fertilizer to soil can be lost to the atmosphere by denitrifying organisms (8). This process is accompanied by the release of gaseous intermediates such as nitric oxide and nitrous oxide which have a significant environmental impact on a global scale. Nitric oxide has been implicated in the destruction of the ozone layer (9) and nitrous oxide is a green house gas several hundred times more powerful than carbon dioxide (10).

1.2.7 Bioenergetic respiration

Microorganisms require energy to perform biological functions. Many microorganisms obtain energy through the oxidation of a variety of substrates, such as organic compounds. In aerobic environments, the electrons released in this process are captured by O₂ as a terminal electron acceptor and reduced to water. In the absence of oxygen, used facultative and obligate
anaerobes use alternative electron acceptors such as nitrate, thiosulfate, carbon dioxide and organic compounds such as fumarate and dimethylsulfoxide (III).

The denitrification pathway is analogous to oxygen-based respiration: both are linked to the energy-conserving NADH dehydrogenase and cytochrome $bc_1$ complexes (4). During denitrification, only turnover by nitrate reductase (NaR) results in a net proton gradient (2); all the other enzymes are involved in accepting electrons generated during organic carbon oxidations in the form of NADH and thus help restore the pool of NAD$^+$.  

1.3 The denitrification pathway and its enzymes

The current understanding of the denitrification pathway and the organization of its enzymes with respect to the cell membrane in Gram-negative bacteria are summarized in Figure 1-3. The overall organization of denitrification in gram-positive bacteria has not been well studied and will not be discussed in this thesis.
Figure 1-3 Cellular localization of the bacterial dissimilatory denitrification enzymes.

Nitrate reductase can exist either as a soluble periplasmic enzyme (NaP, PDB code 2NAP) or in a membrane bound form (NaR, PDB code 1Y4Z). Nitrite reductase (NiR, PDB code 1GJQ (cd1NiR) and 1SNR (CuNiR)) is a periplasmic enzyme that can bind either heme or copper. Nitric oxide reductase (NOR, PDB code 1JFC) and nitrous oxide reductase (NoS, PDB code 2IWF) carry out the final two steps of bacterial denitrification.
In the denitrification pathway, the nitrate to nitrite couple has a relatively high standard reduction potential (Table 1-1), and thus nitrate is a preferred electron acceptor to nitrite. The reduction product, nitrite, is also an effective acceptor. Subsequently, nitrite is reduced to nitric oxide, nitrous oxide (N₂O), and dinitrogen (N₂) in three separate reactions (Table 1-1). The four steps of denitrification are accomplished by four distinct enzymes. The first step (eq. 3), the reduction of nitrate to nitrite, is catalyzed by a molybdenum-containing nitrate reductases: NaR (membrane bound, active in the cytoplasm) or NaP (soluble, active in the periplasm). Nitrite is further reduced to NO in the second step (eq. 4) that can be catalyzed by either cytochrome cd₁ or copper-containing nitrite reductases. In a third step (eq. 5), two nitric oxide molecules are conjugated to form nitrous oxide and water. This step is accomplished by a nitric oxide reductase (NOR) that contains heme c, heme b and non-heme iron cofactors. Finally, a copper-containing enzyme (nitrous oxide reductase, NoS) is responsible for the last step (eq. 6) where nitrous oxide is reduced to dinitrogen.

Table 1-1 Chemical reactions of denitrification and standard reduction potentials measured at pH 7.0

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$E^\circ$ (V)</th>
<th>Equation number</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \text{NO}_3^- + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$</td>
<td>+0.42</td>
<td>4</td>
</tr>
<tr>
<td>$\text{NO}_2^- + \text{e}^- + 2 \text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O}$</td>
<td>+0.37</td>
<td>5</td>
</tr>
<tr>
<td>$2 \text{NO} + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$</td>
<td>+1.17</td>
<td>6</td>
</tr>
<tr>
<td>$\text{N}_2\text{O} + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O}$</td>
<td>+1.33</td>
<td>7</td>
</tr>
</tbody>
</table>

Note: The table was adapted from reference (11).

Nitrate reductases are diverse enzymes in terms of active site constitution, subunit structure and cell localization (4). All are molybdenum-containing enzymes and are classified into four groups: eukaryotic nitrate reductases, assimilatory nitrate reductases, respiratory nitrate reductases, and dissimilatory nitrate reductases.
reductase, and periplasmic nitrate reductases. Only the last two are involved in dissimilatory denitrification. Nitrate reduction and oxidation of the electron transfer molecule quinol by the membrane bound NaR generates a net transmembrane gradient. These proteins are constituted by three subunits: NarG (α), NarH (β), and NarI (γ) \((11, 12)\). NarG contains the active site of the enzyme whereas the remaining subunits contain electron transfer centers. The NarGH complex is located in the cytoplasm but remains anchored to the inner surface of the cytoplasmic membrane by the transmembrane NarI subunit.

As nitrate is turned over by NaR in the cytoplasm, the product (nitrite) is transported across the cytoplasmic membrane presumably to protect the organism from potential toxic effects. In addition, nitrite reductases are found in the periplasm. Both of these factors would require a bacterium to synthesize a transport system. Nitrate/nitrite transport proteins have been identified in organisms capable of denitrification such as *Pseudomonas aeruginosa*, *P. stutzeri* and *Paracoccus pantotrophus* \((13-15)\). For example, the *narK2* gene is required as a nitrate/nitrite transporter in *Pseudomonas aeruginosa*. In addition, in a bioinformatics analysis, this gene was found to cluster with the structural genes for the nitrate reductase enzyme complex \((13)\). A deletion of the gene resulted in almost no growth under denitrifying conditions, and a gene complementation completely rescued the mutant \((13)\). The periplasmic NaP is a 80 kDa multi-subunit enzyme \((16)\). The N-terminal amino acid sequence contains a cysteine motif \((\text{Cys-X}_2\text{-Cys-X}_3)\) involved in the coordination of the \([4\text{Fe}-4\text{S}]\) cluster. In addition, the enzyme contains a bis-molybdopterin guanine dinucleotide cofactor \((4)\).

The enzyme nitrite reductase catalyses the committed step in bacterial denitrification, the conversion of nitrite to nitric oxide. This is the key step by which a mineral form of nitrogen is converted to a gaseous form. Two distinct NiRs exist that contain either heme or copper
cofactors. The two enzymes perform the same reaction; however, they share no significant sequence identity, have different structures and employ distinct mechanisms. Heme and copper-containing nitrite reductases have never been found to coexist in the same microorganism. Also, there is no correlation between the microbial taxonomy of a microorganism and the type of NiR present. For example, a heme containing NiR is present in a strain of *Alcaligenes faecalis* while a copper-containing NiR is found in the closely related *Alcaligenes faecalis* S-6. Interestingly, the activity of the heme cdNiR in *Pseudomonas stutzeri* can be complemented by a copper-containing NiR demonstrating that the two types of enzyme perform the same function in vivo (17). NiRs will be discussed in greater detail in sections 1.3.1 and 1.3.2.

It is now clear that in most denitrifying organisms reduction of nitrate and nitrite occurs with NO as an obligatory intermediate (18). A major concern with NO as an intermediate is its toxicity, particularly its reactivity with heme and non-heme iron-containing proteins. NO is kept at low steady-state levels so that toxic levels are never reached (19, 20). This is accomplished by a very low apparent $K_m$ and a higher $V_{max}$ of the nitric oxide reductase relative to those of the nitrite reductase. Channelling of NO from nitrite reductase to nitric oxide reductase through protein-protein interactions may be another factor limiting NO toxicity. Cytochrome $c'$ is a periplasmic heme-containing protein found in a metabolically diverse set of proteobacteria. In denitrifiers, cytochrome $c'$ has been proposed to have a role in mediating NO transfer either out of the cell, thus protecting the organism from potentially toxic levels of NO that may otherwise accumulate (21), or it could be involved in relaying the NO produced by nitrite reductase to nitric oxide reductase, thus preventing exposure of NO to the reactive environment of the periplasm.
During denitrification, an N-N bond formation takes place and is performed by nitric oxide reductases. These enzymes are integral membrane proteins which are either heterodimers containing NorC and NorB subunits to form a cNOR (cytochrome c derived electrons), or are composed of a single polypeptide qNOR (quinol derived electrons) (22). A c-type cytochrome in the bacterial cNORs is thought to accept electrons from in vivo donors, and a b-type cytochrome coupled to an adjacent iron atom forms a dinuclear cluster in the catalytic site that binds two molecules of NO.

Nitrous oxide, produced by the reduction of two nitric oxide molecules, is the substrate used by the enzyme nitrous oxide reductase. The enzyme catalyzes the two-electron reduction to dinitrogen and represents the final step in denitrification. The functional enzyme is a dimer with 4 copper atoms per monomer. Crystallographic studies show that NoS possesses a tetranuclear Cu$_z$ cluster (23). During catalysis, electrons are passed from a dinuclear copper site to the tetranuclear copper site which is the active site of the enzyme (1).

1.3.1 Cytochrome cd$_1$NiR

Cytochrome cd$_1$NiRs have been found in the periplasmic space in two thirds of isolated denitrifiers. These enzymes consist of two identical subunits with molecular masses of 60 kDa, each containing one heme c prosthetic group covalently linked to the polypeptide chain and one heme d$_i$ moiety non-covalently associated with the protein. These enzymes have been described as bi-functional as they catalyze not only the one-electron reduction of nitrite to nitric oxide, but also the four-electron reduction of dioxygen to water (24, 25). High resolution crystal structures are available for the proteins from *Thiosphaera pantotropha* and *Pseudomonas aeruginosa* (26, 27) (Figure 1-4). Intramolecular electron transfer occurs between the heme c, that serves as the electron uptake site from electron transfer proteins (ETP) such as soluble cytochrome c$_{551}$ or azurin (9), and heme d$_i$ as the nitrite reduction site. The observed electron transfer rates for the
cd1NiRs differ by orders of magnitude. For example, the observed rate constant for *P. pantotrophus* is 1.4x10^3 s⁻¹ and the one found for *P. aeruginosa* is 1 s⁻¹ (11).

During catalysis, binding of nitrite to the reduced *d*₁ heme occurs through the nitrogen atom (Figure 1-5) (24). Subsequently, transfer of an electron and two protons to the nitrite results in the release of water and the formation of nitrosyl species. The protons are likely provided by two highly conserved histidine residues. The formation of an unstable nitrosyl intermediate (Fe²⁺-NO⁺ ⇌ Fe³⁺-NO⁻) is suggested to decompose and release NO, or is replaced by a nearby tyrosine residue. Subsequently, the *d*₁ heme is reduced by an electron transfer from the *c* heme and the tyrosine residue is displaced. The intramolecular electron transfer completes the catalytic cycle. A dead end product, Fe²⁺-NO⁻, is formed if the electron transfer occurs before the release of NO or when the released NO rebinds to the oxidized enzyme (28). Electron paramagnetic resonance (EPR) (29) and x-ray crystallography (24, 27) indicate that NO is bound to the ferric form of the metal via the nitrogen. The Fe-NO bond angle is 131° and the Fe-N distance is 2.0 Å (27).
Figure 1-4 Crystal structure of cytochrome cd₁ nitrite reductase from *T. pantotropha* (PDB code 1AOM). The two separate monomers are coloured in salmon and purple. Each monomer is comprised of two domains, one containing the c heme (smaller one, top) and the other containing the d₁ heme. The heme cofactors are drawn in ball-and-stick; nitrogen atoms are coloured in blue, oxygen atoms are coloured in red and carbon atoms are coloured in yellow. Figure was created with PyMol (30).
Figure 1-5 Catalytic mechanism of cdNiRs. Consecutive steps of the mechanism are numbered 1, 2, 3 and 4. In step 1, nitrite binds to Fe(II) via the N atom. Step 2 shows rearrangement at the active site to produce Fe(II)-NO heme (step 3). Finally, NO is displaced by a tyrosine residue prior to electron transfer (step 4). Figure adapted from reference (27).
1.3.2 Copper-containing nitrite reductases

1.3.2.1 Diversity

NiRs have been identified primarily in Gram-negative denitrifying soil bacteria but also occur in Gram-positive bacteria (31). Denitrifiers with NiRs comprise one-third of the denitrifiers isolated from soil and include species from Pseudomonas, Alcaligenes, Corynebacterium, Bacillus, Rhizobium, Agrobacterium, and Rhodobacter. NiRs have also been isolated from the archael species Haloferax denitrificans (32) and from eukaryotic microorganisms such as the fungus Fusarium oxysporum (33). The presence of a NiR (AniA) has been shown to be essential for anaerobic growth of the pathogen Neisseria gonorrhoeae (34). Notably, antibodies against AniA have been found in patients suffering from gonorrhoea or pelvic inflammation. In addition, the expression of AniA has been shown to provide protection against killing by human sera (35).

1.3.2.2 Biological function and regulation

The primary biological function of NiRs is the reduction of nitrite to nitric oxide under oxygen limiting conditions. In an aerobic environment, NiR reacts with oxygen to form peroxide which through an unknown mechanism inactivates the enzyme (36). Although NO is the main product of NiRs, in vitro experiments performed with the use of chemical reductants showed that production of nitrous oxide could also be detected. Reduction of NO₂⁻ by the NiR from Achromobacter cycloclastes produces NO as the primary product initially, but as NO accumulates, N₂O production becomes significant (37). If nitric oxide is not removed from the reaction vessel, it can react with available nitrite or NO produced from nitrite to form N₂O (37). Recently, however, Wijma et al showed evidence suggesting that in vivo the enzyme is unable to carry out the reduction of NO to N₂O since upon build up of NO, the reaction equilibrium is
most likely pushed towards the production of nitrite and reduced pseudoazurin rather than the production of nitrous oxide (38).

Denitrification is a process that is utilized by bacteria when oxygen levels are low and nitrogen oxides are present as terminal electron acceptors. Microorganisms have evolved sensors to detect low oxygen levels and nitrogen oxide species. Most of the characterized regulators involved in denitrification belong to the FNR (fumarate and nitrate reductase regulator), DNR (dissimilative nitrate respiration regulator) and NnrR (nitrite and nitric oxide reductase regulator) subgroups of the CRP/FNR superfamily of transcription factors (39). These regulators act mainly on the nir (respiratory nitrite reductase) and nor (nitric oxide reductase) operons (40). In addition, the induction of denitrification by oxygen depletion requires the transcription factor ANR (anaerobic regulation of arginine deaminase and nitrate reduction) (39). ANR induces the expression of both the DNR family of regulators and NnrR proteins which are responsible for maintaining NO homeostasis (39).

1.3.2.3 Structural and spectroscopic characteristics of NiRs

Copper-containing nitrite reductases show strong structural similarity to the ‘classical blue multi-copper oxidases’, such as laccase or ascorbate oxidase, although they lack the binuclear type 3 copper site. NiRs are homotrimers in their native state with chain lengths of each monomer varying between 340 and 379 amino acids. Each monomer of the trimer consists of two domains with a cupredoxin fold. The cupredoxin fold is relatively small (10-18 kDa), built out of $\beta$-strands that are arranged in two $\beta$-sheets, forming a $\beta$-barrel (Figure 1-6). There are either no $\alpha$-helices or the $\alpha$-helical content is very low (restricted to a single $\alpha$-helix). Typically, they contain a single type 1 copper atom located at the hydrophobic core between the two $\beta$-sheets (41).
The crystal structure of the Achromobacter cycloclastes enzyme was the first copper-containing NiR to be solved (42). From this study the molecular composition was determined to be a homotrimer with subunits of approximately 37 kDa. Subsequently, the crystal structures of the NiR from Alcaligenes faecalis S-6 (AfNiR) (43) and from Alcaligenes xylosoxidans (44) were solved. The extensive sequence identity between these three different NiRs (> 60%) is consistent with the high degree of structural similarity (45).

AfNiR is a 110 kDa soluble, periplasmic homotrimer with each monomer comprising of N-terminal (residues 1-160) and C-terminal (residues 170-339) domains (Figure 1-6, Figure 1-7). Each domain is folded into a Greek key β-barrel motif of the cupredoxin fold, and the N- and C-terminal domains are connected though a short linker region (residues 161 to 169). The N-terminal domain of each monomer is located on the exterior of the trimer while the C-terminal domain surrounds the three-fold axis at the trimer core. A small helix in the C-terminal domain (residues 306-314) extends beyond the main monomer-monomer interface and packs against the N-terminal domain of the adjacent monomer. In addition, the last β-strand of the C-terminal domain (residues 331-338) forms a part of the β-sheet in the C-terminal domain of an adjacent monomer. Extensive inter and intra subunit hydrogen bond networks contribute significantly to the overall rigidity of the molecule. No significant structural changes were observed in the enzyme between the pH 4.0 to 6.5, consistent with high stability of the molecule (46, 47). The N-terminal domain of the monomer has a conserved copper site similar to that of other cupredoxin folds. The C-terminal domain of NiRs lacks a copper site, but has a second copper site at the interface between the two monomers.
**Figure 1-6 The cupredoxin fold of NiR.** The N-terminal β strand is colored in blue and the last C-terminal β strand is colored in red. The topology diagram (bottom) follows the color scheme of the cupredoxin fold of the NiR monomer (top). Figure was created with PyMol (30).
Figure 1-7 Secondary structure representation of NiR. Top panel: NiR monomer and bottom panel: *A. faecalis* S-6 NiR trimer. The N-terminal domain of each monomer is shown in blue and the C-terminal domain shown in purple. Copper atoms are shown as spheres and colored in magenta. Figure was generated with Molscript (48) and Raster3D (49).
The copper-binding sites of proteins are generally categorized as type 1, type 2 and type 3 according to their spectroscopic and magnetic properties. Each monomer of NiRs contains one type 1 and one type 2 copper site situated approximately 12.5 Å apart and connected by a His-Cys bridge for efficient electron transfer (Figure 1-8, top). The type 1 Cu or ‘blue copper’ is buried within the N-terminal domain of each monomer approximately 6 Å below the surface of the protein. In the oxidized form, Cu(II), the site exhibits an intense absorption band near 600 nm (ε=2000 to 5000 M⁻¹ cm⁻¹) which is attributed to an allowed ligand-to-metal charge transfer transition [S(Cys) —>• Cu(II)] (50). The EPR spectrum of the type 1 Cu site gives small hyperfine coupling constants (3-7 mT) (57). In NiRs, the type 1 Cu center is characterized by distorted tetrahedral or trigonal bipyramidal geometry and is coordinated by two histidine ligands, a cysteine ligand and an axial methionine ligand. This site serves as an electron acceptor site from electron transfer proteins such as azurin and pseudoazurin.

In AfNiR, site-directed mutagenesis studies have identified the charged molecular surface proximal to the type 1 copper site as the docking site for the donor pseudoazurin. Basic residues on pseudoazurin and acidic residues on AfNiR were identified which affect the kinetic parameters of the electron transfer between the two proteins (52). At pH 6.5, the measured $K_m$ for reduced pseudoazurin of AfNiR is ~ 80 μM with a $k_{cat}$ of 1.0 x 10³ s⁻¹ (38, 52). The second-order electron-transfer rate constant between AcNiR (53) or AfNiR (54) and pseudoazurin was measured by cyclic voltametry at the optimal pH of 6.2, and was found to be similar for both systems (7.3 x 10⁵ M⁻¹ s⁻¹ and 1.8 x 10⁶ M⁻¹ s⁻¹, respectively).

The type 1 Cu accepts electrons and donates them to the type 2 Cu, which is the reaction center. In the resting, oxidized state of the enzyme the type 2 copper is coordinated by 3 histidine residues and a water molecule. Two of the histidine residues, His100 and His135, (AfNiR
numbering) come from one monomer and the third histidine (His306) is supplied by an adjacent monomer (Figure 1-8). Since the type 2 copper is not coordinated by a cysteine ligand, it does not produce significant absorbance in the visible spectrum. Thus, this site exhibits a weak visible band at 700 nm (45). The paramagnetic signal of the type 2 copper as measured by EPR spectroscopy shows large hyperfine coupling constants of 12 - 20 mT (55). The similar reduction potentials of the type 1 and type 2 coppers range between +240 to +260 mV as measured by pulse radiolysis and cyclic voltametry (54).

Table 1-2 Properties of type 1 and type 2 copper centers in AfNiR.

<table>
<thead>
<tr>
<th></th>
<th>$A_z$ (hyperfine coupling constant, G)</th>
<th>$g_z$ value</th>
<th>Visible absorption peaks</th>
<th>Reduction potential (mV)</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>73-74</td>
<td>2.254</td>
<td>460 nm (s)</td>
<td>+240 to +260</td>
<td>His 95</td>
</tr>
<tr>
<td>type 1</td>
<td></td>
<td></td>
<td>600 nm (s)</td>
<td></td>
<td>His145</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Met150</td>
</tr>
<tr>
<td>Oxidized</td>
<td>129-130</td>
<td>2.394</td>
<td>700 nm (w)</td>
<td>+240 to +260</td>
<td>His100</td>
</tr>
<tr>
<td>type 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>His135</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>His306</td>
</tr>
</tbody>
</table>

* s-strong, w-weak absorption
Figure 1-8 Type 1 and type 2 copper sites in AfNiR. Top panel represents the location of the type 1 and type 2 coppers with respect to the secondary structures surrounding the sites and surface of the protein. Bottom panel represents the ball-and-stick model of the copper sites showing ligand residues. Carbon atoms are colored in yellow, nitrogen atoms in blue, sulfur atoms in green, copper in brown and water molecules in cyan. Figure was generated with Molscript (48), Raster3D (49) and PyMol (30).
1.3.2.4 Catalytically important residues in AfNiR

The water molecule (W503, Figure 1-9) that coordinates to the type 2 copper forms a hydrogen bond with the side chain of a nearby aspartate residue (Asp98). In the nitrite-soaked AfNiR structure, the water ligand is displaced by nitrite, which also forms a hydrogen bond with the side chain of Asp98 (47). Mutation of this residue reduces enzyme activity to less than 1%, establishing the importance of Asp98 in the reaction mechanism (16-18). In an Asp98Asn AfNiR mutant, the lack of interaction between Asn98 and the ligand water is consistent with a model of Asp98 not being protonated, favouring the binding of substrates that can act as proton donors (56). Furthermore, FTIR spectroscopy of the CO complex (57) and the crystal structure of the Asp98Asn variant with nitrite bound (16, 19) further support the idea that Asp98 is deprotonated (19).

A second residue, His255, forms a hydrogen bond with Asp98 via a solvent molecule (W1, Figure 1-9). This bridging water is hypothesized to donate a proton to the Asp98 Oδ2 atom and is a proton acceptor from the His255 Ne2 atom. The side chains of Asp98 and His255 are the only two residues that can be protonated in the active site and are believed to provide the protons needed during catalysis (46, 47). Site-directed mutations of His255 showed that this residue plays a catalytic role in the mechanism of AfNiR, possibly through donation of a proton via the bridging water to Asp98 (56). In addition to Asp98 and His255, mutagenesis studies have identified a highly conserved Ile257 in the native enzyme, which partially occludes the active site and is a key molecular determinant in directing a catalytically competent mode of nitrite binding (58).
Figure 1-9 Catalytically important residues surrounding type 2 Cu. The type 2 copper site of AfNiR is coordinated by three histidine residues and a water molecule (W503). Ligand bonds are represented as solid lines. Three important residues are situated in the proximity of the site – Asp98, His255 and Ile257. The bridging water connecting Asp98 and His255 is labelled W1 and is colored in cyan. Oxygen atoms are colored in red, nitrogen atoms are colored in blue, carbons are in yellow and copper is colored brown. Hydrogen bonds are drawn as dashed lines and distances are in Å. Figure was generated with PyMol (30).
1.3.2.5 Kinetic studies and proposed mechanisms for NiRs

To measure the catalytic activity of NiR, either a biological electron donor such as pseudoazurin \( (E^\circ = 230-305 \text{ mV}) \) (38), or a chemical reductant such as dithionite or peroxymonosulfate can be used (59, 60). With either chemical or biological electron donor, the maximum activity for NiRs is observed at pH 6.2 with a \( k_{\text{cat}} \) value of \( 1.5 \times 10^3 \text{ s}^{-1} \) (for AfNiR) and \( 1.6 \times 10^3 \text{ s}^{-1} \) (for AxNiR) (60).

An important mechanistic question that has been under debate with respect to the mechanism employed by NiRs is whether nitrite binds to the oxidized type 2 copper first, after which the electron is transferred to the type 2 copper site (46, 47, 61, 62), or whether the type 2 site is first reduced after which the nitrite binds to the copper (9, 63-65). Recently, the results of kinetic studies were fit to a random-sequential mechanism, in which at low nitrite concentration reduction of the type 2 site precedes nitrite binding and at high substrate concentration the reverse occurs (Figure 1-10) (66). In the starting configuration the oxidized Cu(II) carries water as the fourth ligand. Replacement of the water molecule by nitrite and subsequent electron transfer from the type 1 site lead to a nitrite-bound reduced type 2 copper. These two steps constitute the B route. The A route consists of electron transfer from type 1 to type 2 (step 2) and a subsequent nitrite binding (step 4) which leads to the same nitrite-bound reduced type 2 site. Conversion of nitrite to NO, dissociation of NO, and binding of water return the enzyme to the initial state. Step 5 leads to an inactive reduced form of the enzyme.
Figure 1-10 Random-sequential mechanism for NiR. A (step 1 and 3) and B (step 2 and 4) represent the possible routes of nitrite binding to the type 2 copper of NiR. Step 5 represents an inactive form of the reduced enzyme. P stands for product release. See text for more detail.

Figure adapted from reference (66).

A second important question associated with NiRs is the nature of the copper-nitrosyl formation by the enzyme. Hulse and Averill (9, 63) proposed a catalytic model in which reduction to nitric oxide proceeds through the formation of an electrophilic nitrosyl intermediate.
(Cu(I) – NO\(^+\)), similarly to the heme cd/NiR mechanism. The nitrosyl intermediate was suggested to be N-coordinated, which requires nitrite and nitric oxide to be N-coordinated to the type 2 copper. Crystal structures of biomimetic compounds bound to NO\(^2\) and NO via the nitrogen atom support these proposals (Figure 1-11). Interestingly, nitrite has been observed bound to Cu via the oxygen atom as well (67), however only N-coordinated nitrite results in stoichiometric NO production (68).

Nitrite binds to an oxidized type 2 copper atom of NiR in an asymmetric manner via one of the oxygen atoms. The O-coordinated nitrite binding in NiR was shown in ENDOR spectroscopy (69), EXAFS spectroscopy (62, 70) and crystallography experiments (47). Figure 1-12 shows a previously proposed catalytic mechanism, which has a protonated nitrite molecule displacing the ligand water. Reduction of the type 2 site is followed by the formation of a transient complex in which a hydroxyl group and nitric oxide are simultaneously bound to a pentacoordinated copper. This transient complex suggests the existence of a nitrosyl intermediate coordinating to the copper via the oxygen atom. However, this kind of interaction is chemically unprecedented (9, 37, 65, 71) and would require an unlikely NO rebound mechanism or flipping of an intermediate from O to N coordination to the Cu.
Figure 1-11 NO bound to copper in a biomimetic model of NiR. The crystal structure of mononuclear copper-nitrosyl complex, Tp\textsuperscript{RR'}CuNO (Tp\textsuperscript{RR'} = tris(3-R,5-R’-pyrazolyl)hydroborate: R = t-Bu, R' = H) (67). This biomimetic model of NiR shows N-coordination of the NO molecule to reduced copper ion. Figure used with permission from J. Am. Chem. Soc.
Figure 1-12 Proposed catalytic mechanism for NiR. Figure adapted from reference (56). The catalytic intermediate in this proposed mechanism shows NO interacting with the metal as a transient complex.

1.3.2.6 Structures of mononuclear copper-nitrosyls in chemistry and biology

Few copper-nitrosyl complexes are well characterized, presumably because of their instability and sensitivity to oxygen (67, 72). Prior to my thesis research, the only enzyme copper-nitrosyl characterized by crystallography was that of amine oxidase (73). Soaking crystals of reduced amine oxidase with NO to mimic dioxygen resulted in a copper-nitrosyl close to the TPQ cofactor. In this complex, the Cu-N-O angle is 117° and the Cu-NO distance is 2.5 Å (Figure 1-13). As discussed earlier (see Figure 1-11), another monomeric copper-nitrosyl crystal structure is that of a NiR biomimetic model compound: tris (3 t-butylpyrazolyl) hydroborate
Cu(I)-NO (67). In this model compound, the complex is formally described as Cu(I)-NO•, with the NO N-coordinated to the Cu and bent 163°. Notably, side-on Cu-O₂ complexes have been characterized structurally (74-76).

Figure 1-13 Nitric oxide bound to amine oxidase (PDB code 1D6Y). The NO molecule is 2.5 Å from the copper ion and is not considered a ligand to the metal. The orientation of the NO molecule is likely stabilized by a hydrogen bond to the TPQ cofactor and a nearby water molecule. The figure was generated with PyMol (30).
1.3.2.7 Terminology used in this thesis

Linear molecules such as NO, N₂O and N₃ can interact with the metal via one or more than one atom. Often the literature on small molecule interactions with metals uses interchangeably end-on and bent coordination regardless of the M-A-B angle. Figure 1-14 defines the main modes of binding to a copper ion as defined in this thesis.

Figure 1-14 End-on, bent and side-on coordination to a metal (M). Letters A and B represent the atoms of a linear ligand.
1.4 Goals of my Ph.D. thesis research

Copper-containing nitrite reductase (NiR) is a crucial enzyme in the terrestrial nitrogen cycle. It catalyzes the conversion of a mineral form of nitrogen (nitrite) into a gaseous form (nitric oxide) which is a key environmental step in the global nitrogen cycle. Despite the importance of this enzyme, the mechanism of NiRs remains poorly defined, with preliminary evidence suggesting a mechanism distinct from the cd/NiRs. In contrast to the well-accepted mechanism of the cd/NiRs, the structure of the presumptive NO-coordinated intermediate for the NiRs was uncharacterized and controversial owing to the paradox that arises from the apparent conflict between the N-coordinated binding of a copper-nitrosyl implicit in a biomimetic complex and the observed O-coordinated NO\textsubscript{2}\textsuperscript{−} binding in the enzyme.

This thesis focuses on the following main goals: (a) To solve the structure of a copper-nitrosyl in AfNiR; (b) to revise the catalytic mechanism for NiRs; (c) to understand the role of type 1 site and Asp98 in the structure of copper-nitrosyl; and (d) to understand the kinetic mechanism of inhibition by small molecules. To achieve these goals, new methods were developed to expose AfNiR crystals and AfNiR in solution to NO. The primary methods used in this thesis work were EPR spectroscopy, x-ray crystallography, and enzyme kinetics.
Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Chemical supplies and media

All chemicals were purchased from Fisher Scientific and Sigma-Aldrich Inc. unless otherwise specified. IPTG was purchased from Invitrogen Co. Factor Xa and Thrombin used to remove the six-histidine (His\textsubscript{6}) tag from recombinantly expressed proteins was purchased from Haematologies Technologies Inc. Acrylamide and other electrophoresis reagents were obtained from Bio-Rad Laboratories Inc. Nickel Sepharose 6 Fast Flow resin, Resource Q column and CM resin were purchased from GE Healthcare. Bacterial media components were purchased from DIFCO Laboratories. Luria-Bertani (LB) broth with appropriate antibiotics was used for overnight inoculation of bacteria; 2 x YT (yeast, tryptone) media was used for bacterial protein expression. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml.

2.1.2 Bacterial strains and plasmids

Table 2-1 lists all the strains of Escherichia coli used in this study. The E. coli strain DH5α was used as the host for genetic manipulations. E. coli strains HMS174 (λDE3) and BL21 (λDE3) (Novagen) were used for protein expression. Bacterial stocks were stored at -80 °C in LB medium containing 15% glycerol. Table 2-2 lists bacterial plasmids used in this study. pBluescript II SK- and the pET vectors were obtained from Stratagene and Novagen, respectively.
### Table 2-1 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>General host strain used for plasmid propagation</td>
<td>Novagen</td>
</tr>
<tr>
<td>BL21 (λDE3) and</td>
<td>Protease deficient <em>E. coli</em> strains. DE3, a λ</td>
<td>(77)</td>
</tr>
<tr>
<td>HMS174 (λDE3)</td>
<td>prophage carrying the T7 RNA polymerase gene.</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2-2 Bacterial plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript® II SK-</td>
<td><em>E. coli</em> cloning vector used in genetic manipulations, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET28a&lt;sup&gt;®&lt;/sup&gt;</td>
<td><em>E. coli</em> expression vector, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET24c&lt;sup&gt;®&lt;/sup&gt;</td>
<td><em>E. coli</em> expression vector, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pAfNiR28a</td>
<td><em>afnr</em> cloned in pET28a (NcoI / XhoI) in frame with a C-terminal His&lt;sub&gt;6&lt;/sub&gt; tag</td>
<td>(56)</td>
</tr>
<tr>
<td>pD98N28a</td>
<td>D98N <em>afnr</em> variant in pET28a (NcoI / XhoI) in frame with a C-terminal His&lt;sub&gt;6&lt;/sub&gt; tag</td>
<td>(78)</td>
</tr>
<tr>
<td>pH145A28a</td>
<td>H145A <em>afnr</em> variant in pET28a in frame with a C-terminal His&lt;sub&gt;6&lt;/sub&gt; tag</td>
<td>(79)</td>
</tr>
<tr>
<td>pPaz24c</td>
<td><em>Paz</em> cloned into pET24c (NheI / XhoI) without a His&lt;sub&gt;6&lt;/sub&gt; tag</td>
<td>(80)</td>
</tr>
</tbody>
</table>
2.2 General experimental methods

2.2.1 DNA manipulation

Heat shock competent cell preparation. An overnight bacterial culture 500 µl, DH5α (for cloning) and BL21 (λDE3) for protein expression was inoculated into 500 ml of LB broth and incubated at 37 °C in a shaking incubator until an OD₆₀₀ = 0.6 was reached. Cells were transferred to two sterile centrifuge bottles, cooled on ice for 30 min, and harvested by centrifugation at 2,000g for 15 min at 4 °C. The supernatant was decanted and each bottle of cells was resuspended gently in 20 ml cold sterile solution consisting of 100 mM RbCl₂, 10 mM CaCl₂, 30 mM potassium acetate, 50 mM MgCl₂, 15% glycerol, pH 5.8. The bacterial suspensions were incubated on ice for 15 min and centrifuged at 1,500g for 15 min at 4 °C. Each cell pellet was resuspended in 3.5 ml cold sterile solution consisting of 75 mM CaCl₂, 10 mM RbCl, 10 mM MOPS, 15% glycerol, pH 6.8. The cells were cooled on ice for 15 min, aliquoted (150 µl), flash frozen in liquid nitrogen and stored at -80 °C.

Electrocompetent cell preparation. The BL21 (λDE3) cells were streaked on an LB plate and grown at 37 °C overnight. A single colony was picked to inoculate 5 ml of LB medium. The cells were grown at 37 °C overnight. Half of the 5 ml overnight culture was used to inoculate 250 ml of LB media. The cells were grown at 37 °C until OD₆₀₀ was between 0.6 and 0.75. The cells, sterile deionised water and 10% glycerol solution were kept on ice from this step on. The culture was spun for 10 min at 2,000g at 4 °C and the supernatant was discarded. 100 ml of cold sterile deionised water were added and the cells were resuspended. The cells were centrifuged for 10 min at 2000g at 4 °C. The resuspension and centrifugation steps were repeated. The pellet was resuspended in 10 ml of 10% glycerol and cell suspension was transferred to a 50 ml sterile centrifuge tube. The cells were centrifuged for 10 min at 2000g and the supernatant discarded. Resuspension in glycerol and spinning of the cells were repeated. Finally, the pellet was
resuspended in 200 μl of 10% glycerol by gently pipetting. 50 μl aliquots of the cells were frozen in liquid nitrogen and stored at -80 °C.

Transformation (heat shock method). The competent cells were thawed and 7.5 μl of ligation mix (or 2 μl of purified plasmid) were combined with 66 μl of competent cells. The mixture was incubated on ice for 30 min after which it was put in a water bath at 37 °C for 90 seconds. After the heat shock, the cells were put on ice immediately for at least 2 min. The transformed cells were left to recover at 37 °C for 45 min before spreading on plates supplemented with the appropriate antibiotic.

Transformation (electroporation method). Competent cells were retrieved from -80 °C and placed in a chilled electroporation cuvette (1 mm gap). 1 μl of plasmid was added to the cells and the mixture was pulsed for 5 ms at 1250 V. LB was added to the cells which were allowed to recover for 1 hour at 37 °C and subsequently grown overnight on an LB plate supplemented with appropriate antibiotic.

2.3 Protein manipulation

2.3.1 Cytoplasmic expression and purification of wild-type and mutant AfNiR

Five ml of 2 x YT media supplemented with kanamycin was inoculated with a freshly transformed BL21 (λDE3) cells and grown overnight at 30 °C. The following day the 5 ml culture was used to inoculate 1L of 2 x YT media which was grown to OD_600=0.9 at 30 °C. The cells were then transferred to 25 °C and induced with IPTG to a final concentration of 0.5 mM. The cultures were grown at 25 °C for 12 hours. Cells were harvested and resuspended in a buffer consisting of 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8. Prior to lysis, the cells were supplemented with 10 μM CuSO_4. Cells were lysed at 4 °C using an Avestin EmulsiFlex-C5 homogenizer with pressure of 5000 psi. The insoluble debris was removed by ultra centrifugation with a fixed angle rotor 70 Ti (Beckman) for 45 min at 37000 g. The
supernatant was loaded onto Ni Sepharose column equilibrated with the cell lysis buffer and the purification was run with an AKTA FPLC system (GE Healthcare). The column was washed with a buffer containing 20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0 and the protein was eluted with the same buffer supplemented with imidazole. The protein eluted at a concentration of about 350 mM imidazole. To remove the His\textsubscript{6} tag, the protein was dialyzed in 20 mM Tris-HCl, 100 mM NaCl at pH 8 and digested with thrombin. A Resource Q column was used to further purify the protein following digestion. The column was equilibrated with 20 mM Tris-HCl pH 8 and the protein was eluted using a NaCl gradient. The protein eluted at 150 mM NaCl concentration. This protocol yielded approximately 150 mg of purified protein / L of culture.

2.3.2 Cytoplasmic expression and purification of pseudoazurin

The gene for pseudoazurin from \textit{A. faecalis} S-6 was cloned in a pET24a vector (80). Protein expression was as follows. Freshly transformed BL21 (\lambda\textsubscript{DE3}) cells were grown overnight at 37°C on plates supplemented with kanamycin. The following day a 5 ml LB culture was started and grown overnight. The 5 ml culture was used to inoculate 1 L of 2 x YT media which was grown to OD\textsubscript{600} of 2 at 30°C. The temperature was lowered to 25°C at which point the cells were induced with 0.5 IPTG. Following harvesting by centrifugation, the cells were resuspended in a buffer (20 mM potassium phosphate pH 6, 10 \mu M CuSO\textsubscript{4}) and lysed with at 4°C using an Avestin EmulsiFlex-C5 homogenizer with pressure of 5000 psi. The cell lysate was loaded onto carboxymethyl cellulose (CM) column equilibrated with 20 mM potassium phosphate pH 6 and eluted with a gradient of NaCl in the same buffer. The final yield was >200 mg/L of culture.
2.3.3 Basic protein characterization

**SDS-PAGE.** Samples were heated at 95°C in 5x sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min. After denaturation, samples were resolved on 12-15% polyacrylamide gels electrophoresed in running buffer (Tris base 30 g, glycine 144 g, SDS 10 g in total of 2 L pH 8.3) at 40 mA for approximately 45 min. Protein bands were visualized following staining of the gel in Coomassie blue R solution followed by destaining in boiling water.

**Bradford standard assay.** Purified protein samples were diluted to an appropriate concentration, estimated by absorption at 280 nm, and quantified by the method of Bradford (81) using the Bio-Rad Protein Assay Dye Reagent Concentrate as described by the manufacturer. Highly purified samples of bovine serum albumin (BSA) purchased from Sigma-Aldrich were used as the standards in the Bradford assay.

**Mass spectrometry.** Mass spectrometry was used to determine the molecular weight of the recombinantly expressed proteins before and after digestion with thrombin. Samples of protein in 20 mM Tris buffer pH 7.0 were prepared to a concentration of 10-35 mg/ml, prior to injection onto a reverse phase column interfaced to an electrospray mass spectrometer. Dr. Shouming He, in the laboratory of Dr. Stephen Withers, kindly carried out all mass spectrometry measurements.

**Copper content determination.** Copper content of native and variant AfNiR was measured by the 2,2'-bichinonicinic acid (BCA) method after complete reduction of the sample (82). Briefly, in this assay 2 molecules of bichinonicinic acid bind to Cu(I) which is either reduced by the protein or by the added ascorbic acid. BCA is highly sensitive and specific for Cu(I) and rapidly forms an intense purple complex. The complex has peak absorbances at 562 and 354 nm. Interference by other metal ions, pH and detergents is minimal.
The preparation of standard solutions and samples was achieved as follows. Standard solutions containing 5, 10, 15, 20, 25 μM copper concentrations were prepared from a purchased standard Cu(II) sulphate solution (0.16019 M). 375 μl of each standard were mixed with 250 μl of 1.83 M trichloroacetic acid. The solutions were vortexed and centrifuged at 13000 rpm for 5 min. The top 500 μl of each standard were combined with 100 μl of 2 mM L-dihydroascorbic acid and mixed thoroughly. 400 μl of 0.17 mM bicinechoninic acid, 0.9 M NaOH, 0.66 M HEPES buffer pH 7.5 were added to each standard. The absorbance was measured at 562 nm. Enzyme solutions with an estimated copper content of ~ 25 μM were prepared similarly to the standards and measured at A<sub>562</sub> to determine copper content.

2.4 AfNiR kinetic parameter determination

2.4.1 Enzymatic assays

The AfNiR-catalyzed reduction of nitrite was measured by following the oxidation of pseudoazurin at 593 nm using a Cary Varian 5000 spectrophotometer equipped with a Pelletier cooling device operated at 25°C. The standard assay was performed in a final volume of 150 μL containing 0.23 nM AfNiR, 40 – 800 μM nitrite, saturating amounts of reduced pseudoazurin (>315 μM), and 50 mM MES, pH 6.5. For reactions performed at pH 4, the assay contained 12 nM AfNiR, 2.5 – 100 μM nitrite, and 10 mM benzoate. Dilutions of AfNiR were supplemented with 0.1 mg/ml BSA to stabilize the trimeric form of the enzyme. Reduced pseudoazurin was prepared in a dry box maintained at < 2 ppm of O<sub>2</sub>. Pseudoazurin was reduced using ascorbate. Excess ascorbate was removed by passing the sample over a small column of Sephadex G-25 (GE Healthcare) equilibrated with the reaction buffer. The concentration of pseudoazurin was determined by completely oxidizing the protein with K<sub>3</sub>[Fe(CN)<sub>6</sub>] and using an extinction coefficient of 2,900 M<sup>-1</sup>cm<sup>-1</sup> at 593 nm (38). Inhibition parameters were evaluated using the standard assay conditions. Inhibitor concentrations were varied from 0.5 mM to 10 mM for azide
and from 10 to 500 mM for each of acetate, nitrate and formate. Steady-state kinetic parameters were calculated using initial rates determined over 5 s. Initial rates were corrected for the non-enzymatic rate of oxidation of pseudoazurin by nitrite, which never exceeded 10% of the enzymatic rate. Steady-state rate equations were fit to data using the least-squares and dynamic-weighting options of LEONORA (83).

2.4.2 Analysis of kinetic data

All reported kinetic constants were calculated from initial rates. Steady-state rate equations were fit to data sets using the least-squares and dynamic-weighting options of the program LEONORA (83). To determine the fit of the data to either of competitive, uncompetitive or mixed inhibition, the following equations were used:

\[ \nu = \frac{V_{\text{max}}[S]}{K_m(1 + \frac{[I]}{K_c}) + [S]} \]

Competitive inhibition

\[ \nu = \frac{V_{\text{max}}[S]}{K_m + [S](1 + \frac{[I]}{K_{ic}})} \]

Uncompetitive inhibition

\[ \nu = \frac{V_{\text{max}}[S]}{K_m(1 + \frac{[I]}{K_c}) + [S](1 + \frac{[I]}{K_{ic}})} \]

Mixed inhibition

where \( \nu \) is the initial rate of the reaction at a given substrate [S] and inhibitor [I] concentrations, \( V_{\text{max}} \) and \( K_m \) were estimated with LEONORA, and \( K_c, K_{ic} \) are the competitive and uncompetitive inhibitor constants, respectively.
2.5 Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded at X-band frequencies on a Bruker ESP 300E electron paramagnetic resonance spectrometer equipped with a Hewlett Packard Model 532B frequency counter in the laboratory of Dr. Mauk. Liquid nitrogen spectra were collected at 77 K. Wild type and variant NiR samples were concentrated to approximately 1 mM and exchanged into either Buffer A (50 mM MES, 50 mM acetate pH 4), Buffer B (50 mM HEPES, 50 mM acetate, pH 7) or Buffer C (10 mM benzoate, pH 4). Parameters used for data collection were: modulation frequency 9.450 GHz, microwave power 0.5-10 mW and modulation amplitude 1 - 10 G.

2.5.1 Anaerobic, NO-exposed EPR sample preparation

Protein solutions contained ~60 mg/ml A1NiR, 22 mg/ml D98N or 80 mg/ml H145A variants in 20 mM Tris buffer, pH 7.0. Each protein sample was dialyzed overnight in a glove box against Buffer A, B or C. A 300 µl aliquot was used for the collection of a reference spectrum without further treatment. The reduced state of each solution was achieved by dialysis in the glove box for 2 hours against Buffer A, B or C supplemented with 20 mM ascorbate. To ensure complete reduction of each protein sample, a 300 µl aliquot was collected for a control spectrum (data not shown). Excess reductant was removed by two successive dialysis steps in Buffer A, B or C for 2 hours each performed anaerobically. Subsequently, the protein was dialyzed for 20 min in NO-saturated Buffer A, B or C. Samples were transferred into precision EPR tubes (Wilmad, NJ), sealed with an airtight rubber cap, removed from the glove box and submerged immediately in liquid nitrogen.

2.5.2 Spin quantification

Spin quantification was used to determine the amount of oxidized copper present after reduced and oxidized H145A variant protein samples were exposed to NO. The standard solution used for these experiments was a 1.00 mM CuCl₂ solution. Equal volumes of protein sample and
standard were used in either case and the readings were performed in the same high precision EPR tube (Wilmad, NJ). The spectra were integrated and the area underneath the curve calculated. Based on the assayed copper content of each sample as described in Section 2.3.3 and the amount measured by EPR, the amount of oxidized metal in each sample was estimated.

2.6 Structure determination by x-ray crystallography

2.6.1 Crystal growth and substrate soaking of mutant and wild-type AfNiR

Crystals of the native and mutant forms of AfNiR were grown at room temperature by the hanging drop vapor diffusion method with equal volumes (1 μl) of reservoir and protein solution. The reservoir solution for AfNiR consisted of 0.1 M sodium acetate pH 4 and 6-11% polyethylene glycol 4000. The protein stock used in crystallization was 35-50 mg/ml buffered in 20 mM Tris pH 7.0. These conditions resulted in crystals that grew typically to dimensions 0.1 x 0.1 x 0.2 mm within three days. Crystals of the H145A variant were grown similarly to wild type with a reservoir consisting of 6-11% polyethylene glycol 6000, 10 mM sodium acetate pH 4.5, 100 mM ammonium sulfate. The protein stock used in crystallization was 40 mg/ml buffered in 20 mM Tris pH 7.0. Crystals grew as needles with dimensions 0.1 x 0.1 x 0.5 mm within a week. Crystals of the D98N were grown in 10 mM sodium acetate pH 4, 75 mM acetamide, 7-12% polyethylene glycol 4000 and the protein stock solution used was 25 mg/ml.

Nitrite-soaked oxidized AfNiR crystals were obtained by placing the crystals in reservoir solution supplemented with 5 mM sodium nitrite for 30 min at room temperature. The crystals were transferred to a fresh mother liquor solution supplemented to 30% glycerol as cryoprotectant prior to freezing in liquid nitrogen.

2.6.2 Exposing crystals to NO-saturated solutions

All work involving the use of NO was performed anaerobically. To achieve this,
solutions and crystals were degassed with argon and placed in an Mbraun Labmaster anaerobic glove box maintained at 2 ppm of O₂ or less. The NO gas was passed through a NaOH column to eliminate higher nitrogen oxide species. The NO-saturated solutions were purged initially with argon and then with NO at 1 atm for 30 min. Oxidized crystals of AfNiR and H145A variant were placed in NO-saturated solution for 20 min and were subsequently transferred to fresh reservoir solution supplemented to 30% glycerol as a cryo-protectant. The crystals were submerged in liquid nitrogen anaerobically in the glove box.

Preparation of the reduced, NO-exposed AfNiR, H145A and D98N crystals were obtained as described above with the following modifications. Complete reduction of the crystals was achieved by soaking in reservoir solution supplemented with 20 mM ascorbate for 30 min in an anaerobic environment. Reduced crystals were transferred with a loop to a fresh reservoir solution to remove excess reductant. Subsequently, the reduced crystals were transferred to an NO-saturated solution for 20 min. 30% glycerol was used as a cryo-protectant before the crystals were immersed in liquid nitrogen in the glove box.

2.6.3 Inhibitor-bound structures of AfNiR

The AfNiR crystals were soaked aerobically for 30 min in reservoir solution supplemented with 20 mM azide, formate or nitrate. The concentration of acetate used in the acetate-bound AfNiR structure was 100 mM, which is a component of the reservoir solution, and the manipulation of these crystals was performed anaerobically. Crystals of the nitrous oxide-bound NiR were also manipulated anaerobically as described previously (84). Both reduced and oxidized AfNiR crystals were soaked in nitrous oxide saturated solutions. Notably, after refinement only the oxidized crystals showed density for an nitrous oxide molecule bound to the type 2 copper of AfNiR. The nitrous oxide solubility in aqueous solutions is 25 mM at 1 atm,
25°C (85). Prior to immersing in liquid nitrogen, all crystals were transferred to reservoir solution supplemented to 30% (v/v) glycerol and diffraction data were collected at 100 K.

2.6.4 Data collection

X-ray data were collected either at a home source or at a synchrotron. The home source data were collected on a Mar345 image plate system (MarResearch) with CuKα radiation generated by a Rigaku RU 300 rotating anode operating at 100 mA and 50 kV and focused with Osmic confocal Max-Flux optical mirrors. The diffraction data frames were exposed for 10-15 min with a crystal oscillation angle of 0.5-0.75°. The synchrotron data were collected at SSRL (Stanford Synchrotron Radiation Laboratory) on beam lines 7-1, 11-3, 1-5, 9-1 equipped with a Mar345, an ADSC Quantum-315 or an ADSC Quantum-4 detector. Remote data collection was possible with the use of the control software Blu-Ice 4. Typically, the data frames were exposed for 1-5 s with a crystal oscillation angle of 0.5°. Diffraction data were processed with the programs DENZO (auto-indexing refinement and integration) and scaled with SCALEPACK (data merging and scaling) or HKL2000 (86). The HKL2000 software package brings together DENZO, Xdisplay, and SCALEPACK using a single graphical user interface. For each data set, an initial frame was collected and processed. Using the indexed data frame, the program STRATEGY (87) was used to calculate spatial positions of the axes in the crystal. Data collection was started on a crystal axis (whenever possible) to maximize data collection efficiency.

2.6.5 Structure solution and refinement

The wild-type and mutant forms of AfNiR crystallized in space group P2₁2₁2₁ with the functional trimer in the asymmetric unit. For the oxidized, nitrite-soaked wild-type AfNiR, the highest resolution structure (PDB code 1J9Q) was used as a starting model for molecular replacement (78). Molecular replacement was performed with the program Molrep following the
removal of the solvent atoms and the side chain of residue 98 (84). The nitrite-bound AfNiR structure to 1.4 Å resolution (PDB code 1SJM) was used as the starting model for refinement of the reduced, NO-exposed AfNiR structure. For all subsequent structures, the highest resolution structure to 1.3 Å (PDB code 1SNR) was used as the starting model for refinement. For the NO-soaked and inhibitor-bound AfNiR structures, all the water molecules, exogenous ligands and the side chain of Asp98 were removed from the starting model. In addition, the side chain of either His145 or Asp98 was removed from the initial model prior to refinement of the H145A and D98N complex structures, respectively. The final structure of each monomer begins at Ala4 and ends at Glu339 or Thr340. Five percent of the data were set aside for the calculation of the free $R$-factor (88). Standard maximum likelihood positional and $B$-factor refinement was carried out using the program REFMAC (89). Solvent was added with the ARP/wARP program from the CCP4 package (90), resulting in an $R_{\text{work}}$ below 19% and an $R_{\text{free}}$ of less than 22% for all structures. During refinement, the interactions between copper and the polypeptide chain were not restrained. Manual intervention was accomplished using the visualization program O (91). Over 95% of the residues in each structure occupy the most favourable position with the remaining residues in the allowed regions in the Ramachandran plot as described by PROCHECK (92).
In protein crystallography, coordinate error is due to the uncertainty of atomic positions due to data not being measured precisely and due to the atomic model being imperfect (93). The refinement program REFMAC provides several methods for coordinate error calculation such as sigmaA ((94)), Cruickshank’s Diffraction-component precision index (DPI) (93) and directly from the maximum likelihood (ML) refinement. The latter two methods are briefly discussed in this section.

The Cruickshank’s DPI is calculated by the formula:

\[
DPI = \sqrt{\frac{N_{\text{atom}}}{(N_{\text{refl}} - N_{\text{param}}) R_{\text{factor}} \times D_{\text{max}} \times \text{compl}^{-1/3}}},
\]

where \( N_{\text{atom}} \) is the number of the atoms included in the refinement, \( N_{\text{refl}} \) is the number of unique reflections included in the refinement, \( R_{\text{factor}} \) is the overall R-factor, \( D_{\text{max}} \) is the maximum resolution of reflections included in the refinement and \( \text{compl} \) is the completeness of the observed data.

Comparing the coordinate errors calculated by the two methods indicates that the value using Cruickshank’s DPI is consistently larger than the that derived from the ML refinement. For example, for the reduced AfNiR exposed to NO structure (resolution to 1.3 Å), the DPI method gives an error of 0.05 Å, while the ML method results an error of 0.02 Å. The error estimates derived from the maximum likelihood refinement are used in this thesis since these coordinate errors are included in the Protein Databank file and thus are more readily available for comparison among structures.
3.1 Introduction

Nitric oxide (NO) is one of the smallest and simplest of biologically active molecules. In mammals, NO is produced from arginine by isoforms of nitric oxide synthase, and it functions in signal transduction and as a cytoprotective or cytotoxic agent (95). In bacteria, NO is produced by nitrite reductase, and it is an obligatory intermediate in the bacterial dissimilatory denitrification pathway. The proposed mechanism for NiR has been controversial. A paradox arises from the apparent conflict between the implicit N-coordinated binding of a copper-nitrosyl complex observed in a biomimetic compound of NiR (Figure 1-11) (68) and the observed O-coordinated NO$_2^-$ binding in the enzyme (47, 71).

To determine how O-coordinated NO$_2^-$ gives rise to a copper-nitrosyl intermediate, the NO$_2^-$ and NO bound forms of AfNiR were produced and the crystal structure of each determined at high resolution. The structures provide insight into the catalytic mechanism and may resolve a mechanistic paradox associated with NiR.

3.2 Results

3.2.1 X-ray data collection and structure refinement

X-ray data were collected to 1.4 Å resolution for the AfNiR-NO$_2^-$ structure and to 1.3 Å resolution for the AfNiR-NO structure on beam line 7-1 at SSRL. The highest resolution structure of AfNiR available to date (PDB code 1J9Q) was used as the starting model for the AfNiR- NO$_2^-$ structure after deleting the side chain of residue 98 (78). The final model of the AfNiR- NO$_2^-$ structure consists of three peptide chains (A, 5-339; B, 4-340; C, 4-339), 3 nitrite molecules, 8 acetate ions, 1 Tris, and 1107 water molecules. The AfNiR- NO$_2^-$ structure with removal of nitrite was used as the starting model for the AfNiR-NO structure. The final model of
the AfNiR-NO structure contained the three monomers (A, 4-339; B, 4-340; C, 4-339), 3 nitric oxide molecules, 5 acetate ions, 1 Tris, and 1144 water molecules. Near the end of refinement hydrogen atoms were added to the structure using the program REFMAC (89) and non-hydrogen atoms were refined with anisotropic B-factors. The final $R_{\text{work}}$ and $R_{\text{free}}$ are less than 12 and 14%, respectively, in both structures. Statistics of the data processing and structure refinement are presented in Table 3-1.

All metal ligand interactions were unrestrained including those with NO and NO$_2^-$. The NO and NO$_2^-$ were not sufficiently ordered in the crystals to remove covalent bond restraints. The definition for refinement purposes of an ideal N-O bond length in the NO ligand is limited by the absence of a side-on copper-nitrosyl model complex. The bond lengths of diatomic molecules coordinated side-on to transition metals are observed frequently to have elongated bond lengths in comparison to end-on complexes (96). The recently characterized side-on copper-O$_2$ complex was used as a model in which an O-O bond length of 1.44 Å is observed as a model for copper-NO refinement (74). In contrast, the bond length of an end-on copper-NO complexes is 1.15 Å (48). In the copper-NO AfNiR crystal structure, the N-O bond of nitric oxide refined to 1.45-1.46 Å (for trimer). The anisotropic refinement indicates that the N and O atoms show greater motion or disorder in the plane of the N-O bond. The disorder in the N-O plane may explain the lengthening in the N-O bond.
<table>
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<tr>
<th></th>
<th>Oxidized AfNiR- NO$_2^-$</th>
<th>Reduced AfNiR-NO</th>
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<td>0.125/0.141</td>
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<td>0.009</td>
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<tr>
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<td>PDB code</td>
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<td>1SNR</td>
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Note: Numbers in parentheses correspond to those in the highest resolution shell.

$^1$ Coordinate error calculated from maximum likelihood refinement.
3.2.2 Crystal structure of the substrate-bound AfNiR

The structure to 1.8 Å resolution of substrate-soaked AfNiR crystals revealed an asymmetric O-coordination of nitrite to the copper (47). In the crystal structure of this complex extended to 1.4 Å resolution (Figure 3-1), the nitrite is coordinated to the copper primarily by the \( \text{O}_c \) atom (2.04 – 2.08 Å), whereas, the \( \text{O}_{nc} \) atom interacts more weakly with the metal center (2.29 – 2.38 Å) and thus is termed non-coordinating. The N atom is also positioned 2.31 – 2.36 Å from the type 2 copper so that the plane defined by the metal and the two O atoms and the plane defined by the N and the two O atoms form a 75° angle (Figure 3-2). Previous structures of NiR-nitrite complexes also show a bent O-coordination (46, 71). However, the extension to 1.4 Å resolution reveals an almost face-on interaction of the nitrite with the copper. A complete list of copper-ligand distances is given in Table 3-2.

A second interaction of nitrite with AfNiR occurs with the side-chain carboxylate of Asp98. A hydrogen bond between the \( \text{O}^{\delta1} \) atom of Asp98 and the protonated \( \text{O}_c \) atom of the nitrite (2.53 – 2.66 Å) stabilizes the orientation of the substrate. The binding mode of the nitrite also places the N atom close to Asp98 \( \text{O}^{\delta1} \) (2.51 – 2.63 Å) which may facilitate NO formation. Asp98 forms a water-bridged hydrogen bond to His255, which is another essential residue for AfNiR activity (44, 56). Neither His255 nor the bridging water interacts directly with the bound nitrite.

3.2.3 Crystal structure of the product-bound AfNiR

Upon reduction with ascorbate, the green AfNiR crystals turned colorless, but after removal of excess reductant and exposure to an NO-saturated solution, they became green within 2 min, presumably indicating formation of a copper-nitrosyl complex. A difference electron density map \((F_o - F_c)\) reveals elongated density at the apical position of the type 2 copper site.
approximately in the same location as nitrite. The long axis of the density is tangential to the copper atom and is modeled as NO bound side-on (Figure 3-1). After refinement at full occupancy, the average crystallographic $B$-factor of the three NO molecules in the asymmetric unit is $29 \text{ \AA}^2$, similar to that observed for the bound nitrite ($27 \text{ \AA}^2$). The side-on orientation of the NO is confirmed by an $F_{\text{nitric oxide}} - F_{\text{nitric oxide}}$ electron density map which reveals a $5 \sigma$ ($0.55 \text{ e}^2/\text{\AA}^3$) positive peak at the position of the second oxygen of the nitrite (Figure 3-3). The N ($1.93 - 1.97 \text{ \AA}$) and O ($1.92 - 2.06 \text{ \AA}$) atoms of NO are equidistant from the copper and the average Cu – N – O angle is $71^\circ$. The proximity of NO to the carboxylate of Asp98 (Oδ1 – N distance of $2.5 - 2.65 \text{ \AA}$) (Table 3-2) indicates a likely hydrogen bond and suggests an orientation for the NO molecule. The copper-nitrosyl could be formally described as Cu(I)-NO• or Cu(II)-NO**, either of which is consistent with the crystal structure. Protonation of the latter form to give Cu(II)-HNO allows the NO to act as a proton donor to Asp98. His255 and Ile257 are important for nitrite binding and may also influence the mode of NO binding.
Figure 3-1 Crystal structures of the substrate- and product-bound AfNiR. Stereo-views of the type 2 copper sites of subunit A of AfNiR with bound nitrite (A) and nitric oxide (B). In each panel, the electron density represented in grey is a $2F_o-F_c$ map contoured at 1.2 $\sigma$. Omit
difference maps of the nitrite and nitric oxide ligands are coloured in green and contoured at 4 $\sigma$
and 5 $\sigma$, respectively. Carbon atoms (orange), oxygen atoms (red), nitrogen atoms (blue), type 2
copper (brown) and catalytically important waters (red) are coloured as indicated. (C) Overview
of the essential features of the active sites of AfNiR bound with nitrite (left) and with nitric oxide
(right). Metal-ligand bonds (solid) as well as hydrogen bonds and other electrostatic interactions
(dashed) are shown as grey lines of the type indicated. Figure was generated with Molscript (48)
and Raster3D (49).
Table 3-2 Ligand bond distances in substrate- and product-bound AfNiR (Å)

<table>
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<th>Ligand</th>
<th>AfNiR-NO$_2^-$</th>
<th>AfNiR-NO</th>
</tr>
</thead>
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<td><strong>Type 1 Copper Site</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95Nδ1</td>
<td>2.03-2.06</td>
<td>2.03-2.05</td>
</tr>
<tr>
<td>136Sy</td>
<td>2.05-2.08</td>
<td>2.05-2.10</td>
</tr>
<tr>
<td>145Nδ1</td>
<td>2.04-2.06</td>
<td>2.05-2.07</td>
</tr>
<tr>
<td>150Sδ</td>
<td>2.27-2.36</td>
<td>1.92-2.06</td>
</tr>
<tr>
<td>503O (O$_{nc}$)</td>
<td>2.03-2.09</td>
<td>--</td>
</tr>
<tr>
<td>503O (O$_c$)</td>
<td>2.29-2.37</td>
<td>1.93-1.97</td>
</tr>
</tbody>
</table>

Note: 503 ligand number corresponds to either nitrite or nitric oxide.

1 The range of distances observed per trimer.
Figure 3-2 Face-on interaction of nitrite with the copper atom. Schematic representation of the angular orientation of nitrite bound to the type 2 copper site of AfNiR. The gray rectangle represents the plane defined by the two oxygen atoms and the copper atom. The nitrogen atom is out of the plane and forms a 75° angle with the plane defined by the nitrogen and the two oxygen atoms. Resonance structures of nitrite are omitted for clarity.
Figure 3-3 $F_{\text{nitrite}} - F_{\text{nitric oxide}}$ electron difference map. Stereo image of an $F_{\text{nitrite}} - F_{\text{nitric oxide}}$ electron density map contoured at 3.5 $\sigma$ shows a clear positive difference peak for the second nitrite oxygen. Atoms are colored as described in Figure 3-1.
3.2.4 EPR spectroscopy sample preparation and data collection

Preparation of EPR samples of the reduced AfNiR-NO complex corresponding to Figure 3-4. Upon reduction in 20 mM ascorbate, the protein became colorless and remained such during the removal of excess reductant. Dialysis was continued against Buffer B (50 mM HEPES, 50 mM acetate, pH 7) purged with NO. During dialysis, the color of the protein solution changed from colorless to green within 2 min and after 20 min, began aggregating at the membrane surface, at which point the sample was transferred into an EPR tube. The tube was sealed with an airtight rubber cap, removed from the glove box and immediately submerged in liquid nitrogen.

The EPR spectrum of the oxidized AfNiR exhibits hyperfine splitting for both type 1 and type 2 copper centers (79, 97). The type 1 site has an $A_z$ of 73 G and $g_z$ value ~ 2.190, while the type 2 values for $A_z$ and $g_z$ are 128 G and 2.337, respectively (Figure 3-4). Anaerobic incubation of the ascorbate reduced AfNiR with NO at pH 7.0 results in a green solution. The spectrum of the reduced, NO-bound NiR shows features typical of a type 2 copper site an $A_z$ value of 125 and a $g_z$ value of 2.301 (Figure 3-4). No strong features corresponding to an oxidized type 1 copper center were observed suggesting that the type 1 copper is reduced. The formation of a copper-nitrosyl is further supported by the presence of a nine-line superhyperfine splitting pattern at $g_y$ ~ 2.058, which is attributed to the coordination of four-nitrogen donors (nuclear spin I=1) to the copper (Figure 3-4). From the crystal structure, three of the nitrogen atoms belong to the histidine ligands and the fourth is the NO nitrogen. Oxygen ligands (I=0) do not contribute to the superhyperfine splitting.
Figure 3-4 EPR spectra of oxidized and reduced, NO-exposed AfNiR. (A) Expansion of the oxidized type 2 signal. (B) Oxidized wild-type AfNiR. (C) Reduced AfNiR reacted with NO. (D) Expansion of the superhyperfine structure of reduced AfNiR reacted with NO (upper scale).
3.3 Discussion

3.3.1 Crystallographic characterization of copper-nitrosyl complexes

The copper-nitrosyl complex of AfNiR reported here is the first such complex in a biological system to be characterized with side-on coordination of a diatomic molecule. The only other enzyme copper-nitrosyl characterized by crystallography is that of amine oxidase (73). Soaking crystals of reduced amine oxidase with NO to mimic dioxygen resulted in a bent copper-nitrosyl close to the TPQ cofactor. The Cu-N-O angle is 117°, approaching a side-on interaction and the Cu-NO distance is 2.5 Å. A second monomeric copper-nitrosyl crystal structure is that of a NiR biomimetic model compound: tris (3 t-butylpyrazolyl) hydroborate Cu-NO (67). In this model compound, the complex is formally described as Cu(I)-NO•, with the NO N-coordinated to the Cu and bent 163°.

Side-on NO and O₂ coordination to Fe is better known. For example, a ferric heme-NO complex is observed in nitrophorin 4 (98) in which the Fe-N and the Fe-O distances are 2.0 and 2.6 Å, respectively, resulting in an Fe-N-O angle of 110°. This binding mode is attributed to steric hindrance and to the hydrophobic nature of the distal pocket. Side-on binding of dioxygen to iron was reported recently for naphthalene dioxygenase (99), where the distances between the oxygen atoms and the iron are 2.2 and 2.3 Å. In a non-biological system, Coppens et al have shown that when photoexcited, NO can bind side-on to Fe in nitroprusside with Fe-N and Fe-O distances of 1.89 and 2.07 Å, respectively (100). The current structure of a copper-nitrosyl broadens the precedent for side-on coordination to transition metals in biology.

3.3.2 Spectroscopic characterization of copper-nitrosyl complexes

Electron paramagnetic resonance spectra were collected to interrogate the copper sites of oxidized AfNiR and the product obtained by treating the reduced protein with NO. The absence of strong features corresponding to a type 1 copper center from the EPR spectrum of the reduced,
product-bound AfNiR indicates that this copper center remains reduced after treatment with NO.

The observation of signals typical of a type 2 copper site suggests electron transfer to the NO radical to yield a relatively stable copper species which were formally described as Cu(II)-NO$^\cdot$.

The nature of reaction of nitric oxide with other multi-copper oxidases varies from case to case. The EPR spectrum of fully oxidized ceruloplasmin following reaction with NO is that of a type 2 Cu(II) center with a seven-line superhyperfine pattern that is attributable to coordination by three nitrogen donors \(j01\). On the other hand, the reaction of NO with fully reduced ceruloplasmin or tree laccase results in NO coordination to the reduced enzyme followed by oxidization of a metal center \(j02, j03\). In the presence of excess reductant and NO, tree laccase turns over to produce nitrous oxide via a two-electron reduction. In contrast, NiR incubated in the presence of NO and excess reductant is not catalytically productive \(j37\). NO is a potent inhibitor of nitrite reductase and reaches a steady state concentration of about 80 nM. During the NiR-catalyzed reduction of nitrite to NO, N$_2$O is produced if NO is not removed from the reaction vessel. Under these conditions, a copper-nitrosyl intermediate is proposed to accumulate and to react with NO$_2^\cdot$ or with NO derived from NO$_2^-$ to yield N$_2$O. Under our experimental conditions, nitrite and excess reductant are not available for N$_2$O formation.

**3.3.3 Revised catalytic mechanism for NiRs**

Crystallographic, spectroscopic, and kinetic studies show that NO$_2^-$ binds preferentially to the oxidized type 2 site of NiR and that the mode of binding is O-coordinated \(j46, j47, j69, j70\). The detection of a copper-nitrosyl intermediate that is presumably N-coordinated would require an unlikely NO rebound mechanism or flipping of an intermediate from O to N coordination to the Cu, because an O-coordinated nitrosyl group is without precedent \(j9, j37, j65\). Although some biomimetic models of the NiR type 2 site bind O-coordinated NO$_2^-$, NO-evolving models bind NO$_2^-$ through the N atom and proceed through a copper-nitrosyl intermediate \(j67, j68\).
The NO and NO$_2^-$ bound AfNiR crystal structures suggest a revised mechanism (Figure 3-5). NO$_2^-$ binds O-coordinated to an oxidized copper site (type 2). NO$_2^-$ is shown formally to be protonated; however, the proton could be associated with Asp98. Electron transfer from the type 1 Cu site reduces the active site Cu to trigger a rearrangement of NO$_2^-$ to release water and form a Cu(I)-NO$^+$ intermediate. The proximity of NO$_2^-$ N to the Cu in the substrate-soaked structure may facilitate this rearrangement step. While the oxidation states and thus the detailed structural attributes of the Cu(II)-NO$^-$ and Cu(I)-NO$^+$ may differ, an analogy between them may nonetheless be drawn (Figure 3-6), leading to the proposal that side-on bonding occurs in the Cu(I)-NO$^+$ intermediate. This rearrangement reaction obviates the need for a rebound mechanism in which released NO displaces a water ligand to give an N-coordinated copper-nitrosyl (37, 47). In the crystals structure, a hydrogen bond between the presumed HNO and the O$_{61}$ of Asp98 would stabilize this interaction. Analogously, the proposed Cu(I)-NO$^+$ intermediate may be stabilized by a salt bridge formation with the negative charge on Asp98. Furthermore, this copper-nitrosyl intermediate may react with an additional NO$_2^-$ molecule to produce nitrous oxide, as originally proposed by Jackson et al. (37). Finally, NO is displaced from the copper site by water to return the enzyme to the resting state Cu(II)-OH$_2$. 
Figure 3-5 Revised catalytic mechanism for NiRs. Major steps of the mechanism are shown as 1, 2, 3 and 4. Step 1 represents the resting state of the enzyme where a water molecule is bound to the type 2 copper. Step 2 shows displacement of the water by nitrite, which coordinates to the metal via one oxygen atom ($O_c$). Electron transfer (ET) from the type 1 site triggers rearrangement (step 3) and the formation of a copper-nitrosyl intermediate (step 4).
3.3.4 Relevance of copper-nitrosyl complex in AfNiR to other Cu proteins

Interaction of nitric oxide with copper has been proposed in models of some neurodegenerative diseases. *In vitro* studies show that the formation of a copper-nitrosyl in variant forms of superoxide dismutase (SOD) yields highly reactive radicals that are implicated in some types of amyotrophic lateral sclerosis (ALS) (104). Interestingly, the type 2 copper sites of NiR and SOD share structural and functional features (43, 70). In the reduced state, the SOD copper is coordinated by three histidyl residues in an approximately tetrahedral arrangement as is the case for the type 2 copper of NiR. A fourth histidine of SOD, which links the Cu and Zn sites, superimposes with His255 of NiR. Furthermore, NiR from *Alcaligenes xylosoxidans* is reported to have SOD activity (70). The unusual interaction of NO with NiR may serve as a model for the binding of NO and superoxide to the type 2 copper site of SOD. More recently, copper binding to prion proteins was linked to the production of nitric oxide that autocatalyzes

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**Figure 3-6** Hydrogen bond network in the proposed catalytic intermediate of NiR (left) and the crystal structure of the reduced NiR exposed to NO (right).
the removal of glypican 1-heparin sulphate side chains, a process associated with the formation of amyloid deposits (105).
Chapter Four: Copper-Nitrosyls in Wild-Type and Mutant Forms of Nitrite Reductase

4.1 Introduction

AfNiR readily catalyzes the oxidation of NO to nitrite such that exposure of the oxidized enzyme to NO in the presence of an electron acceptor yields nitrite at a rate of 64 s$^{-1}$ at pH 7. In addition, $K_{eq}$ is dependent on pH and decreases with increasing pH such that at pH 8, the formation of nitrite and reduced pseudoazurin is favored by a factor of $10^3$ (38). The side-chain carboxylate of Asp98 forms hydrogen bonds to both product and substrate bound to the type 2 copper (47, 84). Mutating Asp98 to an asparagine (D98N variant) results in the loss of the hydrogen bond between Asp98 and $O_c$ of nitrite. This interaction is suggested to explain the 100-fold decrease in specific activity of the D98N variant (78). A mutation of the type 1 copper ligand histidine to an alanine (H145A) results in an increase of the reduction potential of the site such that the copper remains in the reduced state (79). In this inactive mutant, internal electron transfer from the reduced type 1 to the oxidized type 2 site does not occur irrespective of whether potential exogenous type 1 ligands are present (79).

In this study, both the D98N and H145A variants were examined for copper-nitrosyl formation by the addition of exogenous NO. The D98N variant is used to determine the role of Asp98 in defining the binding mode of NO. The H145A variant was used to study the interactions between the type 2 copper of NiR and NO. Crystal structures were obtained of oxidized AfNiR exposed to NO, reduced and oxidized H145A variant exposed to NO, and of reduced D98N variant exposed to NO. For each x-ray structure, complementary EPR data were collected to ascertain the oxidation states of the copper sites and to gain further insight into the electronic structure of the copper-nitrosyls. The data are interpreted in terms of the proposed catalytic mechanism for NiR.
4.2 Results

The overall protein folds of the NO-treated AfNiR, and the NO-treated D98N and H145A variants were essentially identical when compared to the native AfNiR crystal structure (PDB entry 1AS7) as revealed by a r.m.s. deviation between the Cα atoms of less than 0.2 Å. For all the structural complexes presented here, the major structural differences other than the introduced mutations, are localized around the type 2 copper sites. Data collection and refinement statistics are presented in Table 4-1.
Table 4-1 Data collection and refinement statistics for wild-type and variant AfNiR exposed to NO.

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<tr>
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<th>Reduced D98N-NO</th>
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<td>2PPD</td>
<td>2PPE</td>
</tr>
</tbody>
</table>

Note: numbers in parenthesis correspond to the highest resolution shell.

¹ Coordinate error derived from maximum likelihood.
4.2.1 Crystal structures of oxidized AfNiR and H145A variant exposed to NO

In the oxidized, NO-exposed AfNiR crystal structure, a well-defined omit electron density map in the region of the type 2 copper site revealed density in the apical position that could best be modeled as a non-linear molecule of three atoms (Figure 4-1, A). This density was modeled as nitrite coordinating to the type 2 copper via the O\textsubscript{c} oxygen atom (average Cu-O\textsubscript{c} distance 2.06 Å, Table 4-2). A refined average $B$-factor of 32 Å\textsuperscript{2} indicates that the nitrite molecule is well ordered. The nitrite molecule is oriented almost face-on with respect to the copper ion, such that both the nitrogen (2.48 Å) and second oxygen (2.31 Å) atoms were in close proximity to the metal. A hydrogen bond is formed between the O\textsubscript{c} atom of nitrite and the O\textsubscript{81} atom of Asp98 (2.63 Å). Nitrite is observed coordinating to the type 2 copper in each of the three monomers in the asymmetric unit. In subunits B and C, additional electron density is observed for a fourth atom. These sites were refined with both nitrite and acetate molecules coordinated to the type 2 center at 50% occupancy each (Figure 4-2, B and C). In addition, the proximal Asp98 showed partial disorder in the B and C sites and was refined in two predominant conformations at 50% occupancy each. As in subunit A, when nitrite is bound to the copper, Asp98 forms a hydrogen bond to the substrate and is pointed towards the copper. However, when acetate is bound at the active site, the side chain of the Asp98 is displaced to point away from the metal.

The space freed by this alternate conformation of Asp98 is filled partially by a solvent molecule. This water molecule forms two hydrogen bonds: one to the bridging water (W1), which in the native structure connects Asp98 and His255 (Figure 1-9), and the other to the O\textsubscript{c} atom of acetate.

In the structure of oxidized, NO-exposed H145A variant (Figure 4-1, B and Figure 4-2, C), extra electron density observed in the apical position could accommodate at most two atoms. In subunits A and B, this density was modeled as nitric oxide coordinated side-on to the type 2 copper such that the nitrogen and the oxygen atoms are equidistant from the copper ion. Even
though it is not possible to distinguish crystallographically between the nitrogen and the oxygen atoms of the NO molecule, as assigned, the NO molecules in the three monomers of the asymmetric unit refine to an average $B$-factor of 31 Å$^2$ and average ligand bond lengths of 1.99 Å for Cu-N and 2.09 Å for Cu-O (Table 4-2). The assignment of the N and O atoms was led by the possibility of a hydrogen bond formation with Asp98. Previous studies suggest that Asp98 is likely deprotonated (57, 78) such that a hydrogen bond can form between the protonated N atom of NO and the O61 atom of Asp98 (2.63 Å). In the refinement of monomer C, a single atom is sufficient to accommodate the electron density observed at the apical position of the type 2 copper site (Figure 4-2, D). The density was modeled as a water molecule located 2.01 Å away from the Cu(II) ion and 3.51 Å from Oδ1 of Asp98. This water ligand superimposes with the water bound to the active site Cu(II) ion in the crystal structure of the enzyme in its resting state (43). The observation of a water molecule bound to subunit C is likely due to the difference in solvent accessibility in the crystal based on crystal packing.

4.2.2 Crystal structure of reduced, NO-exposed H145A and D98N variants

In the reduced, NO-exposed H145A structure, the NO molecule is refined at full occupancy in subunit B such that the N and O distances to the type 2 copper are 2.00 and 1.95 Å, respectively (Figure 4-1, C and Table 4-2). At the type 2 copper atom of subunit A, weak density connected two peaks (Figure 4-3, A). The peaks in subunit A were refined with two water molecules at 50% occupancy each. The water molecules are located ~2.01 and ~2.19 Å away from the copper ion and are separated by ~2.38 Å. In subunit C extra electron density at the type 2 copper can accommodate a single atom and was refined as a water molecule (Figure 4-3; B). This water molecule is 1.95 Å from the type 2 copper and 3.54 Å from Oδ1 of Asp98.

In the reduced D98N variant structure, the NO molecule bound at each type 2 copper site shows partial disorder. In subunits A and B side-on coordination to the metal is predominant.
(Table 4-2, Figure 4-1, D and Figure 4-3, C), with N-Cu and O-Cu average bond distances of 2.16 Å and 2.36 Å, respectively. NO is refined at full occupancy in these subunits with final B-factors of 29 Å². In subunit C, the electron density observed at the type 2 copper site is interpreted as NO bound to the metal in two predominant conformations, bent and side-on (Figure 4-3, D). Reduced D98N crystals were colorless but turned partially green upon NO exposure, suggesting partial reoxidation of the type 1 copper. Disorder is also observed in the conformation of the side chain of Asn98 such that the predominant conformation in all three subunits is with the side chain pointing away from the copper.
Figure 4-1 Crystal structures of wild-type and mutant forms of AfNiR exposed to NO. (A) oxidized, wild-type AfNiR with a nitrite molecule coordinating to the copper (subunit A); (B) oxidized H145A variant with NO bound to the copper (subunit A); (C) reduced H145A variant with a NO molecule bound side-on to the copper (subunit B); (D) reduced D98N variant exposed to NO (subunit A). $2F_o-F_c$ (grey) and $F_o-F_c$ (green) electron density maps of type 2 copper site are contoured at 1.0 and 5 σ, respectively. The carbon atoms are colored in orange, the nitrogen atoms are colored in blue and the oxygen atoms are in red. Type 2 copper is colored in brown and a catalytically important water molecule is colored in light blue. This figure was created with the programs Molscript (48) and Raster3D (49).
Figure 4-2 Oxidized, NO-exposed wild type AfNiR and oxidized, NO-exposed H145A variant. Panels (A) and (B) represent the active sites of subunits B and C of the wild type AfNiR exposed to NO. A difference electron density map (green) is computed after nitrite was modeled in at 100% occupancy. The map is contoured at 4 σ and is indicative of mixed species bound to the type 2 copper. The final structure is refined with 50% of the molecules having acetate bound to the metal and 50% having nitrite. Asp98 is disordered and is modeled in 2 predominant conformations. Panels (C) and (D) represent subunits B and C of the oxidized H145A-NO structure, respectively. Panel C shows the difference electron density map of nitric oxide bound side-on to the type 2 copper. Subunit C was refined with a water molecule bound to the metal.
Figure 4-3 Reduced H145A and D98N variants exposed to NO. (A) Subunit C of the reduced, NO-exposed H145A structure showing two distinct modes of binding of a water molecule. (B) Subunit B of the reduced, NO-exposed D98N structure shows disorder at the active site for both NO and the side chain of Asn98. (C) Subunit C of the reduced, NO-exposed D98N variant, which shows NO modeled in two predominant conformations – bent and side-on with Asn98 predominantly pointing outward.
Table 4-2 Nitrite and NO bond distances in wild-type and mutant forms of AfNiR exposed to NO (Å).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oxidized wild-type AfNiR bound to NO&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reduced H145A-NO&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Oxidized wild-type AfNiR, exposed to NO (NO&lt;sub&gt;2&lt;/sub&gt; bound)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reduced H145A-NO&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Reduced D98N-NO&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>n/a</td>
<td>1.99 (0.01)</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>503N-Cu&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>1.99 (0.02)</td>
<td>2.48 (0.05)</td>
<td>1.99 (0.04)</td>
<td>2.00 (0.03)</td>
</tr>
<tr>
<td>503O&lt;sub&gt;nc&lt;/sub&gt;-Cu&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.34 (0.05)</td>
<td>2.0 (0.1)</td>
<td>2.3 (0.1)</td>
<td>2.1 (0.1)</td>
<td>1.95 (0.06)</td>
</tr>
<tr>
<td>503O&lt;sub&gt;c&lt;/sub&gt;-Asp98 Oδ1&lt;sup&gt;e&lt;/sup&gt; (H-bond)</td>
<td>2.57 (0.05)</td>
<td>2.58 (0.07)</td>
<td>2.63 (0.07)</td>
<td>2.6 (0.07)</td>
<td>2.89 (0.2)</td>
</tr>
</tbody>
</table>

Nitrite is residue 503. <sup>a</sup> From reference (84).<sup>b</sup> Deviation from mean bond distance. <sup>c</sup> Ligand distances for nitrite only. <sup>d</sup> Subunits A and B. <sup>e</sup> Subunit B only.
4.2.3 EPR spectra

The EPR spectrum of oxidized AfNiR collected at pH 4 exhibits the typical hyperfine splitting of both the type 1 and type 2 copper centers of NiR despite the mildly acidic pH (Figure 4-4) (97, 106, 107). The spectra of the oxidized D98N and H145A variants recorded at pH 4 (Figure 4-4 and Figure 4-5, respectively; Table 4-3) also resemble those of the corresponding NiR variants from different species and recorded at different temperature or pH (14, 22-24).

Anaerobic dialysis of AfNiR and variants in solutions containing 20 mM sodium ascorbate resulted in EPR spectra that are devoid of any features indicating that the proteins are fully reduced under these conditions (data not shown). Also, reduction of AfNiR and the D98N variant is evident from the disappearance of the green color associated with the oxidized type 1 copper sites of these proteins. Some green color of AfNiR and D98N variant reappeared within several minutes of exposure to NO and was stable prior to freezing the samples in liquid nitrogen. Reaction of the reduced H145A variant with NO resulted in a weak blue color. At high concentrations (1 mM) the H145A variant displays light blue color suggesting partial oxidation of the copper site in the sample. More importantly, the EPR spectra of these samples are devoid of a type 1 copper signal. The four-line hyperfine pattern at \( g_z \sim 2.34 \) with a coupling \( A_z \sim 134 \, G \) in the EPR spectra of the three proteins indicates that reaction of the reduced protein with NO resulted in re-oxidation of the type 2 copper centers (Figure 4-4 for AfNiR and D98N, and Figure 4-5 for H145A; Table 4-3). Binding of NO to the type 2 copper center in the three proteins gives rise to a nine-line superhyperfine pattern between 320 and 335 G (\( g_y = 2.05, A_y = 16 \, G \); Figure 4-6) indicative of binding of NO to these copper sites. An additional set of features are observed in the EPR spectrum of AfNiR (indicated with arrows in Figure 4-4). These features will be discussed further in Section 0 of this chapter.
Anaerobic dialysis of the H145A variant in an NO-saturated buffer solution for 20 min without previous reduction with sodium ascorbate resulted in an EPR spectrum (Figure 4-5) with the hyperfine splitting of an oxidized type 2 copper site ($g_z = 2.39, A_z = 132$ G). The EPR properties of this species are similar to those identified in the spectra of the reduced wild-type AfNiR exposed to NO (Table 4-3). Reaction of the protein with NO under these conditions also resulted in the emergence of a second type 2 copper species ($g_z \approx 2.35, A_z = 170$ G), which on prolonged exposure to NO (~90 min) became the predominant form of the protein (Figure 4-5).
Figure 4-4 X-band EPR spectra of wild-type AfNiR and D98N variant at pH 4.

(A) Reduced, NO-exposed wild-type AfNiR; (B) Oxidized, wild-type AfNiR; (C) Reduced, NO-exposed D98N variant; (D) oxidized D98N. Arrows indicate extra features that correspond to additional species present in the sample. For \( A_z \) and \( g_z \) values refer to Table 4-3.
Figure 4-5 X-band EPR spectra of H145A variant in 50 mM acetate, 50 mM MES, pH 4. (A) oxidized H145A; (B) oxidized H145A, NO-exposed for 20 min; (C) oxidized H145A, NO-exposed for 1.5 hours; (D) reduced H145A, NO-exposed for 20 min; (E) reduced H145A, NO-exposed for 1.5 hours. Asterisks indicate samples for which the spin of the oxidized copper atoms was quantified. For $A_z$ and $g_z$ values refer to Table 4-3.
Table 4-3 EPR parameters for the two copper sites in AfNiR

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<td></td>
<td></td>
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<td>$g_z$</td>
</tr>
<tr>
<td>Oxidized AfNiR</td>
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</tr>
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<tr>
<td>Oxidized D98N</td>
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<tr>
<td>Reduced D98N</td>
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</tr>
<tr>
<td>Oxidized H145A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized H145A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized H145A</td>
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<tr>
<td>Reduced H145A</td>
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</tr>
<tr>
<td>Reduced H145A</td>
<td>90</td>
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</table>

Note: All samples were buffered in 50 mM MES, 50 mM sodium acetate pH 4.
Figure 4-6 Superhyperfine splitting spectra at pH 4. (A) Reduced wild-type AfNiR exposed to NO; (B) Reduced D98N variant, exposed to NO; (C) Reduced H145A variant, exposed to NO. In all cases a 9-line superhyperfine pattern is indicative of 4 magnetically equivalent nitrogen atoms coordinating to the type 2 copper atom. Three of these nitrogen atoms belong to histidine ligands; the fourth belongs to NO.
4.3 Discussion

4.3.1 The reverse reaction (the oxidation of nitric oxide to form nitrite)

Wijma et al. characterized the pH dependence of the kinetics of AfNiR activity in both the forward and reverse directions (38). Although the forward reaction is favored below pH 7, the reverse reaction was also observed to proceed under conditions where NO and an electron acceptor are present in sufficient excess. Exposure of oxidized AfNiR crystals at pH 4 to a saturated NO solution produces nitrite, which remains bound to the active site copper (Figure 4-1, A). The binding mode of nitrite and its interaction with Asp98 is the same as those observed in the structure of nitrite-soaked, oxidized AfNiR (84). The fate of the electron for the reverse reaction is unclear. The crystals remain green indicating that the type I site either does not receive the electron or, more likely, that it quickly transfers the electron to another acceptor. Because NO is present in a substantial excess (~1.9 mM, (108)), it may serve as the oxidant in a dismutation reaction to give both nitrite and NO− (or HNO), two molecules which combine rapidly in solution to form N2O (Scheme I, Figure 4-7) (109, 110). Hulse and Averill proposed a catalytic mechanism in which reduction of nitrite to nitric oxide proceeds through the formation of an electrophilic nitrosyl intermediate Cu(I)−NO+ (63, 111). Similarly, upon binding of NO to an oxidized type 2 copper atom in CuNiR, electron transfer from the radical to the metal ion also can yield an electrophilic nitrosyl intermediate (Cu(I)-NO+). This intermediate is susceptible to attack by nucleophiles such as water, azide or hydroxylamine (63, 111). Reaction with water to give nitrite is the reverse of the dehydration step in the proposed mechanism (84).

The requirement for electron transfer to the type I copper in the reverse reaction is evident from the crystal structure of the oxidized H145A variant exposed to NO (Figure 4-1, B). This variant exhibits <1% of the activity of the wild-type enzyme due to a mutation-induced increase in the reduction potential of the type I copper center that eliminates electron transfer to
the type 2 copper center (79). Instead of producing nitrite, the addition of excess NO yields a
type 2 copper that is predominantly bound by NO side-on (Figure 4-1, B). This side-on binding
mode was observed previously when reduced wild-type AfNiR crystals were exposed to NO
(84). Accordingly, the EPR spectrum of the oxidized H145A variant exposed to NO at the same
pH reveals a type 2 copper signal (Figure 4-5, B) similar to that observed for the reduced wild-
type enzyme exposed to NO (84). This observation is particularly noteworthy since the oxidized,
NO-exposed H145A variant is expected to be EPR silent (due to the coupling of the Cu(II) and
NO•). Furthermore, NO has greater affinity for Cu(I) (67). Exposure of the reduced H145A
variant to NO under similar conditions also results in side-on binding to the type 2 copper atom
(Figure 4-1, C) and a similar EPR spectrum (Figure 4-5, D). The high similarity in both the
binding mode observed in crystals and the EPR spectra in solution suggest that oxidized and
reduced H145A and reduced wild-type samples yield the same stable copper-nitrosyl species
proposed to be Cu(II)-NO•.

4.3.2 Stable copper-nitrosyl formation (Cu(II)-NO•, cupric-nitroxyl)

Prolonged exposure (20 min) of the H145A variant to nitric oxide yields the same stable
cupric-nitroxyl complex (Cu(II)-NO•) regardless of the starting oxidation state of the enzyme
(Schemes II and III, Figure 4-7). In both the crystal and solution experiments, the oxidized type 2
copper center of the H145A variant can be reduced by an equivalent of NO to yield the
electrophilic nitrosyl species proposed by Hulse and Averill (63) so that upon reaction with
water, nitrite is generated. Immediately upon release of this product, another NO molecule binds
to the nascent type 2 Cu (I) to yield the stable copper-nitrosyl that is also generated when starting
with the fully reduced variant. Spin quantification of EPR samples prepared with the reduced and
oxidized H145A protein solutions exposed to NO indicates that 60-85% of the copper atoms in
the protein solution are oxidized, confirming that these are not minor but rather the predominant
species in solution.

Subtle differences are observed in the superhyperfine splitting patterns of the EPR spectra obtained from the reduced versus oxidized protein samples (Figure 4-6). When starting with a reduced protein, the EPR spectra of the wild-type, H145A and D98N variants exhibit nine-line superhyperfine splitting indicative of four magnetically equivalent nitrogen atoms coordinating to the metal. In all cases, $A_z$ is ~16 G and the $g_z$ is 2.04 - 2.05. The lack of a similar superhyperfine signal of the cupric-nitroxyl in spectra of the oxidized, NO-exposed H145A variant could be due to broadening of the lines such that no signal is observed (data not shown).

Side-on interactions of NO with metal centers are stabilized by donation of electron density from the $\sigma$ orbital on NO to the metal and by back-donation of electron density from the occupied metal $d$-orbitals to the $\pi^*$ antibonding orbitals of the NO ligand (112). The copper-nitrosyl species observed in this work differ from the complexes recently characterized by EPR-ENDOR spectroscopy (113). When samples of reduced NiR from Rhodobacter sphaeroides were frozen within 1 min of adding exogenous NO, signals associated with the NO• radical were the principal signals detected by EPR (113). ENDOR studies on the complex were interpreted as arising from end-on coordination of NO to the metal. Exactly how a radical can interact with a metal ion without prompt electron transfer remains unclear even if the species formed are short lived. Furthermore, the presence of additional equivalents of reductant (113) may have led to secondary electron-transfer events to make definitive interpretation of the results ambiguous. In the current study, the impossibility of electron transfer from the type 1 site of the H145A variant and the removal of excess ascorbate reductant limit secondary reactions to those involving NO. Under these conditions, the side-on NO interaction observed could best be described as Cu(II)-NO•. When both the reduced and oxidized H145A protein samples were dialyzed versus NO-
saturated buffer for periods longer than one hour, an EPR spectrum of a third, previously
uncharacterized species was observed ($A_z$ value > 170 G). The larger splitting is consistent with
an oxidized type 2 copper center. After close examination, similar features are present as minor
species in spectra of the reduced wild-type AfNiR and H145A variant EPR samples after 20 min
of exposure to NO (Figure 4-4, Figure 4-5, Table 4-3).

4.3.3 Role of Asp98 in defining the NO binding mode

Of the residues in close proximity to the type 2 copper in AfNiR, Asp98 is the least
ordered in the absence of substrate (47). Direct hydrogen bonding to the substrate (78) and
product (84) result in a decrease in the $B$-factors of Asp98. Side-on coordination of NO to the
type 2 copper is observed in the D98N crystal structure (Figure 4-1). However, difference
electron density maps indicate that both the NO and the side chain of Asn98 are less well ordered
than those observed in the wild-type enzyme. In addition to the predominant side-on mode of
binding, partial end-on coordination of the NO to the metal is modeled in subunit C (Figure 4-3).
The EPR spectrum of the reduced, NO-exposed D98N complex is similar to the spectrum of the
reduced wild-type AfNiR-NO complex (Figure 4-4, C). These spectra are characterized by $A_z$ and
g$_z$ of 130 G and 2.31, respectively, which is indicative of an oxidized type 2 copper. Taken
together, the available data indicate that the hydrogen bond to Asp98 is not required for side-on
binding by NO to the Cu.

4.3.4 Insight into the catalytic mechanism of NiRs

Both Asp98 and electron transfer by the type 1 copper site are essential for nitrite
reductase activity. The Oδ1 atom of Asp98 forms a hydrogen bond with both nitrite and NO
bound to the type 2 copper (56, 84). An analogous hydrogen bond is not observed in the same
complexes prepared with the D98N variant (78) (Figure 4-1, D). The greatly diminished activity
of this variant (60, 78) suggests that Asp98 may also interact with intermediates throughout the catalytic cycle. Characterization of variants involving substitution of ligands at the type I copper site has established that this site relays electrons to the catalytic center from the physiological electron donor, pseudoazurin (97, 114). For these variants, addition of chemical reductants partially restores nitrite reductase activity presumably by direct reduction of the type II catalytic center. Exposure of oxidized H145A variant crystals to exogenous NO did not yield the structure of the proposed catalytic intermediate (Cu(I)-NO$^+$). Rather, the presence of excess NO resulted in a complex with one more electron (Cu(II)-NO$^-$). In this case, exposure to NO as a substrate probably results in a single turnover that produces nitrite and reduces the type II copper. A second molecule of NO then binds to form a copper-nitrosyl complex that in the absence of electron transfer from the type I copper is kinetically stable, thereby preventing further reaction.

In one of the three monomers of the structure of the reduced H145A-NO complex, the coordination environment of the type II copper is modeled with two coordination positions partially occupied by water molecules. A single water molecule may occupy these two positions or two water molecules are present to form a pentacoordinate catalytic center. The positions of these water molecules suggest a potential model for NO displacement by water in the catalytic cycle. The formation of NO at the type II site could be followed by a transient pentacoordinate intermediate with a water molecule ultimately leading to NO release and generation of the resting state.

Presumably, the catalytic intermediate (Cu(I)-NO$^+$) is short lived relative to the time frame of our experiments. Within the 20 min of NO exposure of the oxidized crystals, either electron transfer to the type I site occurs resulting in nitrite formation in the wild-type enzyme or a second NO equivalent reduces the type II site in the H145A variant. Trapping the copper-
nitrosyl intermediate formed during the catalytic cycle of Cu NiR may require the development of single turnover techniques in crystal.
Figure 4-7 Possible electron transfer and NOx interactions in wild type AfNiR and its H145A variant in the presence of excess NO. See text for details.
Chapter Five: Inhibition of Alcaligenes faecalis nitrite reductase by substrate and product analogues

5.1 Introduction

Powerful insights into enzyme mechanisms can often be obtained by studying the interaction of substrate and product analogues with the enzyme (115). To gain insight into the catalytic mechanism of AfNiR, the interaction of the enzyme with a number of small molecules was investigated. In the nitrite-bound NiR complex, the nitrite binding to the type 2 Cu(II) is often termed bidentate even though one of the oxygen atoms, O₁₃₂, is ~2.3 Å away from the copper and is likely non-coordinating (44, 47). In this study, substrate analogues were chosen based on the presence of at least two oxygen atoms for potential bidentate coordination to the type 2 copper, and included acetate, formate and nitrate. In addition, azide and nitrous oxide were chosen based on the presence of nitrogen atoms and the linearity of the molecules. Interactions of the studied small molecules with fully oxidized AfNiR were examined by x-ray crystallography and steady-state kinetics. An analysis of these data provides insight into the determinants of substrate specificity in NiR.

5.2 Results

5.2.1 Active site structure and inhibitor binding

The overall protein folds of the inhibitor-bound AfNiR was essentially identical to the native AfNiR crystal structure (PDB entry 1AS7) as revealed by a r.m.s. deviation between the Cu atoms of less than 0.2 Å. For all the complexes presented here, the major structural differences are localized around the type 2 copper sites. Data and refinement statistics for all inhibitor-bound structures are presented in Table 5-1.
Table 5-1 Data collection and refinement statistics for inhibitor-bound AfNiR

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<th>AfNiR-azide</th>
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</tr>
<tr>
<td>(R_{merge}^{a}) (%)</td>
<td>5.2 (20.2)</td>
<td>3.7 (45.1)</td>
<td>8.8 (42.8)</td>
<td>8.6 (36.1)</td>
<td>12.7 (37.8)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(R_{work}/R_{free})</td>
<td>16.0/18.0</td>
<td>16.4/20.1</td>
<td>16.9/19.5</td>
<td>15.3/17.9</td>
<td>19.1/22.4</td>
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<tr>
<td></td>
<td>(19.0/23.0)</td>
<td>(28.7/38.5)</td>
<td>(27.7/33.0)</td>
<td>(20.2/23.3)</td>
<td>(25.7/31.2)</td>
</tr>
<tr>
<td>Overall (B)-value (Å²)</td>
<td>14.8</td>
<td>19.1</td>
<td>21.3</td>
<td>19.5</td>
<td>23.6</td>
</tr>
<tr>
<td>Coordinate error(^1) (Å)</td>
<td>0.04</td>
<td>0.05</td>
<td>0.08</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>r.m.s.d. from ideality</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.006</td>
<td>0.010</td>
<td>0.012</td>
<td>0.010</td>
<td>0.009</td>
</tr>
<tr>
<td>Bond angles (deg)</td>
<td>1.096</td>
<td>1.288</td>
<td>1.330</td>
<td>1.342</td>
<td>1.308</td>
</tr>
<tr>
<td>PDB code</td>
<td>2E86</td>
<td>2PP8</td>
<td>2PP9</td>
<td>2PP7</td>
<td>2PPA</td>
</tr>
</tbody>
</table>

Note: Numbers in parenthesis correspond to the highest resolution shell.

\(^1\) Coordinate error derived from maximum likelihood refinement.
The $F_c-F_c$ difference map of the azide-exposed oxidized AfNiR crystal structure to 1.5 Å resolution revealed an elongated difference electron density in close proximity to the active-site Cu(II), which was assigned to an azide anion (Figure 5-1, A). This ion was modeled coordinating to the type 2 copper via one of its nitrogen atoms with a Cu-N distance of 2.04 Å. Azide also interacts with residues surrounding the active site. The distance between the Oδ1 atom of Asp98 and the coordinating N atom of azide is 3.37 Å. The bound azide is bent (Cu-N-N angle of 119°) (Figure 1-14), apparently due to a steric clash with Ile257 (~3.3 Å). The nitrogen atom of azide farthest from the copper forms a hydrogen bond (~3.0 Å) with a water molecule (Figure 5-1, W2), which in turn forms a hydrogen bond to the Oδ1 atom of Asp98 (~2.5 Å). Azide was refined at full occupancy at each active site and refined with a final $B$-factor of 27 Å².

In subunit A of the oxidized AfNiR crystals exposed to formate, a difference map revealed electron density that can accommodate at most 3 atoms in a non-linear fashion (Figure 5-1, B). Formate was modeled at full occupancy and refined coordinating to the type 2 copper via one of its oxygen atoms (Oc-Cu distance of 2.19 Å). In the final model, the second oxygen and carbon atoms are situated 2.39 and 2.52 Å from the copper ion, respectively. The side-chain of Asp98 is disordered and is modeled in two distinct conformations at 50% occupancy each. In the inward position, the carboxylate group is pointing towards the copper and may form a hydrogen bond with formate. A rotation of approximately 80° about the $\chi_2$ torsional angle gives an outward conformation resulting in the Oδ2 atom being displaced about 3.75 Å. The displacement of the side-chain of Asp98 results in a pocket which is occupied by a solvent molecule (W3) 50% of the time. The two conformations of Asp98 are also observed in subunits B and C. These two subunits show greater disorder in the binding of formate, with two predominant conformers. In addition to the binding mode observed in subunit A, subunits B and
C show monodentate coordination to the metal with an O$_{nc}$-Cu distance of 2.44 Å (Figure 5-2, Table 5-2). The second formate conformation may be stabilized by a hydrogen bond with a nearby water molecule (2.69 Å) which is also hydrogen bonded to the Oδ1 atom of the Asp98 (2.6 Å). The two formate conformers were refined at 50% occupancy each and resulted in an average $B$-factor of 28 Å$^2$. 
Figure 5-1 Inhibitors bound to type 2 Cu of AfNiR. Stick diagram of the coordination geometries at the type 2 copper center showing A) azide binding, B) formate binding, C) nitrate binding, D) acetate binding, E) nitrous oxide binding. The $2F_o-F_c$ electron density map is colored in grey and contoured at 1 $\sigma$. The omit difference electron density map is colored in green and contoured at 4 $\sigma$. The residues surrounding the copper atom are labelled in panel A. Nitrogen atoms are colored in blue, oxygen atoms are colored in red, carbon atoms are colored in yellow and water molecules are colored in cyan. Ile257 has been omitted from panel C for clarity. Molscript (48) and Raster3D (49) were used to prepare the figure.
Figure 5-2 Formate-bound AfNiR subunits B and C show disorder of both formate and Asp98. $F_o-F_c$ difference map is shown in green and contoured at 4 $\sigma$. The $2F_o-F_c$ map is colored in gray and contoured at 1 $\sigma$. 
A nitrate molecule was modeled in the difference electron density observed at the type 2 copper site of AfNiR exposed to nitrate (Figure 5-1, C). The nitrate molecule is interacting with the metal via two of its three oxygen atoms, situated at 2.19 and 2.23 Å from the metal. The nitrate molecule forms an angle of 25° with the plane defined by the oxygen atoms and the copper ion. Due to the bulkiness of the inhibitor molecule, the side-chain of Asp98 is displaced to point away from the copper. A hydrogen bond network comprising water molecules surrounds the bound anion. One of these water molecules, W3, occupies the free space created by the displacement of the Asp98 carboxylate and is situated 2.65 Å from one of the oxygen atoms of nitrate (Figure 5-1, C). In addition, W3 stabilizes the bridging water (W1) by a hydrogen bond (2.74 Å) such that no changes are observed in the orientation of His255. The water network stabilizing the binding of nitrate to the type 2 copper extends to two other water molecules, W2 and W4 that are situated at 3.31 Å and 2.72 Å from nitrate and Asp98, respectively.

When crystals of oxidized AfNiR were manipulated anaerobically, acetate was observed bound to the active site of the enzyme. Acetate is part of the crystallization buffer but in the resting state of the enzyme under aerobic conditions, a water molecule occupies the fourth coordination position of the type 2 copper (43). However, when manipulated anaerobically, the difference electron density map is indicative of an acetate molecule bound to the Cu(II) instead of a water molecule (Figure 5-1, D). It is unclear how oxygen availability determines binding specificity between acetate and water. The acetate molecule is refined at full occupancy coordinating to the copper via its two oxygen atoms that are situated 2.14 Å and 2.39 Å away from the metal. Binding of acetate at the active site requires that the side-chain of Asp98 swings away from the copper. A hydrogen bond is formed between the acetate and a nearby water molecule, W3 (2.77 Å). No disorder in the binding mode of acetate bound to the active site is
observed and the three acetate molecules were modeled in at full occupancy and refined to a low average $B$-factor of 26 Å$^2$.

Crystals of the reduced and oxidized AfNiR exposed to nitrous oxide were also examined by x-ray diffraction methods. The reduced crystals exposed to nitrous oxide revealed no difference peaks at the active site of the enzyme. In the oxidized crystals, a difference map revealed density that can accommodate a linear molecule comprising at most three atoms. A nitrous oxide molecule was refined bound side-on to the copper (Figure 5-1, E). The x-ray data were insufficient to distinguish between the oxygen and the nitrogen atoms. Subunits A and B have a nitrous oxide molecule bound to the metal ion. By contrast, the density in subunit C was modeled as an acetate molecule bound at the active site. The presence of acetate in these crystals is not surprising since the crystals were manipulated anaerobically in the presence of 100 mM acetate. In subunits A and B, the side-chain of Asp98 is displaced to point away from the copper ion to accommodate the bound nitrous oxide. The average $B$-factor of the nitrous oxide molecules at full occupancy in subunits A and B is 31 Å$^2$. 
Table 5-2 Inhibitor bond distances to the type 2 copper of AfNiR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oxidized wild-type AfNiR</th>
<th>Reduced wild-type AfNiR bound to NO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>AfNiR - formate</th>
<th>AfNiR - acetate</th>
<th>AfNiR - nitrate</th>
<th>AfNiR - azide</th>
<th>AfNiR - nitrous oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>503O&lt;sub&gt;c&lt;/sub&gt;-Cu</td>
<td>2.06 (0.02)</td>
<td>n/a</td>
<td>2.19</td>
<td>2.14</td>
<td>2.2 (0.1)</td>
<td>n/a</td>
<td>2.10 (0.07)</td>
</tr>
<tr>
<td>503N-Cu</td>
<td>2.33 (0.03)</td>
<td>1.95 (0.02)</td>
<td>2.52 (0.02)</td>
<td>2.51 (0.02)</td>
<td>2.50 (0.02)</td>
<td>2.04 (0.06)</td>
<td>2.2 (0.2)</td>
</tr>
<tr>
<td>503O&lt;sub&gt;nc&lt;/sub&gt;-Cu</td>
<td>2.34 (0.05)</td>
<td>1.99 (0.07)</td>
<td>2.39 (0.03)</td>
<td>2.39 (0.06)</td>
<td>2.23 (0.06)</td>
<td>n/a</td>
<td>2.6 (0.1)</td>
</tr>
<tr>
<td>503O&lt;sub&gt;c&lt;/sub&gt;-O81</td>
<td>2.57 (0.05)</td>
<td>2.58 (0.07)</td>
<td>2.41 (0.06)</td>
<td>n/a</td>
<td>n/a</td>
<td>3.4 (0.4)</td>
<td>n/a</td>
</tr>
<tr>
<td>Asp98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.98 (0.4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> From reference (84). <sup>b</sup> Numbers in parenthesis show deviation from average bond distances for all three monomers. <sup>c</sup> Ligand distance for carbon atom, subunit A. <sup>d</sup> Distance between Asp98 and N<sub>c</sub> atom of azide. <sup>e</sup> Average for subunits A and B only. <sup>f</sup> Ligand distance for the second nitrogen in nitrous oxide.
5.2.2 Steady-state kinetics analysis of nitrite reduction

The turnover of AfNiR was analyzed using steady-state kinetics at each of pH 4 and 6.5 based on the oxidation of reduced pseudoazurin. Initial rates were corrected for the non-enzymatic oxidation of pseudoazurin by nitrite. This non-enzymatic oxidation was 10-fold faster at pH 4 than 6.5 but in no case exceeded 10% of the enzyme-catalyzed oxidation of pseudoazurin.

At both pH 4 and 6.5, the oxidation of pseudoazurin obeyed Michaelis-Menten kinetics. Although the turnover of AfNiR was approximately 60-fold higher at the higher pH, the enzyme’s $K_m$ for nitrite was approximately 20-fold higher, such that the difference in $k_{cat}/K_m$ at the two pHs was only 3-fold (Table 5-3).

5.2.3 Evaluation of small molecule inhibitors

Azide, formate, nitrate and acetate each inhibited the AfNiR-catalyzed oxidation of pseudoazurin. This inhibition was studied as a function of inhibitor and nitrite concentrations and was analyzed by fitting the data to each of three inhibition models described in Chapter 2. Overall, azide inhibited AfNiR approximately two orders of magnitude more strongly than any of other three small anions (Table 5-3). Moreover, based on the quality of the fits, azide was the only anion investigated that competitively inhibited AfNiR with respect to nitrite, consistent with the parallel lines obtained in the Cornish-Bowden plot (Figure 5-4, Figure 5-5). Similar analyses revealed that of the other three inhibitors, formate and acetate inhibited AfNiR in a mixed fashion and nitrate inhibited the enzyme uncompetitively (Figure 5-4). Nevertheless, even the inhibition parameter for azide, the best inhibitor, was about an order of magnitude greater than the enzyme’s $K_m$ for nitrite.
### Table 5-3 Inhibition kinetic parameters

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ (nitrite) (μM)</th>
<th>$K_{ic}$ (mM)</th>
<th>$K_{iu}$ (mM)</th>
<th>$k_{cat}/K_m$ ($10^6$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AfNiR at pH 6.5</td>
<td>620 ± 10</td>
<td>150 ± 10</td>
<td></td>
<td></td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>AfNiR at pH 4.0</td>
<td>10.5 ± 0.3</td>
<td>8 ± 1</td>
<td></td>
<td></td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>AfNiR + azide</td>
<td>540 ± 20</td>
<td>100 ± 10</td>
<td>2.0 ± 0.1</td>
<td></td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>AfNiR + formate</td>
<td>610 ± 10</td>
<td>140 ± 10</td>
<td>150 ± 10</td>
<td>53 ± 2</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>AfNiR + nitrate</td>
<td>430 ± 20</td>
<td>110 ± 10</td>
<td></td>
<td>61 ± 5</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>AfNiR + acetate</td>
<td>770 ± 30</td>
<td>230 ± 20</td>
<td>160 ± 20</td>
<td>72 ± 6</td>
<td>3.4 ± 0.1</td>
</tr>
</tbody>
</table>

Note: Inhibition experiments were performed in 50 mM MES buffer, pH 6.5. Other details are provided in Chapter 2. $K_m$ is the Michaelis-Menten constant, $K_{ic}$ is the competitive inhibition constant, $K_{iu}$ is the uncompetitive inhibition constant, and $k_{cat}/K_m$ is the specificity constant.
Figure 5-3 Steady-state turnover of AfNiR at pH 6.5 (A) and pH 4 (B). Initial rates were based on pseudoazurin oxidation. The curves represent the fit of the Michaelis-Menten equation to the data (squares).
Figure 5-4 Cornish-Bowden plots showing the inhibition of AfNiR by azide (A) and formate (B). The reaction mixtures contained 230 pM AfNiR, 315 μM reduced pseudoazurin together with the following concentrations of nitrite: ■, 40 μM; △, 80 μM; ●, 120 μM; ○, 200 μM; ▲, 400 μM; ◀, 800 μM. at 25°C.
Figure 5-5 Cornish-Bowden plots showing the inhibition of AfNiR by nitrate (A) and acetate (B). Refer to Figure 5-4 for further detail.
5.3 Discussion

The presented crystallographic and steady-state kinetic data demonstrate that a number of small molecules interact with the oxidized type 2 copper site of AfNiR and inhibit the enzyme. More specifically, the structural data show that formate, acetate, nitrate, azide and nitrous oxide bind to the catalytic centre of AfNiR. Consistent with the structural data, those small molecules that were tested inhibited the pseudoazurin-dependent reduction of nitrite by AfNiR. The kinetic data indicated that none of the inhibitors interact as strongly with the enzyme as either nitrite or nitric oxide: the inhibition parameters were one to two orders of magnitude greater than the $K_m$ for nitrite. As discussed in more detail below, the structural data are consistent with these kinetic results inasmuch as the poorer inhibitors bind at a greater distance from the copper ion and have fewer specific interactions with active-site residues.

5.3.1 Binding of substrate analogues to the type 2 copper of AfNiR

The interactions of formate, acetate and nitrate with AfNiR are of particular relevance as these anions are structurally similar to nitrite. It is therefore not surprising that each of the three inhibitors bound to AfNiR in a manner similar to the substrate. In the crystal structures of these inhibitor-bound AfNiR complexes, two oxygen atoms of the bound anion were directed towards the type 2 copper in a bidentate-like manner and the water molecule that occupies the fourth coordinating position in the resting state enzyme (43) was displaced. Moreover, one of the Cu-O distances was longer than the other. This asymmetric binding is in contrast to what is observed in model complexes. In one such series of compounds, formate and acetate bind in a bidentate but symmetric manner, in which the Cu-O bond distances are ~2 Å (116, 117). In another series of compounds, nitrite and acetate bound the copper ion in a monodentate fashion (68). These results
suggest that the protein environment of NiR determines the mode of binding of these small inhibitors, and that these same factors help determine the substrate specificity of the enzyme.

In light of the underlying similarities of inhibitor and nitrite binding in AfNiR, it is striking that the enzyme has such a low affinity for the former. More specifically, the inhibition constants for formate, acetate and nitrate were >50 mM (Table 5-3). By contrast, both the $K_m$ and the $K_d$ of NiR for nitrite (64) are two orders of magnitude lower. The relatively low affinity of AfNiR for nitrate is consistent with pulse radiolysis and EPR spectra of NiRs from *Achromobacter cycloclastes* and *Alcaligenes xylosoxidans*. Exposing AcNiR and AxNiR to a 16 mM solution of nitrate alters the type 2 copper EPR spectrum and reduction potential (107).

Comparison of the structures of the inhibitor-bound AfNiR complexes with that of the nitrite-bound AfNiR reveals two elements of the active site that apparently tune the enzymes specificity for nitrite (Figure 5-6, A). First, the Cu-O$_c$ distances in the inhibitor complexes are ~0.15 Å longer than in the nitrite complex (Table 5-2). Second, Asp98 is orientated differently in the inhibitor and substrate complexes. These differences are most clear in the nitrate and acetate complexes, in which the side chain of Asp98 is orientated away from the bound inhibitor such that hydrogen-bond formation is not feasible. This alternate orientation is likely due at least in part to the fourth non-hydrogen atom that is present in nitrate and acetate versus nitrite and formate. Examination of the nitrite-bound AfNiR structure indicates that a fourth atom can not be accommodated in the active site without displacement of the Asp98 side chain. Nevertheless, even in the formate-bound AfNiR structure, Asp98 clearly occupies two conformations suggesting that this residue does not interact with the inhibitor as it does with the substrate.

Formate and acetate show mixed inhibition and nitrate shows uncompetitive inhibition of AfNiR in the presence of nitrite. These results suggest the presence of an anion binding site that
affects the activity of the enzyme, however is not the nitrite binding site. Upon close examination of the crystal structures, eight such sites were identified. The anion bound at these sites is modeled as acetate which is present at 100 mM in the crystallization buffer. The majority of the acetate binding sites were found at the surface of the protein distant from the type 1 or type 2 copper sites. However, three of the anion binding sites are conserved positionally within the trimer and are 11.2 Å and 13.5 Å from the nearest type 1 and type 2 Cu atoms, respectively. In addition, these sites are located at the opening of the 12 Å cleft leading to the active site. This observation suggests that during inhibition experiments inhibitor molecules could bind at these sites of the enzyme-substrate complex and uncompetitively inhibit catalysis.
Figure 5-6 Superposition of inhibitor-bound AfNiR structures. A) Substrate analogues. The carbon atoms of the formate-bound structure are colored in purple, of the nitrite structure are colored in orange, of the acetate structure are in pink and nitrate structure are in green. The oxygen and nitrogen atoms are represented as red and blue spheres, respectively. B) Azide and nitrous oxide. The carbon atoms of the nitrous oxide structure are colored in grey, the NO structure is in yellow, the azide structure are in pink and the resting state of the enzyme is colored in green. Water molecule coordinating to Cu(II) in the resting state of the enzyme is colored in cyan.
5.3.2 The importance of Asp98

The current study is consistent with previous work establishing the key catalytic role of Asp98 in AfNiR (78). In the proposed mechanism (Figure 3-5), Asp98 is deprotonated in the resting state enzyme, thereby favouring the binding of protonated substrates. Even though its \( pK_a \) is 3.4, nitrite binds in its protonated form (Figure 5-7) (56), and forms a hydrogen bond with O\( \delta_1 \) of Asp98. The proposed protonation state of Asp98 is supported by FTIR spectroscopic studies of carbon monoxide binding to reduced forms of wild-type and D98N variant (57). In the reduced product-bound AfNiR complex, electron transfer from the copper ion to NO likely results in the formation of HNO which can form a hydrogen bond with Asp98 (84) as suggested by density functional theory (DFT) calculations (118). The protonation states of the NiR-bound formate, nitrate and acetate are unknown. However, the orientation of the Asp98 side chain in the inhibitor complexes indicates that these small molecules do not form a hydrogen bond with the residue. In particular, the disorder of the side chain in the formate-bound AfNiR structure is strikingly similar to that observed in the oxidized D98N variant complexed to nitrite (78).

![Figure 5-7 Protonation states of nitrite and Asp98 at the active site of AfNiR.](image)

Asp98 is deprotonated and nitrite is the hydrogen bond proton donor. Asp98 is connected to His255 via a water bridge. Figure adapted from (56).

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5.3.3 Azide and nitrous oxide interactions with type 2 copper

The higher affinity of AfNiR for azide versus the other inhibitors likely reflects the higher intrinsic affinity of the type 2 copper for this ligand. Significantly, the bent binding mode of azide (Figure 1-14) is unique among the tested inhibitors and is very similar to that observed in the type 2 copper site of Cu,Zn superoxide dismutase (SOD). In the reduced state, the SOD copper ion is coordinated by three histidyl residues in an approximately tetrahedral arrangement (43, 62), as is the type 2 copper of NiR. A fourth histidine of SOD that bridges the copper and zinc sites, superimposes with His255 of NiR (43, 62). Consistent with the similar structure of the copper sites of these enzymes, AxNiR has SOD activity (62). An X-ray crystallographic study revealed that azide binds bent as a fourth ligand to the Cu(II) form of yeast SOD in a manner thought to mimic superoxide (119). In the azide-bound SOD structure, the Cu-Nc distance was 2.15 Å. EXAFS studies of an bovine azide-bound SOD structure revealed a Cu-Nc distance of 1.99 Å, in very close agreement with that observed in the AfNiR-azide complex. However, the azide was bound at more of an angle in SOD (Cu-N-N < 135°) (120) than in NiR (168°) (Figure 5-1). Interestingly, FTIR studies on the reduced Cu(I) bovine SOD indicated that azide not bind directly to the metal (121). Finally, NMR studies indicated that formate binds to SOD but does not interact directly with the copper ion (122).

The binding of nitrous oxide to AfNiR clearly resembles the side-on binding of nitric oxide, and not the bent binding of azide, despite the fact that the latter and nitrous oxide are linear and triatomic. The binding of nitrous oxide to NiR is consistent with the observation that AcNiR can catalyze the further reduction of nitric oxide to nitrous oxide in the presence of a chemical reductant (37). It is not possible to distinguish between the distal N and O atoms of the nitrous oxide molecule in the crystal structure. Nevertheless, the side-on binding of the nitrous
oxide molecule is very similar to nitric oxide (Figure 5-6, B) except that the former binds at a slightly further distance from the metal than the latter (~0.15 Å, Table 5-2). Finally, the disorder in the Asp98 side chain further suggests the critical role of this residue in determining the substrate specificity of AfNiR.

In conclusion, the studies with small molecule inhibitors illustrate the importance of the protein environment for tuning the specificity of the copper site of NiR for reacting with nitrite. Specifically, Asp98 and Ile257 form a pocket that limits the binding of exogenous ligands. Asp98 places a requirement for a hydrogen bond partner and Ile257 places steric constraints on bound ligands. Inhibitor studies with AfNiR derivatives mutated at Asp98 and Ile257 would further confirm the importance of these residues in discriminating against binding of non-catalytic small molecules to the type 2 copper.
Chapter Six: Overview and future directions

6.1 In search of the copper-nitrosyl intermediate

A copper-nitrosyl intermediate is proposed as part of the catalytic mechanism for NiRs. Averill et al. assigned the intermediate with the formal description of Cu(I)-NO⁺, which would be formed by the exposure of Cu(II) to NO⁺. Copper-nitrosyl complexes in the wild-type and variant forms of AfNiR were prepared and characterized by x-ray crystallography and EPR spectroscopy. New techniques were developed to address the challenging manipulation of exposing crystals and protein solutions to NO (Sections 2.5.1 and 2.6.2). Although none of the characterized complexes correspond precisely to the proposed copper-nitrosyl intermediate, exposing reduced AfNiR to NO led to the characterization of the first side-on copper-nitrosyl complex in an enzyme and to further mechanistic insights.

Chapter 3 describes the complex formed when the reduced enzyme was exposed to NO. The crystallographic results reveal that NO⁺ binds side-on to Cu(I) to form Cu(I)-NO⁺ or Cu(II)-NO⁻, the latter of which is consistent with EPR spectroscopic data (Figure 3-4). However, the crystallographically observed system has one extra electron when compared to the proposed catalytic intermediate. A revised catalytic mechanism is proposed with the assumption that the NO-exposed type 2 copper in either oxidation state would result in a structurally similar side-on copper-nitrosyl (Figure 3-5). This assumption is also supported by DFT calculations where the side-on coordination to Cu(I) is energetically favored to the end-on binding. In the reverse reaction, NiR binds NO and oxidizes it to nitrite. As expected, when an oxidized AfNiR crystal was exposed to NO, nitrite was observed bound to the type 2 Cu(II). The observation of a
nitrite molecule bound at the active site of AfNiR indicates that the copper-nitrosyl intermediate could not be trapped, likely due to the fact that the enzyme remains active in the crystal.

Chapter 4 focuses on the interactions of two AfNiR variants with NO. The H145A variant has a type 1 Cu(I) ligand mutated from a histidine to an alanine, which results in an elevated reduction potential and inactive enzyme (79). In the H145A variant, electron transfer to the type 1 copper site is not possible thus this mutant was used to trap the catalytic Cu(I)-NO⁺ intermediate. Irrespective of the oxidation state of the type 2 copper, spectroscopic and crystallographic results indicate that the H145A variant forms the same stable species formally assigned as Cu(II)-NO⁻. The most likely explanation for these observations is multiple reactions between NO and the type 2 copper. These multiple reactions are possibly due to the prolonged exposure to excess NO (~20 min). In the substrate- and product-bound wild-type AfNiR, the Asp98 forms a hydrogen bond to both the substrate and product. In the D98N variant, the lack of a hydrogen bond with the substrate results in 100-fold decrease in enzymatic activity (78, 79). Exposing D98N crystals to NO results in disorder in both the side chain of Asn98 and NO, and further supports the idea of the importance of Asp98 in stabilizing the bound NO to the copper.

So far, only two other groups have published data on the characterization of a copper-nitrosyl intermediate in NiR. EPR-ENDOR studies on the Cu(I)-NO system generated by either a single turnover experiment or a short (<1 min) exposure to exogenous NO resulted in a species formally described as Cu(I)-NO⁺ (113). However, it is unclear how a Cu(I) can bind NO⁺ without immediate electron transfer to the radical. Also, the presence of a 2-fold molar excess of reductant (PMS) in these experiments could result in additional side reactions (113). The second group attempted to characterize a copper-nitrosyl in NiR crystallographically, using the AfNiR copper-nitrosyl crystal structure (84) to interpret a complex mixture of substrate and product.
bound to AcNiR (124). The crystals were grown and kept in air and it is not clear how a Cu(I)-
NO• complex can exist in an oxic environment as suggested by the authors (124). Thus, the
structural details of the copper-nitrosyl formed by NiRs during catalysis remain controversial and
a definitive experiment is elusive.

6.1.1 Complexity of the NiRs system

Several aspects of working with NiR have been obstacles to mechanistic investigation. First, the product of the reaction is NO – a gas, highly reactive with O2. Therefore, most studies with respect to product formation and release have to be performed anaerobically. In addition, NiR has two copper centers. The active site type 2 copper is silent to UV-visible spectroscopy and many of the spectroscopic tools used to investigate the mechanism of the heme cd1NiR are not accessible. The copper sites are connected by a His-Cys bridge for efficient electron transfer. Thus, isolating interactions of NO with either the type 1 or type 2 copper sites, in either oxidation state is challenging. Additional reactions of NiR with O2 to produce H2O2 (125), reduction of NO to N2O (37) and the existence of a fully reduced inactive state (66) contribute to the complexity of working with NiR.

6.2 Revised catalytic mechanism for NiRs

Ruggiero et al. proposed a catalytic scheme for NiRs in which, regardless of the initial oxidation state of the type 2 copper, upon exposure to nitrite, the same Cu(I)-NO2− intermediate is formed (67). Wijma et al. supported this hypothesis by demonstrating that AfNiR undergoes a random-sequential mechanism with respect to nitrite and the oxidation state of the type 2 copper (66). These experiments show that nitrite can bind to either reduced or oxidized type 2 copper and that the catalytic path is dependent on nitrite concentrations and pH (66). In accordance with these results, the catalytic mechanism for NiR was adapted (Figure 6-1). In the mechanism, the
resting state of the enzyme is oxidized with a water molecule bound to the type 2 Cu(II) (step 1). The water/hydroxyl is displaced by nitrite either before or after electron transfer from the type 1 copper site (step 2). Rearrangement occurs at the active site and a copper-nitrosyl intermediate is formed (step 3). Even though cupric-nitrooxyls of AfNiR were shown to be side-on (Figure 3-1, Figure 4-1), these are not the actual reaction intermediates and the mechanism is drawn with the N atom as a copper ligand and the O atom close but not coordinating to the metal. The orientation of the NO molecule in the proposed mechanism is assigned such that the nitrogen atom (carrying the positive charge) would be closer to the side chain of Asp98 (negatively charged) and the NO molecule is stabilized by the formation of a salt bridge with Asp98. How NO is released from the copper is not known. NO either diffuses away from the active site or the Cu(I)-NO$^+$ intermediate is unstable and a water is able to displace NO from the active site (step 4).

6.3 Inhibition of AfNiR

Several small molecules were tested for their ability to inhibit AfNiR (Chapter 5). The inhibition studies show that none of the tested small molecules were potent inhibitors of the enzyme. The strongest inhibitor was azide with a $K_{ic}$ of 2 mM. These results suggest that the enzyme is highly specific for its substrates and that it is able to discriminate against small molecules with the use of active site residues. This specificity is useful for an enzyme located in the periplasm where small molecules such as $N_2O$, CHOO$^-$ and NO$^-$ are present.

Crystallographic data show that all inhibitors can bind to the active site copper of AfNiR. However, the kinetic studies demonstrate that only azide is a competitive inhibitor. Formate and acetate perform mixed inhibition, and nitrate is an uncompetitive inhibitor to AfNiR. The ability
of formate and nitrate to bind to forms of the enzyme other than the resting state could be useful for regulating the activity of NiR \textit{in vivo}.

6.4 Future work

In 1994 Jackson \textit{et al.} (37) showed that at a concentration of 80 nM NO is an inhibitor to NiR; the inhibitory effects of NO have not been explored further. More importantly, the inhibitory effects of NO with the use of a biological electron donor have not been studied previously. One area for future research is the direct quantification of NO product formation. So far NiR activity has been measured by the disappearance of nitrite (using the Griess reagent), the reoxidation of an electron donor such as pseudoazurin (spectroscopically) or by direct electrochemistry. These methods do not determine quantitatively the amount of NO produced. In addition, a technical challenge associated with these experiments has been the availability of equipment sensitive enough to measure NO in the nM range.

EPR is a powerful technique that could aid in understanding the interactions of NO with NiR. All the data to date suggest time-dependent interactions of NO with NiR. NiR protein samples can be exposed to NO and frozen in liquid nitrogen. Sealing the EPR tube can allow for thawing of the sample and letting the interaction to proceed for longer periods of time without exposing the protein to air. A single EPR sample can be used for the collection of multiple spectra at different temperatures. Furthermore, EPR experiments show that NO can be trapped to a reduced wild-type NiR. Thus, using labelled $^{15}$NO could further support my proposal of the source of the signal. Lowering the temperature of the EPR data collection has also been suggested as a useful means to measure the fast relaxation of the Cu(I)-NO complex.
Figure 6-1 Overall catalytic mechanism for NiRs. See text for details.
References


