CHARACTERIZATION OF NOSX FROM ACHROMOBACTER CYCLOCLASTES

AND CO-CRYSTALLIZATION OF N₂OR AND PAZ

by

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Fumihiro Ijima

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TABLE OF CONTENTS

1. INTRODUCTION .............................................................. ................................................... .....................1

Nitrogen Cycle and Denitrification .......................................................... ................................................... ..................1
Metalloenzymes of Denitrification .......................................................... ................................................... .................3
Nitrate Reductase (NaR) .................................................................................................................. .................4
Nitrite Reductase (NiR) .................................................................................................................. .................6
Nitric Oxide Reductase (NOR) ...................................................................................................... .....10
Nitrous Oxide Reductase (N$_2$OR) ................................................................................................. 14
Pseudoazurin (PAz) .................................................................................................................. .................23
Accessory Proteins for N$_2$OR ........................................................................................................... 26
NosR as the Regulator of the Nos Gene ............................................................................................ 29
NosD, NosF, and NosY as the Cu$_2$ Site Assembly System .......................................................... 31
Copper Chaperon Protein NosL ........................................................................................................ 32
Flavoprotein NosX .......................................................................................................................... 34
Other Nos Proteins .......................................................................................................................... 36
Research Goals .................................................................................................................................. 39

2. PRODUCTION AND PURIFICATION OF NOSX: GENE STRUCTURE
REFINEMENT, AND OPTIMIZATION OF THE CULTURE AND
PURIFICATION CONDITIONS FOR NOSX .................................................................................. 40

Introduction ....................................................................................................................................... 40
Materials and Methods .................................................................................................................... 41
Growth and Purification of NosX ........................................................................................................ 41
Promoter Refinement for NosX .......................................................................................................... 43
Subcloning of nosX Gene from pMLClustXst ........................................................................... 43
Isolation of Promoters of AcNiR and AcPAz .............................................................................. 46
Assembly of nosX Gene and Promoters of AcNiR and AcPAz on p5 plasmid ......................... 47
Optimization of Culture Conditions for NosX ................................................................................ 49
Optimization of Purification Conditions for NosX ......................................................................... 50
Results .............................................................................................................................................. 51
Promoter Refinement for NosX ........................................................................................................ 51
Optimization of Culture Conditions for NosX ................................................................................ 53
Optimization of Purification Conditions for NosX ......................................................................... 58
Discussion ......................................................................................................................................... 59
Promoter Refinement for NosX ........................................................................................................ 60
Culture Conditions for NosX ............................................................................................................ 62
Purification Conditions for NosX ....................................................................................................... 65
Conclusions ....................................................................................................................................... 66
### TABLE OF CONTENTS - CONTINUED

#### 3. CHARACTERIZATION OF NOSX FROM *ACHROMOBACTER CYCLOCLASTES*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>68</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>69</td>
</tr>
<tr>
<td>- Confirmation of the DNA Sequence of <em>nosX</em> Gene</td>
<td>69</td>
</tr>
<tr>
<td>- Molecular Weight and Quaternary Structure of <em>AcNosX</em></td>
<td>69</td>
</tr>
<tr>
<td>- Cofactor Identification</td>
<td>70</td>
</tr>
<tr>
<td>- Redox Potential</td>
<td>71</td>
</tr>
<tr>
<td>- Secondary and Tertiary Structure Predictions</td>
<td>72</td>
</tr>
<tr>
<td>- Crystallization trials</td>
<td>73</td>
</tr>
<tr>
<td>Results</td>
<td>74</td>
</tr>
<tr>
<td>- Confirmation of the DNA Sequence of <em>nosX</em> Gene</td>
<td>74</td>
</tr>
<tr>
<td>- Molecular Weight and Quaternary Structure of <em>AcNosX</em></td>
<td>74</td>
</tr>
<tr>
<td>- Cofactor Identification</td>
<td>75</td>
</tr>
<tr>
<td>- Redox Potential</td>
<td>77</td>
</tr>
<tr>
<td>- Secondary and Tertiary Structure Predictions</td>
<td>79</td>
</tr>
<tr>
<td>- Crystallization Trials</td>
<td>84</td>
</tr>
<tr>
<td>Discussion</td>
<td>85</td>
</tr>
<tr>
<td>- Identity Confirmation of Purified <em>AcNosX</em></td>
<td>85</td>
</tr>
<tr>
<td>- Characterization of the Cofactor in <em>AcNosX</em></td>
<td>85</td>
</tr>
<tr>
<td>- Structural Aspects of <em>AcNosX</em></td>
<td>87</td>
</tr>
<tr>
<td>Conclusions</td>
<td>90</td>
</tr>
</tbody>
</table>

#### 4. INVESTIGATIONS ON THE FUNCTION OF NOSX FROM *ACHROMOBACTER CYCLOCLASTES*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>91</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>93</td>
</tr>
<tr>
<td>- Expression and Purification of Pseudoazurin from <em>Achromobacter cycloclastes</em></td>
<td>93</td>
</tr>
<tr>
<td>- Expression and Purification of <em>N₂OR</em> from <em>Achromobacter cycloclastes</em></td>
<td>95</td>
</tr>
<tr>
<td>- Electron Transfer from <em>AcNosX</em> to <em>AcN₂OR</em></td>
<td>98</td>
</tr>
<tr>
<td>- Regeneration of the Cu₂⁺ State in <em>AcN₂OR</em> with <em>AcNosX</em></td>
<td>101</td>
</tr>
<tr>
<td>- Electron Transfer from Reduced <em>AcNosX</em> to Oxidized <em>AcPAz</em></td>
<td>102</td>
</tr>
<tr>
<td>- Catalytic Reaction of <em>AcN₂OR</em> with Reduced <em>AcNosX</em></td>
<td>102</td>
</tr>
<tr>
<td>- Identification of Inhibitory Factor for the <em>N₂OR</em> Catalytic Cycles with <em>AcPAz</em></td>
<td>103</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS - CONTINUED

- AcNosX Stabilization in Solution ................................................................. 104
- Results .................................................................................................................. 105
- Electron Transfer from AcNosX to AcN₂OR .................................................... 105
- Regeneration of the Cu₂⁺ State in AcN₂OR with AcNosX .................................... 111
- Electron Transfer from Reduced AcNosX to Oxidized AcPAz .............................. 113
- Catalytic Reaction of AcN₂OR with Reduced AcNosX ......................................... 114
- Identification of Inhibitory Factor for the N₂OR Catalytic Cycles with AcPAz ....... 117
- AcNosX Stabilization in Solution ........................................................................ 121
- Discussion ......................................................................................................... 122
- Function of AcNosX as an Electron Donor for AcN₂OR ....................................... 122
- Function of AcNosX for Regeneration of the Cu₂⁺ State in AcN₂OR ..................... 126
- Function of AcNosX as an Electron Donor for AcPAz ......................................... 128
- Identification of Inhibitory Factor for the N₂OR Catalytic Cycle ......................... 128
- Function of AcNosX in the N₂OR Catalytic Cycle .............................................. 133
- Stability of AcNosX in Vitro ............................................................................ 134
- Substrate Bound N₂OR Structure from Pseudomonas stutzeri ............................ 136
  - New Hypothesis on the Electron Transfer from Cu₄ to Cu₂⁺ ............................. 136
  - New Definition of the Cu₂⁺ State ....................................................................... 137
- Conclusions ....................................................................................................... 138

5. CO-CRYSTALLIZATION OF N₂OR AND PSEUDOAZURIN FROM 
   
   ACHROMOBACTER CYCLOCLASTES .................................................................. 140

- Introduction ....................................................................................................... 140
- Materials and Methods ..................................................................................... 142
  - Screening of Crystallization Conditions .......................................................... 142
  - Optimization of Crystallization Conditions ..................................................... 142
  - Crystal Check for Protein or Salt .................................................................... 143
  - X-ray Measurements of the Co-crystals .......................................................... 144
  - Crosslink of AcN₂OR and AcPAz .................................................................. 146
- Results ............................................................................................................... 148
  - Screening and Optimizations of Crystallization Conditions ............................ 148
  - Crystal Check for Protein .............................................................................. 151
  - X-ray Measurements of the Co-crystals .......................................................... 152
  - Crosslink of AcN₂OR and AcPAz ................................................................. 155
# TABLE OF CONTENTS - CONTINUED

Discussion .......................................................................................................................... 157
Conclusions ....................................................................................................................... 160

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS ........................................... 161

APPENDICES .................................................................................................................... 164

APPENDIX A: Supporting Information for Chapter 1 .................................................. 165
APPENDIX B: Supporting Information for Chapter 2 .................................................. 168
APPENDIX C: Supporting Information for Chapter 3 .................................................. 171
APPENDIX D: Supporting Information for Chapter 4 .................................................. 174

REFERENCES CITED ....................................................................................................... 181
LIST OF TABLES

Table                           Page

1.1. Structural types of N_2OR variants.................................................................38

2.1. Variables tested for effects on the yield of NosX ........................................50

2.2. The NosX yields from the refined expression systems                      
            with the LB from RPI........................................................................52

2.3. The NosX yields from the refined expression systems                      
            with the LB from Fisher.....................................................................53

2.4. The NosX yields with different LB media brands (RPI vs. Fisher).........54

2.5. The NosX yields with or without riboflavin.............................................55

2.6. The NosX yields with different shaking speeds (100 vs. 250 rpm).........55

2.7. NosX yields following 24 or 48 hours culture post induction................56

2.8. NosX yields from the original and optimized                      
            culture conditions.........................................................................58

3.1. Secondary structure predictions of AcNosX by JUFO, 
            PSIpred, and PAPIA ........................................................................80

4.1. Spin quantification of EPR spectra of AcN_2OR.......................................109

4.2. Redox state assignments of each form of AcN_2OR .................................110

4.3. AcPAz oxidation during the N_2OR catalytic cycle in                      
            various buffers.............................................................................119

5.1. The initial crystallization conditions for AcN_2OR-AcPAz co-crystal......148

5.2. Band intensities on the SDS-PAGE gel in Figure 5.9 ..............................157
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. A schematic view of the nitrogen cycle</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Crystal structures of nitrate reductase A complex from <em>E. coli</em></td>
<td>5</td>
</tr>
<tr>
<td>1.3. Crystal structures of AcCuNiR and cd1NiR from <em>Paracoccus pantotrophus</em></td>
<td>6</td>
</tr>
<tr>
<td>1.4. Coordination around the Cu sites in AcNiR</td>
<td>7</td>
</tr>
<tr>
<td>1.5. The proposed catalytic mechanism for AcCuNiR</td>
<td>9</td>
</tr>
<tr>
<td>1.6. Crystal structure of nitric oxide reductase from <em>Pseudomonas aeruginosa</em></td>
<td>11</td>
</tr>
<tr>
<td>1.7. Proposed structures of hyponitrite intermediate in <em>trans</em> - and <em>cis</em>- mechanism for NOR</td>
<td>13</td>
</tr>
<tr>
<td>1.8. Crystal structure of a monomeric form of AcN2OR and a schematic model of the dimeric form of AcN2OR</td>
<td>15</td>
</tr>
<tr>
<td>1.9. Coordinations around the CuA and CuZ sites of AcN2OR</td>
<td>16</td>
</tr>
<tr>
<td>1.10. Calculated CuZ–N2O complex and proposed catalytic mechanism for AcN2OR</td>
<td>20</td>
</tr>
<tr>
<td>1.11. [4Cu:2S] form of the CuZ site in N2OR from <em>Pseudomonas stutzeri</em></td>
<td>22</td>
</tr>
<tr>
<td>1.12. Crystal structure of AcPAz and coordination environment of the Cu center</td>
<td>23</td>
</tr>
<tr>
<td>1.13. Calculated docking structures of AcN2OR and AcPAz, and putative electron transfer pathways from the T1Cu of AcPAz to the CuA of AcN2OR</td>
<td>25</td>
</tr>
<tr>
<td>1.14. The nos cluster from <em>Achromobacter cycloclastes</em></td>
<td>26</td>
</tr>
<tr>
<td>1.15. Distribution of the Nos proteins from <em>Achromobacter cycloclastes</em></td>
<td>27</td>
</tr>
<tr>
<td>1.16. Structural elements of unprocessed NosR and mutated NosR</td>
<td>30</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES – CONTINUED

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.17. NMR structure of apo-AcNosL</td>
<td>34</td>
</tr>
<tr>
<td>1.18. The nos gene cluster from <em>Wolinella succinogenes</em> and distribution of the Nos proteins</td>
<td>37</td>
</tr>
<tr>
<td>2.1. Schematic view of the assembling process of a new NosX expression system</td>
<td>44</td>
</tr>
<tr>
<td>2.2. NosX yield and cell amount vs. A&lt;sub&gt;600&lt;/sub&gt; at induction</td>
<td>57</td>
</tr>
<tr>
<td>2.3. SDS-PAGE and western blot for NosX purification</td>
<td>59</td>
</tr>
<tr>
<td>3.1. Quaternary structure analysis of AcNosX with a gel filtration column</td>
<td>75</td>
</tr>
<tr>
<td>3.2. Normalized fluorescence spectra of AcNosX cofactor, FAD and FMN with or without PDE</td>
<td>76</td>
</tr>
<tr>
<td>3.3. Stepwise photo-reductions of AcNosX and anthraquinone 2-sulfonate</td>
<td>78</td>
</tr>
<tr>
<td>3.4. Titration measurement of redox potential of AcNosX</td>
<td>79</td>
</tr>
<tr>
<td>3.5. AcNosX model based on ApbE and surface electrostatic potential</td>
<td>81</td>
</tr>
<tr>
<td>3.6. Hydrophobicity of the AcNosX model</td>
<td>81</td>
</tr>
<tr>
<td>3.7. Sequence alignment of NosX proteins and ApbE</td>
<td>82</td>
</tr>
<tr>
<td>3.8. Distribution of the conserved amino acids and potential FAD binding residues in the AcNosX model</td>
<td>83</td>
</tr>
<tr>
<td>3.9. Pictures of putative crystals from condition #1</td>
<td>84</td>
</tr>
<tr>
<td>4.1. Schematic view of sample preparation for the EPR measurements</td>
<td>99</td>
</tr>
<tr>
<td>4.2. UV/Vis spectra of reduced AcNosX, oxidized AcN&lt;sub&gt;2&lt;/sub&gt;OR, and AcNosX reduced AcN&lt;sub&gt;2&lt;/sub&gt;OR</td>
<td>106</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES – CONTINUED

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3</td>
<td>UV/Vis spectra and differential spectrum of AcN₂OR samples for EPR</td>
<td>107</td>
</tr>
<tr>
<td>4.4</td>
<td>EPR spectra of AcN₂OR samples at 14 K</td>
<td>108</td>
</tr>
<tr>
<td>4.5</td>
<td>Photo-reduction of AcN₂OR in the absence of AcNosX</td>
<td>112</td>
</tr>
<tr>
<td>4.6</td>
<td>Photo-reduction of AcN₂OR in the presence of AcNosX</td>
<td>112</td>
</tr>
<tr>
<td>4.7</td>
<td>UV/Vis spectra of reduced AcNosX, oxidized AcPAz, and AcNosX reduced AcPAz</td>
<td>114</td>
</tr>
<tr>
<td>4.8</td>
<td>Oxidation of AcNosX during the N₂OR catalytic cycle and time course at 460 nm</td>
<td>115</td>
</tr>
<tr>
<td>4.9</td>
<td>Time course for the oxidation of free FAD during the N₂OR catalytic cycle</td>
<td>116</td>
</tr>
<tr>
<td>4.10</td>
<td>UV/Vis spectra of AcPAz oxidation during the N₂OR catalytic cycle with buffer #1 and time course at A₅₉₄ in buffer #1 - 3</td>
<td>117</td>
</tr>
<tr>
<td>4.11</td>
<td>Time course for the oxidation of AcPAz during the N₂OR catalytic cycle in buffer #1 - 5</td>
<td>118</td>
</tr>
<tr>
<td>4.12</td>
<td>Time course for the oxidation of AcPAz during the N₂OR catalytic cycle in buffer #6 and 7</td>
<td>119</td>
</tr>
<tr>
<td>4.13</td>
<td>The N₂OR activity in buffer #1, 2, and 6</td>
<td>120</td>
</tr>
<tr>
<td>4.14</td>
<td>UV/Vis spectra of AcNosX with spermine, NaCl, and flowthrough</td>
<td>121</td>
</tr>
<tr>
<td>4.15</td>
<td>The Cuₐ, Cu₉, and chloride binding sites in the C-terminal domain of AcN₂OR (iodide bound form)</td>
<td>132</td>
</tr>
<tr>
<td>5.1</td>
<td>Crystals obtained from conditions #1 - 4</td>
<td>149</td>
</tr>
<tr>
<td>5.2</td>
<td>Crystal shape transition with 150 and 250 mM lithium sulfate (condition #1)</td>
<td>150</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>5.3. Crystals obtained from modified condition #3</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>5.4. Crystals obtained from modified condition #4</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>5.5. Crystal checks with the Izit dye and SDS-PAGE for condition #1 and #4</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>5.6. Diffractions from the crystals with flash and overnight introduction of cryoprotectant</td>
<td>154</td>
<td></td>
</tr>
<tr>
<td>5.7. SDS-PAGE and band quantification for AcN_{2}OR-AcPAz crosslink</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>5.8. AcN_{2}OR-AcPAz crosslink with activated AcPAz or AcN_{2}OR</td>
<td>156</td>
<td></td>
</tr>
<tr>
<td>5.9. AcN_{2}OR-AcPAz crosslink in the coupling buffer with or without NaCl</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>5.10. Expected crosslink sites of AcPAz and AcN_{2}OR</td>
<td>159</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

A. cycloclastes or Ac: Achromobacter cycloclastes
AA: Atomic absorption
ABC: ATP binding cassette
AcN2OR: Nitrous oxide reductase from Achromobacter cycloclastes
AcNosX: NosX from Achromobacter cycloclastes
AcPAnz: Pseudoazurin from Achromobacter cycloclastes
Amp: Ampicillin
APBS: Adaptive Poisson-Boltzmann solver
AS: Anthraquinone 2-sulfonate
ATP: Adenosine triphosphate
BCA: Bicinchoninic acid
BjNosX: NosX from Bradyrhizobium japonicum
BLAST: Basic local alignment search tool
BSA: Bovine serum albumin
Buffer P: 100 mM phosphate, 150 mM NaCl, 10 % glycerol, pH 7.0
Buffer PE: 100 mM phosphate, 150 mM NaCl, 10 % glycerol, and 10 mM EDTA, pH 7.0
Buffer W: 100 mM Tris, 150 mM NaCl, 10 % glycerol, and 1 mM EDTA, pH 8.0
BV: Benzyl viologen
CASH: Carbohydrate binding proteins and sugar hydrolases
CBB: Coomassie Brilliant Blue
CD: Circular dichroism
cd1NiR: Heme cd1 containing nitrite reductase
CuNiR: Cu containing nitrite reductase
CV: Column volume
DFT: Density functional theory
DT: Dithionite
E. coli: Escherichia coli
EDC: 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
EDTA: Ethylenediaminetetraacetic acid
EPR: Electron paramagnetic resonance
ESI: Electrospray ionization
FAD: Flavin adenine dinucleotide
FMN: Flavin mononucleotide
Gm: Gentamicin
GST: Glutathione S-transferase
IPTG: Isopropyl β-D-1-thiogalactopyranoside
LMCT: Ligand-metal charge transfer
M. hydrocarbonoclasticus: Marinobacter hydrocarbonoclasticus
MBP: Maltose binding protein
MPD: 2-methyl 2,4-pentanediol
MV: Methyl viologen
LIST OF ABBREVIATIONS – CONTINUED

MW: Molecular weight
N\textsubscript{2}OR: Nitrous oxide reductase
Nap: periplasmic nitrate reductase
NaR: Nitrate reductase
Nas: Assimilatory nitrate reductase
NHS: N-hydroxysuccinimide
NiR: Nitrite reductase
NOR: Nitric oxide reductase
p5: pBBR1MCS-5
PAz: Pseudoazurin
PCR: Polymerase chain reaction
PDB: Protein databank
PDE: phosphodiesterase I
\textit{PdNosX}: NosX from \textit{Paracoccus denitrificans PD1222}
PEG: Polyethylene glycol
PEG mme: PEG monomethylether
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
\textit{SmNosX}: NosX from \textit{Sinorhizobium meliloti}
SSRL: Stanford Synchrotron Radiation Lightsource
T1Cu: Type I copper
T2Cu: Type II copper
\textit{W. succinogenes} or \textit{Ws}: \textit{Wolinella succinogenes}
WT: wild type
Nitrous oxide (N\textsubscript{2}O) is known as a greenhouse gas and an ozone-depleting substance. Denitrifying bacteria such as \textit{Achromobacter cycloclastes} emit N\textsubscript{2}O into the atmosphere during the denitrification process. Up to seventy percent of the total N\textsubscript{2}O emission on the earth is derived from this process. N\textsubscript{2}O is a very stable substance and its lifetime in the atmosphere is 114 years. Investigation of N\textsubscript{2}O reductase (N\textsubscript{2}OR) and its accessory proteins may ultimately be useful in the design of catalytic systems, either biological or chemical, for the control of N\textsubscript{2}O level.

The first aim of this research is to characterize NosX, which is one of the accessory proteins for N\textsubscript{2}OR. Although NosX was shown to be involved in the activation of N\textsubscript{2}OR, the structure and function of NosX and the mechanism of NosX on N\textsubscript{2}OR activation are still unknown. Furthermore, NosX has never been isolated and probed. In this study, culture conditions for NosX expression in \textit{A. cycloclastes} (AcNosX) were optimized to obtain adequate amounts of AcNosX (chapter 2), and AcNosX was characterized with UV/Vis, fluorescence, EPR, and MS spectrometry (chapter 3). The structure of AcNosX was investigated by computational and modeling methods (chapter 3). Crystallization trials were also undertaken. The mechanism of N\textsubscript{2}OR activation by AcNosX was studied with UV/Vis and EPR spectrometry (chapter 4). We demonstrated that AcNosX was redox active and donated electrons to the Cu sites of AcN\textsubscript{2}OR. The information revealed from this research will help to fully understand the activation mechanism of N\textsubscript{2}OR with NosX.

The second aim of the research is to investigate the interaction between AcN\textsubscript{2}OR and its potential physiological electron donor, pseudoazurin (PAz) (chapter 5). X-ray crystallographic techniques were utilized to determine the structure of the AcN\textsubscript{2}OR-AcPAz complex and conditions were identified that grew crystals that included both AcN\textsubscript{2}OR and AcPAz. Although the crystals showed diffraction patterns, further optimization is required to identify the complex structure. The information from this research will be important to elucidate the docking mechanism for electron transfer between N\textsubscript{2}OR and PAz.
CHAPTER 1

INTRODUCTION

Nitrogen Cycle and Denitrification

Nitrogen circulates within the air, soil, water, and organic matter. This process is called the nitrogen cycle and is composed of nitrogen fixation, nitrification, and denitrification as represented in Figure 1.1 (1; 2).

![Figure 1.1. A schematic view of the nitrogen cycle.](image)

Denitrification is a process that reduces nitrate ($\text{NO}_3^-$) to dinitrogen ($\text{N}_2$) in four steps ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) (3) and is a respiratory system that uses nitrogen oxides as electron sinks under anaerobic/micro-aerobic conditions.
Denitrification is performed by bacteria and archaea in soils, rivers, oceans, and under extreme environments (e.g. high pressure, pH, and/or temperature) (4). The denitrification process is important because it releases fixed nitrogen to the atmosphere, ultimately balancing nitrogen distribution among air, soil, and water (5). Each step in the denitrification process is catalyzed by distinct metalloenzymes (see next section).

Denitrification directly influences the environment (6). Denitrifying bacteria such as *Achromobacter cycloclastes* (*Ac*) reduce surplus nitrogen oxides in contaminated soil and water. It is reported that nitrate and nitrite from fertilizer are the major water pollutants, which are leached mainly from farmlands (6; 7). From this perspective, it is significant to study the bacterial denitrification pathway that can effectively decrease excess nitrogen oxides in the environment.

Denitrifying bacteria may also emit nitrous oxide (N₂O) into the atmosphere during this process (Figure 1.1). It is estimated that ~ 70 % of the total N₂O emission on the earth is derived from the denitrification process (8). To date, N₂O is the third most abundant (9 %) greenhouse gas in the atmosphere following carbon dioxide (72 %) and methane (18 %) (6; 9). Although the abundance ratio of N₂O in the atmosphere is less significant than CO₂, N₂O still has a large impact on the environment since the global-warming potential of N₂O is ~ 300 times higher than that of CO₂ (10; 11). N₂O is also known as an ozone-depleting substance (6). N₂O is reported to be the most alarming ozone-depleting substance since N₂O emission is expected to be the most abundant among ozone-depleting compounds throughout the 21st century (10). In addition, N₂O is very stable and its lifetime in the atmosphere is 114 years (10). Thus, it is important to
study the mechanism of N₂O reduction by N₂OR and its accessory proteins for possible biological or chemical catalytic systems to remove N₂O from the atmosphere.

Metalloenzymes of Denitrification

The equation below (Eq.1) represents the denitrification process (7):

$$2\text{NO}_3^- + 10e^- + 12H^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O} \quad (\text{Eq. 1})$$

This equation is a summation of the four successive reactions below (Eq. 2 - 5):

$$\text{NO}_3^- + 2e^- + 2H^+ \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \quad (\text{Eq. 2})$$

$$\text{NO}_2^- + e^- + 2H^+ \rightarrow \text{NO} + \text{H}_2\text{O} \quad (\text{Eq. 3})$$

$$2\text{NO} + 2e^- + 2H^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \quad (\text{Eq. 4})$$

$$\text{N}_2\text{O} + 2e^- + 2H^+ \rightarrow \text{N}_2 + \text{H}_2\text{O} \quad (\text{Eq. 5})$$

These reactions are catalyzed by nitrate reductase (NaR) (Eq. 2), nitrite reductase (NiR) (Eq. 3), nitric oxide reductase (NOR) (Eq. 4), and nitrous oxide reductase (N₂OR) (Eq. 5), respectively (Figure 1.1). The conversion of N₂O to N₂ is exergonic ($\Delta G^{\circ} = -339.5$ kJ/mol) and energetically favorable (12).

Nitric oxide (NO) is a toxic metabolite in denitrification which is produced by NiR and detoxified by NOR (Eq. 3 & 4). The expressions and activity levels of NiR and NOR are known to be tightly regulated to keep the NO concentration low in the cell (13). In contrast, nitrous oxide (N₂O), which is another metabolite in denitrification (Eq. 4 & 5), does not show any obvious toxicity to the cell (3). Thus, there is not a strict requirement for the cell to regulate N₂OR expression and activity (14). Some denitrifying bacteria do not always express N₂OR even though they contain an intact nos gene cluster,
which codes for proteins to express active N$_2$OR (15). Also, other denitrifying bacteria can survive without the *nos* gene cluster (15). In addition, when the environment becomes anaerobic and denitrification respiration starts, the N$_2$OR expression from the *nos* gene cluster is induced far later than the expressions for the other denitrifying related reductases (NaR, NiR, and NOR) (14). Thus, N$_2$O is transiently accumulated in cultures. N$_2$OR activity in whole cells becomes obvious 20 ~ 40 hours after the conditions become anaerobic (14; 16). These observations imply that the reduction of N$_2$O does not seem to be an absolute requirement for the denitrifying organisms (14). This seems to be a factor of N$_2$O emission from the denitrifying organisms in the presence of excess nitrogen oxides, which potentially cause the environmental problems such as global warming and ozone layer destruction. An overview of each metalloenzyme will be described below.

**Nitrate Reductase (NaR)**

Nitrate reductases are molybdenum containing enzymes that catalyze the reduction of nitrate to nitrite (Eq. 2). The physiological significance of nitrate reductases for organisms has been reported as respiration (to generate energy) and assimilatory ammonification (to incorporate nitrogen into a biomolecule) (7; 17). According to the function and its location, nitrate reductases are categorized in several types such as respiratory nitrate reductase (NaR), assimilatory nitrate reductases (Nas), and periplasmic nitrate reductases (Nap). NaR is a cytoplasmic membrane bound protein and responsible for denitrification (7; 17; 18). Nas is responsible for nitrate assimilation whereas the function of Nap, which is a soluble protein located in the periplasm, is still unclear (18).
Although the molecular structure of NaR from *A. cycloclastes* (*AcNaR*) has not been identified, several crystal structures of NaR are available in the protein databank (PDB). Nitrate reductase A from *E. coli* (PDB code: 1Q16) is a cytoplasmic membrane-bound quinol-nitrate oxidoreductase (Figure 1.2) (19). According to the BLAST (Basic Local Alignment Search Tool) search, the structure of *AcNaR* is expected to be homologous to the structure in Figure 1.2.

![Figure 1.2. Crystal structures of nitrate reductase A complex (NarGHI) from *Escherichia coli.*](image)

NaR is composed of three subunits (NarG, NarH, and NarI) (Figure 1.2). NarG has one Mo binding site, which is the active center, and one [4Fe-4S] cluster (FS0). NarH includes one [3Fe-4S] cluster (FS4) and three [4Fe-4S] clusters (FS1, FS2, and FS3), and
NarI includes two $b$-type hemes ($b_p$ and $b_d$). Electrons that are required to reduce nitrate on the active site are expected to be provided from the quinol pool in the cell membrane via these redox active metal sites (quinol $\rightarrow b_d \rightarrow b_p \rightarrow FS4 \rightarrow FS3 \rightarrow FS2 \rightarrow FS1 \rightarrow FS0 \rightarrow Mo \rightarrow$ nitrate) (7). The mechanism of nitrate reduction at the active site of NaR is still under investigation.

Nitrite Reductase (NiR)

There are two distinct types of nitrite reductases that contain either copper (CuNiR) or heme (cd$_1$NiR) as cofactors. Both NiRs catalyze the same reaction, namely the reduction of nitrite ($NO_2^-$) to nitric oxide (NO) (Eq. 3). NiR is an important enzyme in agriculture since it releases nutrient elements (nitrogen oxides) present in farm land to the atmosphere (7).

Figure 1.3. Crystal structures of AcCuNiR (left) and cd$_1$NiR from Paracoccus pantotrophus (right).
CuNiR from A. cycloclastes (AcCuNiR) is a homotrimer (~ 40 kDa per monomer) and includes two copper ions (type I and II coppers) per monomer (20), whereas cd1NiR from Paracoccus pantotrophus is a homodimer (~ 60 kDa per monomer) and has one heme c and one heme d1 per monomer (21) (Figure 1.3). Both proteins are located at the periplasmic space. It is reported that typical denitrifying organisms have either CuNiR or cd1NiR exclusively (7). For instance, A. cycloclastes contains CuNiR but not cd1NiR. However, both types of NiR are observed in Pseudomonas and Alcaligenes (7). The structure and proposed mechanism of AcCuNiR will be further described.

AcCuNiR has two coppers per monomer. One copper site is classified as type I (T1Cu) and the other is type II (T2Cu) (Figure 1.3, 1.4).

![Coordination of Cu sites in AcCuNiR](image)

Figure 1.4. Coordination around the Cu sites in AcCuNiR.

The type I Cu (T1Cu) site in AcCuNiR is coordinated with two histidines (His95 and His145), one cysteine (Cys136), and one methionine (Met150) (Figure 1.4). The
geometry of T1Cu is an axially flattened tetrahedron (7) and is stable in both Cu(I) and Cu(II) states. Therefore, this Cu site is suitable for electron relay, and it transfers electrons to the active center (7; 22). The type II Cu (T2Cu) site in AcCuNiR works as the active center, and is located at the boundary surface of two monomers. This site is coordinated with three histidines (His100 and His135 from one monomer, and His306 from a neighboring monomer) and one water molecule (Figure 1.4) (7). Asp98 and His255, which are located above the copper center, are expected to be involved in the catalytic reaction (see below for detail).

The T1Cu and T2Cu centers are categorized by spectroscopic (UV/Vis and EPR) properties that are derived from ligands and its geometries around the coppers (22). In the UV/Vis spectrum, the oxidized T1Cu center (e.g. pseudoazurin from *A. cycloclastes*) shows a strong absorption peak around 600 nm due to ligand-metal charge transfer (LMCT) transitions from the thiolate group of cysteine to Cu(II) (22; 23). According to the absorption spectrum, the T1Cu protein shows a blue color and it is called a “blue copper protein” (24). Once the Cu is reduced, however, the LMCT band is abolished since the d orbitals of Cu(I) are fully occupied with electrons thereby preventing LMCT (25). In contrast, both oxidized and reduced T2Cu centers do not show the LMCT absorption peak because there is no thiolate ligand on the T2Cu site (26). In the EPR spectra, both oxidized T1Cu and T2Cu centers (e.g. NiR from *Alcaligenes xylosoxidans* and laccase from *Rhus vernicifera*) show small hyperfine splittings whereas reduced forms of the centers become EPR silent since Cu(I) is S = 0 (26; 27).
The mechanism of the catalytic reaction by CuNiR has been proposed based on the crystal structure (Figure 1.5) (28). Structural analysis of AcCuNiR reveals a channel that leads the substrate to the T2Cu active site located ~ 12 Å from the protein surface (28). Since the entry site of the channel is negatively charged, nitrite is expected to enter the active site as a protonated form (HNO₂) to avoid electrostatic repulsion (28).

![Diagram](image)

Figure 1.5. The proposed catalytic mechanism for AcCuNiR (28).

In the resting state, the substrate binding site on the T2Cu is occupied with a water molecule (Figure 1.5A). Deprotonated Asp98, protonated His255, and the T2Cu are connected with water molecules via hydrogen bonds (Figure 1.5A). When the substrate
(HNO$_2$) comes into the active center, the water molecule on the T2Cu is replaced by the substrate, and HNO$_2$ binds to the T2Cu via O-coordination (Figure 1.5B). After the substrate binds to the T2Cu site, the Cu is reduced by an electron from the T1Cu, and the coordination of the substrate is changed (Figure 1.5C). The proton on His255 is transferred to the activated substrate to form NO and H$_2$O (Figure 1.5D) (28). The product (NO) leaves from the active center and a water molecule binds to the oxidized T2Cu ion (Figure 1.5A).

In CuNiR from *Pseudomonas chlororaphis*, redox potentials of the T1Cu and T2Cu have been determined to be 298 mV and 172 mV at pH 7.6, respectively (29). According to the relative redox potentials, the electron transfer from T1Cu to T2Cu is energetically unfavorable (29). However, the redox potential of T2Cu is increased in the presence of the substrate, which facilitates the electron transfer (29). This observation indicates that there is a redox regulation mechanism by substrate binding in this NiR. In contrast, such a mechanism will not be required in AcCuNiR since the redox potentials of T1Cu and T2Cu without the substrate are already favorable for electron transfer (T1Cu = 240 mV, T2Cu = 250 mV at pH 7.0) (30).

**Nitric Oxide Reductase (NOR)**

Nitric oxide reductase is a membrane bound protein. It catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N$_2$O) (Eq. 4) (8). NORs have been categorized in three classes (cNOR, qNOR, and qCuNOR) according to the kinds of electron donor sources (electron transfer proteins or quinols) and acceptor sites (heme or Cu$_A$ sites) bound to the proteins (31). The structures of the active sites among the different classes
are expected to be homologous (7). To date, only one structure of NOR (cNOR from *Pseudomonas aeruginosa*) has been determined with X-ray crystallography (PDB code: 3O0R) (Figure 1.6) (32).

![Crystal structure of nitric oxide reductase from *Pseudomonas aeruginosa*.](image)

Figure 1.6. Crystal structure of nitric oxide reductase from *Pseudomonas aeruginosa*.

cNOR has been found in denitrifying bacteria such as *Pseudomonas stutzeri* (33), *Paracoccus denitrificans* (34), *Pseudomonas aeruginosa* (35), and *Marinobacter hydrocarbonoclasticus* (transferred from *Pseudomonas nautica*) (17). NOR from *A. cycloclastes* (AcNOR) was also expected to be categorized as a cNOR since almost all the denitrifying bacteria (except *Ralstonia eutropha* (36)) have been found to have this type of NOR to date (7). Although characterization of AcNOR has not been reported, protein sequence alignment analysis of AcNOR by Clustal W showed close similarity to those of cNORs (Appendix A: Figure S1.1 and S1.2).
cNOR is composed of two subunits, NorB and NorC (Figure 1.6). cNOR receives electrons from electron transfer proteins such as cytochrome c and/or cupredoxin (T1Cu containing electron transfer protein such as pseudoazurin) in the periplasm. NorB includes three iron sites, heme b, heme b₃, and Fe₉ while NorC has one heme c. The heme c in NorC works as an electron acceptor from the electron donor proteins in the periplasm. The heme b in NorB receives the electron from the heme c and transfers it to the binuclear active site (electron donor → Heme c → Heme b → heme b₃ and Fe₉).

qNOR has been isolated from pathogens (37). These pathogens do not have the other denitrifying related enzymes such as NaR and NiR (7). Macrophages produce NO as a part of the immunological response to kill pathogens (38). These facts imply that qNOR acts in the detoxification of NO rather than respiration to nitrogen oxides (8; 39). Since the amino acid sequence of qNOR is homologous to NorC in the N-terminal region and NorB in the C-terminus region, qNOR is hypothesized to arise through the gene fusion of NorC and NorB (7). qNOR has also been proposed to be the ancestor of all three types of NOR since the electron transfer system with quinones is thought to be older than the system with cytochromes (31). This class of NOR functions as a monomer and uses quinol for the electron source (40). This enzyme does not have a heme c site to receive electrons. Instead, the heme b directly receives an electron from the quinol pool in the membrane and transfers it to the active center.

NOR from *Bacillus azotoformans*, which is a gram-positive soil bacterium, was classified as qCuNOR (41). To date, this is the only NOR that is categorized in this class. *Bacillus azotoformans* is a denitrifier and has membrane-bound types of NaR, NiR, NOR,
and N$_2$OR (42). qCuNOR is composed of two subunits as well as cNOR. Although the catalytic subunit is homologous to NorB in cNOR, the smaller subunit that is similar to NorC has a dinuclear copper center; a Cu$_A$ site serves as the electron transfer site instead of heme c. qCuNOR can utilize both menaquinol and membrane bound cytochrome $c_{551}$ as electron sources (43). The NOR activity with menaquinol is 4 times higher than the activity using cytochrome $c_{551}$ as an electron source (43).

Several catalytic mechanisms have been proposed for NOR and these are still controversial because some of the key experimental data conflicts with one another (7). The proposed mechanisms are referred to as trans- and cis-mechanisms (Figure 1.7). In the trans-mechanism, one NO molecule binds to the heme $b_3$ site and the other NO molecule binds to the Fe$_B$ site (Figure 1.7, left). Alternatively, two NO molecules bind to the Fe$_B$ site in the cis-mechanism (Figure 1.7, right).

![Figure 1.7](image)

**Figure 1.7.** Proposed structures of hyponitrite intermediate in trans- and cis-mechanism for NOR (8).

Several studies on cNOR from *Pseudomonas aeruginosa* with EPR and ultra-rapid freeze quench techniques have shown data that are consistent with the trans-mechanism (8; 35; 44). The signals for the Fe$_B$ (II)–NO and the penta-coordinated heme $b_3$ Fe (II)–NO complexes were observed in the EPR spectra (35). The cis-mechanism is supported by a
study on cytochrome bo (heme–CuB protein) from E. coli, which data indicated two NO molecules were bound to the CuB site (45).

Nitrous Oxide Reductase (N2OR)

Conversion of N₂O to N₂ is catalyzed by N₂OR, which is the last step in the denitrification pathway (Eq.5). In the absence of oxygen, N₂O is used as the terminal electron acceptor in the anaerobic respiratory chain (4; 46). N₂O respiration has been observed in a number of species in a wide variety of environments, such as soil bacteria, phototrophic and chemolithotrophic bacteria, pathogens, marine organisms, piezophiles (high pressure-like deep sea bacteria and archaea), sulfur- and halorespirers, thermo- and psychrophilic (cold-like) bacteria, and extremophilic (extreme condition-like) archaea (4; 12; 47; 48; 49). Subsequently, the nos gene cluster that is required for the active N₂OR expression has been found in many bacteria and archaea (50; 51; 52; 53; 54). The initial research on Pseudomonas stutzeri suggested that N₂OR was a Cu containing enzyme since Cu specifically induced N₂OR expression under anaerobic conditions (4).

N₂OR has a conserved twin-arginine sequence in the N-terminal signal peptide region, which is recognized by the Tat (twin arginine translocation) pathway that transports folded proteins across the cytoplasmic membrane (50; 55). The mutation of the twin-arginine motif in N₂OR from Pseudomonas stutzeri resulted in accumulation of unprocessed N₂OR in the cytoplasm, which did not contain any copper (56). The result indicated that the CuA and CuZ sites were assembled in the periplasm. In contrast, N₂OR from Wolinella succinogenes has a sec-type signal peptide for the Sec (secretory) pathway that transports unfolded proteins through the cytoplasmic membrane (4; 51).
Most N$_2$ORs including from *A. cycloclastes* are soluble head-to-tail homodimers (Figure 1.8) located in the periplasmic space, although there have been several membrane-associated N$_2$ORs reported (57; 58). N$_2$OR is O$_2$ sensitive and exposure of N$_2$OR from *A. cycloclastes* (AcN$_2$OR) to air results in both loss of Cu content and irreversible enzymatic activity (11). However, N$_2$OR from *Marinobacter hydrocarbonoclasticus* (formerly *Pseudomonas nautica*) maintains a relatively high activity even under aerobic conditions, and what activity is lost can be reversibly restored with a standard activation process with reduced methyl viologen (MV) (55).

![Crystal structure of a monomeric form of AcN$_2$OR (59) and a schematic model of the dimeric form of AcN$_2$OR (60).](image)

Figure 1.8. Crystal structure of a monomeric form of AcN$_2$OR (59) and a schematic model of the dimeric form of AcN$_2$OR (60).
To date, N$_2$OR structures from *Marinobacter hydrocarbonoclasticus* (61), *Paracoccus denitrificans* (62), *Achromobacter cycloclastes* (59) and *Pseudomonas stutzeri* (63) have been solved. These structures are highly homologous (12). N$_2$OR is typically composed of two domains, the C-terminal domain that includes the Cu$_A$ site and the N-terminal domain that holds the Cu$_Z$ site (see below for details).

The C-terminal domain of N$_2$OR contains a cupredoxin motif that contains one Cu$_A$ center (Figure 1.9, left) (64). This domain is homologous to the Cu$_A$ center of bovine cytochrome $c$ oxidase (65). In the Cu$_A$ center in AcN$_2$OR, two copper ions are bridged by two conserved cysteines (Cys578 and Cys582), and the oxidized Cu$_A$ site exists in a mixed-valence state [Cu(1.5) – Cu(1.5)] which is energetically more favorable than the [Cu(I) – Cu(II)] state (4; 55).

Figure 1.9. Coordinations around the Cu$_A$ (left) and Cu$_Z$ sites (right) of AcN$_2$OR (59).
The Cuₐ site can exist either in an oxidized form [Cu(1.5) – Cu(1.5)] or reduced form [Cu(I) – Cu(I)] following electron transfer, whereas the [Cu(II) – Cu(II)] form has never been observed for any Cuₐ center to date (4). The Cuₐ site is expected to relay an electron from electron donors (such as pseudoazurin and cytochrome c) to the Cuₐ site (7; 55). The reorganization energy of the Cuₐ site during the redox reaction is estimated to be lower than the one of typical T1Cu site (4). Therefore, the binuclear Cuₐ sites in cytochrome c oxidase and N₂OR are thought to be more suitable for electron transfer than the mononuclear T1Cu site (4). In addition, the Cuₐ domain is important for the structural stability of N₂OR from Pseudomonas stutzeri according to results from a domain study (4). No N₂OR expression was observed when this domain was deleted, although the corresponding nosZ transcript was detected in Northern blot analysis (66). Since the redox potentials of Cuₐ in cytochrome c oxidase from murine and N₂OR from Paracoccus pantotrophus have been determined as 250 mV (67) and 260 mV (68), respectively, the redox potential of the Cuₐ site in AεN₂OR is also expected to be in this range.

The large N-terminal domain of N₂OR consists of a seven-bladed β-propeller fold which contains the Cuₐ center (Figure 1.9, right) (61). To date, the Cuₐ center has been found only in N₂OR, and it is known as the first biologically active Cu-S cluster (61; 69). The four coppers in the cluster are labeled as Cu_I (upper side of the sulfide), Cu_II (lower), Cu_III (right), and Cu_IV (left) (Figure 1.9, right). The substrate (N₂O) is expected to bind on Cu_I and Cu_IV and will be described later (70; 71). The presence of sulfide in the Cuₐ center was determined with ³⁴S isotope labeling of N₂OR from Pseudomonas stutzeri and
resonance Raman spectroscopy (72; 73). The N₂OR structure from *Paracoccus denitrificans* solved to 1.6 Å resolution confirmed that the Cu₇ site is a unique μ₄-sulfide bridged tetracopper center (74). Additionally, a chloride ion is bound close (6.2 Å) to Cu₁ in the Cu₇ site in all four N₂OR crystal structures (61; 62; 59; 63). This chloride shares one of the ligands of the Cu₁ in N₂OR from *Paracoccus denitrificans* (74). It is suggested that this chloride probably influences the redox potential of Cu₁ so that this copper is stabilized in the Cu(II) state, which is preferable for N₂O binding (74). The N-terminal domain of N₂OR is responsible for dimerization and the residues at the dimer interface are well conserved (4). The dimerization of N₂OR is facilitated by ionic and hydrophobic interactions between the monomers, and chelating interactions of two calcium ions which are located at the dimer interface (61; 74). The dimerization is thought to be essential for the catalytic reaction. The distance between the Cu₆ and Cu₇ sites within a monomer unit is 40 Å, which is too far for rapid electron transfer. However, the distance between the Cu₆ and Cu₇ sites across the dimer interface is 10 Å, implying that electron transfer reactions between subunits are favorable (Figure 1.8) (75; 76). The redox potential of Cu₇ from *Paracoccus pantotrophus* was determined to be 60 mV, which is not suitable for the electron transfer from the Cu₆ site (~ 250 mV) (68). The mechanism of electron transfer from the Cu₆ site to the Cu₇ center is still unknown.

Three redox states of N₂OR have been observed; the dithionite-reduced form, the fully reduced form, and the oxidized form (70; 77; 78). When AcN₂OR is isolated from the cell extract, dithionite is added to the sample during the purification to avoid oxidation by O₂ contamination (11). Thus, the AcN₂OR is purified as the dithionite-
reduced form and is referred to as the “as-purified” form or the resting form. In the
dithionite-reduced N$_2$OR the Cu$_Z$ site is partially reduced ([1Cu(II)•3Cu(I)], $S = 1/2$),
while the Cu$_A$ site is fully reduced (Cu(I) – Cu(I), $S = 0$) (79). The Cu$_Z$ site can be further
reduced by prolonged incubation (~ 3 hours) with reduced MV in vitro (70; 77). This
state is catalytically active and is referred to as the fully reduced form or the active form
([4Cu(I)], $S = 0$) (70). In the oxidized form of N$_2$OR, the Cu$_Z$ site is assigned to [2Cu(II)
•2Cu(I)], and is EPR silent ($S = 0$) because of antiferromagnetic coupling between the
coppers (80). This form can be observed after the fully reduced form is reacted with N$_2$O
or oxidized with potassium ferricyanide (77; 11). The Cu$_A$ site is also oxidized (Cu(1.5) –
Cu(1.5), $S = 1/2$) with potassium ferricyanide.

The catalytic mechanism of N$_2$O reduction at the Cu$_Z$ site has been proposed
based on the crystal structures and density functional theory (DFT) calculations (70; 71).
In the resting state of Cu$_Z$ ([1Cu(II)•3Cu(I)]), N$_2$O binds to Cu$_I$ and Cu$_{IV}$. The substrate is
expected to bind to the Cu$_Z$ site as the “µ-1,3-N$_2$O bridging mode” (N atom binds to Cu$_I$
and O atom binds to Cu$_{IV}$, Figure 1.10) (79). When the Cu$_Z$–N$_2$O complex is reduced to
the fully reduced form ([4Cu(I)]), the geometry of N$_2$O on the Cu$_Z$ site is altered. The
N–N bond and N–O bond distances are stretched 0.03 and 0.07 Å, respectively, and the
N–N–O bond angle bends from 180 ° to 139 ° (Figure 1.10, step 1) (70). This N$_2$O
geometry shift facilitates the two-electron transfer from Cu$_I$ and Cu$_{IV}$ to the substrate (70).
After the N–O bond is cleaved and product (N$_2$) is released (Figure 1.10, step 2), the
remaining oxygen atom from the substrate will be subsequently protonated (Figure 1.10,
step 3) and released as H$_2$O (Figure 1.10, step 5). The conserved Lys397 in N$_2$OR from

*Marinobacter hydrocarbonoclasticus* (Lys412 in AcN2OR), which is 4 Å away from the Cu$_{IV}$ atom, is proposed to facilitate the catalytic reaction by acting as a proton source (81).

Figure 1.10. Calculated Cu$_{Z}$–N$_2$O complex and proposed catalytic mechanism for N$_2$OR (7; 71).
Two states (CuZ and CuZ*) of the CuZ center have been characterized (68). The CuZ state is considered to be enzymatically active, whereas the CuZ* state is inactive and redox inert (68; 69). The CuZ* state is generated by O₂ exposure of the enzyme (68) and gene inactivation of nosR and/or nosX, as will be described later (82; 83). In AcN₂OR, both CuZ and CuZ* states seem to have almost identical absorption peaks at 640 nm in UV/Vis spectra (11; 77). In N₂OR from *Pseudomonas stutzeri*, the CuZ and CuZ* states show peaks at 382 and 384 cm⁻¹ in resonance Raman spectra, which are excited at 647 nm (73). These spectroscopic features of the CuZ and CuZ* states indicate that the structures of these states are highly similar, although the redox behavior of the states is significantly different. In N₂OR from *Marinobacter hydrocarbonoclasticus*, the CuZ* state is associated with an absorption peak at 640 nm in the UV/Vis spectrum as the protein is purified in the presence of oxygen (84). Although the CuZ* state in AcN₂OR cannot be reduced with MV, the CuZ* state in N₂OR from *M. hydrocarbonoclasticus* can be fully reduced with MV and shows almost identical activity to that of the CuZ state (84). The mechanisms of O₂ resistance and reversibility from the CuZ* to the CuZ state of N₂OR from *M. hydrocarbonoclasticus* are still unclear.

Recently, a structural relationship between the CuZ and CuZ* states was proposed in N₂OR from a crystal structure of substrate bound N₂OR from *Pseudomonas stutzeri* (63). This structure revealed the presence of an extra sulfide ion bound at the CuZ site ([4Cu:2S] form) (Figure 1.11, left) (63). The authors suggested that the CuZ and CuZ* states corresponded to the [4Cu:2S] and [4Cu:1S] forms, respectively (63). The 2nd sulfide in the CuZ site was located at the place where the substrate and inhibitor (iodide
anion) were thought to be bound (59).

They found that the 2nd sulfide was highly oxygen sensitive and was lost when the enzyme was exposed to air. This observation was consistent with the fact that the Cu∗ state was generated following O2 exposure. The N2OR structures that had been determined previously were all crystallized under aerobic conditions, whereas N2OR from *Pseudomonas stutzeri* was purified and crystallized under strictly anaerobic conditions. Therefore, it is resumed that the other N2ORs (from *Marinobacter hydrocarbonoclasticus*, *Paracoccus denitrificans*, and *A. cycloclastes*) also have the 2nd sulfide in the CuZ site under strictly anaerobic conditions. The substrate bound form of the crystal structure revealed that N2O was located between the CuA and CuZ sites (Figure 1.11, right) (59; 63). The distance between the O atom of N2O and the CuII of the CuZ site was 3.2 Å, whereas the distance between this O atom and His626, which is the ligand of...
the Cu_{A} site, is 3.5 Å. The authors propose that the substrate receives electron(s) directly from the Cu_{A} site, instead of receiving the electrons from the Cu_{Z} site as described in Figure 1.10 (7; 71).

Pseudoazurin (PAz)

Pseudoazurin is a T1Cu containing protein located in the periplasm (Figure 1.12, PDB code: 1BQR) (85). PAz works as a common electron transfer protein for the denitrifying enzymes such as CuNiR (86), cd_{1}NiR (87; 88), NOR (89), and N_{2}OR (90; 91). PAz has a mononuclear copper center, and exists in two redox states, Cu(I) (reduced state) and Cu(II) (oxidized state). Expression levels of PAz in *Paracoccus denitrificans* are regulated by the transcriptional activator FnrP, which is responsible for protein expression under anaerobic conditions and will be further described in the next section (88).

Figure 1.12. Crystal structure of AcPAz (1BQR) and coordination environment of the Cu center.
Since PAz is required to interact with several structurally different proteins for electron transfer, the donor-acceptor interactions are achieved in a “pseudo-specific” manner (87). The pseudo-specific interaction is based on a combination of a hydrophobic patch and surrounding positively or negatively charged patches on the surface of two proteins, which is less specific than a structure-based interaction (87). In the case of the N$_2$OR-PAz complex, electron transfer is facilitated by the hydrophobic patches on N$_2$OR and PAz, and several positive residues (Lys) on PAz and negative residues (Glu/Asp) on N$_2$OR that are located adjacent to the hydrophobic patches (see chapter 5) (87).

Recently, putative docking structures of AcN$_2$OR and AcPAz were calculated in our lab (91). The top three candidates of the AcN$_2$OR-AcPAz docking structures are shown in Figure 1.13A. The three AcPAz structures in orange, green and blue in Figure 1.13 indicate the binding orientations of AcPAz on AcN$_2$OR. Additionally, the electron transfer pathways from the T1Cu of AcPAz to the Cu$_A$ site of AcN$_2$OR were also calculated based on the three docking structures (Figure 1.13B). The calculations indicated that AcPAz interacted with AcN$_2$OR through the hydrophobic patch as mentioned above. The calculated electron transfer pathways from AcPAz to AcN$_2$OR were consistent with the previous studies on N$_2$OR and PAz from Paracoccus pantotrophus (92) and on N$_2$OR and cytochrome $c_{552}$ from M. hydrocarbonoclasticus (93).

Pseudoazurin from Paracoccus pantotrophus (formerly Thiosphaera pantotropha) donates electrons to N$_2$OR in vitro (90). Additionally, several possible docking sites for electron transfer between N$_2$OR and PAz from Paracoccus pantotrophus were calculated
and the plausible electron transfer pathways from PAz to CuA in N2OR are identified (92). In additional investigations in *Paracoccus pantotrophus*, oxidized PAz is shown to dramatically enhance the nitrite reduction by cd1NiR and facilitate the release of product NO (94). This result shows that PAz works not only as an electron donor but also as a “facilitator” of its binding partner’s function, as possibly mediated by electron exchange between Cu in PAz and heme in cd1NiR.

![Calculated docking structures of AcN2OR (2IWF) and AcPAz (1BQR), and (B) putative electron transfer pathways from the T1Cu of AcPAz to the CuA of AcN2OR (91).](image)

Figure 1.13. (A) Calculated docking structures of AcN2OR (2IWF) and AcPAz (1BQR), and (B) putative electron transfer pathways from the T1Cu of AcPAz to the CuA of AcN2OR (91).
Accessory Proteins for N\textsubscript{2}OR

Nitrous oxide reductase (N\textsubscript{2}OR) requires accessory proteins to be expressed and activated (12; 55). Although Nos proteins have been characterized in several denitrifying bacteria, the functions of the accessory proteins on N\textsubscript{2}OR are still somewhat unclear. For example, NosX from *Paracoccus denitrificans* was shown to be involved in the activation of N\textsubscript{2}OR *in vivo* (83). However, the mechanism by which NosX activates N\textsubscript{2}OR is still unknown. Investigations on the roles of each accessory protein are important in order to understand the assembly of the cofactors (Cu\textsubscript{A} and Cu\textsubscript{Z}) in N\textsubscript{2}OR and the activation process of N\textsubscript{2}OR for reduction of N\textsubscript{2}O. This study is mainly focused on the expression and characterization for function and structure of NosX from *Achromobacter cycloclastes* (AcNosX) *in vitro*. An overview of the accessory proteins for N\textsubscript{2}OR will be described.

The nitrous oxide reductase gene cluster (*nos* cluster) in *A. cycloclastes* was identified in 1998 (53). Seven protein coding regions including *nosR, nosZ* (structural N\textsubscript{2}OR gene), *nosD, nosF, nosY, nosL*, and *nosX* were identified and arranged in four transcriptional units consisting of *nosR, nosZ, nosDFYL*, and *nosX* (Figure 1.14) (52).

![Figure 1.14. The nos cluster from Achromobacter cycloclastes.](image-url)

The Nos proteins from these genes are thought to be expressed across the periplasmic and cytoplasmic regions (Figure 1.15). The proteins that structures have already been solved
are shown with the structures whereas the proteins without the structural information are represented by circles (soluble proteins) and squares (membrane bound proteins).

The nos clusters have also been isolated from denitrifying bacteria such as *Pseudomonas stutzeri* (nosRZDFYL) (95; 50), *Paracoccus denitrificans* (nosRZDFYLX) (96), *Sinorhizobium meliloti* (nosRZDFYLX) (97; 52), *Pseudomonas aeruginosa* (nosRZDFYL) (98), and *Bradyrhizobium japonicum* (nosRZDFYLX) (99). The nosRZDFYL gene set is the most frequently observed sequence (55). In several species, the nosX gene has not been identified. To date (as of January 2012), 351 of nosZ (N₂OR) genes have been registered in the NCBI database, whereas only 92 of nosX genes have been identified in the nos clusters.

Figure 1.15. Distribution of the Nos proteins from *Achromobacter cycloclastes*. 
In the non-denitrifying organism *Pseudomonas putida*, it is shown that the expressions of *nosRZDFY* genes, but not *nosL* and *X*, are sufficient to construct a holo-N$_2$OR (100). It is believed that NosD, NosF, and NosY are responsible for biosynthesis of the Cu$_2$Z site (50). The functions of the Nos proteins will be described in this section.

Under denitrifying conditions, *nos* gene expression is activated by transcriptional factors that are the FNR-CRP family proteins. It has been shown that three transcriptional factors are involved in the *nos* gene expression; Fnr, Dnr, and Nnr. Fnr is responsible for the protein expression in response to anaerobic conditions, whereas Dnr and Nnr upregulate the protein expression in the presence of NO (101). Dnr in *Pseudomonas aeruginosa* and DnrD in *Pseudomonas stutzeri* regulate the *nos* gene expression (55; 102; 103), whereas both FnrP and NNR equally contribute to the whole cell N$_2$OR activity in *Paracoccus denitrificans* (101). In *A. cycloclastes*, it is still unclear which transcriptional factor regulates the *nos* cluster expression.

According to the mechanisms for the *nos* gene expression, *nosR* promoter activity is increased with NO, but not N$_2$O, in *Pseudomonas aeruginosa* (98). Additionally, NO shows strong upregulation for the expressions of the *nosR*, *nosZ*, and *nosDFYL* in *Pseudomonas stutzeri* (103). The NO signal transduction pathway in *Pseudomonas stutzeri* is summarized as follows; NO produced by NiR activates DnrD, which facilitates the expression of *nosR*, and NosR further stimulates expression of *nosZ* and the *nosDFYL* operon: NO $\rightarrow$ DnrD $\rightarrow$ *nosR*/NosR $\rightarrow$ *nosZ* and *nosDFYL* (55; 50). It is still unclear whether the expression of *nosX* is also upregulated by the transcriptional factors because the effects of transcriptional factors on *nos* cluster expression have only been investigated
on the bacteria that do not have the nosX gene. Even though anaerobic conditions are required for the nos gene expression and N$_2$OR activity, a low level of O$_2$ is also required for maximum nos gene expression and/or cell growth in Pseudomonas stutzeri and Sinorhizobium meliloti (104; 105). Micro-aerobic conditions were also required to culture A. cycloclastes in our lab (Bollinger, J. et al. unpublished results).

NosR as the Regulator of the Nos Gene Expressions and the Activator of N$_2$OR

NosR from A. cycloclastes (GenBank code: AAD09156.1) is expected to be an 80 kDa membrane bound protein (53). The nosR gene is shown to be essential for expression of N$_2$OR in Pseudomonas stutzeri (82), in Pseudomonas aeruginosa (98), and in Bradyrhizobium japonicum (99). The nosR expression is regulated with the transcription factor DnrD in response to NO in Pseudomonas stutzeri, as mentioned earlier (103). NosR is a transmembrane protein containing a flavin mononucleotide (FMN) binding domain on the periplasmic side and two [4Fe-4S] binding motifs on the cytoplasmic side (82; 106) (Figure 1.15). The Fe content of recombinant NosR from Pseudomonas stutzeri is determined as 7.9 atoms per molecule, and acid-labile sulfur is detected in the protein (82). These results confirm the presence of two [4Fe-4S] clusters in NosR. NosR and DnrD are essential to activate transcriptions of nosZ and nosDFYL in Pseudomonas stutzeri (50; 106). However, the precise mechanism of NosR is still unclear since NosR does not have any features of transcription factors (82). To date, it is presumed that NosR might interact with DnrD for nosZ expression (82).

NosR is also shown to be essential in maintaining N$_2$OR activity in vivo (82).
Whole cell N$_2$OR activity is observed only in the presence of both the periplasmic FMN domain and the cytoplasmic [4Fe-4S] domain of NosR. Either the FMN or [4Fe-4S] domain truncated NosR expressions in *Pseudomonas stutzeri* resulted in loss of N$_2$OR activity in the cell (Figure 1.16, b and c).

![Diagram](image)

Figure 1.16. Structural elements of unprocessed NosR (a) and mutated NosR (b-d) (82).

Further, Cys $\rightarrow$ Val mutations of one of the [4Fe-4S] sites, which is located between the fourth and fifth transmembrane helices and exposed to the cytoplasmic side, show that this site is indispensable for N$_2$OR activity (Figure 1.16, d) (82). Since a low initial N$_2$OR activity is observed, it is suggested that the N$_2$OR inactivation is due to the absence of electron supply after the first catalytic turnover, which further indicates that NosR might serve as an electron source for N$_2$OR. This observation also indicates that NosR is not involved in the biosynthesis of the active center of N$_2$OR (Figure 1.15). Indeed, Cu contents of N$_2$ORs from the strains which express intact or truncated NosRs were almost the same (82). It is suggested that NosR donates electrons to N$_2$OR through
electron transfer proteins such as pseudoazurin, c-type cytochromes, and/or NosX, as will be discussed in chapter 4 (82; 83). Additionally, the CuZ* proportion in N2OR is increased in the presence of truncated nosR mutants (82). This suggests that NosR helps maintain the CuZ site in a catalytically active state and/or regenerates the CuZ* state to the CuZ state.

NosD, NosF, and NosY as
the CuZ Site Assembly System

NosD, NosF, and NosY are essential for biosynthesis of the CuZ site (100). Deletion of either nosD, nosF, or nosY resulted in loss of the Cu content in N2OR from Pseudomonas stutzeri (95; 107). Sequence analyses of NosD, NosF, and NosY reveal that these proteins are homologous to the bacterial ATP binding cassette (ABC) transporter proteins (108). The typical ABC transporter system is composed of three units; a cytoplasmic ATP/GTP binding protein (analogous to NosF), a transmembrane protein (NosY), and a periplasmic protein (NosD) (109). This system transports small molecules and/or proteins across the cell membrane using the energy generated by ATP/GTP hydrolysis. Since CuZ site assembly requires four coppers and a sulfur atom, the NosDFY complex is expected to transport sulfide and/or Cu from the cytoplasm to N2OR in the periplasm (Figure 1.15) (100; 109).

NosD from A. cycloclastes (AcNosD, GenBank code: AAD09158.1) is predicted to be a 46 kDa periplasmic protein (53; 110). Amino acid sequence analysis of AcNosD identified two CASH (carbohydrate binding proteins and sugar hydrolases) domains, although the function of these domains was unclear (111). Recombinant AcNosD has
been overexpressed in *E. coli* and purified for characterization in our lab (53). However, recombinant NosD was expressed in inclusion bodies. Purification of NosD from the overexpressed strain Ac/pMLnos, in which whole Nos proteins were expected to be overexpressed, has also been tried several times, but was not successful (Bollinger, J. *et al.* unpublished results).

NosF from *A. cycloclastes* (GenBank code: AAD09159.1) is a 34 kDa cytoplasmic protein (Figure 1.15) (53). An ATP/GTP binding site was identified by sequence analysis (53). Tagging of a maltose binding protein (MBP) to NosF from *Pseudomonas stutzeri* enabled expression of stable NosF in an *E. coli* over-expression system (50). The ATP/GTPase activity assay of the MBP-NosF fusion protein show that NosF can hydrolyze both ATP and GTP, although with varying efficiency, whereas no CTPase or ADPase activity is detected (50). This result indicates that CuZ assembly is coupled with ATP/GTP hydrolysis.

According to sequence analysis, NosY from *A. cycloclastes* (AcNosY, GenBank code: AAD09160.1) is expected to be a six-transmembrane protein with a size of 28 kDa (Figure 1.15) (53). AcNosY is composed of 273 amino acids (aa) and is highly hydrophobic. The alanine and leucine contents of AcNosY are 59 aa (21.6 %) and 53 aa (19.4 %), respectively. Since few studies on NosY have been reported, there is little additional information on this protein.

**Copper Chaperon Protein NosL**

NosL from *A. cycloclastes* (AcNosL, GenBank code: AAD09161.1) is a periplasmic protein (18.5 kDa) whose N-terminal end is expected to be anchored to the
outer membrane (Figure 1.15) (53; 108). The N-terminal domain of this protein includes Leu-(Ala/Ser)-(Gly/Ala)-Cys motif which is a Type-II signal peptidase recognition sequence and leads the protein to the outer membrane (53; 112). Recombinant AcNosL was overexpressed in *E. coli* for characterization (108). AcNosL is a monomeric copper binding protein, which has selective affinity to Cu(I) but not to Cu(II) (108). The copper on AcNosL will be released as the Cu(I) is oxidized to Cu(II). This observation indicates that NosL delivers copper ions to the Cuₐ and/or Cu₂ sites of N₂OR, although it is still unclear whether NosL works independently or in concert with the NosDFY complex.

Although NosL is assumed to be a metallochaperone for the biosynthesis of the Cu sites in N₂OR, NosL is not required to produce catalytically active holo-N₂OR in the non-denitrifying organism *Pseudomonas putida* (100). Inactivation of NosL in *Pseudomonas stutzeri* also shows no apparent effect on N₂OR synthesis (113). These observations indicate that there is an alternative system to transport coppers to N₂OR in the host (112). It is likely that NosL has some functional role during N₂OR biosynthesis because many denitrifiers have the conserved nosDFYL gene set in common (55).

The apo form of AcNosL structure has been determined with NMR (112). AcNosL is composed of two sequential ββαβ motifs whose structure is homologous to the mercury binding protein MerB, although amino-acid homology of the two proteins is very low. The structural analysis of apo-AcNosL revealed that there are disordered regions in both the N-terminal (Met1 - Pro34) and C-terminal (Asp161 - Gly175) ends (112). One of the ligands for Cu(I) in AcNosL is expected to be Met109 (Met127 in the PDB data), which is well conserved among NosL sequences from various species (Figure
1.17) (112). Cys24 and Met26 are also assumed to be the Cu ligands, but the structure is not identified since these are in the N-terminal disordered region (112).

![Figure 1.17. NMR structure of apo-AcNosL (PDB code: 2HQ3).](image)

**Flavoprotein NosX**

The *nosX* gene was originally found in *Sinorhizobium meliloti* in 1997 (52). Gene inactivation experiments imply that NosX interacts with NosR (52). Although the *nosX* gene has been identified in many denitrifying bacteria (55), NosX has never been isolated and characterized *in vitro* prior to our work. The *nosX* gene is shown to be essential for N$_2$O reduction activity in *Sinorhizobium meliloti* (52) and *Paracoccus denitrificans* (114). Inactivation of the *nosX* gene in *Paracoccus denitrificans* results in loss of N$_2$O reduction *in vivo*, even though N$_2$OR from the cells can be activated *in vitro*, as described below (83). This result suggests that NosX is required for N$_2$OR activation. In contrast, the *nosX* gene has not been found in *Pseudomonas stutzeri* (115), *Pseudomonas aeruginosa* (116), and *Pseudomonas fluorescens* (117). This observation suggests that
these strains may have other cellular components that can substitute for the NosX function.

The function of NosX has been discussed in several papers. In 2000, Saunders et al. proposed that NosX was involved in CuA biosynthesis in Paracoccus denitrificans (114). NirX, which was a product of nirX in the nir (nitrite reductase) gene cluster, was homologous to NosX (37% identity and 49% similarity) and was only found in Paracoccus denitrificans. NosX and NirX were thought to be functional homologues and each protein could be substituted with the other protein. Thus, single mutation of either nosX or nirX did not change any apparent phenotype. However, once both nosX and nirX genes were knocked out, the N2OR activity in whole cells was diminished. The authors measured EPR and UV/Vis spectra of crude cell extracts that were obtained from either WT or the double mutant strain. The signals from the CuA site of N2OR were observed in the sample from WT, but not from the mutant strain. They concluded that the functions of NosX and NirX were involved in the biosynthesis of the CuA site. In yeast, the CuA site in cytochrome c oxidase is constructed with a copper chaperone protein termed Sco1 (118). The authors’ hypothesis indicated that NosX corresponds to Sco1 for the CuA biosynthesis in denitrifying bacteria. However, no metal binding site has been identified among NosX sequences (53; 99; 52).

Another hypothesis regarding NosX function was proposed in 2005 by Wunsch et al (83). They also investigated the nosX nirX double knock out mutant in Paracoccus denitrificans and found that purified N2OR from the mutant strain possessed the CuA site. Although the N2OR activity in vivo was deficient as Saunders et al observed, the isolated
N₂OR was catalytically active *in vitro*. The result indicated that NosX was not a Cu chaperone for Cu₆₇ biosynthesis, but instead maintained N₂OR activity. Additionally, the Cu₂⁺ state proportion in purified N₂OR was apparently increased in the absence of NosX expression. This phenotype was similar to the nosR mutants as described before, and it implied the existence of an electron transfer pathway from NosR to N₂OR via NosX to sustain N₂OR activity (83; 82).

Structural characterization of NosX has not been reported to date. The BLAST search of the NosX sequence from *A. cycloclastes* (AcNosX, GenBank code: AAD09162.2) revealed that the AcNosX sequence resembles the ApbE superfamily proteins, for which structural information is available in the PDB. Although the function of the ApbE protein is still ambiguous, it has been proposed to be involved in thiamine (vitamin B) biosynthesis (119) and Fe-S cluster metabolism (120). Recently, the crystal structure of the ApbE protein from *Salmonella enterica* was solved (PDB code: 3PND) and revealed that it bound FAD as a cofactor (121). This structure has an unique FAD-binding motif which can be applied to NosX and will be described in chapter 3.

Overexpression trials of AcNosX in *E. coli* have been attempted in our lab. The purified recombinant AcNosX from *E. coli* did not include any bound cofactor. Reconstitution of FAD, which was identified as the cofactor for NosX in chapter 3, into NosX by dialysis was unsuccessful (Bollinger, J. *et al.* unpublished results).

**Other Nos Proteins**

Several different types of N₂OR and its *nos* accessory proteins have been reported (4). *Wolinella succinogenes* (Ws) is a close relative of *Helicobacter pylori*, although this
strain is not pathogenic. The genome sequence of *Ws* revealed that it had an unusual N2OR and accessory Nos proteins (51). The *nos* cluster in *Ws* included *nosZDFYL* and four new *nos* genes, *nosG, nosC1, nosC2*, and *nosH* (Figure 1.18A). *nosR* and *nosX* genes were not observed in this cluster. There were also several potential open reading frames in the cluster labeled as *Ws0918, Ws0925*, and *Ws0928* in Figure 1.18. In addition to the typical domains for the Cu₄ and Cu₂ sites, *WsN₂OR* included a cytochrome *c* domain in the C-terminal region (Figure 1.18B), which had never been observed in any other denitrifying bacteria.

Figure 1.18. The *nos* gene cluster from *Wolinella succinogenes* (A) and distribution of the Nos proteins (B) (51).
The sequence analysis reveals that NosG and NosH are homologous to NapG and NapH, which are accessory proteins for periplasmic nitrate reductase (Nap). NosH is a membrane bound protein (Figure 1.18) and shows similarity to the C-terminal domain of NosR (Fe-S cluster motifs on the cytoplasmic side), which implies that NosH substitutes for the function of NosR. NosG also has conserved Fe-S cluster binding motifs, and is expected to form a complex with NosH which transports electrons from menaquinol to N₂OR (Figure 1.18) (51). According to the unprecedented heme domain of WsN₂OR and these unique accessory proteins, the WsN₂OR is categorized in a variant group as “H-type”, whereas the typical N₂ORs have been categorized as “Z-type” (Table 1.1) (4).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Organisms</th>
<th>Catalytic site</th>
<th>Domain order</th>
<th>Accessory proteins</th>
</tr>
</thead>
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<tr>
<td>Z</td>
<td><em>Paracoccus denitrificans</em></td>
<td>Cu-S</td>
<td>Tat signal, beta-propeller, cupredoxin</td>
<td>NosR(X) NosDFYl</td>
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<td></td>
<td><em>Achromobacter cycloclastes</em></td>
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<td>etc.</td>
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<tr>
<td>H</td>
<td><em>Wolinella succinogenes</em></td>
<td>Cu-S</td>
<td>Sec signal, beta-propeller, cupredoxin, C-domain</td>
<td>NosGHC NosDFYl</td>
</tr>
<tr>
<td></td>
<td><em>Magnetospirillum magnetotacticum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td><em>Haloferax volcanii</em></td>
<td>Cu-S?</td>
<td>Unknown</td>
<td>NosDFYl</td>
</tr>
<tr>
<td>(hypothetical)</td>
<td><em>Pyrobaculum aerophilum</em></td>
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</table>

Table 1.1. Structural types of N₂OR variants (4).

In addition to the Z- and H-types, hypothetical N₂ORs that are categorized as “A-type” have been reported in two archaea (Table 1.1) (4). *Haloferax volcanii* and *Pyrobaculum aerophilum* are identified as denitrifiers since genome analyses reveal that these archaea have NiR and NOR, and *nosDFYL* genes. There is also evidence that
*Pyrobaculum aerophilum* produces NO, N\(_2\)O, and N\(_2\) from nitrate (122). However, no *nosZ* gene is identified in the genomes of these archaea. These results suggest that there is a different type of N\(_2\)OR in these archaea. Since these archaea have *nosDFYL* genes which are responsible for Cu\(_Z\) site biosynthesis, the unknown N\(_2\)OR is expected to have a similar Cu-S cluster (Table 1.1).

**Research Goals**

The focus of this research was to establish expression conditions and purification methodologies for NosX and to characterize the structure and function of NosX from the soil bacterium *Achromobacter cycloclastes*. Although several mutational experiments on the *nosX* gene *in vivo* have been reported (83; 114), no investigation prior to these studies had been carried out on the properties of isolated NosX. This is the first study that identifies the conditions to obtain sufficient amounts of NosX for biochemical characterization. The information revealed from this research will help to elucidate the activation mechanism of N\(_2\)OR with NosX and control of N\(_2\)O metabolism. It also will be a basis for future applications that could be relevant to such broad environmental issues as water pollution, ozone depletion, and global warming (123).

The interaction between N\(_2\)OR and its potential physiological electron donor pseudoazurin was also investigated with co-crystallization techniques to determine the docking structure of AcN\(_2\)OR and AcPAz, and to understand the mechanism of the electron transfer reaction. The information from this project is important in clarifying the docking and electron transfer mechanisms between N\(_2\)OR and PAz.
CHAPTER 2

PRODUCTION AND PURIFICATION OF NOSX:
GENE STRUCTURE REFINEMENT, AND OPTIMIZATION OF
THE CULTURE AND PURIFICATION CONDITIONS FOR NOSX

Introduction

The overexpression of the gene products from the nos cluster of *A. cycloclastes* was facilitated by subcloning of the entire gene cluster into the multi-copy, broad host range vector pML10 (124; 125). The plasmid was transferred into *A. cycloclastes* for homologous expression. The plasmid strain is referred to as Ac/pMLnos. The yield of AcN$_2$OR from Ac/pMLnos was 10 ~ 100-fold higher than the yield from Ac without the plasmid (126). For NosX purification, a factor Xa protease site (-Ile-Glu-Gly-Arg-), an Ala-Ser linker, and a strep-tag II sequence (-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) were inserted on the 3’ end of the nosX gene in the pMLnos plasmid (125). The strain including this plasmid is referred to as Ac/pMLClustXst.

Initial expression levels of NosX in Ac/pMLClustXst were significantly lower compared to that of AcN$_2$OR. A typical yield of each protein from 10 g of cells was about 10 ~ 20 mg for AcN$_2$OR and less than 0.1 mg for NosX. It was critical to improve the NosX yield for characterization and experiments. While it was reported that nosR gene and NosR protein regulate the expression levels of nosZ and nosDFYL (106; 50), the regulation mechanism for nosX gene expression was still unclear. A weak promoter sequence for nosX transcription had been identified in the upstream flanking region of the
The nosX gene from A. cycloclastes (Bollinger, J. et al. unpublished results). It was also observed that the expression levels of NiR and PAz in Ac was high under denitrification conditions, which was indicative of strong promoter sequences for these protein expressions. Therefore, to improve NosX production, we replaced the weak promoter for NosX with either the AcNiR or AcPAz promoters. Factors and conditions that were thought to influence NosX expression and yield were also investigated. In addition, the purification protocol for NosX was modified to remove biotinylated proteins from Ac cell extract.

Materials and Methods

Growth and Purification of NosX

Ac/pMLClustXst was streaked on a LB plate with 50 µg/mL gentamicin (Gm50) and incubated at 30 °C for 3 days. A single colony from the plate was used to inoculate 10 mL LB media (Gm50) which was left overnight at 30 °C and shaking at 250 rpm. The culture was inoculated to 200 mL LB (Gm50) culture for 8 hours and further scaled-up to 2 L × 4 flasks until an A600 = 1.0 ~ 1.4 was reached. Expression of Nos proteins was induced by the addition of 20 mL of 1 M KNO3 (final conc. 10 mM) and 4 mL of 50 mM CuSO4 (final conc. 100 µM) per flask. The shaking speed was reduced to 100 ~ 125 rpm for micro-aerobic conditions. After 24 hours, the cultures were harvested by centrifugation for 10 minutes at 4 °C at 10800 × g (Sorvall GS3 rotor). The cell pellet (~30 g) obtained was frozen at -80 °C immediately or kept at 4 °C overnight for further
purification. Conditions of each culture and yields of cell and NosX are listed in APPENDIX B (Table S2.1) with culture number (#1 - 85).

The following steps for NosX purification were performed on ice or at 4 °C, under aerobic conditions unless otherwise noted. Twenty to thirty grams of cell pellet was re-suspended in 20-30 mL of 100 mM Tris, 150 mM NaCl, 1 mM EDTA, and 10 % (w/v) glycerol at pH 8.0 (Buffer W). A small amount of DNaseI was added to the suspension. To lyse the cells, the suspension was passed through a French pressure cell twice, at a pressure between 60 and 70 MPa. The cell lysate was centrifuged for 60 min at 39000 ×g (Sorvall SS-34 rotor). As a modified purification protocol (see discussion), 40 % ammonium sulfate precipitation was applied to the supernatant, prior to avidin treatment. Ammonium sulfate was added to the supernatant (40 % saturation) and incubated for 1 hour while stirring (~ 400 rpm). After centrifuging and re-suspension of the precipitate with ~ 20 mL of Buffer W, avidin (12.5 U for every 10 g of cells) was added to the re-suspended solution and incubated for at least 30 min while stirring (~ 200 rpm). The solution was centrifuged for 30 min at 39000 ×g. The supernatant was decanted and loaded onto a strep-tactin sepharose column (2 mL, 10 × 25 mm; IBA-GmbH) equilibrated with Buffer W. The column was washed with five column volumes of Buffer W. Proteins bound to the column were eluted with Buffer W plus 2.5 mM desthiobiotin (IBA-GmbH) and collected in 1 mL fractions. NosX was identified by its yellow color and UV/Vis spectra. The eluted NosX was concentrated using an Amicon Ultrafree-4 10K centrifugal filter (Millipore). A typical yield of NosX was 1~2 mg from 30 g cells from optimized culture conditions (see “Optimization of Culture Conditions for NosX”
section). Stocks of NosX were stored at -20 °C. After several purifications, all NosX stocks were thawed, concentrated to 1~2 mL (~ 6 mg/mL) and loaded onto a Superdex 200 gel filtration column (16 × 600 mm; GE Healthcare). The column was equilibrated with 100 mM phosphate, 150 mM NaCl, 10 % (w/v) glycerol, with or without 10 mM EDTA, pH 7.0 (Buffer P (without EDTA) or Buffer PE (with EDTA)) depending on the experiment. The NosX containing fraction eluted between 80 and 100 min with a flow rate at 1 ml/min. The eluted sample was concentrated with an Amicon Ultra Centrifugal Filter 10 K (Millipore) and stored at -20 °C if not used immediately. To determine the purity of NosX, 12.5 % gels were used to run SDS-PAGE analysis (Bio-Rad) and stained with Coomassie Brilliant Blue (CBB).

Promoter Refinement for NosX

Subcloning of nosX Gene from pMLClustXst. The constructions of the new NosX expression system with AcNiR or AcPAz promoters are summarized in Figure 2.1. The plasmid pMLClustXst was purified from Ac/pMLClustXst with a QIAprep Spin Miniprep Kit (Qiagen). Since the yield of plasmid from Ac was very low, the purified plasmid was transferred into the E. coli strain, Top10F’ (Invitrogen) by electroporation for plasmid propagation. Colony screening was carried out with an LB plate with 10 µg/mL gentamicin (Gm10). A colony was picked up and cultured for further plasmid purification. The purified plasmid was used for the following experiments. The strep-tagged nosX gene from the nos cluster in the plasmid was amplified with PCR using two primers (Figure 2.1A). The primer sequences were as follows;
Figure 2.1. Schematic view of the assembling process of a new NosX expression system
Primer #1: 5' - ATATCGAAGCTTGCAACGTGGGACCAGCCCGG -3'

\textit{HindIII} recognition site

Primer #2: 5' - ATATCGGAAATTCCTTTCTCGATGGGCGCGGAACAG -3'

\textit{EcoRI} recognition site

The settings for PCR were programmed as follows; Step 1, 98 °C for 1 min (initial denaturation), Step 2, 98 °C for 30 sec (denaturation), Step 3, 70 °C for 30 sec (annealing), Step 4, 72 °C for 30 sec (extension), 30 cycles for Step 2 - Step 4, Step 5, 72 °C for 5 min (final extension). The product (1457 bps) including \textit{HindIII} and \textit{EcoRI} recognition sites in 5’ and 3’ ends were inserted into the cloning vector pBBR1MCS-5 (referred to as p5 plasmid) (Figure 2.1B) and transferred to Top10F’ by electroporation. The colonies were incubated on an LB plate (Gm10) at 37 °C for overnight. Colony PCR screening was carried out to find the colony that included the \textit{nosX} inserted p5 vector (p5-\textit{nosX}, 6213 bps). The plasmids were extracted from several colonies and amplified with PCR using a Taq polymerase (Bio-Rad), primers #1 and #2. The settings for colony PCR follow; Step 1, 94 °C for 2 min 30 sec (initial denaturation), Step 2, 94 °C for 1 min (denaturation), Step 3, 62 °C for 1 min (annealing), Step 4, 72 °C for 1 min 30 sec (extension), 40 cycles for Step 2 - Step 4, Step 5, 72 °C for 5 min (final extension). The colony that showed the right size of PCR product (1457 bps) on a gel was selected and used for further experiments. The p5-\textit{nosX} plasmid was purified from the colony, and the promoter region was removed by inverse PCR using a high fidelity Phusion DNA polymerase (Bio Labs) and two primers (Figure 2.1C). The primer sequences follow;

Primer #3: 5' - CGTTGCAAGCTTATCGATACCGTCG -3'

\textit{HindIII} recognition site
Primer #4: 5’- P-ATGCTGCTGACCCGACGCCG -3’
Phosphorylated primer on 5’ side

The settings for inverse PCR follow; Step 1, 100 °C for 2 min (initial denaturation), Step 2, 100 °C for 30 sec (denaturation), Step 3, 64 °C for 30 sec (annealing), Step 4, 72 °C for 3 min (extension), 30 cycles for Step 2 - Step 4, Step 5, 72 °C for 5 min (final extension). The PCR product (5918 bps) was further processed with HindIII for ligation with the promoter of AcNiR or AcPAz (Figure 2.1D).

Isolation of Promoters of AcNiR and AcPAz. The DNA sequences prior to AcNiR and AcPAz genes were expected to be efficient promoters under denitrifying conditions because the expression levels of these proteins were high under the conditions. To replace the original promoter for NosX with these promoters, the DNA regions were isolated from Ac genomic DNA.

The promoter of AcNiR was amplified by PCR and two primers. Gene sequences of AcNiR and AcPAz were obtained from NCBI nucleotide database: AcNiR gene code is Z48635, and AcPAz gene code is Z48669. The primer sequences were designed as follows;

Primer #5: 5’- GTCCAAGCTTGAAGCCATAGGAGAAGAC -3’  
\( \text{HindIII recognition site} \)

Primer #6: 5’- P-CTTGCTCTCCTCGGAGC -3’  
Phosphorylated primer on 5’ side

The settings for PCR were programmed as follows; Step 1, 98 °C for 1 min (initial denaturation), Step 2, 98 °C for 30 sec (denaturation), Step 3, 70 °C for 30 sec (annealing), Step 4, 72 °C for 30 sec (extension), 40 cycles for Step 2 - Step 4, Step 5,
72 °C for 5 min (final extension). The product (484 bps) was processed with HindIII for ligation with p5-nosX without its promoter (Figure 2.1E).

The promoter of AcPAz was amplified from Ac genome DNA as the AcNiR promoter was amplified. The primer sequences are as follows;

Primer #7: 5’- ATGCAAGCTTGCTCATGCGTCAATTATAGG -3’
HindIII recognition site

Primer #8: 5’- P-CGTCTTCTCCATTTGAGCGACC -3’
Phosphorylated primer on 5’ side

The settings for PCR are as follows; Step 1, 98 °C for 1 min (initial denaturation), Step 2, 98 °C for 5 sec (denaturation), Step 3, 67 °C for 30 sec (annealing), Step 4, 72 °C for 15 sec (extension), 40 cycles for Step 2 - Step 4, Step 5, 72 °C for 5 min (final extension). The product (165 bps) was processed with HindIII for ligation with the p5-nosX (Figure 2.1E).

Assembly of nosX Gene and Promoters of AcNiR and AcPAz on p5 plasmid. The HindIII processed AcNiR or AcPAz promoters (Figure 2.1E) were assembled to p5-nosX plasmid with ligation. The AcNiR or AcPAz promoter was mixed with p5-nosX (without its own promoter) and incubated with T4 DNA ligase at 16 °C overnight (Figure 2.1F). The assembled plasmids were referred to as p5-Np-nosX (with AcNiR promoter, 6402 bps) and p5-Pp-nosX (with AcPAz promoter, 6076 bps). These plasmids were transferred into the E. coli strain DH10β by the heat shock method. The colonies were cultured on a LB plate (Gm15) at 37 °C overnight. Several colonies for both plasmids were selected and checked with the colony PCR screening method using the Taq polymerase and primer #5 (for AcNiR promoter) or
#7 (for AcPAz promoter) and #2 (for nosX gene). The colony PCR was carried out to confirm whether the selected colonies included the plasmid with the expected DNA sequence. The settings for the colony PCR were programmed as follows; Step 1, 94 °C for 2 min 30 sec (initial denaturation), Step 2, 94 °C for 1 min (denaturation), Step 3, 56 °C for 1 min (annealing), Step 4, 72 °C for 1 min 30 sec (extension), 40 cycles for Step 2 - Step 4, Step 5, 72 °C for 5 min (final extension). The colonies that showed the right size of PCR products (1638 bps for AcNiR promoter + nosX, and 1312 bps for AcPAz promoter + nosX) on a gel were selected and the plasmids were purified for DNA sequencing. Three primers were used to confirm the sequence. The sequences follow:

Primer #9: 5’- CCATGATTACGCCAAGCGCGCAATTAACCC -3’
Primer #10: 5’- CGCTTCGATCCGACGGTACAGCCGCTC - 3’
Primer #11: 5’- CCCCTCTCGGCACGACCTTCGATCAAGC - 3’

Primers #9 - #11 were designed to sequence different regions of the nosX gene on p5 plasmid. Primer #9 was for the upstream range of p5-nosX gene. Primer #10 was for the middle range of nosX, and primer #11 was for the downstream range. Sequence-confirmed plasmids were transferred into A. cycloclastes using electroporation. Ac/p5-Np-nosX and Ac/p5-Pp-nosX were cultured individually to check the NosX expression levels (n = 1). After induction, the shaking speed was reduced to 125 rpm and the media was cultured for 24 hours. Cells were harvested and purified with a strep-tactin sepharose column. Additionally, Ac/p5-Np-nosX and Ac/p5-Pp-nosX were cultured using LB media from Fisher brand and the NosX yields were compared (n = 1) because of the observation in the next section.
Optimization of Culture Conditions for NosX

The growth conditions of Ac/pMLClustXst were modified to identify potential factors that may have affected the NosX yield. Harvested cell amounts and NosX yields from each modified condition were compared to the amounts and yields from the original culture conditions to determine the effectiveness of the modification. The following conditions were considered:

1. LB media (purchased from RPI (n = 7) or Fisher (n = 7))
2. Riboflavin (with (n = 6) or without 50 mg/flask (n = 8))
3. Aerobic/micro-aerobic condition (250 (n = 5) or 100 rpm after induction (n = 7))
4. Incubation time after induction (24 (n = 5) or 48 hours (n = 7))
5. A_600 at induction (A_600 = 0.8 ~ 3.8 (n = 16))

“n=” stands for the number of trials performed. For example, “RPI (n = 7)” indicates that Ac/pMLClustXst was cultured with LB media purchased from RPI for seven trials and the harvested cell amount and the NosX yield were measured.

Ac/pMLClustXst was cultured with modified conditions as described above. Only a single modification was made to the original culture condition to permit systematic comparisons. Unless otherwise noted, once a factor was revealed to be effective in increasing the NosX yield, the factor was used for following optimization steps. The culture conditions that were used in each optimization step are summarized in Table 2.1.
Table 2.1. Variables tested for effects on the yield of NosX

<table>
<thead>
<tr>
<th>#</th>
<th>LB</th>
<th>Riboflavin</th>
<th>Shaking speed</th>
<th>Time</th>
<th>A&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Average yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>RPI</td>
<td>100 rpm</td>
<td>24 h</td>
<td>N/A</td>
<td>0.25 ± 0.13 mg</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Fisher</td>
<td>100 rpm</td>
<td>24 h</td>
<td></td>
<td>1.32 ± 0.74 mg</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>Fisher</td>
<td>0 mg</td>
<td>100 rpm</td>
<td>24 h</td>
<td>1.2-1.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Fisher</td>
<td>50 mg</td>
<td>100 rpm</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>Fisher</td>
<td>-</td>
<td>100 rpm</td>
<td>24 h</td>
<td>1.1-1.9</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Fisher</td>
<td>-</td>
<td>250 rpm</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>Fisher</td>
<td>-</td>
<td>250 rpm</td>
<td>24 h</td>
<td>1.1-3.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Fisher</td>
<td>-</td>
<td>250 rpm</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fisher</td>
<td>-</td>
<td>250 rpm</td>
<td>48 h</td>
<td>1.0</td>
<td>3.21 - 5.98 mg</td>
</tr>
<tr>
<td></td>
<td>Fisher</td>
<td>-</td>
<td>250 rpm</td>
<td>48 h</td>
<td>3.8</td>
<td>2.54 mg</td>
</tr>
</tbody>
</table>

Optimization of Purification Conditions for NosX

The purification process of NosX was monitored by western blot using streptactin-AP (alkali phosphatase) that would detect the strep-tagged protein (NosX) and biotinylated proteins. To remove biotinylated proteins in the Ac cell extract, a 40% ammonium sulfate precipitation method was applied before the sample was loaded onto the strep-tactin column. The effectiveness of this additional purification step was determined by western blot. Samples were selected from the following purification steps: the supernatant after cell lysis and centrifugation, supernatant and precipitate from 40% ammonium sulfate precipitation, and the elution fraction from the strep-tactin sepharose column. Each sample was denatured at 100 °C for 5 min in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol, and two identical SDS-PAGE gels (12.5%) were prepared (200 V, 30 min). One of the gels was stained with CBB for comparison with the result from the western blot. The other gel was electro-blotted to a PVDF membrane (Millipore) (90 V, 60 min) at 4 °C. The membrane was washed with 20
mL of TBS buffer (Tris 1.5 g, NaCl 4.0 g, KCl 0.1 g in 500 mL H₂O). The membrane was further treated with 10 mL of blocking solution (TBS + 2 % BSA) for 1 hour, and 10 mL of TTBS (TBS + 0.05 % Tween 20) for 10 min. The membrane was then soaked into 20 mL of antibody solution (TTBS + 2 % BSA + 2 µL strep-tactin-AP) for 1.5 hours. The residual strep-tactin-AP on the membrane was washed with 10 mL × 3 of TBS and the membrane was developed with BCIP/NBT solution (Sigma) for 10 min.

Results

Promoter Refinement for NosX

The nosX gene from pMLClustXst and the promoter genes of NiR and PAz from Ac genome DNA were amplified with PCR. Since the GC content in the nosX gene was relatively high, several modifications of the PCR settings and the GC reaction buffer (BioLabs, for high GC content DNA) were required for successful amplification. Those DNA fragments (AcNiR promoter + nosX gene (Np-nosX) and AcPAz promoter + nosX gene (Pp-nosX)) were successfully assembled on p5 plasmid and the sequences were confirmed. The putative Dnr/DnrD binding sites (TTG AT-CT-ATCAA for AcNiR promoter and TTGAC-GCGC-ATCAA for AcPAz promoter) were observed at 292 bps upstream of nosX gene in p5-Np-nosX and at 90 bps upstream of nosX gene in p5-Pp-nosX, respectively. The typical DNR binding motif is TTGA(T/C)-N₄-(A/G)TCAA (127).

Ac/p5-Np-nosX and Ac/p5-Pp-nosX were cultured with 2 L ×4 flasks of LB media (purchased from RPI, Gm50) and purified to see if expression level of NosX was enhanced (n = 1). The cell yields of the new expression systems were relatively higher.
(36.8 g for Ac/p5-Np-nosX and 31.0 g for Ac/p5-Pp-nosX) than the cell yield of Ac/pMLClustXst (average of culture #1 - 19 was 23.9 ± 5.8 g) (Table 2.2). The NosX expression levels (per 10 g of cell) in Ac/p5-Np-nosX and Ac/p5-Pp-nosX were 0.10 and 0.13 mg/10 g cell respectively, which were almost the same degree of Ac/pMLClustXst (0.10 ± 0.08 mg/10 g cell) (Table 2.2). Since the cell yields were higher with the refined plasmids, the total NosX yields were slightly higher in both Ac/p5-Np-nosX (0.37 mg) and Ac/p5-Pp-nosX (0.40 mg) than Ac/pMLClustXst (0.28 ± 0.26 mg) (Table 2.2). In total, the NosX yields from Ac/p5-Np-nosX and Ac/p5-Pp-nosX were increased 1.32-fold and 1.43-fold from the yield from Ac/pMLClustXst under the original culture conditions. No significant difference between Ac/p5-Np-nosX and Ac/p5-Pp-nosX was observed in terms of cell amounts and NosX yields.

<table>
<thead>
<tr>
<th>Culture #</th>
<th>Strain/plasmid</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 19</td>
<td>Ac/pMLClustXst</td>
<td>23.9 ± 5.8</td>
<td>0.28 ± 0.26</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>20</td>
<td>Ac/p5-Np-nosX</td>
<td>36.8</td>
<td>0.37</td>
<td>0.10</td>
</tr>
<tr>
<td>21</td>
<td>Ac/p5-Pp-nosX</td>
<td>31.0</td>
<td>0.40</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 2.2. The NosX yields from the refined expression systems with the LB from RPI

In additional experiments, the expression levels of NosX in Ac/p5-Np-nosX and Ac/p5-Pp-nosX, which were cultured with LB media from Fisher, were also examined and compared (n = 1). As will become apparent in the next section, the LB media from Fisher significantly (~ 5-fold) enhanced the NosX yield from Ac/pMLClustXst (0.25 ± 0.13 (RPI) vs. 1.32 ± 0.74 (Fisher), Table 2.4). Thus, the same conditions were applied to culture with the new NosX expression systems. With the LB media from Fisher, the
NosX yields from the new expression systems were 0.43 mg and 0.34 mg respectively (Table 2.3), which were almost the same level of the yields with the LB media from RPI (0.37 mg and 0.40 mg) (Table 2.2). No significant increase of the NosX yields was observed in either Ac/p5-Np-nosX or Ac/p5-Np-nosX with the LB media purchased from Fisher (Table 2.3). Since the effectiveness to obtain NosX using Ac/pMLClustXst and the LB media from Fisher was more significant (~ 5-fold) than using the new expression systems (1.32-fold and 1.43-fold), following optimization of culture conditions for NosX expression was carried out using Ac/pMLClustXst and the LB media from Fisher.

<table>
<thead>
<tr>
<th>Culture #</th>
<th>Strain/plasmid</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-34 Ac/pMLClustXst</td>
<td>23.0 ± 6.0</td>
<td>1.20 ± 0.66</td>
<td>0.54 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>29 Ac/p5-Np-nosX</td>
<td>26.3</td>
<td>0.43</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>28 Ac/p5-Pp-nosX</td>
<td>21.0</td>
<td>0.34</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. The NosX yields from the refined expression systems with the LB from Fisher

Optimization of Culture Conditions for NosX

Culture conditions were optimized for NosX expression based on five factors: LB media (purchased from RPI or Fisher), riboflavin addition, aerobic or micro-aerobic condition (100 or 250 rpm after induction), incubation time after induction (24 or 48 hours), and A600 at induction (0.8 - 3.8).

The LB media purchased from Fisher brand showed the most significant effect on the NosX expression based on the five factors investigated. Ac/pMLClustXst was cultured with 2 L × 4 flasks of the LB media from RPI (culture #1-5, 8, 14, n = 7) or
Fisher (culture #22, 24, 25, 30-33, n = 7), and harvested cell amounts and purified NosX yields were compared (Table 2.4). Although cell yield with the LB from Fisher was lower (21.6 ± 4.8 g) than one with the LB from RPI (28.7 ± 4.0 g), NosX expression level per cell with the Fisher brand was ~ 6 times higher than that with media purchased from RPI (Table 2.4, 0.62 ± 0.28 mg/10g cell for Fisher vs. 0.09 ± 0.05 mg/10g cell for RPI). The factor(s) in the LB media from Fisher, which enhanced the NosX yield, has not been identified. Due to the significant enhancement on the NosX yield with the LB media from Fisher brand, this LB media was decided to use in the following optimization steps as mentioned in the method section (Table 2.1).

<table>
<thead>
<tr>
<th>LB media</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPI</td>
<td>28.7 ± 4.0</td>
<td>0.25 ± 0.13</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>Fisher</td>
<td>21.6 ± 4.8</td>
<td>1.32 ± 0.74</td>
<td>0.62 ± 0.28</td>
</tr>
</tbody>
</table>

Table 2.4. The NosX yields with different LB media brands (RPI vs. Fisher). Condition that showed higher NosX yield was highlighted.

It was expected that the cofactor biosynthesis for NosX in *A. cycloclastes* might be a limiting factor for the NosX expression. The cofactor of NosX has been confirmed to be FAD as will be described in chapter 3. Riboflavin composes a part of FAD and thus it was added in culture media for supporting FAD biosynthesis. *Ac/pMLClustXst* was cultured in comparable conditions with (culture #44, 47, 49, 50-52, n = 6) or without 50 ~ 100 mg/flask of riboflavin (culture #37, 39-43, 46, 53, n = 8). 12 L (2 L × 6 flasks, instead of 4 flasks) of LB media was used for following cultures for efficient purification. Although varying amount (50 ~ 100 mg) of riboflavin was added to each flask, 2 L of the
media was saturated with 50 mg of riboflavin and the rest of it was precipitated because of low solubility. No apparent difference was observed in terms of the amount of riboflavin per flask. Cell yield from the culture with riboflavin was relatively lower (26.5 ± 6.4 g) than without riboflavin (34.4 ± 9.3 g) (Table 2.5). No significant positive effect of riboflavin on the NosX yield was observed.

<table>
<thead>
<tr>
<th>Riboflavin</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>26.5 ± 6.4</td>
<td>0.48 ± 0.40</td>
<td>0.16 ± 0.10</td>
</tr>
<tr>
<td>-</td>
<td>34.4 ± 9.3</td>
<td>0.63 ± 0.28</td>
<td>0.18 ± 0.07</td>
</tr>
</tbody>
</table>

Table 2.5. The NosX yields with (+) or without riboflavin (–). Condition that showed higher NosX yield was highlighted.

The shaking speed after induction was either reduced to 100 rpm (micro-aerobic conditions, culture #37, 39-43, 46, n = 7) or kept at 250 rpm (aerobic conditions, culture #54-56, 61-62, n = 5) after induction to elucidate the effect of oxygen on NosX yield. Unexpectedly, the NosX expression level was constant under both aerobic and micro-aerobic conditions (Table 2.6, 0.20 ± 0.06 for 100 rpm vs. 0.23 ± 0.14 for 250 rpm). With shaking speed set at 250 rpm, the total NosX yield became twice the cell yield of that obtained at 100 rpm (Table 2.6).

<table>
<thead>
<tr>
<th>Speed (rpm)</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>35.8 ± 9.1</td>
<td>0.70 ± 0.22</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>250</td>
<td>75.3 ± 7.7</td>
<td>1.57 ± 0.87</td>
<td>0.23 ± 0.14</td>
</tr>
</tbody>
</table>

Table 2.6. The NosX yields with different shaking speeds (100 vs. 250 rpm). Condition that showed higher NosX yield was highlighted.
Incubation time (24 hours (n = 5) or 48 hours (n = 7)) after induction was examined to investigate whether the NosX yield was enhanced with prolonged culture (Table 2.7). The results showed that the cell amount and the NosX yield per cell were increased ~ 35 % and ~ 40 % respectively in the 48-hour culture compared to the 24-hour culture. In total, the NosX yield increased about two-fold in the 48-hour culture (Table 2.7).

<table>
<thead>
<tr>
<th>Culture</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>75.3 ± 7.7</td>
<td>1.57 ± 0.87</td>
<td>0.23 ± 0.14</td>
</tr>
<tr>
<td>48 h</td>
<td>102.4 ± 4.5</td>
<td>3.23 ± 1.20</td>
<td>0.32 ± 0.12</td>
</tr>
</tbody>
</table>

Table 2.7. NosX yields following 24 or 48 hours culture post induction. Condition that showed higher NosX yield was highlighted.

Typically, proteins are expressed at a rapid rate in “log phase” during cell growth and at a slow rate during “stationary phase”. Thus, a mid-log phase (A$_{600}$ = 0.6 - 1.0 in the case of _E. coli_) is known to be the best timing for induction. In _A. cycloclastes_, the correlation of absorption at 600 nm and the growth phase is still not clear, thus induction was carried out at several different A$_{600}$ values to determine the best time to induce to maximize NosX yield. The expression of denitrification proteins, including NosX, were induced through addition of nitrate and copper when A$_{600}$ was between 0.8 and 3.8 (culture #63-67, 72-80, 82-83, n = 16). Correlation between the NosX yield and the A$_{600}$ at induction time was investigated by comparing the cell yield and NosX yield from each culture. No clear correlations among A$_{600}$ at induction, the harvested cell amount, and the
NosX yield were observed (Figure 2.2). However, the highest NosX yield was obtained when $A_{600}$ was 1.0.

![Graph showing NosX yield and cell amount vs. $A_{600}$ at induction](image)

Figure 2.2. NosX yield and cell amount vs. $A_{600}$ at induction

The optimized culture conditions were determined as follows;

1. LB media purchased from Fisher brand enhanced NosX yield six-fold.
2. Riboflavin did not have any positive effect on NosX yield.
3. Incubation with 250 rpm increased NosX yield two-fold.
4. 48-hour incubation increased NosX yield two-fold.
5. Induction at $A_{600} = 1.0$ seemed to be the best for NosX yield.

The NosX yield under the original growth conditions was $0.28 \pm 0.26$ mg (culture #1 - 19, n = 19). After optimization, yield was improved to $5.22 \pm 0.54$ mg in the best case (culture #79-80, 84, n = 3) (Table 2.8). On average, the optimized culture conditions
produced ~ 19-fold higher amount of NosX when compared to the original growth conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell yield (g)</th>
<th>NosX Total yield (mg)</th>
<th>mg/10g cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>23.9 ± 5.8</td>
<td>0.28 ± 0.26</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>Optimized</td>
<td>76.8 ± 6.6</td>
<td>5.22 ± 0.54</td>
<td>0.68 ± 0.06</td>
</tr>
</tbody>
</table>

Table 2.8. NosX yields from the original and optimized culture conditions

**Optimization of Purification Conditions for NosX**

During the NosX purifications, binding capacity of the strep-tactin column was irreversibly decreased by biotin (vitamin B7) in the Ac cell extract. It has been known that A. cycloclastes has several biotinylated proteins which potentially interfere with the purification using the strep-tag affinity system (Bollinger, J. et al. unpublished results). Therefore, it was desirable to remove biotinylated proteins from the cell extract before loading it on the strep-tactin column. An ammonium sulfate fractionation method was applied. The results of the SDS-PAGE and western blot of NosX purification are shown in Figure 2.3. The labels (#1 - 4) in Figure 2.3 indicate the supernatant after cell breakage and centrifugation (#1), supernatant and precipitate from 40 % ammonium sulfate precipitation (#2 and #3), and the elution fraction from the strep-tactin sepharose column (#4). Marker proteins (M) were pre-stained in red (72 kDa) and blue (130, 95, 55, 43, 34, 26, 17, and 11 kDa). Thus, these marker bands are also visible on the western blot membrane (Figure 2.3, right). In preliminary experiments, it was found that NosX was precipitated at 40 % saturation of ammonium sulfate, which was also apparent in Figure
2.3 (#2) since no NosX band was observed in the supernatant of 40 % ammonium sulfate precipitation. Three biotinylated proteins (130, 70, and 20 kDa) and NosX (31 kDa) were detected by the western blot. These four proteins were observed in the cell extract from Ac/pMLClustXst (#1), although minimally. The 130 kDa biotinylated protein was successfully removed by the 40 % ammonium sulfate precipitation (#2, #3).

![Figure 2.3. SDS-PAGE (left) and western blot (right) for NosX purification. #1, cell extract; #2 and #3, supernatant and precipitate from 40 % ammonium sulfate precipitation; #4, elution fraction; M, marker](image)

In this purification process, however, the 70 and 20 kDa biotinylated proteins could not be removed from the NosX containing fraction before loading the sample onto the streptactin affinity column (#3). The 70 kDa protein was observed in both #3 and #4, whereas the 20 kDa protein was not observed in the elution fraction (#4).

**Discussion**

The expression level of NosX in Ac/pMLClustXst was very low using the original culture conditions and it became necessary to improve yields in order to carry out
biochemical characterization of this protein. Overexpression of NosX in *E. coli* and co-factor reconstitution trials for the heterologously expressed NosX had failed in our lab (Bollinger, J. *et al.* unpublished results). Thus, we attempted to enhance the NosX yield from the *Ac* homologous expression system (pMLClustXst) by modifying the plasmid and optimizing the culture conditions for further characterization of NosX (chapter 3 and 4). Additionally, the NosX purification procedure with the strep-tag was also modified for sustainable purification.

**Promoter Refinement for NosX**

The expression level of NosX was low, suggesting that the native NosX promoter in the *nos* cluster was not potent. In *A. cycloclastes*, the expression level of PAz (AcPAz) was revealed to be high (91). It was also observed that the expression level of NiR in *A. cycloclastes* (AcNiR) was significant (Ijima, F. *et al.* unpublished observation). These observations indicated that the promoters of AcPAz and AcNiR were expected to be potent. As the expression levels of AcNiR and AcPAz were known to become high under denitrifying conditions, these promoters included putative Dnr/DnrD binding motifs which were not observed in the native NosX promoter. Thus, the NosX promoter was replaced with the promoters of AcNiR and AcPAz to enhance the NosX expression level. Unexpectedly, the expression levels of NosX between p5-(Np/Pp)-*nosX* and pMLClustXst expression systems were almost the same (Table 2.2, Ac/p5-Np-*nosX* (0.10 mg/10 g cell), Ac/p5-Pp-*nosX* (0.13 mg/10 g cell), and Ac/pMLClustXst (0.10 ± 0.08 mg/10 g cell)). It is still unknown why the expression level of NosX was not enhanced by these potent promoters. There are several possibilities to explain this observation. First, it
might be possible that mRNAs of nosX in the cell are unstable and NosX expression does not reach expected levels. Second, it is possible that NosX in the cell is unstable and degrades quickly before isolation. As will be discussed in chapters 3 and 4, NosX is unstable \textit{in vitro}. This observation might imply that there are additional protein components that stabilize NosX \textit{in vivo}. If the amounts of these additional components are insufficient in the NosX overexpressed cell, it might result in degradation of NosX. Third, the promoters of AcNiR and AcPAz might not be effective for NosX overexpression from the plasmids. To evaluate these hypotheses, additional investigations will be required.

The combination of Ac/pMLClustXst and the LB media purchased from Fisher resulted in much higher NosX efficiency (~ 5-fold) relative to Ac/p5-(Np/Pp)-nosX (1.3 - 1.4-fold) (Table 2.4). Interestingly, the LB media from Fisher did not show a positive effect for the NosX yield on p5-(Np/Pp)-nosX expression systems (Table 2.3). One of differences between Ac/pMLClustXst and Ac/p5-(Np/Pp)-nosX was that the whole set of Nos proteins were expected to be overexpressed in Ac/pMLClustXst whereas only NosX was overexpressed in Ac/p5-(Np/Pp)-nosX. Therefore, the overexpressed Nos proteins might be involved in the enhancement effect on NosX expression levels with the unknown factor in the LB from Fisher. As mentioned in chapter 1, it has been reported that NosR acts as a positive regulatory element for the expressions of the other Nos proteins (106; 50). Hence, in this hypothesis, overexpressed NosR in Ac/pMLClustXst would be the most plausible candidate that would enhance NosX expression. Although the expression level of NosR in Ac/pMLClustXst was not monitored directly, it was
plausible that NosR was also overexpressed because active N\textsubscript{2}OR (NosZ) in $Ac/pMCLustXst$ was 10 - 100-fold overexpressed compared to $Ac$ without the plasmid (125). Further, this enhancement mechanism with the unknown factor in LB media from Fisher was expected to be independent (or could be remotely related) from transcriptional factor Dnr/DnrD for the following reason. $Ac/p5-(Np/Pp)-nosX$, which had Dnr/DnrD binding sites in the promoter, did not show the enhancement of the NosX expression, whereas the \textit{nosX} gene in $Ac/pMCLustXst$, which did not have Dnr/DnrD binding site in the promoter, showed the enhancement.

**Culture Conditions for NosX**

Culture conditions were optimized to increase the NosX yield from $Ac/pMCLustXst$. Three conditions (LB media, aeration speed, and prolonged incubation time) were identified as positive factors on the NosX yield. Since there were still fluctuations on the yield after the optimizations, existence of another unknown factor on the NosX expression is implied.

In the presence of oxygen, aerobic respiration is expected to occur preferentially rather than denitrification (3). Although dissolved oxygen in the LB media was not monitored during the cultures, the media agitated at 250 rpm was assumed to be aerobic because significant increase of the cell yield was observed (Table 2.6, 35.8 ± 9.1 g for 100 rpm vs. 75.3 ± 7.7 g for 250 rpm). It was reasonable that the cell yield was increased under the aerobic conditions (250 rpm) because the aerobic respiration system is more efficient with regard to energy generation for cell proliferation than nitrate respiration (denitrification). However, the NosX expression level was consistent under both aerobic
and micro-aerobic conditions (Table 2.6, 0.23 ± 0.14 mg/10 g cell for 250 rpm and 0.20 ± 0.06 mg/10 g cell for 100 rpm). Initially, it was expected that expression of NosX would be decreased under the aerobic conditions since the expression of Nos proteins were controlled by the transcriptional factor Dnr/DnrD and NosR. It was reported that transcription of dnrD was activated in O₂-limited cells in Pseudomonas stutzeri (128) and nos gene expression was activated under anaerobic conditions (55). It was also known that NosR had Fe-S clusters in the cytoplasmic region that were expected to regulate expression of the other Nos proteins (82; 106). Since the Fe-S cluster is oxygen sensitive, the regulation ability of NosR is expected to be perturbed in aerobic conditions. In contrast, it was also reported that denitrification could occur even under aerobic conditions, which was referred to as aerobic denitrification (129). Although no advantage on nitrate respiration under aerobic conditions was known, this phenomenon has been observed in denitrifying bacteria such as Paracoccus denitrificans (130) and Pseudomonas stutzeri (105). The observation on the NosX expression under aerobic conditions might suggest that aerobic denitrification by Achromobacter cycloclastes is occurring. It was also reported that a small amount of N₂OR was expressed constitutively in Pseudomonas stutzeri even under aerobic conditions (105; 50). The consistent expression of NosX could be also explained as NosX was expressed constitutively at a low level in Ac.

Currently, there are two hypotheses to explain the result that the expression level of NosX was constant under the aerobic and micro-aerobic conditions. The first hypothesis is that the DnrD/NosR regulation systems for the Nos protein expression in Ac
might be relatively insensitive to oxygen. This hypothesis is supported by the facts that denitrification can occur under aerobic conditions and N₂OR was constitutively expressed under aerobic conditions. If the DnrD/NosR could function under aerobic conditions as well, it explained why the expression level of NosX was consistent under both aerobic and micro-aerobic conditions. The second hypothesis is based on the traditional concepts for denitrification, that is, denitrification does not function in the presence of abundant oxygen. This hypothesis is supported by the fact that many proteins related to denitrification are sensitive to oxygen. In this hypothesis, the expression of NosX should be independent, at least in part, from the oxygen sensitive DnrD/NosR regulation systems. Instead, there might be another expression mechanism for NosX that is oxygen resistant, although there has been no report supporting this hypothetical expression mechanism to date.

The protein expression levels vary with time, and therefore the incubation time needs to be optimized to maximize the particular protein yield. It was reported that there was a lag between the expressions of NiR and NOR and the expression of the *nos* genes (14). This can be explained as follows: after potassium nitrate was added to the LB media, denitrifying bacteria start using nitrate for respiration. As described in chapter 1, nitrate will be converted to nitrite and NO by NaR and NiR in the denitrification process (Figure 1.1). Once NO starts to be generated, it strongly enhances the expression of the *nos* genes via Dnr/DnrD (102; 55; 99), subsequently resulting in the expression of N₂OR and its accessory proteins are started to be expressed. Therefore, a considerable time lag is expected for expression of the Nos proteins. Indeed, the results showed that the NosX
expression level per cell was increased in the elongated (48-hour) cultures (Table 2.7), consistent with this expectation.

Purification Conditions for NosX

Protein purification with an affinity tag is a commonly known technique to isolate target proteins with one step. The strep-tag, which is a widely used affinity purification system, was attached to the C-terminal part of NosX (125). As will be revealed in chapters 3 and 4, NosX requires a high salt concentration (150 mM NaCl) for its stabilization. Thus, affinity techniques are essential for the purification of NosX because standard ion exchange techniques that require low salt conditions cannot be used.

Biotin is a critical contaminant that interrupts the purification process using the strep-tactin column. In *E. coli*, the total amount of biotin in cell lysate is negligible (~ 1 nmol in 1 g cell) and only 20 µg (per 1 g cell) of avidin, which binds to biotin and inhibits the irreversible binding between biotin and the strep-tactin resin, was required for column maintenance (131). Thus, the strep-tag is an excellent option for protein purifications from *E. coli*. However, this affinity purification system was found to be not suitable for the protein purification from *Achromobacter cycloclastes* because there are a considerable amount of biotinylated proteins (20, 70, and 130 kDa proteins, Figure 2.3) in the cell extract. Indeed, even with the 4-fold amount of avidin treatment (12.5 U for 10g cell (~ 86 µg/g cell)) compared to the prep from *E. coli*, the binding capacity of the strep-tactin column was gradually and irreversibly reduced.

Although the 130 kDa protein was successfully removed from the NosX containing fraction by the ammonium sulfate precipitation, the 20 and 70 kDa proteins
could not be removed with the present purification protocol. According to the western blot (Figure 2.3), the 70 kDa protein was observed in both #3 and #4, indicating that this protein still had affinity to the column, but the interaction between this protein and the column resin was reversible and thus could be eluted. In contrast, the 20 kDa protein was not observed in the elution fraction (#4) and it indicated that this protein irreversibly bound to the column. Therefore, the 20 kDa protein was likely contributing to the reduction of the column capacity. The reason that avidin could not neutralize biotin in the 20 kDa protein was probably that bulky avidin (MW = 66 kDa) could not access biotin in the 20 kDa protein because of steric hindrance. For further investigations, the protocol needs to be improved to remove the 20 kDa protein prior to loading the sample to the strep-tactin column. Ion exchange and hydroxylapatite columns could not separate the biotinylated proteins from the NosX containing fraction under high salt conditions (data not shown). Alternatively, GST (Glutathione S-Transferase) or MBP (Maltose-Binding Protein) tag systems might be better choices for both purification and protein stabilization of NosX, since these tags, which are very stable and soluble proteins, may significantly improve the stability of the fused protein in solution. As mentioned in chapter 1, NosF was successfully purified and characterized as a MBP tagged protein (50).

Conclusions

Two new expression systems for NosX (Ac/p5-(Np/Pp)-nosX) were successfully constructed with strong promoters for AcNiR (Np) and AcPAz (Pp). The expression levels of NosX in Ac with the new expression systems were examined and compared. The
new systems showed 1.3 ~ 1.4-fold higher yields of NosX than Ac/pMLClustXst. In addition, we determined the NosX yield was significantly enhanced (5 - 6-fold) when Ac/pMLClustXst was cultured with the LB media purchased from Fisher bland. Five factors during culture were optimized for NosX expression using Ac/pMLClustXst. After the optimizations, the NosX yield was ~ 19-fold increased compared to the original conditions.
CHARACTERIZATION OF NOSX FROM ACHROMOBACTER CYCLOCLASTES

Introduction

Several papers have explored the function of NosX by deleting the nosX gene in vivo (114; 52; 83). In Paracoccus denitrificans, the function of NosX has been connected to activation of N₂OR in vivo (83). However, NosX has not previously been isolated and fully investigated. Gaining functional and structural information of NosX is essential to understanding the putative mechanism of N₂OR activation by this protein.

Further, it is known that several proteins interact with more than one protein in the denitrification process. For example, NirX in Paracoccus denitrificans was shown to interact with N₂OR although this protein was expected to work on NiR because it was coded in the nir gene cluster (83). NosX in Paracoccus denitrificans was implied to interact with both N₂OR and NiR (132). Pseudoazurin and cytochrome c₅₅₀ are also known to donate electrons to several enzymes such as NiR, NOR, and N₂OR (88; 91; 86; 89). These observations indicate that NosX from A. cycloclastes (AcNosX) may indeed interact with several proteins other than N₂OR. Characterization of the biochemical properties of AcNosX will provide critical insight into potential interactions between NosX and other proteins of the denitrification system.

In the previous chapter, the culture conditions were successfully optimized to enhance AcNosX yields, which enabled us to carry out the studies in the remainder to this
thesis. In this chapter, the properties of AcNosX were investigated in vitro. The structure of AcNosX was also investigated by crystallization and homology modeling methods.

**Materials and Methods**

**Confirmation of the DNA Sequence of nosX Gene**

The nosX gene in pMLClustXst and pTrcClust were sequenced to confirm the sequence that was registered in the GenBank. The pTrcClust plasmid included whole nos cluster from the Ac genome DNA and was the original template for the other derivative such as pMLClustXst. The primer sequences were as follows;

NosX sequence primer #1: GACGCCGGTGAGACCGGCTCGG
NosX sequence primer #2: CGCTTCGATCCGACGGTACAGCCGCT
NosX sequence primer #3: CCCCTCTCGGCACGACCTTCGATCAAGC

The three primers were designed to read anterior region, middle region, and posterior region of nosX sequence. 1 µg of DNA and 10 pmol of primer were mixed and sent to the University of Iowa for sequence.

**Molecular Weight and Quaternary Structure of AcNosX**

The molecular weight of AcNosX was measured with micrOTOF-MS (Bruker Daltonics) with an electrospray ionization (ESI) source. 4 µL of AcNosX (150 µM in Buffer W) was mixed with 4.5 µL of 0.1 % formic acid and injected to a Luna C8 cartridge column (Phenomenex Inc.) that was connected to the micrOTOF-MS at a flow rate 200 µL/min. AcNosX in the column was eluted with buffer A (0.1 % formic acid, 99.9 % H₂O) and buffer B (0.1 % formic acid, 99.9 % acetonitrile). Subsequently, the
mass spectra of AcNosX were measured in the 300-3000 m/z range in the positive mode. The data was analyzed with the Bruker Daltonics DataAnalysis version 3.3.

The quaternary structure of AcNosX in solution was determined using a gel filtration column as previously described (121). The column was equilibrated with Buffer W. The calibration was conducted using a gel filtration standard (Bio-Rad) containing thyroglobulin (bovine, 670 kDa), gamma globulin (bovine, 158 kDa), ovalbumin (chicken, 44 kDa), myoglobin (horse, 17 kDa), and vitamin B$_{12}$ (1.35 kDa). The void volume (Vo) was determined with thyroglobulin. The molecular weight of AcNosX was calculated with a plot of log molecular weight of the standard versus the elution volumes over the void volume (Ve/Vo). The quaternary structure of AcNosX was deduced from the molecular weight.

Cofactor Identification

The identity of the cofactor bound to AcNosX was determined using a fluorescence spectroscopy method, as previously described (133). The AcNosX concentration was determined with absorption at 450 nm and the molecular extinction coefficient of $\epsilon_{450} = 11570 \text{ M}^{-1} \text{ cm}^{-1}$ (134). 1000 µL of 10 µM AcNosX in 20 mM Tris (pH 8.0) was denatured by incubation at 100 °C for 10 min. The denatured protein was removed by centrifugation at 14000 rpm for 10 min. 15 µL of phosphodiesterase I (PDE) solution (362 mU) was added to the supernatant that included the AcNosX cofactor. Fluorescence and UV/Vis spectra of AcNosX, the extracted AcNosX cofactor, and the cofactor with PDE were measured and compared. The samples were excited at 455 nm, and the emission spectra were measured between 470 and 670 nm. Positive and negative
controls were measured where the same amount of PDE solution was added to 1000 µL of either 10 µM FAD or FMN in 20 mM Tris (pH 8.0). Fluorescence and UV/Vis spectra were measured before and after addition of PDE to the samples.

**Redox Potential**

The redox potential of AcNosX was determined using the method described by Massey (135) with slight modifications. As preliminary experiments, AcNosX and anthraquinone 2-sulfonate (AS, redox dye, $E = -225$ mV) were individually photo-reduced and monitored by UV/Vis spectroscopy to determine the molecular extinction coefficients of oxidized and reduced forms under the experimental conditions. Also, the appropriate incubation time following photo-reduction, which was necessary for equilibration among the electron transfer reactions involving AcNosX, AS, and benzyl viologen (BV), was determined by monitoring UV/Vis spectra.

AcNosX, a mixture of AS and BV in 100 mM phosphate, 150 mM NaCl, 10 % glycerol, 10 mM EDTA, pH 7.0 (Buffer PE), and an aerobic cuvette were individually purged with Ar gas for at least 1 hour on ice. The oxygen purge for the AcNosX solution was carried out by gently blowing Ar gas on the surface of the protein solution. The sample was tapped once every 10 to 15 min. The buffer with reagents was intensively purged with Ar gas to remove dissolved oxygen in the solution. After anaerobic conditions were established, AcNosX and reagents were injected in the cuvette. Final concentrations of AcNosX and AS were 10 µM, and BV was 1 µM. AS, BV, and EDTA were used as an internal standard, a mediator in electron transfer, and electron source in the photochemical reaction, respectively. The cuvette was illuminated in the ice/water
bath for 15 sec using a Xe arc lamp (175 W, Lambda-LS/17, Sutter Instrument Company) and an IR cut-off filter. The cuvette was incubated for 10 to 15 min in the dark while stirring at 10 °C to let the both redox pairs of NosX<sub>ox</sub>/NosX<sub>red</sub> and AS<sub>ox</sub>/AS<sub>red</sub> reach equilibrium. After incubation, the UV/Vis spectrum was recorded. The light exposure and UV/Vis measurements were repeated until no more changes were observed in the spectra. At each equilibrium state, the concentrations of oxidized and reduced AcNosX ([NosX<sub>ox</sub>], [NosX<sub>red</sub>]) were calculated with the equation [NosX<sub>red</sub>] = [NosX<sub>total</sub>] - [NosX<sub>ox</sub>] and using the Lambert-Beer equation (A = ε<sub>red</sub>[NosX<sub>red</sub>] + ε<sub>ox</sub>[NosX<sub>ox</sub>]). The concentrations of oxidized and reduced AS ([AS<sub>ox</sub>], [AS<sub>red</sub>]) were also calculated similarly. The data were analyzed with the Nernst equation as previously described (136). Measurements were completed in triplicate.

Secondary and Tertiary Structure Predictions

The secondary structure of AcNosX was predicted from the sequence using three different methods: JUFO (137), PSIPred (v3.0) (138), and PAPIA (139). The secondary structures of AcPAz (PDB code: 1BQR) and AcN<sub>2</sub>OR (PDB code: 2IWF) were also predicted by those sequences to check the accuracy of the methods. Structural ratios of helix, sheet, and random coil content in AcNosX were calculated by the three methods and compared with a homology model (described below).

A three-dimensional AcNosX structure was generated by homology modeling with the ApbE protein from Salmonella enterica (121) (PDB code: 3PND). Sequences of both AcNosX and ApbE were aligned by T-Coffee (140) and a homology model was
generated with the aligned sequence and Swiss-Model (alignment mode) (141). The coordinates of FAD from ApbE were superimposed onto the model structure.

The electrostatic potential and hydrophobicity of the AcNosX model surface were calculated by PyMOL using the Adaptive Poisson-Boltzmann Solver (APBS) (142) and rTools (143) plugins. Additionally, the sequences of AcNosX (Genbank accession code: AAD09162), Paracoccus denitrificans PD1222 (PdNosX, CAB53356), Sinorhizobium meliloti (SmNosX, AAB69119), and Bradyrhizobium japonicum (BjNosX, CAA05524) were aligned by Clustal W 2.1 (144) and compared with the sequence and structure of ApbE protein to identify possible correlations between conserved regions and the FAD binding motif. The results were displayed with BoxShade 3.21 (145). The conserved amino acids among NosX proteins and potential FAD binding residues were mapped on the homology model of AcNosX to identify candidates for functionally important regions in AcNosX.

**Crystallization Trials**

Crystallization of AcNosX was attempted for structural studies. The AcNosX concentration was adjusted to 5 mg/mL in 5 mM Tris, 150 mM NaCl, and 10 % glycerol (pH 8.0). Screening reagents (96 × 7 plates = 672 conditions) were purchased from Hampton research and Emerald BioSystems Inc. Screening plates were set up with a Honeybee protein crystallization system (Genomic Solutions). The samples were crystallized with the sitting drop method under 4 °C. The conditions that showed crystal-like structures were used for further optimizations using a 24-hole plate and the hanging drop method. Optimization of the conditions was carried out by changing concentrations
of salt, buffer, and precipitant. The amount of crystallization solution in the reservoir was 1 mL, and 2 µL each of the solution and AcNosX were mixed on a cover glass and incubated at 4 °C.

Results

Confirmation of the DNA Sequence of nosX Gene.

The nosX gene in pMLClustXst and pTrcClust were sequenced to determine if there was an error in the sequence of AcNosX. The results showed that both pMLClustXst and pTrcClust had A159 (GCT) whereas V159 (GTC) was registered in the Genbank. The data in the Genbank was obtained from the sequence of pTrcClust and was obviously misread. The corrected sequence fitted closely to the measured molecular weight.

Molecular Weight and Quaternary Structure of AcNosX

The molecular weight of AcNosX was determined to be 30806.58 Da by the micrOTOF-MS, which was different from the theoretical molecular weight of 30835 Da. This result indicated that there was an error in the sequence from either the pMLClustXst plasmid or the sequence data in the Genbank database (code: AAD09162.2). To confirm, the nosX gene in pMLClustXst and pTrcClust were sequenced (see next section for details). The result indicated that V159 (GTC) (V183 with the signal peptide) in the Genbank should be A159 (GCT). This correction shifted the theoretical molecular weight to 30807 Da, which was very close to the measured value of 30806.58 Da.
The quaternary structure of \textit{AcNosX} was measured using a gel filtration column and standard markers. \textit{AcNosX} and the standard markers were individually applied to the column and the elution times were monitored. The molecular weights of the standards and \textit{AcNosX} were plotted versus the elution volume over void volume (\(Ve/Vo\)) (Figure 3.1). The elution position of the purified \textit{AcNosX} (\(Ve/Vo = 1.76\)) is indicated by an empty square that is labeled as NosX in Figure 3.1. The calculated molecular weight of \textit{AcNosX} in Buffer W from the plot was 31583 Da which was close to the molecular weight of one holo-\textit{AcNosX} with one FAD molecule bound (31592.6 Da). This result indicated that \textit{AcNosX} was monomeric under the experimental conditions.

![Figure 3.1. Quaternary structure analysis of AcNosX with a gel filtration column](image)

**Cofactor Identification**

PDE converts flavin adenine dinucleotide (FAD) to flavin mononucleotide (FMN) by breaking a phosphodiester bond. The fluorescence intensity of FMN is \(\sim 10\)-fold
stronger than FAD (133). In the control experiments, FAD showed a significant increase of fluorescence intensity with PDE (Figure 3.2B) whereas the intensity of FMN did not change, as expected (Figure 3.2C). The fluorescence spectrum of the extracted cofactor from AcNosX showed a significant increase of intensity in the presence of PDE (Figure 3.2A, blue). The result was almost identical to the control experiment for FAD, and clearly showed that the cofactor bound to AcNosX was FAD.

Figure 3.2. Normalized fluorescence spectra (10 μM) of (A) AcNosX cofactor, (B) FAD and (C) FMN with or without PDE.
Redox Potential

AcNosX and anthraquinone 2-sulfonate (AS) were individually photo-reduced and monitored by UV/Vis spectroscopy. The absorption peaks at 374 and 460 nm of oxidized AcNosX were diminished as the protein was reduced (Figure 3.3A). Oxidized AS showed a peak at 330 nm, which decreased upon reduction, whereas peaks at 381 and 400 nm concomitantly increased (Figure 3.3B). Isosbestic points were observed at 338 nm for AcNosX (Figure 3.3A) and 355 nm for AS (Figure 3.3B). The concentrations of AcNosX and AS during the photo-reduction were determined using the absorptions at 355 nm for AcNosX and 338 nm for AS. The molecular extinction coefficients of AcNosX at 355 nm were calculated as $\varepsilon_{355, \text{ox}} = 9626 \text{ M}^{-1} \text{ cm}^{-1}$ (fully oxidized form) and $\varepsilon_{355, \text{red}} = 5184 \text{ M}^{-1} \text{ cm}^{-1}$ (fully reduced form). The molecular extinction coefficients of AS at 338 nm were also calculated as $\varepsilon_{338, \text{ox}} = 4673 \text{ M}^{-1} \text{ cm}^{-1}$ and $\varepsilon_{338, \text{red}} = 1458 \text{ M}^{-1} \text{ cm}^{-1}$. It should be noted that any increasing peak around 540 - 700 nm, which is indicative of the semiquinone form of FAD (146), was not observed during the stepwise photo-reduction of AcNosX (Figure 3.3A). It was also confirmed that 10 min of incubation was sufficient to reach a state of equilibrium for the electron transfer reaction among AcNosX, AS, and BV since no spectral change was observed in the UV/Vis spectra after 10 min (data not shown).

The redox potential of AcNosX was determined by photo-reduction in the presence of AS as an internal standard. Typical UV/Vis spectra of the sample at various equilibrium states are shown in Figure 3.4. The plotting of $\log([\text{NosX}_{\text{ox}}]/[\text{NosX}_{\text{red}}])$ versus $\log([\text{AS}_{\text{ox}}]/[\text{AS}_{\text{red}}])$ according to Minnaert (146) gave a linear line with a slope of
0.95 and intercept of 0.201 (Figure 3.4, inset). The redox potential of AcNosX under the experimental conditions was calculated to be -231 ± 0.6 mV.

Figure 3.3. Stepwise photo-reductions of AcNosX (A) and anthraquinone 2-sulfonate (B)
This method to determine the redox potential using a redox dye was based on the premise that there was no significant binding between the protein (AcNosX) and the dye (AS) (136). After redox measurements, the samples were injected onto a gel filtration column and AcNosX and AS were clearly separated based on information from the elution profile and UV/Vis spectra (data not shown). This result showed that AcNosX did not bind AS significantly under our experimental conditions.

![Titration measurement of redox potential of AcNosX](image)

**Figure 3.4.** Titration measurement of redox potential of AcNosX

**Secondary and Tertiary Structure Predictions**

Comparison of the secondary structure predictions of AcNosX by JUFO, PSIpred, and PAPIA are shown in Table 3.1. The accuracies of each program were evaluated using the sequences and structures of AcPAz and AcNzOR. Among the three programs, PSIpred
showed the highest accuracy on secondary structure prediction (93.3 % for AcPAz and 85.4 % for AcN₂OR). The predicted values for AcNosX structure with PSIPred (helix: 32 %, sheet: 24 %, and coil: 44 %) were very close to the ratio of each component (helix: 36 %, sheet: 22 %, and coil: 42 %) in the generated AcNosX model, suggesting the model structure was reasonable.

<table>
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<th>JUFO</th>
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<th>PAPIA</th>
<th>AcNosX model</th>
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Table 3.1. Secondary structure predictions of AcNosX by JUFO, PSIPred, and PAPIA (upper) and calculated values of PAz and N₂OR by PSIPred (lower)

The homology model of AcNosX generated with ApbE and its surface electrostatic potential are shown in Figure 3.5. The electrostatic potential map on the surface of AcNosX model showed a putative FAD binding pocket that was negatively charged. This negatively charged pocket was also observed in the FAD binding site of ApbE protein (Appendix C, Figure S3.1). The Tyr46 (Tyr70 with the signal peptide) residue which was well conserved in NosX from various species was located close to the
isoalloxazine ring of FAD for a pi stacking interaction in the model (Figure 3.5). This observation indicated that Tyr46 might be important to hold the FAD cofactor in AcNosX.

Figure 3.5. AcNosX model based on ApbE and surface electrostatic potential

Figure 3.6. Hydrophobicity of the AcNosX model
Figure 3.7. Sequence alignment of NosX proteins and ApbE

NosX from *Achromobacter cycloclastes* (AcNosX), *Paracoccus denitrificans* (PdNosX), *Sinorhizobium meliloti* (SmNosX), *Bradyrhizobium japonicum* (BjNosX) and ApbE from *Salmonella enterica* were aligned with Clustal W (Figure 3.7). Conserved
amino acids among the NosX proteins are highlighted in blue and light blue, and the amino acids involved in FAD binding in ApbE protein are highlighted in yellow and underlined. Well conserved Tyr70 was emphasized with “*” (will be described in discussion).

Figure 3.8. Distribution of the conserved amino acids (green) and potential FAD binding residues (red) in the AcNosX model

The conserved amino acids of AcNosX are also highlighted in green in the AcNosX model (Figure 3.8). The residues that interact with FAD in ApbE protein (Met41, Gly42, ASP72, Ser76, Ala119, Glu121, Asp181, Ser183, Glu187, Arg267 and Ile272) correspond to the conserved residues in the AcNosX sequences (Leu12 (L36 with the signal peptide in Figure 3.7), Gly13 (G37), Glu40 (E64), Ser44 (S68), Tyr46 (in Figure 3.5, Y70), Asp87 (D111), Thr140 (T164), Asn142 (N166), Gln146 (Q170), Arg216 (R240) and Leu221 (L245)). The potential FAD binding residues are highlighted in red
(Figure 3.8). This mapping on the model reveals that many of the conserved amino acids are located in the plausible FAD binding pocket, and the small hydrophobic patch (Figure 3.6) was composed of well conserved amino acids.

**Crystallization Trials**

Crystallization conditions for AcNosX were screened with reagent kits and a screening robot. Since AcNosX starts precipitating from ~ 6 mg/mL (~ 200 μM) and at room temperature within an hour, protein concentration for the screening was adjusted to 5 mg/mL (158.2 μM) and the plate was incubated at 4 °C. At days 1, 7, and 14 after the plate preparation, similar wedge-like structures were observed in three conditions at E-11 in plate #1, 2, and 4.

![Figure 3.9. Pictures of putative crystals from condition #1](image)

The first condition was 100 mM HEPES, 20 mM MgCl₂, 22 % (w/v) Poly(acrylic acid sodium salt) 5100, pH 7.5 and the second condition was 100 mM Bis-Tris propane, 500 mM succinic acid, pH 7.0. The third condition was 4% (v/v) Tacsimate (pH 5.0) and 12 % (w/v) PEG3350. Since the crystals were observed in the same spot (E-11) in different plates, it is possible that the crystals were due to the contamination of the buffer.
injection needle for E-11. However, further crystallization trials under the first conditions produced additional crystals (~ 10 × ~ 10 × ~ 70 μm, Figure 3.9) on a large scale plate. These crystals were dissolved after several weeks. To date, the crystal formation process has been poorly reproducible.

Discussion

Identity Confirmation of Purified AcNosX

Identity of the purified yellow protein was confirmed to be AcNosX with several aspects. DNA sequencing of the nosX gene from Ac indicated that the molecular weight of apo-AcNosX was 30807 Da, which was almost identical to the measured value of the purified protein (30806.58 Da). Further, the strep-tag that was attached on the C-terminal end of AcNosX was detected from the purified protein in chapter 2 by Western blot (Figure 2.2). Additionally, N-terminal amino acid sequence of the purified protein has shown that the first 12 amino acids were identical to the expected sequence from nosX gene sequence (134). These results, together with the previous data, confirmed that AcNosX was expressed and purified as intended.

Characterization of the Cofactor in AcNosX

The cofactor of AcNosX was determined to be FAD (Figure 3.2). One AcNosX molecule was expected to have one FAD molecule according to the following observations: (1) The molecular weight of holo-AcNosX was estimated at 31583 Da by the gel filtration column (Figure 3.1), which was close to the sum (31592.5 Da) of apo-AcNosX (30807 Da) and one FAD (785.5 Da). (2) The fluorescence intensity of PDE
treated AcNosX cofactor (10 µM) was comparable intensities of PDE treated FAD and FMN (10 µM) (Figure 3.2), which indicated that 10 µM of AcNosX included 10 µM of FAD.

FAD was expected to be bound to AcNosX non-covalently. The micrOTOF-MS detected the apo form of AcNosX (30807 Da). The ESI process of the micrOTOF-MS is sufficiently mild to keep covalent bonds intact. Thus, if AcNosX and FAD were bound covalently, the holo form should be detected. Additionally, FAD in AcNosX was extracted to the supernatant after boiling of the protein sample (Figure 3.2A). Further, addition of spermine to AcNosX resulted in ejection of FAD to solution (Figure 4.11, in chapter 4). These results consistently indicated that FAD was non-covalently bound to AcNosX.

There are three redox states for FAD in electron transfer reactions; oxidized, semiquinone, and reduced forms. Thus, the total amount of AcNosX during the redox reaction could be expressed with the following equation: $[\text{NosX}_{\text{total}}] = [\text{NosX}_{\text{ox}}] + [\text{NosX}_{\text{semi}}] + [\text{NosX}_{\text{red}}]$. The UV/Vis spectra during the experiments for the redox determination were measured at the equilibrium state. It was possible that the semiquinone form was transiently generated during the electron transfer reaction. However, the semiquinone form was not detected in the equilibrium state (Figure 3.3A). This observation indicated that the semiquinone form of AcNosX was unstable and it immediately became either oxidized or reduced form. Thus, the $[\text{NosX}_{\text{semi}}]$ term was negligible at least in the equilibrium state. This observation supported that it was
adequate to use the equation \([\text{NosX}_{\text{total}}] = [\text{NosX}_{\text{ox}}] + [\text{NosX}_{\text{red}}]\) for the calculations to determine the AcNosX concentrations.

The redox potential of AcNosX was determined as -230 mV at pH 7.0. AcPAz reduced only the Cu\(_A\) site (~ 250 mV (67)) in AcN\(_2\)OR \textit{in vitro} (see chapter 4, Figure 4.2). This was probably because the redox potential of AcPAz was 260 mV (147) and it was not potent enough to reduce the Cu\(_Z\) site (~ 60 mV (68)). Since the redox potential of AcNosX was revealed to be lower than those values, AcNosX might be able to donate electrons to the Cu\(_Z\) site as well as the Cu\(_A\) site in AcN\(_2\)OR. The electron transfer between AcNosX and AcN\(_2\)OR \textit{in vitro} will be further investigated and discussed in chapter 4.

\textbf{Structural Aspects of AcNosX}

Four FAD-binding protein families have been reported (148). Recently, ApbE was shown to have a new FAD-binding motif (121). According to the sequence identity (23.5 \%) and similarity (38.7 \%) between AcNosX and ApbE (Figure 3.7), NosX is also expected to have this new FAD-binding motif.

AcNosX structure was investigated with computational and crystallization methods. Homology modeling of AcNosX, sequence-based secondary structure predictions for AcNosX, and comparative analysis of NosX proteins and ApbE sequences provided structural insights for AcNosX. The crystallization trials for AcNosX required further optimization of the conditions for structure determination. The rationale for selecting NosX sequences from \(Ac\), \(Sm\), and \(Bj\) for the comparative analysis was that these bacteria were relatively well studied with respect to denitrification and located on different branches in a phylogenetic tree, which was generated based on the NosX
sequences (Appendix C, Figure S3.2). Well conserved amino acids among such distantly related NosX proteins are expected to be important for the function and/or structure of NosX. Indeed, the sequence alignment showed good agreement between the well conserved residues in NosX proteins and the residues that formed the FAD binding pocket in ApbE (Figure 3.7). These conserved amino acids were located close to or within the FAD binding pocket in the AcNosX model (Figure 3.8), indicating that the corresponding conserved amino acids were likely to be involved in cofactor recognition.

Tyr46 (Tyr70 with the signal peptide), which was one of the well conserved amino acids among the species, which is indicated with “*” in Figure 3.7, was located close to the isoalloxazine ring of the FAD in the model (Figure 3.5). The ring stacking between FAD and aromatic amino acids has been well observed in many FAD binding proteins (149). In ApbE protein, the same type of ring stacking was observed between FAD and Tyr78 (121). This Tyr78 residue was also well conserved and found to be essential to FAD binding (121). These observations indicate that Tyr46 in AcNosX is likely to play a similar role in cofactor binding. Further, Gly13 (Gly37 with the signal peptide) in AcNosX is also well conserved, which corresponds to well conserved Gly42 in ApbE protein (Figure 3.7). This residue was shown to function in FAD binding as well (121).

NosX from *Paracoccus denitrificans* was involved in the N\textsubscript{2}OR activation *in vivo* (83). Also, we found that AcNosX had a redox active cofactor, FAD (Figure 3.2, 3.4). These results lead us to propose a hypothesis that AcNosX donates electrons to AcN\textsubscript{2}OR (see chapter 4 for detail). It has been revealed that the pseudo-specific interaction
between $AcPAz$ and $AcN_2OR$ is important for electron transfer (see detail for chapter 1) (87; 93). According to the pseudo-specific electron transfer mechanism (87), it was expected that $AcNosX$ should have a hydrophobic patch and surrounding positively charged patches to interact with $AcN_2OR$ for electron transfer, if $AcNosX$ binds to the similar place where $AcPAz$ binds for electron transfer. The electrostatic potential and hydrophobicity on the surface of the $AcNosX$ model showed that there was a small hydrophobic patch composed of well conserved residues, and it was located close to the putative FAD binding site (Figure 3.6, 3.8). Further, positively charged residues were observed next to the hydrophobic patch (Figure 3.5). These results suggested that $AcNosX$ may bind and donate electrons to $AcN_2OR$ similarly to $AcPAz$.

$NosX$ has been suggested to interact with the membrane bound protein, $NosR$ (52; 83). The electron transfer pathway from $NosR$ to $N_2OR$ through $NosX$ in *Paracoccus denitrificans* has been implied (83). There is a large hydrophobic patch on the back side of the FAD binding site in the $AcNosX$ model (Figure 3.6). Since there are almost no conserved amino acids in the hydrophobic patch (Figure 3.6, 3.8), this patch was less likely to be responsible for protein interaction with partner proteins. Instead, this hydrophobic patch might interact with the membrane surface during the electron transfer between $AcNosX$ and $AcNosR$ since $AcNosX$ would get closer to the membrane to interact with $AcNosR$. $NosR$ from *Pseudomonas stutzeri* was reported to have an FMN binding domain in the periplasm (82). $AcNosX$ was expected to interact with this domain to receive electrons. Since the redox potential of $AcNosX$ was -230 mV, the redox
potential of FMN in AcNosR would be a similar or lower value than -230 mV for energetically favored electron flow.

Conclusions

The data revealed from this project supported and complemented the hypothesis by Wunsch et al (83), which stated that NosX is involved in N₂OR activation. To the best of our knowledge, this is the first time that NosX was purified and characterized. It was determined that AcNosX was a soluble, FAD containing protein and was redox active. Computational analysis of AcNosX structure suggested that well conserved amino acids were involved in the cofactor binding and the protein-protein interaction with AcN₂OR. The large hydrophobic patch might be important to facilitate the protein-protein interaction with AcNosR by interacting with the membrane. Together with previous data, these observations suggest that AcNosX is a physiological electron donor for AcN₂OR in sustaining N₂OR activity and potentially mediates electron transfer from AcNosR to AcN₂OR.
CHAPTER 4

INVESTIGATIONS ON THE FUNCTION OF NOSX FROM ACHROMOBACTER CYCLOCLASTES

Introduction

In denitrifying bacteria, pseudoazurin (PAz) and cytochrome c have been reported to be common electron donors for the denitrifying enzymes NiR, NOR, and N₂OR (88; 86; 89; 87). The electron transfers from PAz and cytochrome c to N₂OR were reported in several bacteria such as Paracoccus pantotrophus (PAz and cytochrome c₅₅₁) (90), Marinobacter hydrocarbonoclasticus (formerly Pseudomonas nautica) (cytochrome c₅₅₂) (150), Paracoccus denitrificans (PAz and cytochrome c₅₅₀) (89). We also reported that PAz from A. cycloclastes (AcPAz) could donate electrons to A. cycloclastes N₂OR (AcN₂OR) (91). Furthermore, PAz and cytochrome c were shown to sustain the catalytic reaction of N₂OR (150; 91).

Two states of the Cu₉ site in N₂OR have been characterized; the Cu₉ state (enzymatically active) and the Cu₉* state (inactive) (68; 69). The Cu₉* state may be generated with O₂ exposure of N₂OR and/or mutations of several Nos proteins (see below for detail) (82; 68; 83). Therefore, the Nos proteins (NosX and NosR) are expected to display reactivity towards the Cu₉* state. Both the Cu₉ and Cu₉* states in AcN₂OR show the absorption peak at 640 nm in the UV/Vis spectrum (11; 77). The structural difference between two states has been unclear. Recently, the structure of N₂OR from Pseudomonas stutzeri was solved (63). The Cu₉ site in this structure included an extra sulfide (Figure
It was hypothesized that the difference between the CuZ state and the CuZ* state was derived from the presence or absence of the extra sulfide. That is, the CuZ state is the [4Cu:2S] form and the CuZ* state is the [4Cu:1S] form.

The function of NosX was shown to be involved in the activation of the CuZ site in *Paracoccus denitrificans* (83). Deletion of the nosX and nirX genes resulted in a loss of N₂OR activity and an increase of the CuZ* state *in vivo* (83). Although it was not mentioned in the paper, PAz and cytochrome c₅₅₀ were expressed as WT and could donate electrons to N₂OR in the double mutant strain. Thus, the results suggested that NosX has a function that is distinct from the functions of PAz and cytochrome c₅₅₀, since the N₂OR activity was inhibited even in the presence of PAz and cytochrome c₅₅₀. Isolated N₂OR from the double mutant strain could be activated with reductants *in vitro* (83). The results suggested that NosX was involved in the activation of the catalytic center (the CuZ site) of N₂OR.

As presented in chapter 3, NosX from *A. cycloclastes* (AcNosX) is a redox active FAD protein (*E* = -230 mV). This observation together with the previous data lead us to the hypothesis that AcNosX donates electrons to AcN₂OR to activate the catalytic center and/or to regenerate the inactive CuZ* state to the catalytically active CuZ state. In this chapter, the function of AcNosX was investigated to probe the possible activation mechanism for AcN₂OR. It is important to investigate the N₂OR activation process to fully understand the mechanism of N₂O reduction by N₂OR. As mentioned in chapter 1, this information may be a basis for future applications that could help reduce broad environmental issues that are caused by uncontrolled N₂O emission (123). Additionally,
the electron transfer reaction between AcNosX and AcPAz was also examined. The electron transfer between AcNosX and AcN₂OR was measured by UV/Vis spectroscopy and confirmed by electron paramagnetic resonance (EPR) in vitro. The regeneration of the Cu₂⁺ state was also attempted with photo-reduction in the presence of AcNosX in vitro. Further, the N₂OR catalytic reaction was monitored in the presence of an excess amount of AcNosX to see if AcNosX was able to sustain catalytic turnover.

**Materials and Methods**

Expression and Purification of Pseudoazurin from *Achromobacter cycloclastes*

PAz from *A. cycloclastes* (AcPAz) was overexpressed and purified from *E. coli* (JM109/pTrc99A-paz). The plasmid pTrc99A-paz was provided from Prof. T. Kohzuma (Ibaraki University, Japan) (151). The plasmid was inserted to *E. coli* JM109 strain with electroporation. JM109/pTrc99A-paz was streaked on LB plate with 100 µg/mL of ampicillin (Amp100) and incubated at 37 °C for overnight. A single colony from the plate was suspended to 200 mL LB media (Amp100) and cultured for overnight at 37 °C while shaking at 250 rpm. The culture (30 mL) was inoculated to 900 mL of LB media (Amp100) in a 3 L baffled flask. 6 flasks of the inoculated LB media were incubated at 37 °C for ~ 2 hours until A₆₀₀ became 0.6. 1.5 mL of 300 mM IPTG (final conc. 500 µM) and 1.8 mL of 50 mM CuSO₄ (final conc. 100 µM) per a flask were added to the media to induce the PAz expression. After 8 hours, the cultures were centrifuged with 10800 × g (Sorvall GS3 rotor) for 10 minutes at 4 °C to collect the cell. The cell pellet (~ 25 g) was kept at 4 °C overnight for purification or -80 °C for storage.
Overexpressed AcPAz in *E. coli* was isolated from the harvested cell. The cell pellet (~ 25 g) was suspended to an equal amount (~ 30 mL) of 50 mM Phosphate buffer (pH 6.0). The overexpressed AcPAz was extracted from the cell with a French pressure cell at ~ 70 MPa. The cell debris was removed with centrifugation at 39000 ×g (Sorvall SS-34 rotor) for 60 min. The AcPAz containing fraction was separated from the crude supernatant with the ammonium sulfate precipitation method (40 % saturation). The sample with ammonium sulfate was incubated for 1 hour while stirring (~ 400 rpm) at 4 °C. After centrifuging the sample for 30 min at 39000 ×g, the supernatant (~ 50 mL) was dialyzed with 5 L of 5 mM Phosphate (pH 6.0) for 3 ~ 5 hours and with 5 L of 20 mM Phosphate (pH 6.0) with a dash of potassium ferriyanide for overnight. AcPAz was fully oxidized with the oxidant to facilitate the following purification process by making AcPAz visible. The sample was centrifuged for 30 min at 39000 ×g to remove proteins that precipitated during dialysis. The supernatant was loaded onto a SP-sepharose column (20 mL, 10 × 260 mm) which was equilibrated with 50 mM Phosphate (pH 6.0). After washing the column with five column volumes of 50 mM Phosphate (pH 6.0), proteins on the column were eluted with 500 mM Phosphate (pH 6.0). Fractions that included AcPAz were detected with A$_{600}$ and collected. The AcPAz containing fraction (~ 30 mL) was dialyzed with 5 L of 20 mM Phosphate (pH 6.0) for overnight. After centrifuging the sample for 30 min at 39000 ×g, the supernatant was loaded onto the SP-sepharose column which was equilibrated with 50 mM Phosphate (pH 6.0). AcPAz was eluted with a linear gradient (50 – 500 mM Phosphate, pH 6.0) method, and concentrated with an Amicon Ultra Centrifugal Filter 3K (Millipore). The purity of AcPAz was checked with
SDS-PAGE analysis using 12.5 % gels and Coomassie Brilliant Blue (CBB) staining. On average, ~ 100 mg of AcPAz could be obtained from 25 g of cells. The concentration of AcPAz and Cu content were calculated using a BCA protein assay kit (Pierce) and an atomic absorption spectrometer (AA, SpectrAA220, Varian), respectively. When the Cu content of AcPAz was lower than 80 %, the sample was dialyzed with Cu containing buffer to reconstitute the Cu site of apo-AcPAz. The sample was dialyzed with 1 L of 20 mM Tris (pH 8.0) with 1 mM CuSO$_4$ for 4 ~ 5 days. The sample was further dialyzed with 5 L of 20 mM Tris (pH 8.0) without CuSO$_4$ for 2 ~ 3 days to remove excess free Cu in the sample. Typically, the Cu content of AcPAz after dialysis became over 90 %.

Concentration of Cu loaded AcPAz was estimated with A$_{594}$ and ε$_{594}$ = 3700 M$^{-1}$ cm$^{-1}$ (147). The ratio of absorptions at 280 nm over 594 nm ($A_{280}/A_{594}$) of pure and fully Cu loaded AcPAz was about 1.4.

Reduced AcPAz was obtained by incubation of purified AcPAz with 100-fold excess of dithionite. To prevent protein precipitation, oxidized AcPAz (~ 5 mL) was gradually put into 30 mL of 100 mM Phosphate (pH 7.0) with dithionite. The residual reductant was removed with an Amicon ultrafiltration membrane (YM-5, Millipore) under anaerobic conditions using Ar bubbled (~ 1 hour) Buffer P (100 mM Phosphate, 150 mM NaCl, 10 % (v/w) glycerol, pH 7.0).

Expression and Purification of N$_2$OR
from Achromobacter cycloclastes

N$_2$OR from A. cycloclastes (AcN$_2$OR) was homologously overexpressed in Ac/pMLnos. Ac/pMLnos was cultured as previously described (see chapter 2). The
culture was carried out with 2 L of LB media × 4 flasks for 24 hours under micro aerobic conditions. A typical cell yield was ~ 30 g. The harvested cell pellet was immediately frozen at -80 °C or kept at 4 °C overnight for purification.

\( \text{AcN}_2\text{OR} \) was purified as previously described (11). During the purification process, the sample was kept under anaerobic conditions to minimize \( \text{O}_2 \) exposure which results in low Cu content and low activity. Typically, ~ 10 g of cell was suspended with ~ 20 mL of 20 mM Tris (pH 8.0) with a dash of dithionite. The suspended cells were lysed with the French pressure cell twice at ~ 70 MPa. The cell lysate was ultracentrifuged for 60 min at 45000 rpm (Sorvall Ti50 rotor) at 4 °C, and supernatant was anaerobically transferred to a DEAE fast-flow column (120 mL, 25 × 240 mm), which was equilibrated with 20 mM Tris (pH 8.0). All buffers in this purification were purged with Ar gas at least 30 min right before use and included 250 µM sodium dithionite to remove residual oxygen in buffers. Proteins bound on the column were eluted with a linear gradient method with 20 mM Tris, 500 mM NaCl, pH 8.0. \( \text{AcN}_2\text{OR} \) containing fractions (~ 60 mL) were collected and diluted with 250 mL of 20 mM Tris (pH 8.0) to reduce salt concentration. The sample was loaded onto a DEAE column (34 mL, 25 × 70 mm). \( \text{AcN}_2\text{OR} \) on the column was eluted with 20 mM Tris, 500 mM NaCl, pH 8.0. \( \text{AcN}_2\text{OR} \) containing fractions (~ 15 mL) were collected and diluted with 50 mL of 20 mM Tris (pH 8.0) to reduce salt concentration. The sample was then applied to a Mono Q column (8 mL, 10 × 100 mm) that was equilibrated with 20 mM Tris (pH 8.0). The buffer in the column was exchanged to 20 mM Bis-Tris (pH 6.5) and bound proteins on the column were eluted with a linear gradient method. \( \text{AcN}_2\text{OR} \) was eluted from the column around
30 – 70 min. The sample buffer was exchanged with an Amicon (YM-30 filter) several times to reduce the salt concentration. At the final step, AcN₂OR was applied to a cytochrome c agarose affinity column (8 mL, 10 × 100 mm) and eluted by a linear gradient. The AcN₂OR containing fractions were eluted around 43 – 70 min. The sample buffer was exchanged with an Amicon with 20 mM Tris (pH 8.0, without dithionite) and concentrated to 1 ~ 2 mL. This AcN₂OR sample was called the as dithionite reduced form or the “as purified” form. The protein concentration was estimated with A₂₈₀ and ε₂₈₀ = 105,000 M⁻¹ cm⁻¹. Typical AcN₂OR yield from 10 g cell pellet was 10 ~ 15 mg. Cu content was measured with atomic absorption spectrometer (AA). Typically, the N₂OR samples that were used for the following measurements contained 4.0 ~ 5.0 Cu/monomer.

The N₂OR activity assay was also carried out under anaerobic conditions. 10.5 μM AcN₂OR in 50 mM Phosphate (pH 7.0) was activated in the presence of 2 mM methyl viologen (MV) and 1 mM dithionite (DT) at 25 °C for 3 hours. 2 μL of activated AcN₂OR was injected to the mixture of 2700 μL of 50 mM phosphate, 300 μL of 5 mM BV and 2 μL of 100 mM DT in an anaerobic cuvette with a total volume of 3004 μL. The time course of absorption change at 600 nm was monitored for 2 min to see the background of the auto-oxidation rate of BV. After 2 min, 80 μL of N₂O saturated 50 mM phosphate (bubbled with N₂O gas at least 1 hour at room temperature) was injected to the anaerobic cuvette and to begin the catalytic reaction. Absorption changes, which indicated the oxidation of BV (= electron transfer to AcN₂OR), were measured for 3 min. The activity (U/mg) was calculated by the absorption change between 0.12 and 0.18 min after N₂O was injected as described previously (11). The typical activity of sample was ~
80 U/mg at 25 °C. To investigate the inhibition effect of chloride on the N₂OR activity (see below), the activity assay was carried out with various buffers: 50 mM phosphate (pH 7.0), 50 mM phosphate + 150 mM NaCl (pH 7.0), and 50 mM phosphate + 50 mM Na₂SO₄ (pH 7.0). The procedure was the same as described above. Prior to N₂O injection, N₂OR was incubated in each buffer for 5 min. The BV oxidation was monitored for 5 min in the presence of N₂O.

Fully oxidized AcN₂OR was prepared by the incubation of the “as purified” form of AcN₂OR with a 100-fold excess amount of potassium ferricyanide. The oxidant was removed with gel filtration. PAz reduced AcN₂OR was obtained with the incubation of an excess amount of reduced AcPAz and fully oxidized AcN₂OR in the molar ratio of 30 to 1 (monomer base). AcPAz was removed with a gel filtration column. All samples were exchanged to Buffer P (100 mM phosphate, 150 mM NaCl, 10 % glycerol, pH 7.0) under anaerobic conditions.

**Electron Transfer from AcNosX to AcN₂OR**

The redox state of fully oxidized AcN₂OR or PAz reduced AcN₂OR in the presence and absence of reduced NosX were monitored with UV/Vis spectroscopy and electron paramagnetic resonance (EPR). The following experiments were carried out under anaerobic conditions. AcNosX in Buffer PE (100 mM Phosphate, 150 mM NaCl, 10 % glycerol, 10 mM EDTA, pH 7.0) was photo-reduced by illuminating in the ice/water bath for 3-5 min intervals using a Xe arc lamp (175 W) and an IR cut-off filter. Typically, fully reduced AcNosX was obtained within 15 - 30 min.
The electron transfer from \( \text{AcNosX} \) to \( \text{AcN}_2\text{OR} \) was measured by UV/Vis spectroscopy. An equimolar (17.8 \( \mu \)M) amount of reduced \( \text{AcNosX} \) and fully oxidized \( \text{AcN}_2\text{OR} \) were mixed in an anaerobic cuvette and the UV/Vis spectra were measured at 4 \( ^\circ \text{C} \) to stabilize NosX. As a negative control, Buffer PE was photo-reduced for 15 min in the anaerobic cuvette under the same conditions and mixed with oxidized \( \text{AcN}_2\text{OR} \). The UV/Vis spectrum was measured to check if the illuminated Buffer PE could reduce \( \text{AcN}_2\text{OR} \).

EPR spectra were measured to investigate the redox state of the Cu sites in \( \text{AcN}_2\text{OR} \) which was reduced with \( \text{AcPAz} \) and/or \( \text{AcNosX} \). The sample preparation process for the EPR measurements is summarized in Figure 4.1. Fully oxidized \( \text{AcN}_2\text{OR} \) (#2) and PAz reduced \( \text{AcN}_2\text{OR} \) (#3) were prepared as described in the method section. Dithionite reduced \( \text{AcN}_2\text{OR} \) (#1, as purified) was oxidized with an excess amount (100-fold) of potassium ferricyanide to obtain fully oxidized \( \text{AcN}_2\text{OR} \) (#2, oxidized). Excess amount of ferricyanide was removed.

![Figure 4.1. Schematic view of sample preparations for the EPR measurements](image)

The fully oxidized \( \text{AcN}_2\text{OR} \) (#2) was further reduced with an excess amount (30-fold) of \( \text{AcPAz} \) (#3, PAz reduced). PAz and NosX reduced \( \text{AcN}_2\text{OR} \) (#4, PAz-NosX reduced), and NosX reduced \( \text{AcN}_2\text{OR} \) (#5, NosX reduced) were prepared by mixing either PAz.
reduced AcN$_2$OR (#3) or fully oxidized AcN$_2$OR (#2) with 2× molar excess of reduced AcNosX in a glove box (O$_2$ < 1 ppm, Coy laboratory products Inc.). These samples were prepared sequentially from one sample (#1). The excess amount of AcNosX was not removed from the samples because of the following reasons: (1) Oxidized and reduced forms of AcNosX are diamagnetic. Thus, it would not contribute to the EPR spectra and was negligible for the measurements. (2) If any signal from AcNosX was observed in the EPR spectra, it would indicate the presence of a semi-quinone form of AcNosX. It was desirable to confirm the presence or absence of the semi-quinone form under conditions similar to those used to determine the redox potential. In chapter 3, the equation

$$([\text{NosX}_{\text{red}}] = [\text{NosX}_{\text{total}}] - [\text{NosX}_{\text{ox}}]),$$

which assured the absence of the semi-quinone form under the equilibrated states, was used to determine the redox potential of AcNosX. Recall that the original equation was $[\text{NosX}_{\text{red}}] = [\text{NosX}_{\text{total}}] - [\text{NosX}_{\text{ox}}] – [\text{NosX}_{\text{semi}}]$, but $[\text{NosX}_{\text{semi}}]$ term that corresponds to the concentration of the semi-quinone form of AcNosX was assumed to be negligible. The final concentrations of each N$_2$OR sample were 146.8 µM (as purified, 300 µL), 79.2 µM (oxidized, 300 µL), 50.4 µM (PAz reduced, 300 µL), 36.2 µM (PAz-NosX reduced, [N$_2$OR]:[NosX]=1:2.2, 300 µL), and 67.6 µM (NosX reduced, [N$_2$OR]:[NosX]=1:2.5, 260 µL). Samples were placed in EPR quartz tubes and rapidly frozen with liquid nitrogen. For spin quantification, Cu(triethanolamine) standard solutions (0, 25, 50, 100 µM, 300 µL) were prepared. EPR spectra of the samples and the standards were collected at 14, 20 and 30 K respectively using an EMX X-band spectrometer (Bruker) equipped with a liquid helium cryostat and temperature controller (Oxford instruments). Typical EPR parameters were: microwave
frequency, 9.38 GHz; microwave power, 1.16 mW; time constant, 163.84 msec; sweep
time 83.89 sec. After EPR measurements, UV/Vis spectra of the samples were measured
at room temperature. Each sample was collected from the EPR quartz tube in the glove
box (O₂ < 1 ppm). If necessary, the samples were diluted with Buffer P (100 mM
Phosphate, 150 mM NaCl, 10 % glycerol, pH 7.0) to increase the volumes to measure the
spectra. After the UV/Vis and EPR measurements, the Cu contents of the samples were
measured by AA.

Regeneration of the Cu₉* State
in AcN₂OR with AcNosX

As a preliminary experiment, AcN₂OR was incubated in Buffer PE (100 mM
Phosphate, 150 mM NaCl, 10 % glycerol, and 10 mM EDTA, pH 7.0) for 4 hours to
investigate the effect of EDTA on the Cu sites of N₂OR. 1000 µL of ~ 30 µM AcN₂OR in
Buffer PE was incubated in an anaerobic cuvette at room temperature. The UV/Vis
spectra of the sample were monitored for 4 hours.

Photo-reduction of oxidized AcN₂OR was monitored in the absence and presence
of AcNosX to see if AcNosX was able to reduce the Cu₉* state in AcN₂OR. The sample
preparation was carried out in the glove box (O₂ < 1 ppm). 23 µM of AcN₂OR in Buffer
PE was photo-reduced on ice/water for 5 min in an anaerobic cuvette, and UV/Vis
spectrum was measured. The sample was further exposed to the light for 5 min until no
change was observed spectrally. After AcN₂OR was reduced up to the limit of photo-
reduction system, oxidized AcNosX was added to the sample. The ratio between AcN₂OR
and AcNosX was 1 to 1 (20 µM). The cuvette was further exposed to the light for 10 min and UV/Vis spectra were measured until no further change was observed.

Electron Transfer from Reduced AcNosX to Oxidized AcPAz

The electron transfer pathway from AcNosX to AcPAz was investigated in vitro. The following procedures were carried out under anaerobic conditions since reduced AcNosX was sensitive to oxygen. ~ 500 µL of 105 µM NosX in Buffer PE (pH 7.0) was photo-reduced in an anaerobic cuvette with ice and water. The progress of AcNosX reduction was monitored by UV/Vis where a spectrum was taken every 10 min. After the AcNosX was fully reduced, 171 µL of reduced AcNosX was injected to 429 µL of oxidized AcPAz in Buffer P (pH 7.0) and the electron transfer was monitored by UV/Vis at 4 °C. Final concentrations of AcNosX and AcPAz were 30 µM at a 1:1 ratio.

Catalytic Reaction of AcN₂OR with Reduced AcNosX

The N₂OR catalytic reaction in the presence of an excess amount of reduced AcNosX was monitored. All procedures were carried out under anaerobic conditions. The conditions for the measurements were the same as the N₂OR activity assay with two modifications: AcNosX was used for electron source instead of BV, and Buffer P (pH 7.0) was used to stabilize AcNosX during the measurements instead of 50 mM phosphate (pH 7.1). 10.5 µM AcN₂OR in Buffer P was activated in the presence of 2 mM MV and 1 mM DT at 25 °C for 3 hours. 1330 µL of 100 µM AcNosX in Buffer PE (pH 7.0) was photo-reduced. 2 µL of activated AcN₂OR, 1330 µL of photo-reduced AcNosX, 1668 µL
of Buffer P was mixed in an anaerobic cuvette. The total volume of the sample was 3 mL. Final concentrations of each component were, 6.8 nM AcN₂OR, 44 µM AcNosX, 4.3 mM EDTA, 1.3 µM MV, and 0.7 µM DT. UV/Vis spectra were monitored for 10 min at 10 °C to determine the background auto-oxidation rate of AcNosX. After 10 min, 80 µL of N₂O saturated Buffer P (bubbled with N₂O gas at least 1 hour at room temperature) was injected to the anaerobic cuvette and UV/Vis spectra were further collected for 20 min.

For control experiments, the catalytic reactions of AcN₂OR in the presence of an excess amount of reduced AcPAz or free FAD were also monitored using Buffer P (pH 7.0) or 50 mM phosphate (pH 7.0). AcPAz and FAD were fully reduced with equimolar amounts of dithionite. Final concentrations of each experiment were 75.6 µM AcPAz vs. 6.8 nM AcN₂OR and 72.0 µM FAD vs. 6.8 nM AcN₂OR.

Identification of Inhibitory Factor for the N₂OR Catalytic Cycle with AcPAz

It was determined that Buffer P inhibited the N₂OR catalytic reaction when reduced AcPAz was used as an electron donor instead of reduced BV. To identify which component of the buffer inhibited the reaction, the following buffers were prepared and the N₂OR activity was checked with these buffers; (#1) 50 mM potassium phosphate (pH 7.0), (#2) 50 mM potassium phosphate + 150 mM NaCl (pH 7.0), (#3) 50 mM potassium phosphate + 10 % glycerol (pH 7.0), (#4) 50 mM potassium phosphate + 150 mM KCl (pH 7.0), (#5) 50 mM sodium phosphate (pH 7.0). As negative controls, (#6) 50 mM potassium phosphate + 50 mM Na₂SO₄ (pH 7.0, I = 0.242, the same ionic strength as buffer #2), and (#7) 129 mM potassium phosphate (pH 7.0, I = 0.241) were also applied.
to prove if high ionic strength inhibited the electron transfer reaction. AcN\(_2\)OR was activated as previously described with 50 mM potassium phosphate (pH 7.0). 2 µL of the activated AcN\(_2\)OR, 250 µL of reduced AcPAz (932 µM) in 50 mM potassium phosphate (pH 7.0), and 2752 µL of one of those buffer (#1 - 5) were mixed. For the negative control experiments, 200 µL of reduced AcPAz (932 µM) and 2802 µL of buffer (#6 - 7) were used. The total sample volume was 3004 µL. The final concentrations of AcN\(_2\)OR and AcPAz were 7 nM and 77.6 µM. UV/Vis spectra of the samples were monitored for 5 min to monitor the background auto-oxidation of AcPAz. After 5 min, 80 µL of N\(_2\)O saturated 50 mM potassium phosphate (pH 7.0) was anaerobically injected to the sample to start the catalytic reaction and the UV/Vis spectra were monitored for 20 - 25 min.

**AcNosX Stabilization in Solution**

It was essential to remove NaCl from the AcNosX sample to investigate whether AcNosX could sustain the N\(_2\)OR catalytic cycle. To find conditions that stabilized AcNosX in the absence of NaCl, six reagents (arginine, ammonium sulfate, spermine, spermidine, Triton X-100, and Tween 20) that were known as protein stabilizers (152; 153; 154; 155) were individually added to AcNosX samples. To remove NaCl, AcNosX in Buffer W (100 mM Tris, 150 mM NaCl, 1 mM EDTA, and 10 % (w/v) glycerol, pH 8.0) was applied to a gel filtration column that was equilibrated with 100 mM phosphate, 10 % glycerol, pH 7.0. The eluted AcNosX fraction was gently mixed and aliquotted to 1 mL × 8 tubes. 50 µL of stock solutions of candidate reagents (arginine (4.2 M), ammonium sulfate (2.1 M), spermine (4.1 M), spermidine (4.1 M), Triton X-100 (2.1 %), Tween 20 (2.1 %), and NaCl (3.15 M)) were added to the AcNosX samples immediately.
Final concentrations of each reagent were arginine (200 mM), ammonium sulfate (100 mM), spermine (200 mM), spermidine (200 mM), Triton X-100 (0.1 %), Tween 20 (0.1 %), and NaCl (150 mM). The sample with NaCl was a positive control and the sample without any reagent was a negative control. The eight samples were incubated at 4 °C overnight. Precipitations in the samples were checked visually at 2, 4, and 8 hours. After the incubation, samples were centrifuged for 10 min at 14000 rpm and measured by UV/Vis spectra.

Results

Electron Transfer from AcNosX to AcN₂OR

The electron transfer from reduced AcNosX to oxidized AcN₂OR was monitored by UV/Vis spectroscopy. When an equimolar amount of reduced AcNosX (Figure 4.2, black) and oxidized AcN₂OR (Figure 4.2, red) were mixed, an increase in absorption at 374 and 460 nm from AcNosX and the decrease in absorption between 550 and 800 nm from AcN₂OR were observed (Figure 4.2, blue). The absorption changes in the spectrum indicate the oxidation of AcNosX and the reduction of AcN₂OR. In a negative control, Buffer PE was photo-reduced and mixed with oxidized AcN₂OR. The illuminated Buffer PE did not reduce AcN₂OR (data not shown). The control experiment confirmed that AcNosX but not any other components in Buffer PE reduced AcN₂OR. These results demonstrated that AcNosX donated electrons to AcN₂OR under the experimental conditions.
The various forms of AcN2OR including “as purified” (#1), fully oxidized (#2), PAz reduced (#3), PAz and NosX reduced (#4), and NosX reduced (#5) were measured by UV/Vis and EPR spectroscopy (Figure 4.3, 4.4). The UV/Vis spectra of the EPR samples are shown in Figure 4.3. The spectra were normalized based on Cu concentrations. The spectra were normalized with the N2OR concentration (23.5 µM) for comparison. The spectrum of PAz reduced N2OR (#3) showed that absorption peaks of AcN2OR were decreased compared to the spectrum of fully oxidized N2OR (#2), indicating that AcPAz donated electrons to AcN2OR (Figure 4.3, green). The differential spectrum (Figure 4.3, blue) of fully oxidized (#2) and PAz reduced (#3) N2OR showed peaks at ~ 480, ~ 530, and ~ 810 nm. For clarity, AcNosX containing samples (PAz-NosX reduced N2OR (#4) and NosX reduced N2OR (#5)) were not shown in Figure 4.3.
because absorption from AcNosX was so strong that peaks from AcN₂OR were buried in it. The spectra of #4 and #5 were essentially similar to the blue spectrum in Figure 4.2.

![Figure 4.3. UV/Vis spectra and differential spectrum of AcN₂OR samples for EPR](image)

The EPR spectra of the samples showed the degrees of reduction for the Cu sites in AcN₂OR which were reduced with AcPAz and/or AcNosX (Figure 4.4). The EPR spectra were normalized with the N₂OR concentrations (36 µM) for comparison. The integrated intensities of each spectrum, which are indicative of the amount of oxidized Cu, are summarized in Table 4.1. Amounts of oxidized Cu (per monomer) in the AcN₂OR samples at 14, 20, and 30 K were estimated with spin quantification using Cu standard curves that were measured at each temperature (see Appendix D: Figure S4.1 for EPR spectra and standard curves of the Cu standard). The integrated EPR intensities (×10⁵) of
$AcN_2OR$ samples at 14 K (Figure 4.4) were calculated as follows; 2065 (#2, fully oxidized) > 1379 (#3, PAz reduced) \approx 1271 (#5, NosX reduced) > 692 (#1, as purified) > 396 (#4, PAz-NosX reduced). The oxidized Cu in each sample was calculated as 0.32 (#1, as purified), 1.02 (#2, oxidized), 0.66 (#3, PAz reduced), 0.22 (#4, PAz-NosX reduced), and 0.59 (#5, NosX reduced) (Table 4.1). The intensity of the EPR spectrum for NosX reduced $N_2OR$ (sample #5, $Int = 1271 \times 10^5$ at 14 K) was obviously lower than the intensity of fully oxidized $N_2OR$ (sample #2, $Int = 2065 \times 10^5$ at 14 K), confirming that $AcNosX$ reduced $AcN_2OR$.

Figure 4.4. EPR spectra of $AcN_2OR$ samples at 14 K (normalized by protein concentration at 36 µM)
The Cu contents of the samples were determined by AA after the EPR and UV/Vis measurements. The total Cu amounts per N$_2$OR monomer of each sample were estimated as follows; #1 (as purified) = 4.0, #2 (fully oxidized) = 2.9, #3 (PAz reduced) = 3.2, #4 (PAz-NosX reduced) = 3.5, #5 (NosX reduced) = 2.9. The Cu amounts of #2 (2.9) and #5 (2.9), #3 (3.2) and #4 (3.5) should be comparable since the differences between them were only with or without AcNosX (Figure 4.1). The Cu amount of fully oxidized N$_2$OR (#2, Cu = 2.9/monomer) was significantly lower than “as purified” form (#1, Cu = 4.0/monomer).

<table>
<thead>
<tr>
<th>No.</th>
<th>N$_2$OR sample (normalized to 36 µM)</th>
<th>Temp</th>
<th>Integrated Intensity ($\times 10^5$)</th>
<th>Cu$^{2+}$/monomer</th>
<th>Ave. Cu$^{2+}$/monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>As purified</td>
<td>14 K</td>
<td>692</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 K</td>
<td>566</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 K</td>
<td>395</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Oxidized</td>
<td>14 K</td>
<td>2065</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 K</td>
<td>1570</td>
<td>1.02</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 K</td>
<td>1077</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PAz reduced</td>
<td>14 K</td>
<td>1379</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 K</td>
<td>1061</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 K</td>
<td>709</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PAz-NosX reduced</td>
<td>14 K</td>
<td>396</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 K</td>
<td>496</td>
<td>0.26</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 K</td>
<td>295</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NosX reduced</td>
<td>14 K</td>
<td>1271</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 K</td>
<td>909</td>
<td>0.55</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 K</td>
<td>653</td>
<td>0.57</td>
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</tbody>
</table>

Table 4.1. Spin quantification of EPR spectra of AcN$_2$OR

It was revealed that incubation of “as purified” form of AcN$_2$OR with 100-fold excess amount of ferricyanide resulted in decrease of Cu content in N$_2$OR (Appendix D: Table
The activities of AcN$_2$OR in Table S4.1 (Exp #3) were determined to be 122.3 ± 5.1 U/mg for as purified form and 94.9 ± 4.1 U/mg for oxidized form after incubation with reduced MV.

According to the oxidized Cu amounts derived from the spin quantification with the EPR spectra and total Cu amounts obtained from the AA results, oxidized Cu ratios (oxidized Cu/total Cu) in each AcN$_2$OR sample were calculated as follows; #1 = 8.0 % (oxidized Cu/total Cu = 0.32/4.0 = 8.0 %), #2 = 35.2 % (1.02/2.9 = 35.2 %), #3 = 20.6 % (0.66/3.2 = 20.6 %), #4 = 6.3 % (0.22/3.5 = 6.3 %), #5 = 20.3 % (0.59/2.9 = 20.3 %).

According to the calculated ratio, the redox state of each sample was assigned as in Table 4.2. Theoretical oxidized Cu ratios were calculated as oxidized Cu over fully Cu loaded N$_2$OR (6 Cu/monomer) (e.g. sample #1, oxidized Cu/total Cu = 1/6 = 16.7 %). In sample #2 in Table 4.2, three out of six coppers in AcN$_2$OR were expected to be oxidized. Thus, oxidized Cu ratio was calculated as 50 %. However, two oxidized Cu in the Cu$_Z$ site could not be detected with EPR because two spins in the Cu$_Z$ site were supposed to be canceled out with anti-ferromagnetic coupling (80). Thus, apparent oxidized Cu ratio would be calculated as 16.7 % (oxidized Cu/total Cu = 1/6 = 16.7 %).

<table>
<thead>
<tr>
<th></th>
<th>Cu$_A$</th>
<th>Cu$_Z$</th>
<th>Oxidized Cu (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1. As purified</td>
<td>Cu(I) • Cu(I)</td>
<td>1Cu(II) • 3Cu(I)</td>
<td>Maximum* *</td>
</tr>
<tr>
<td>#2. Fully oxidized</td>
<td>Cu(1.5) • Cu(1.5)</td>
<td>2Cu(II) • 2Cu(I)</td>
<td>16.7</td>
</tr>
<tr>
<td>#3. PAz reduced</td>
<td>Cu(I) • Cu(I)</td>
<td>2Cu(II) • 2Cu(I)</td>
<td>33.3</td>
</tr>
<tr>
<td>#4. PAz-NosX reduced</td>
<td>Cu(I) • Cu(I)</td>
<td>1Cu(II) • 3Cu(I)</td>
<td>16.7</td>
</tr>
<tr>
<td>#5. NosX reduced</td>
<td>Cu(I) • Cu(I)</td>
<td>2Cu(II) • 2Cu(I)</td>
<td>33.3</td>
</tr>
</tbody>
</table>

* Maximum values were calculated with the assigned structures and oxidation states of the Cu sites

Table 4.2. Redox state assignments of each form of AcN$_2$OR
No radical signal from a semiquinone form of AcNosX was observed in the EPR spectra of sample #4 and #5 (Figure 4.4). The result supported the idea that AcNosX exists in either its fully reduced or oxidized form in the equilibrium state. This observation was consistent with the data from the UV/Vis spectra of the stepwise photo-reduction for AcNosX (Figure 3.3, left). Thus, the equation, $[\text{Total NosX}] = [\text{oxidized NosX}] + [\text{reduced NosX}] + [\text{semiquinone NosX}]$, can be approximated to $[\text{Total NosX}] = [\text{oxidized NosX}] + [\text{reduced NosX}]$, which was used to determine the redox potential of AcNosX. The result showed that the approximation was reasonable.

Regeneration of the Cu$_Z^*$ State in AcN$_2$OR with AcNosX

For a control experiment, AcN$_2$OR was incubated in Buffer PE for 4 hours under anaerobic conditions to check the effect of EDTA on AcN$_2$OR. Although a minor decrease in absorption intensity on the UV/Vis spectra was observed, the spectral change was slight and negligible within the time scale of the following measurements (Appendix D: Figure S4.3).

Purified N$_2$OR always includes a certain percentage of the Cu$_Z^*$ state (69). To investigate if AcNosX was able to regenerate the Cu$_Z^*$ state, oxidized AcN$_2$OR was photo-reduced in the absence and presence of AcNosX. 23 µM of oxidized AcN$_2$OR was photo-reduced in Buffer PE under anaerobic conditions (Figure 4.5). The arrows indicate decrease of the absorption that was caused by the reduction of Cu centers in AcN$_2$OR. The sample was gradually reduced and did not show significant absorbance changes after 30 min of light exposure.
Figure 4.5. Photo-reduction of AcN$_2$OR in the absence of AcNosX
Arrows indicate decrease of absorption peaks

Figure 4.6. Photo-reduction of AcN$_2$OR in the presence of AcNosX
Arrows indicate decrease of absorption peaks
The spectrum after photo-reduction still had a shoulder around 540 nm which was assigned to the $2\text{Cu(II)} \cdot 2\text{Cu(I)}$ form in the Cu$_Z$ site. The peak at 640 nm was assigned to the partially oxidized Cu$_Z$ ($1\text{Cu(II)} \cdot 3\text{Cu(I)}$) form and Cu$_Z^*$ form, both of which have the absorption peak at the same wavelength (11; 77). The result shows that photo-reduction can reduce the Cu$_A$ completely and the Cu$_Z$ site partially (Figure 4.5, black).

After reduction of AcN$_2$OR was reached to the limit of photo-reduction system, oxidized AcNosX was anaerobically added to the cuvette. The cuvette was further photo-reduced in the presence of oxidized AcNosX (Figure 4.6). If AcNosX was able to reduce those partially oxidized Cu$_Z$ ($1\text{Cu(II)} \cdot 3\text{Cu(I)}$) and/or Cu$_Z^*$ centers, the peak at 640 nm should decrease. The arrows point to decreases in absorption which indicate a reduction of AcNosX. After 65 min, no more changes were observed by UV/VIS. AcNosX was fully reduced by the light and the absorption peak at 640 nm from the Cu$_Z$ site of AcN$_2$OR did not show any intensity change in the presence of reduced AcNosX.

Electron Transfer from Reduced AcNosX to Oxidized AcPAz

The reduction of AcPAz with AcNosX was investigated with UV/Vis spectroscopy. The UV/Vis spectra of oxidized AcPAz (30 µM), reduced AcNosX (30 µM), and mixture of both proteins (30 µM each) are shown in Figure 4.7. When oxidized AcPAz and reduced AcNosX were mixed under anaerobic conditions, the increase of peaks at 374 and 460 nm and decrease of peaks at 594 and 780 nm were observed (Figure 4.7, blue). These spectral changes clearly indicated the oxidation of FAD in AcNosX and reduction of Cu in AcPAz. The result indicated that AcNosX donated electrons to AcPAz.
Catalytic Reaction of $AcN_2$OR with Reduced $Ac$NosX

Previously, it was shown that an excess amount of reduced $Ac$PAz could sustain the $N_2$OR catalytic cycle by donating electrons to $AcN_2$OR in vitro (91). Since $Ac$NosX was also shown to donate electrons to $AcN_2$OR, the questions of whether $Ac$NosX able to sustain the $N_2$OR catalytic reaction was investigated. The UV/Vis spectra of reduced $Ac$NosX (44 µM) and a catalytic amount of $AcN_2$OR (6.8 nM) in the presence or absence of $N_2$O are shown in Figure 4.8. The time course of the absorption change at 460 nm was monitored for 30 min (Figure 4.8, right). After monitoring the background auto-oxidation rate of $Ac$NosX for 10 min, $N_2$O saturated buffer (80 µL) was injected at 11 min. If $Ac$NosX donated electrons to $AcN_2$OR during the catalytic reaction, the $Ac$NosX
oxidation rate should be significantly increased after N\textsubscript{2}O was injected as observed in Figure 4.9 and 4.10. The oxidation rates of AcNosX before and after N\textsubscript{2}O injection were calculated as slopes of trend lines (linear regression) for the absorption change at 460 nm. The equations are shown in Figure 4.8 (right). The result showed that no significant increase in the oxidation rates of AcNosX was observed between before (0.0012 min\textsuperscript{-1}) and after (0.0013 min\textsuperscript{-1}) the N\textsubscript{2}O injection (Figure 4.8, right). The result indicated that AcNosX did not support the N\textsubscript{2}OR catalytic cycle under the experimental conditions.

Figure 4.8. Oxidation of AcNosX during the N\textsubscript{2}OR catalytic cycle (left) and time course at 460 nm (right)

However, according to the control experiment with reduced AcPAz, the electron transfer from AcPAz to AcN\textsubscript{2}OR was not observed under the same conditions using Buffer P (data not shown). This observation indicated that electron transfer from reduced AcNosX to AcN\textsubscript{2}OR during the catalytic cycle may be inhibited by a component in Buffer P.
Therefore a series of investigations to determine if Buffer P and its components inhibited the catalytic reaction were carried out in the next section.

The N$_2$OR catalytic cycle in the presence of excess free FAD (72.0 µM FAD vs. 6.8 nM N$_2$OR) in 50 mM Potassium Phosphate (pH 7.0) was monitored with UV/Vis spectra (Figure 4.9) as a further control.

![Figure 4.9](image)

Figure 4.9. Time course for the oxidation of free FAD during the N$_2$OR catalytic cycle

The result showed that the electron transfer from reduced free FAD to AcN$_2$OR during the catalytic cycle was observed, but it stopped within 30 min. Another injection of activated AcN$_2$OR (6.8 nM (trial #1) or 13.6 nM (#2)) at 35 min induced further FAD oxidation, but the FAD oxidation stopped within 30 min again. At 66 min, N$_2$O saturated
buffer was injected (Figure 4.9, trial #1), but no further FAD oxidation was observed. At 71 min, the anaerobic cuvette was opened and sample could be further oxidized by air, indicating that there was still enough amount of free FAD in the sample at 66 min.

Identification of Inhibitory Factor for the N₂OR Catalytic Cycle with AcPAz

Buffer P was composed of 100 mM Phosphate, 150 mM NaCl, and 10 % glycerol. Since the N₂OR catalytic cycle with reduced AcPAz was originally observed in 50 mM Phosphate, the concentration of phosphate was reduced to 50 mM in following experiments. The oxidations of AcPAz during the N₂OR catalytic cycle were monitored to determine if NaCl or glycerol was the component that inhibited the catalytic cycle. 50 mM Potassium Phosphate (pH 7.0) (buffer #1), 50 mM Potassium Phosphate with 150 mM NaCl (pH 7.0) (buffer #2), or 10 % glycerol (buffer #3) were used for the measurements (Figure 4.10).

Figure 4.10. UV/Vis spectra of AcPAz oxidation during the N₂OR catalytic cycle with buffer #1 (left) and time course at A₅₉₄ in buffer #1 - 3 (right)
The result showed that buffer #2 (with 150 mM NaCl) inhibited AcPAz oxidation during the N₂OR catalytic cycle, whereas AcPAz was fully oxidized as AcN₂OR received electrons from AcPAz in buffer #1 and #3 (Figure 4.10, right, Table 4.3).

To determine if Na⁺ or Cl⁻ inhibited the catalytic reaction, the AcPAz oxidation during the N₂OR catalytic reaction were also measured with 50 mM Potassium Phosphate + 150 mM KCl (pH 7.0) (buffer #4), 50 mM Sodium Phosphate (pH 7.0) (buffer #5) (Figure 4.11, Table 4.3). The results showed that the chloride ion inhibited AcPAz oxidation during the N₂OR catalytic cycle.

![Figure 4.11. Time course for the oxidation of AcPAz during the N₂OR catalytic cycle in buffer #4 - 5](image)

As control experiments, if ionic strength of buffer affected the electron transfer from AcPAz to AcN₂OR during the catalytic cycle was investigated using 50 mM
Potassium Phosphate + 50 mM Na$_2$SO$_4$ (pH 7.0, $I = 0.242$) (buffer #6), and 129 mM Potassium Phosphate (pH 7.0, $I = 0.241$) (buffer #7) (Figure 4.12). The oxidation of $AcPAz$ was slightly perturbed in both buffer #6 and 7. The behavior of $AcPAz$ oxidations in buffer #6 and 7 were almost identical, suggesting that the slight perturbation of $AcPAz$ oxidation was due to high ionic strength.

Figure 4.12. Time course for the oxidation of $AcPAz$ during the N$_2$OR catalytic cycle in buffer #6 and 7

<table>
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<th>K**</th>
<th>Na**</th>
<th>Cl*</th>
<th>SO$_4$*</th>
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<td>0.241</td>
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</tr>
</tbody>
</table>

(* unit = mM)

Table 4.3. $AcPAz$ oxidation during the N$_2$OR catalytic cycle in various buffers
The N₂OR activities in buffer #1 (50 mM Phosphate), buffer #2 (50 mM Phosphate + 150 mM NaCl), and buffer #6 (50 mM Phosphate + 50 mM Na₂SO₄) were measured to investigate if chloride and ionic strength affected the N₂OR activity. Typical results are shown in Figure 4.13.

![N₂O injection](image)

**Figure 4.13.** The N₂OR activity assay in buffer #1, 2, and 6

N₂O saturated buffer #1 was injected to the cuvette at 5 min and the activity was calculated with the slope between 5.12 and 5.18 min. The N₂OR activities were 147.4 ± 8.70 U/mg in buffer #1, 134.9 ± 6.68 U/mg in buffer #2, and 156.5 ± 4.33 U/mg in buffer #6. The activities in the buffers were almost comparable at the beginning of N₂O catalytic cycle, but the activity in buffer #2 was significantly decreased within 5 min. An increase
in ionic strength did not reduce the activity since the activity in buffer #6, which ionic strength was the same as buffer #2, was not decreased (Figure 4.13).

**AcNosX Stabilization in Solution**

Stabilizing abilities of arginine (200 mM), ammonium sulfate (100 mM), spermine (200 mM), spermidine (200 mM), Triton X-100 (0.1 %), Tween 20 (0.1 %), and NaCl (150 mM) on AcNosX *in vitro* were checked by monitoring protein precipitations after 2, 4 and 8 hours at 4 °C. After two hours, samples with arginine, ammonium sulfate, spermidine, Triton X-100, Tween 20, and sample without reagent showed significant precipitation, whereas samples with spermine and NaCl did not show such turbidity in the solution. After eight hours, samples were centrifuged and UV/Vis spectra were measured (Figure 4.14).

![Figure 4.14. UV/Vis spectra of AcNosX with spermine, NaCl, and flowthrough](image)

Figure 4.14. UV/Vis spectra of AcNosX with spermine, NaCl, and flowthrough
Although the samples with spermine and NaCl also showed minor precipitation, the amount was significantly suppressed compared to other samples. The results indicated that spermine seemed to be an encouraging candidate for an AcNosX stabilizer. However, it turned out that spermine pulled FAD out from AcNosX based on the following observations: (1) the UV/Vis spectrum of AcNosX with spermine showed an absorption peak shift from 460 nm to 450 nm which was identical to free FAD (Figure 4.14, blue). (2) Concentration of AcNosX with spermine by an Amicon ultrafiltration (YM-10) resulted in FAD in the flowthrough (Figure 4.14, green). As a result, only NaCl kept AcNosX intact in solution under the experimental conditions.

**Discussion**

**Function of AcNosX as an Electron Donor for AcN$_2$OR**

To determine the function of AcNosX, electron transfers from AcNosX to AcN$_2$OR (oxidized and PAz reduced form) were investigated. Both AcNosX and AcPAz were shown to reduce the Cu$_A$ site of AcN$_2$OR (Figure 4.2, 4.3). In the UV/Vis spectrum that showed AcNosX reduced AcN$_2$OR (Figure 4.2, blue), the peak at 800 nm that was derived from the Cu$_A$ site (63) was decreased. This observation indicated that AcNosX reduced the Cu$_A$ site in AcN$_2$OR. It was not clear in the spectrum whether AcNosX could donate electrons to the Cu$_Z$ site due to the absorption peak from AcNosX. The differential spectrum of fully oxidized (#2) and PAz reduced (#3) N$_2$OR (Figure 4.3, blue) was almost identical to the Vis spectra of the Cu$_A$ site in N$_2$OR from *Pseudomonas stutzeri* (peaks at 485, 525, and 795 nm) (63) and Cu$_A$ reconstituted AcN$_2$OR expressed in *E. coli*
that did not have the CuZ site (peaks at 484, 531, and 796 nm, Fujita et al. unpublished results). If AcPAz could reduce the CuZ site, there should be additional peak(s) around 550 and 640 nm, which were derived from the CuZ site, in the differential spectrum (63). However, no such peak or shoulder was observed in the spectrum (Figure 4.3, blue). Thus, it was indicated that AcPAz reduced only the CuA site, but not the CuZ site. Also, it further indicated that electron transfer from CuA to CuZ did not occur under the experimental conditions. AcN2OR was reduced with 30-fold excess amount of reduced AcPAz. The ratio between AcPAz and AcN2OR was physiologically realistic since the abundance ratio of PAz vs. N2OR in WT of A. cycloclastes was shown to be ~ 40:1 (91).

AcNosX appeared to be capable of reducing the CuZ site of AcN2OR. The redox state of the CuZ site in the dithionite reduced N2OR was assigned to 1Cu(II) • 3Cu(I) form with DFT calculations based on experimental data (156; 79). The redox state of the CuZ site in the fully oxidized N2OR was also assigned to 2Cu(II) • 2Cu(I) form (84). Further, the fully reduced form of the CuZ site was determined to be 4Cu(I) form (70; 77). According to the EPR and AA data together with the previous information, the redox states of the CuZ sites in PAz reduced N2OR (sample #3) and PAz-NosX reduced N2OR (sample #4) were assigned to 2Cu(II) • 2Cu(I) form and 1Cu(II) • 3Cu(I) form, respectively (Table 4.2). This result indicated that AcNosX could reduce the CuZ site at least from [2Cu(II) • 2Cu(I)] to [1Cu(II) • 3Cu(I)]. There are two possibilities on the CuZ reduction mechanism by AcNosX. First, AcNosX directly reduces the CuZ site and second, AcNosX facilitates the electron transfer from CuA to CuZ, which AcPAz could not. To
clarify this hypothesis, AcN$_2$OR without the Cu$_A$ site by mutation would be required for further study.

The observations on the electron transfer reactions among AcN$_2$OR, AcPAz, and AcNosX were consistent with the redox potentials of these proteins. Although the redox potentials of the Cu centers in AcN$_2$OR have not been determined yet, it was expected that the redox potentials of the Cu$_A$ and Cu$_Z$ site were $\sim$ 250 mV and $\sim$ 60 mV, respectively (see chapter 1) (68). The redox potentials of AcPAz and AcNosX were determined as 260 mV (147) and -230 mV (see chapter 3). The redox potential of AcPAz (260 mV) was comparable with the one of the Cu$_A$ site ($\sim$ 250 mV), which enabled AcPAz to donate electrons to the site. However, the redox potential of the Cu$_Z$ site was too low for AcPAz to donate electrons to that site. Therefore, AcPAz was expected to donate electrons only to the Cu$_A$ site, but not Cu$_Z$ site. Indeed, the results were as expected (Figure 4.3). In contrast, AcNosX was expected to reduce both the Cu sites because the redox potential of AcNosX (-230 mV) was lower than the redox potentials of the Cu$_A$ (250 mV) and Cu$_Z$ (60 mV) sites. The EPR data confirmed this expectation (Table 4.1, 4.2).

The percentage of oxidized Cu in the fully oxidized N$_2$OR was determined as 35.2 % (Table 4.2). This observation was different from the theoretical value (16.7 %). This result was explained as follows. In the fully oxidized N$_2$OR, the two spins on the Cu$_Z$ site (2Cu(II) • 2Cu(I)) should be cancelled out with anti-ferromagnetic coupling (80), and only one spin on the Cu$_A$ site (Cu(1.5) • Cu(1.5)) should be detectable with the EPR. However, the data from AA indicated that the total Cu amount in the sample #2 was $\sim$ 3
per monomer on average, which was caused by ferricyanide incubation (Table S4.1, Figure S4.2). Thus, it was highly likely that many of the CuZ sites in AcN2OR molecules included less than four coppers, which resulted in the disappearance of the antiferromagnetic interaction. In this case, the spin(s) on the CuZ site would be detectable in the EPR and the percentage of oxidized Cu would increase. Also, it might be possible that the Cu loss by ferricyanide results in higher Cu(II) content.

Although AcNosX was shown to be a more potent reductant than AcPAz, the percentages of oxidized Cu in the sample #3 (PAz reduced N2OR) and #5 (NosX reduced N2OR) were calculated as almost the same level (20.6 and 20.3 %, respectively, Table 4.2). This result appeared to disagree with the conclusion. This observation is explained as follows. In the sample #3, fully oxidized AcN2OR was reduced with 30-fold excess amount of AcPAz, whereas fully oxidized AcN2OR was reduced with only 2-fold amount of AcNosX in the sample #5. Since AcNosX could not be concentrated more than 200 µM, addition of 2-fold AcNosX to the sample was the maximum limit in the experiment. If enough amount of AcNosX could be added to the sample, it was likely that the Cu sites in fully oxidized N2OR (CuA = Cu(1.5) • Cu(1.5), CuZ = 2Cu(II) • 2Cu(I)) would be reduced to CuA = Cu(I) • Cu(I), CuZ = 1Cu(II) • 3Cu(I), which was identical to the sample #1 (as purified) and #4 (PAz-NosX reduced) (Table 4.2).

We determined that incubation of AcN2OR with an excess amount of ferricyanide resulted in decrease of Cu content in AcN2OR (Appendix D: Table S4.1, Figure S4.2). It was not clear how ferricyanide removed Cu from AcN2OR. It is possible that an excess amount of ferricyanide oxidized the Cu clusters in AcN2OR and “super-oxidized” forms
such as [Cu(II)-Cu(II)] and [4Cu(II)] might be generated. Such species might be unstable since the species have never been observed (4). Consequently, the “super-oxidized” clusters might decompose and the Cu content of oxidized N$_2$OR decreased. The oxidized AcN$_2$OR still showed activity ($94.9 \pm 4.1$ U/mg) after incubation with reduced MV, and the UV/Vis spectrum clearly showed absorption peaks from the Cu$_A$ and Cu$_Z$ sites (Figure 4.3). These results indicated that the Cu loss by oxidation with ferricyanide was expected to occur randomly in both Cu$_A$ and Cu$_Z$ sites, rather than either Cu$_A$ or Cu$_Z$ cluster was specifically decomposed.

To date, reduction of the partially oxidized Cu$_Z$ (1Cu(II)$\cdot$3Cu(I)) form to the active form (4Cu(I)) requires a long incubation time ($\sim 3$ hours) with reduced MV, which is not physiologically relevant (70; 77). There should be a more efficient reduction mechanism to reduce 1Cu(II)$\cdot$3Cu(I) form in vivo. AcNosX was one of the candidates. However, the EPR and UV/Vis data suggested that AcNosX could not reduce the 1Cu(II)$\cdot$3Cu(I) form under the experimental conditions (Table 4.2, Figure 4.6). The mechanism that reduces the 1Cu(II)$\cdot$3Cu(I) form in vivo is still unclear.

Function of AcNosX for Regeneration of the Cu$_Z^*$ State in AcN$_2$OR

The N$_2$OR activity in *Paracoccus denitrificans* was diminished in the NosX and NirX double mutant strain although PAz and cyt $c$ should still be able to donate electrons to N$_2$OR (83). Since direct correlation between the Cu$_Z^*$ proportion and NosX deletion was observed, NosX was expected to be involved in the regeneration of the Cu$_Z^*$ state in vivo. However, AcNosX could not regenerate the Cu$_Z^*$ state under the
experimental conditions (Figure 4.6). If reduced AcNosX was able to regenerate the Cu$_Z^*$ state, the peak at 640 nm should decrease. The peak did not change in the presence of reduced AcNosX. It might be possible that AcNosX requires other factor(s) to reduce the Cu$_Z^*$ state in vivo. For instance, it was shown that the deletion or modification of NosR resulted in the same phenotype as the NosX knockout strain in *Pseudomonas stutzeri* (82). This observation imply that the regeneration of the Cu$_Z^*$ state requires both NosX and NosR to form a complex that enables to regenerate the Cu$_Z^*$ state.

Another possibility is that NosX does not regenerate the Cu$_Z^*$ state, but it inhibits the generation of the Cu$_Z^*$ state. This hypothesis can also explain the observations that the proportion of Cu$_Z^*$ state was increased in the absence of NosX (83). In any case, the structure and properties of the Cu$_Z^*$ state are still uncertain, and the current data do not support a direct role for AcNosX in converting Cu$_Z^*$ to Cu$_Z$. As will be discussed later, a new definition of the Cu$_Z^*$ state was proposed based on the new structure of N$_2$OR from *Pseudomonas stutzeri* (63). The authors proposed that the Cu$_Z^*$ state is the [4Cu:1S] form and the Cu$_Z$ state is the [4Cu:2S] form. This hypothesis can be interpreted that the regeneration of the Cu$_Z^*$ state requires re-construction of the Cu$_Z$ site by adding a sulfide ion to the [4Cu:1S] form. If this can be applied to AcN$_2$OR, it is expected that AcNosX cannot regenerate the Cu$_Z^*$ state because it was shown that NosX was not involved in the Cu$_Z$ site construction (100). In such case, it is plausible that AcNosX works as a “cofactor” to keep the Cu$_Z$ site active and inhibit the generation of the Cu$_Z^*$ state.
Function of AcNosX as an Electron Donor for AcPAz

AcNosX donated electrons to oxidized AcPAz (Figure 4.7). The redox potential of AcPAz (260 mV) is very close to the redox potential of the CuA site (~ 250 mV) (157; 68; 147). As shown in previously (Figure 4.2), AcNosX could donate electrons to the CuA site. Therefore, it was expected that AcNosX could also reduce AcPAz. The result suggested that there was an electron transfer network among NosX, PAz, and N2OR in the periplasm of A. cycloclastes. However, the physiological relevance of the electron transfer pathway from AcNosX to AcPAz is still unknown. It was shown that the abundance ratio of PAz and N2OR in WT of A. cycloclastes was ~ 40:1 (91). Although the ratio of AcNosX and AcN2OR in WT has never been monitored, the ratio of AcNosX and AcN2OR in the overexpression strain for Nos proteins (Ac/pMLClustXst) was 1: 25 ~ 50, which was calculated from the yields of the proteins. Assuming the abundance ratio of AcNosX and AcN2OR in WT was the same as the overexpression strain, the ratio among AcPAz, AcN2OR, and AcNosX in the periplasm would be 40:1:0.03. Thus, the total abundance of AcNosX in vivo is expected to be considerably smaller than that of AcPAz. Therefore, the contribution of AcNosX in reducing AcPAz in vivo would be relatively small, unless the turnover of AcNosX re-reduction (perhaps by NosR) would be fast.

Identification of Inhibitory Factor for the N2OR Catalytic Cycle

Chloride in Buffer P was identified as an inhibitory factor of the N2OR catalytic cycle using BV and AcPAz as electron donors (Figure 4.10, 4.11, 4.13). Although N2OR
was incubated in buffer with or without chloride for 5 min prior to N\textsubscript{2}O injection, the initial N\textsubscript{2}O reduction rates, which corresponded to the N\textsubscript{2}OR activities, were almost comparable (147.4 \pm 8.70 U/mg without chloride and 134.9 \pm 6.68 U/mg with chloride, Figure 4.13). If the inhibitory reaction between chloride and the enzyme occurred in the absence of N\textsubscript{2}O, a significant decrease of the initial N\textsubscript{2}O reduction rate should be observed in the buffer with chloride. Therefore, the results indicated that the inhibition of the N\textsubscript{2}OR catalytic cycle by chloride occurred in the presence of N\textsubscript{2}O. The mechanism of the chloride inhibition effect is still unclear. Two straightforward possibilities are that the electron transfer reactions from electron source (BV or AcPAz) to the active site (Cu\textsubscript{Z}) through the Cu\textsubscript{A} site are impacted, or that the catalytic reaction of N\textsubscript{2}O at the Cu\textsubscript{Z} site was inhibited by chloride (see below for more detail).

The electron transfer process from AcPAz to the Cu\textsubscript{Z} site, which chloride ions possibly inhibit, can be categorized in two steps, (1) inter-molecular electron transfer step, involving interaction (binding and releasing) between AcPAz and AcN\textsubscript{2}OR, and (2) intra-molecular electron transfer step (perturbations of the Cu\textsubscript{A}/Cu\textsubscript{Z} site and/or electron transfer from the Cu\textsubscript{A} to the Cu\textsubscript{Z} site).

The inter-molecular electron transfer step might be inhibited by chloride ions. Chloride ions could interrupt the protein-protein interaction between AcPAz and AcN\textsubscript{2}OR by blocking release of oxidized AcPAz from AcN\textsubscript{2}OR. For rapid catalytic turnover, reduced AcPAz needs to bind to AcN\textsubscript{2}OR, donate one electron to the Cu\textsubscript{A} site, and then rapidly dissociate for the next AcPAz-AcN\textsubscript{2}OR interaction. If chloride ions inhibit the release of oxidized AcPAz from AcN\textsubscript{2}OR, the PAz binding site would be blocked and the
catalytic cycle would be blocked by a lack of electrons. However, this is unlikely. In the last step of \( \text{AcN}_2\text{OR} \) purification, a cytochrome \( c \) agarose affinity column was used (see method section). Since it was reported that both PAz and cytochrome \( c \) donated electrons to \( \text{N}_2\text{OR} \) through similar docking sites (92; 150; 91; 87), it was expected that the complex of \( \text{AcN}_2\text{OR} \) and cytochrome \( c \) on the resin was essentially the same as the complex between \( \text{AcN}_2\text{OR} \) and \( \text{AcPAz} \) in solution. In the purification protocol, bound \( \text{AcN}_2\text{OR} \) on the column was eluted by buffer including \( \text{NaCl} \). This fact indicated that chloride ions interrupted the formation of the \( \text{N}_2\text{OR}-\text{cytochrome c} \) complex. Thus, it was unlikely that chloride ion inhibited release of \( \text{AcPAz} \) from \( \text{AcN}_2\text{OR} \). Another possibility of the inhibition effect was that chloride ions occlude the binding sites of \( \text{AcPAz} \) and/or \( \text{AcN}_2\text{OR} \), which inhibits proper protein-protein interaction between \( \text{AcPAz} \) and \( \text{AcN}_2\text{OR} \).

It has been reported that the electron transfer from cytochrome \( c \) to \( \text{N}_2\text{OR} \) from \emph{Paracoccus pantotrophus} was perturbed with \( \text{NaCl} \) (over 50 mM) (75). The authors concluded that high ionic strength perturbed the protein-protein interaction between cytochrome \( c \) and \( \text{N}_2\text{OR} \) (75). Since the interaction of positively and negatively charged amino acids in \( \text{AcPAz} \) and \( \text{AcN}_2\text{OR} \) was important to form a transient electron transfer complex (92; 91; 87), the perturbation of the electron transfer under high ionic strength could be a consequence of perturbing the interaction between the two proteins by charged species in the buffer. However, this possibility is doubtful in our case because electron transfer only requires a “light touch” between donor and acceptor, and even if binding sites of \( \text{AcPAz} \) and \( \text{AcN}_2\text{OR} \) were buried in chloride ions, there still would be many chances for a productive transient complex to form. In this case, chloride might decrease
the reaction rate, but it would seem unlikely that it would completely block the reaction as observed. According to the control experiments (Figure 4.12), the electron transfer from AcPAz to AcN2OR was slightly perturbed under higher ionic strength, but it was not totally inhibited as observed in the presence of chloride, indicating that the inhibition effect was not mainly due to ionic strength. Further, the inhibition was also observed when the small molecule (BV) reductant was used as the electron source (Figure 4.13), suggesting that the inhibition occurred within the N2OR molecule rather than the electron transfer step between BV and N2OR (see below). It is less likely that high ionic strength perturbed the interaction between the small molecule (BV) and the protein (N2OR).

Another possible inhibition mechanism by chloride ions is expected to occur within the N2OR molecule. Previously, it was reported with UV/Vis spectroscopy that chloride interacted with the CuA site in AcN2OR (80). The binding of chloride ion on the CuZ site was also observed and it was hypothesized that the chloride ion influenced the redox potential of the CuZ site (Figure 4.15) (74). The chloride on the CuZ site was observed in all structure determined N2ORs (61; 59; 63; 62). It is possible that the chloride on the CuZ site inhibited the catalytic reaction by changing the redox potential or by interfering with a step or steps in the reduction mechanism. It is also possible that the redox potential of the CuA site would be disturbed with bound chloride. Although no chloride ion was observed around the CuA site in the crystal structures of AcN2OR, three chloride ions were identified around the cupredoxin motif in the C-terminal domain of AcN2OR, which holds the CuA site (Figure 4.15) (59). The CuA does not serve as a binding site for chloride, but the remote (20.0 ~ 23.4 Å) chloride binding sites and the
amino acids for coordination of the Cu_A site were located on the same β-sheets. Thus, it might be possible that the binding of the chloride ions affect either the redox potential of the Cu_A site, the electron transfer from AcPAz/BV to the Cu_A, and/or the electron transfer from Cu_A to the Cu_Z by changing the coordination of amino acids around the Cu_A site.

Figure 4.15. The Cu_A, Cu_Z, and chloride binding sites in the C-terminal domain of AcN_2OR (iodide bound form)

It should be noted that the chloride ions around the Cu_A site were only observed in the iodide bound AcN_2OR (PDB: 2IWK) but not the other structure determined N_2ORs (61; 59; 63; 62). It might be interesting to investigate if the chloride inhibition on the electron transfer from physiological electron donor to N_2OR is only observed in AcN_2OR or not. If the other three N_2OR do not show the inhibition effect, these chlorides observed in
AcN\(_2\)OR will be suggested to be involved in the inhibition mechanism. As mentioned earlier, it was shown that the electron transfer reaction from cytochrome \(c\) to N\(_2\)OR in \textit{Paracoccus pantotrophus} was perturbed by NaCl (75). Further investigation on the chloride inhibition is required on this N\(_2\)OR as well. Also, even though no bound chloride was observed on the Cu\(_A\) site in the crystal structure, it did not mean that there was no chloride on the Cu\(_A\) site. It is possible that loosely bound chloride, for which occupancy was low and undetectable by X-ray crystallography, might be on the Cu\(_A\) site.

**Function of AcNosX in the N\(_2\)OR Catalytic Cycle**

The oxidation of AcNosX during the N\(_2\)OR catalytic reaction was not observed (Figure 4.8). If AcNosX could sustain the catalytic reaction, the time course of the absorption at 460 nm should look like the blue spectrum in Figure 4.11. However, such behavior was not observed. The result indicated that AcNosX could not sustain the catalytic reaction. However, as described above, chloride ions in Buffer P inhibited the N\(_2\)OR catalytic cycle with AcPAz (Figure 4.10, 4.11). This result brought up a possibility that AcNosX was potentially able to sustain the N\(_2\)OR catalytic cycle but the chloride ions from the buffer interfered with the reaction. AcNosX did donate electrons to AcN\(_2\)OR when both proteins were mixed in a 1:1 ratio and in the presence of 150 mM NaCl (Figure 4.2). In addition, as described below, free reduced FAD could sustain the N\(_2\)OR catalytic cycle at least for a while (Figure 4.9). Therefore, it was likely that AcNosX could sustain the catalytic reaction in the absence of chloride ions. However, even if AcNosX would be able to sustain the catalytic reaction, it was hard to believe that this reaction was physiologically significant. Since the abundance of AcNosX in the cell...
was much smaller than AcPAz and AcN2OR as previously described (91), the contribution of AcNosX on the catalytic reaction as a direct electron donor is expected to be very small.

Free reduced FAD could support the N2OR catalytic cycle for some period of time (Figure 4.9). However, the oxidation of FAD was stopped after 30 min. The reaction started again by addition of extra AcN2OR to the cuvette, but not by addition of extra substrate. When the sample was exposed to the air, FAD was further oxidized. These observations indicated that the termination of the catalytic reaction was due to inhibition of the N2OR activity, but not due to the lack of substrate (N2O) or electron source (reduced FAD). Since such behavior was not observed when reduced BV was used as an electron source, free FAD was expected to cause the inhibition. The inhibition mechanism of the N2OR activity by free FAD was still unknown. Since no chloride was added to the sample, this phenomenon was independent from the inhibition effects by chloride (Figure 4.11).

**Stability of AcNosX in Vitro**

AcNosX required both 150 mM NaCl in buffer and low temperature to be stable in vitro (Chapter 3). However, chloride ions were found to inhibit the N2OR catalytic cycle with AcPAz as an electron donor. This result indicates that chloride would also interrupt the N2OR catalytic cycle with AcNosX. Thus, it was required to remove chloride ions from the AcNosX sample to measure the N2OR catalytic cycle with AcNosX. Further, as mentioned in chapter 2, high salt concentration also interrupted AcNosX purification using an ion exchange column. To overcome these obstacles,
screenings for AcNosX stabilization without chloride were carried out using a host of different reagents. Although spermine showed a stabilization effect on AcNosX, it was revealed that FAD was ejected from AcNosX when spermine was added to the sample (Figure 4.14). The pKa of spermine are 11.50, 10.95, 9.79 and 8.90. Thus, spermine is positively charged under the experimental conditions (pH 7.0). It was also indicated that the FAD binding pocket of AcNosX was negatively charged (Figure 3.5). Therefore, it was possible that spermine tended to get into the FAD binding pocket and FAD was replaced with a spermine molecule. Suitable reagent for AcNosX stabilization in vitro was not found.

Although A. cycloclastes grew at 30 °C, AcNosX immediately precipitated at this temperature in vitro. This fact indicates that there should be certain protein(s) and/or small molecule(s) that stabilize AcNosX in vivo. AcNosX was shown to be a relatively hydrophobic protein (Figure 3.6). The existence of the large hydrophobic patch might be one of the reasons that AcNosX was easily precipitated in vitro (Figure 3.6). In addition, NosR has been implied to be the redox partner of NosX (52; 83). Since NosR is a membrane bound protein, this hydrophobic patch on AcNosX might be important for stabilizing the protein-protein interaction with NosR because NosX needs to get close to the hydrophobic membrane surface to interact with NosR. In other words, NosR would be a strong candidate to stabilize NosX in vivo.
Substrate Bound N$_2$OR Structure
from *Pseudomonas stutzeri*

New Hypothesis on the Electron Transfer from Cu$_A$ to Cu$_Z$. As described in chapter 1, the structure of N$_2$OR from *Pseudomonas stutzeri* was recently determined (63). The electron transfer from Cu$_A$ (250 mV) to Cu$_Z$ (60 mV) in N$_2$OR is thermo-dynamically unfavorable and the mechanism that permits the electron transfer *in vivo* has been unknown (83). The substrate bound structure of N$_2$OR from *Pseudomonas stutzeri* implied that the substrate received electron(s) directly from the Cu$_A$ site, instead of receiving two electrons from the Cu$_Z$ site as it has been expected and described in Figure 1.9 (71; 7). This hypothesis can explain our observation that AcPAz was able to sustain the N$_2$OR catalytic cycle (91) even though AcPAz could not donate electrons to the Cu$_Z$ site *in vitro* (Figure 4.3). If the substrate on the Cu$_Z$ site can receive electrons directly from the Cu$_A$ site, AcPAz might be able to sustain the catalytic cycle by donating electrons via the Cu$_A$ site. However, it was also observed that the N$_2$OR activity was directly correlated with the degree of reduction on the Cu$_Z$ site (70). Also, the Cu$_Z$ site was obviously oxidized when the fully reduced N$_2$OR was reacted with the substrate (77). These data indicated that reduction of the Cu$_Z$ site was still important for the catalytic reaction and the Cu$_Z$ site was oxidized during the catalytic reaction.

Another possibility of the mechanism that enables the electron transfer from the Cu$_A$ to the Cu$_Z$ site is that the substrate binding to the Cu$_Z$ site alters the redox potential of the site. Our observation that AcPAz could sustain the N$_2$OR catalytic cycle can be interpreted as AcPAz could donate electrons to the Cu$_Z$ site only in the presence of N$_2$O.
This hypothesis is supported by the fact that the redox potential of the active center in CuNiR from *Pseudomonas chlororaphis* was tuned with the substrate (NO$_2^-$) binding to receive electrons (29).

**New Definition of the Cu$_Z$* State.** The structure of N$_2$OR from *Pseudomonas stutzeri* showed an unprecedented Cu$_Z$ structure that included two sulfide ions (Figure 1.10). Based on the structure, it was hypothesized that the [4Cu:2S] form was the Cu$_Z$ state and the [4Cu:1S] form was the Cu$_Z$* state (63). In AcN$_2$OR, however, no correlation between the N$_2$OR activity and the sulfide content of the sample was observed, although a positive correlation between the N$_2$OR activity and the Cu content was clearly observed (Appendix D; Figure S4.3). The observation indicated that the $2^{nd}$ sulfide was not involved in the catalytic reaction using reduced BV as an electron source, or AcN$_2$OR intrinsically does not have the $2^{nd}$ sulfide in the Cu$_Z$ site. In addition, the Cu$_Z$* state of N$_2$OR from *M. hydrocarbonoclasticus* can be fully restored to the active Cu$_Z$ state by incubation with reduced MV (84), which is unlikely to related with a reaction from [4Cu:1S] (Cu$_Z$*) to [4Cu:2S] (Cu$_Z$). Further, this N$_2$OR is relatively active even under O$_2$, which conflicts with the fact the $2^{nd}$ sulfur is extremely oxygen sensitive (84; 63). Therefore, to date, the relationship among [4Cu:2S] form, the Cu$_Z$/Cu$_Z$* states, and the N$_2$OR activity are still controversial. Further studies to investigate the presence of the $2^{nd}$ sulfide in N$_2$OR from the other species and N$_2$O reduction mechanism by DFT calculation on the structure will be required.
Conclusions

The function of AcNosX on AcN\textsubscript{2}OR was investigated in vitro. It was observed that AcNosX could reduce both the Cu\textsubscript{A} and Cu\textsubscript{Z} sites of AcN\textsubscript{2}OR whereas AcPAz reduced only the Cu\textsubscript{A} site. This observation was consistent with the relative redox potentials of AcNosX (-230 mV), AcPAz (260 mV), and AcN\textsubscript{2}OR (250 mV for the Cu\textsubscript{A} and 60 mV for the Cu\textsubscript{Z}). AcNosX was expected to facilitate the electron transfer from Cu\textsubscript{A} to Cu\textsubscript{Z}, which was thermodynamically unfavorable. AcNosX was also shown to donate electrons to AcPAz. It is still unclear whether this electron transfer pathway is physiologically significant. Further, the question of whether AcNosX is able to sustain the N\textsubscript{2}OR catalytic cycle was investigated. However, it was found that chloride ion that was essential to stabilize AcNosX in vitro inhibited the N\textsubscript{2}OR catalytic cycle in the presence of N\textsubscript{2}O. Satisfactory replacements for NaCl in AcNosX stabilization could not be identified. Thus, the N\textsubscript{2}OR catalytic cycle with AcNosX could not be monitored. However, AcNosX was expected to sustain the N\textsubscript{2}OR catalytic cycle because free FAD could do so. Free FAD also showed an inhibition effect on the catalytic reaction. It was possible that AcNosX structure was important scaffold to avoid this inhibition during electron transfer from FAD in AcNosX to AcN\textsubscript{2}OR. The regeneration of the Cu\textsubscript{Z}\textsuperscript{*} state in AcN\textsubscript{2}OR with AcNosX in vitro failed. If the new definition of the Cu\textsubscript{Z}\textsuperscript{*} state ([4Cu:1S] form) which was proposed recently by Pomowski et al. could be applied to AcN\textsubscript{2}OR, it implies that the regeneration of the Cu\textsubscript{Z}\textsuperscript{*} state with AcNosX by itself was improbable because NosX was not involved in the construction of the Cu\textsubscript{Z} site and unlikely to
reconstitute the CuZ site. In addition, even if it would be possible, AcNosX would require a sulfide source to re-construct the [4Cu:2S] form.

In conclusion, these investigations support in part the hypothesis that AcNosX donates electrons to AcN2OR to activate the catalytic center (CuZ site). Another hypothesis that AcNosX donates electrons to AcN2OR to regenerate the inactive CuZ* state to the catalytically active CuZ state requires further investigation.
CO-CRYSTALLIZATION OF N$_2$OR AND PSEUDOAZURIN FROM
ACHROMOBACTER CYCLOCLASTES

Introduction

Pseudoazurin (PAz) works as an electron shuttle among several different proteins in the denitrification system (87). PAz has been observed to donate electrons to several reductases such as cd$_1$NiR in Paracoccus pantotrophus (158), CuNiR in A. cycloclastes (159) (Figure 1.3), NOR in Paracoccus denitrificans (89), and N$_2$OR in Paracoccus pantotrophus (90). We also showed that PAz from A. cycloclastes (AcPAz) could donate electrons to N$_2$OR from A. cycloclastes (AcN$_2$OR) (Figure 4.2) (91). Although these reductases are structurally different from one another, PAz can donate electrons to the reductases through a pseudo-specific interaction (87). The pseudo-specific interaction between redox proteins was mainly characterized by promiscuous docking motifs based on a hydrophobic patch and surrounding positive/negative amino acids (87). Since hydrophobic interaction is less specific and less directional than the interactions with hydrogen bonds, several proteins that have the docking motifs can interact by the pseudo-specific interaction without any particular structure (87).

Co-crystallization of several proteins is a good way to investigate a binding mode of the proteins in a complex (160). Recently, CuNiR and cytochrome $c_{551}$ from Achromobacter xylosoxidans were co-crystallized and a docking structure of the two proteins was solved (161). The structure showed the electron transfer pathway from
cytochrome $c_{551}$ to CuNiR. Although there is no report on the co-crystal of N$_2$OR and its electron donors (PAz and cytochrome $c$), docking structures of N$_2$OR and the electron donors have been studied by calculations *in silico* using structures of N$_2$OR and PAz/cytochrome $c$ (92; 150; 93). Possible docking structures of AcN$_2$OR and AcPAz for electron transfer and several possible electron transfer pathways from the Cu center of AcPAz to the Cu$_A$ site of AcN$_2$OR were also calculated in our lab (Figure 1.13) (91). However, as of today, there has been no experimental data that confirms the calculated docking structures of N$_2$OR and the electron donors. It is essential to reveal how AcN$_2$OR and AcPAz form the transient complex for electron transfer to fully understand the N$_2$OR catalytic mechanism.

Crosslink of proteins in a crystal helps to solve its structure with X-ray analysis, especially when the proteins in the crystal tended to be out of alignment (162; 163; 164). The interactions between electron donor and acceptor proteins are usually very weak (87). Additionally, DFT calculations indicated that there were several possible binding geometries between electron donor and acceptor (92). These reports suggested that electron transfer proteins in a co-crystal might be disordered. Indeed, X-ray measurements of co-crystals for cytochrome $c$ peroxidase and cytochrome $c$ could not show the electron density of cytochrome $c$ since the cytochrome $c$ molecules were disordered in the co-crystals (165). In such a case, the crosslink technique might fix the alignment of the cytochrome $c$ molecules in the co-crystals. This can happen to the co-crystal of AcN$_2$OR and AcPAz as well, and the cross-link technique might stabilize crystals to be prepared.
In this chapter, co-crystallization trials for AcN2OR and AcPAz are described. Specifically, the co-crystallization of AcN2OR and AcPAz was attempted to determine the docking structure that suggests the electron transfer pathway from AcPAz to AcN2OR. Also, the crosslink conditions for AcN2OR and AcPAz were investigated.

**Materials and Methods**

**Screening of Crystallization Conditions**

Protein solutions were prepared as 10 mg/mL (AcN2OR = 8.35 mg (128.5 µM), AcPAz = 1.67 mg (128.5 µM)) in 10 mM Tris (pH 8.0). The protein solution was prepared in the largest amount possible at one time, and was aliquoted to 55 µL per tube and frozen at -80 °C for further use. High throughput screening for AcN2OR-AcPAz co-crystallization was carried out with an automated screening robot Honeybee961 (Digilab) using 960 screening reagents (96 reagents × 10 plates, Hampton Research and Emerald BioSystems, Inc.). 0.4 µL of protein solution was mixed with the same amount of each crystallization solution per spot. The screening plates were prepared with the sitting drop method and kept at 22 °C. The conditions of the spots that showed crystals were selected for further optimizations.

**Optimization of Crystallization Conditions**

The conditions were optimized to obtain bigger crystals by changing buffer pH, incubation temperature, and concentrations of buffer, salt, and precipitant, respectively. 2 µL of protein stock was mixed with the same amount of crystallization solution and the hanging drop method was used. The amount of reservoir was 1 mL (24 holes in a plate).
For optimization of condition #1 (Table 5.1, 100 mM Tris (pH 8.5), 200 mM lithium sulfate, and 30 % PEG 4000), PEG concentration was changed between 20.5 and 35.0 % in increments of 0.5 %, lithium sulfate concentration was changed between 50 and 350 mM in increments of 50 mM, protein amount was changed between 10 and 4 mg/mL in increments of 1 mg/mL, and at the last step of optimization, additive screening reagents (48 conditions, Hampton Research) were added to the modified condition above. In each spot, 2 µL of protein solution, 1.6 µL of crystallization solution, and 0.4 µL of additive screen reagent were mixed on a coverslip. The plates were incubated at 22 °C and checked with a microscope once a week. For optimization of condition #3 (Table 5.1, 50 mM Bis-Tris propane (pH 5.0), 50 mM citric acid, and 16 % PEG 3350), PEG concentration was changed between 5.0 and 19.0 % in increments of 1.0 %, and pH was changed between 4.5 and 7.0 in increments of 0.5. Optimization of condition #4 (Table 5.1, 100 mM Bis-Tris (pH 6.5), and 20 % PEG monomethylether (mme) 5000) was carried out by changing PEG concentration (10.0 – 35.0 %), pH (5.0 – 8.5), additive screen, temperature (22, 18, or 4 °C), and the micro-seeding method. The crystal for the micro-seeding was obtained from the initial screening plate.

Crystal Check for Protein or Salt

To confirm whether the obtained crystals were made of proteins or salt, the crystals were checked with Izit Crystal Dye (Hampton Research) that specifically dyes protein crystals in blue, and SDS-PAGE. 1 µL of Izit Crystal Dye was added to the crystallization droplet with crystals in condition #4 (Table 5.1). The plate was incubated overnight and color change of the crystals was monitored under a microscope. After the
stained crystals were photographed, the crystals were used for SDS-PAGE samples. The stained crystals were scooped with a cryo-loop from the crystallization droplet under the microscope. The crystals were rinsed with freshly prepared crystallization solution (condition #4) several times and solved in 10 µL of water for SDS-PAGE. As a control, the crystallization droplet was scooped with the cryo-loop and the solution within the loop was diluted with the same manner that the crystals were rinsed. The solution was also diluted with 10 µL of water for SDS-PAGE. The crystals from condition #1 (Table 5.1) were also checked with SDS-PAGE after washing the crystals with the crystallization solution (condition #1).

**X-ray Measurements of the Co-crystals**

The obtained crystals were screened and measured at MSU and Stanford Synchrotron Radiation Lightsource (SSRL). A needle-like crystal (30 × 30 × 500 µm) that was obtained from modified condition #1 (100 mM Tris, 200 mM Lithium sulfate, 24.0 % PEG4000, pH 8.5) was measured with the home source X-ray beam at MSU to see if the crystal showed diffraction patterns. The crystal was scooped with a cryo-loop and frozen with liquid nitrogen immediately. Since condition #1 solution worked as a cryo-protectant, no glycerol was added to the solution prior to crystal freezing. The crystal was measured at 0 ° and 90 ° for 20 min each and 8 hours each at -196 °C.

Several crystals obtained from condition #3 (PEG concentration was modified to 12.0 %, rod shaped crystals, 30 × 30 × 100 µm) and #4 (hexagonal column shaped crystals, 50 × 50 × 50 µm, obtained from the initial screening plate) were incubated in cryo-protectant solutions (crystallization solution with 10 % glycerol) for 0.5 ~ 1 min to
introduce glycerol into the crystals. The crystals were frozen with liquid nitrogen immediately and screened at SSRL (Beam Line 11-1) at 0 °, -45 °, and 90 ° for 10 sec each to see if the crystals showed diffraction patterns.

Crystals from optimized condition #4 (20.0 ~ 21.0 % PEG mme 5000 with micro-seeding) were frozen in the presence of 10 % glycerol. The crystal size was about 100 × 100 × 100 µm, and the crystal shape was a hexagonal column. The crystals were screened as described above at SSRL, and the crystal that showed the highest resolution was selected for data collection for structural analysis. Diffraction patterns from the crystal were obtained from 270 ° to 370 ° in increments of 0.5 °. The data set was analyzed with HKL2000 (HKL Research).

Crystals that were obtained from the optimized condition #4 with micro-seeding were frozen in the presence of either 15 or 20 % MPD (2-methyl 2,4-pentanediol), 15 or 20 % PEG400, and 15 or 20 % glycerol to find optimal cryo-protectant and concentration for the crystal freezing process. The frozen crystals were screened at SSRL and the data set from the crystal that showed the best data quality was obtained from 0 ° to 360 ° in increments of 0.25 ° with 15 sec exposure per flame. The data set was analyzed with HKL2000.

Crystals from the optimized condition #4 with micro-seeding were incubated in the cryo-protectant solution (crystallization solution with 20.0 % glycerol) for overnight before freezing them to improve the mosaicity. The crystallization drops on coverslips were moved from reservoirs with the original crystallization condition (20.0 % PEG mme 5000) to one with 30.0 % PEG mme 5000, and further moved to one with 30.0 % PEG
mme 5000 + 20 % glycerol in a stepwise manner. At each step, the crystals on the plate were incubated for 24 hours to equilibrate the crystallization drops to the condition in the reservoir. This process was called dehydration and it was expected to improve the crystal diffraction as well (162). After the crystallization solutions were equilibrated to the condition that corresponded to 30.0 % PEG mme 5000 + 20 % glycerol, the crystals in the droplets were moved to freshly prepared cryo-protectant solution (100 mM Bis-Tris, 30.0 % PEG mme 5000, 20.0 % glycerol, pH 6.5) and incubated overnight. When the crystals were frozen with liquid nitrogen, an evaporated nitrogen gas layer upon the liquid nitrogen surface was removed by blowing N₂ gas from 30 cm above. This process was reported to alleviate crystal degradation when it was frozen (166). For comparison, several other crystals from the optimized condition #4 with micro-seeding were incubated in the cryoprotectant solution for a few minutes and were also frozen with the same procedure. The crystals were screened at SSRL as described above.

**Crosslink of AcN₂OR and AcPAz**

EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, Thermo) reacts with carboxyl group (Glu or Asp) of protein #1 and forms an amine-reactive O-acylisourea intermediate (167). NHS (N-hydroxysuccinimide, Thermo) stabilizes the activated intermediate in solution until it reacts with amine group (most often Lys) of protein #2 (167). The crosslink reaction occurs when the Glu/Asp of protein #1 and Lys of protein #2 interact with each other.

To obtain higher quality of co-crystals, a crosslink between AcN₂OR and AcPAz was attempted with EDC and NHS. The crosslink reaction was carried out under
anaerobic conditions in the glove box using Ar bubbled buffers. First, as a positive control experiment, both AcN2OR and AcPAz were activated with EDC/NHS and crosslinked at the same time to confirm whether the crosslink reaction occurred. Also, the optimal ratio between [total protein] and [EDC] was examined. 32 µM AcN2OR and 162.4 µM AcPAz ([AcN2OR]:[AcPAz] = 1:5) in coupling buffer (10 mM Na2HPO4, 1.76 mM KH2PO4, 137 mM NaCl (pH 7.4)) was aliquoted in 7 tubes. Each sample was activated and crosslinked with different concentrations of EDC and NHS (the ratio between EDC and NHS was always 1:2.5) for 1 hour at room temperature. The concentration of EDC was changed as 0 mM (control), 0.2 mM (×1 of total protein concentration), 1 mM (×5), 2 mM (×10), 10 mM (×50), 20 mM (×100), and 100 mM (×500). The total volume of each sample was adjusted to 50 µL. The crosslinked protein was monitored with SDS-PAGE. The amounts of crosslinked protein in each condition were calculated with band intensities on the SDS-PAGE gel using Image J (168).

Second, AcN2OR or AcPAz in activation buffer (100 mM MES, 500 mM NaCl (pH 6.0)) was individually activated in the presence of EDC/NHS ([total protein]:[EDC]:[NHS] = 1:10:25). After 1 hour, the activation reaction was stopped with an Amicon using YM-50 membrane (for AcN2OR) and a gel filtration column (for AcPAz, since it was much faster than using the Amicon with YM-3 membrane) by removing the reagents. The sample buffer was exchanged to the coupling buffer at the same time. Then, the other protein (AcPAz for activated AcN2OR, or AcN2OR for activated AcPAz) was added for the crosslink reaction and the samples were incubated for 4 hours. The amounts
of crosslinked protein at 1, 2, 3, and 4 hours were estimated with SDS-PAGE and Image J as described above.

Third, the effect of NaCl on the crosslink reaction was elucidated by comparing efficiency of the crosslink reaction using the coupling buffer with or without NaCl. AcN₂OR was activated in the activation buffer with EDC/NHS for 1 hour ([AcN₂OR] = 5 nmol, [EDC] = 50 nmol, [NHS] = 125 nmol). After the reagents were removed with the Amicon, the buffer was exchanged with the coupling buffer with or without NaCl. 5-fold amount of AcPAz (~ 25 nmol) was added to the activated AcN₂OR and incubated it for 4 hours. The samples at 2, 3, and 4 hours were checked with SDS-PAGE. The crosslink efficiency was calculated with the band intensity of crosslinked AcN₂OR-AcPAz on the SDS-PAGE gel over the sum of band intensities of the proteins between 66.5 and 170 kDa.

**Results**

**Screening and Optimizations of Crystallization Conditions**

The conditions that showed crystals in the initial screening plates were selected and optimized with 24-hole plates. The selected conditions from the initial screenings were listed in Table 5.1.

<table>
<thead>
<tr>
<th>#</th>
<th>Buffer (pH)</th>
<th>Salt</th>
<th>Precipitant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 mM Tris (pH 8.5)</td>
<td>200 mM</td>
<td>Lithium Sulfate</td>
</tr>
<tr>
<td>2</td>
<td>100 mM Sodium phosphate (pH 6.2)</td>
<td>200 mM NaCl</td>
<td>35% 2-ethoxyethanol</td>
</tr>
<tr>
<td>3</td>
<td>50 mM Bis-Tris propane (pH 5.0)</td>
<td>50 mM Citric acid</td>
<td>16% PEG 3350</td>
</tr>
<tr>
<td>4</td>
<td>100 mM Bis-Tris (pH 6.5)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* mme = monomethyl ether

Table 5.1. The initial crystallization conditions for AcN₂OR-AcPAz co-crystal
The initial conditions produced crystals that were shaped like needles (condition #1), branched needles (#2), rods (#3), and hexagonal columns (#4) (Figure 5.1). These crystals were observed at day 7 (#1), day 1 (#2), day 4 (#3), and day 68 (#4) in the initial screening plates. Conditions #1, 3, and 4 were further modified to obtain bigger and higher quality crystals for X-ray measurements. During optimization of condition #1, it was found that both PEG and lithium sulfate concentrations affected the crystal amount and shape. Protein amount affected the crystal growth speed. Additive screens did not show any apparent effect. Crystals were observed between 24.0 and 31.0 % PEG, and between 100 and 250 mM lithium sulfate. In lower concentrations of lithium sulfate (150
mM, 27.0 % PEG), the crystal shapes were feather-like structures (Figure 5.2, left), whereas the crystals became needle-like structures in higher concentration of lithium sulfate (250 mM, 27.0 % PEG) (Figure 5.2, right).

Figure 5.2. Crystal shape transition with 150 and 250 mM lithium sulfate (condition #1)

In optimization of condition #3, crystals were observed between 11 – 15 % PEG. Crystals at higher percentage of PEG (13 – 15 %) were dissolved over time. Changing pH conditions between 4.5 and 7.0 did not show any positive effect on crystal formation. The best condition to date was 50 mM Bis-Tris propane, 50 mM citric acid, 12.0 % PEG3350, pH 5.0 (Figure 5.3). The crystal size was 100 × 30 × 30 µm.

Figure 5.3. Crystals obtained from modified condition #3
In condition #4, the micro-seeding method significantly improved crystal formation. It was revealed that temperature (22 °C) was a critical factor for the crystal growth because the crystals were not observed at 18 and 4 °C. The biggest crystal (100 × 100 × 200 µm) was obtained from 100 mM Bis-Tris, 20.0 % PEG, pH 6.5, 22 °C with the micro-seeding method (Figure 5.4). Several crystals grew as polycrystals (Figure 5.4, left), which were also observed in the initial screening plates (Figure 5.1).

Figure 5.4. Crystals obtained from modified condition #4

Crystal Check for Protein

Salt in crystallization solutions sometimes aggregates as crystals. The crystals obtained from condition #4 were checked with Izit dye to confirm whether they were made of protein or salt. The Izit crystal dye turned the crystals blue, suggesting that the crystals were made of protein (Figure 5.1, and 5.5, left). The crystals from conditions #1 and 4 were also checked with SDS-PAGE to confirm they included both AcN2OR (66.5 kDa) and AcPAz (13.0 kDa) (Figure 5.5, middle and right). “M” in Figure 5.5 indicates marker, “#1” and “#4” are the crystals from the condition # 1 and #4, “Pos” is a positive control (×20 diluted protein stock for crystallization), and “Neg” is a negative control that
shows the proteins around the crystals (#1 and #4) were completely rinsed out. The results confirmed that the crystals from conditions #1 and #4 were made of protein and included both AcN2OR and AcPAz.

Figure 5.5. Crystal checks with the Izit dye and SDS-PAGE for condition #1 and #4

X-ray Measurements of the Co-crystals

A needle-like crystal (30 × 30 × 500 μm) from modified condition #1 did not show any diffraction pattern with a home source X-ray apparatus. Several crystals from condition #3 (rod shaped) and #4 (hexagonal column shaped) were screened at SSRL and showed some diffractions. A crystal from condition #3 showed some diffraction spots around ~ 18 Å, whereas several crystals from condition #4 showed ~ 4 Å resolution data. Preliminary analysis with the screening data from crystals in condition #4 showed the cell parameters as follows; a = 70.16 Å, b = 70.16 Å, c = 552.10 Å, α = 90.00 °, β = 90.00 °, γ = 120.00 °, mosaicity = 1.322 °. Structural analysis from the crystals could not be carried out because the resolution was too low and the mosaicity was too high, but the data
indicated that crystals from condition #4 were highly promising. Therefore, condition #4 was further optimized for the following measurements.

Crystals obtained from the optimized condition #4 were screened and measured at SSRL. The crystal that showed the best resolution (3.5 Å) in the screening was used for the following measurements and the data set obtained was analyzed with HKL2000. The cell parameters were almost the same as the previous preliminary measurement; $a = 69.93$ Å, $b = 69.93$ Å, $c = 552.35$ Å, $\alpha = 90.00$ °, $\beta = 90.00$ °, $\gamma = 120.00$ °, mosaicity = 1.160 °. However, data index failed because of two reasons; (1) the crystal was a polycrystal and two different lattices were overlapped, which precluded further analyses of the data set, (2) the c axis of the cell unit was large (552.35 Å) and diffraction spots from c axis tended to overlap each other, which also hindered further analyses. It was required to obtain single crystals and to reduce mosaicity for further analysis.

To lower the mosaicity, several cryo-protectants (MPD, PEG400, and Glycerol) were applied to the crystal freezing process. Screening and data set collection were carried out at SSRL. The crystal that was incubated with glycerol showed the best mosaicity (0.6 °). The resolution of the data was 3.6 Å. Although the mosaicity had been improved, the data quality was not good enough for analysis and index failed.

Mosaicity usually increases when crystals are freezing. In the previous experiments, the crystals were incubated in the cryo-protectant solutions for 30 seconds to a few minutes prior to freezing. Prolonged cryo-protectant introduction to the crystals might lower the mosaicity. The crystals that were incubated in the cryoprotectant solution for a few minutes or overnight were screened at SSRL to investigate the effect of
incubation time with the cryoprotectant (Figure 5.6). The result showed that the overnight incubation made the diffraction spot shapes crooked (Figure 5.6, right), and the spot size became bigger (= mosaicity became bigger) than the diffraction from the crystals that were incubated for a few minutes (Figure 5.6, left). The analysis (index) of the data failed.

Figure 5.6. Diffractions from the crystals with flash (left) and overnight (right) introduction of cryoprotectant

For the structural analysis of the crystals from condition #4, the mosaicity needed to be less than 0.30 ° to obtain the structural information from the data set (Prof. C. Martin Lawrence, pers. commun.) since the crystals from condition #4 had a large cell unit (c = ~ 550 Å). To date, 0.60 ° was observed as the lowest mosaicity. The freezing condition needs to be further optimized to reduce the mosaicity. Alternatively, optimizations of the other conditions (#1, 2, and 3) might produce appropriate crystals for the structural analyses.
Crosslink of AcN$_2$OR and AcPAz

Crosslink between AcN$_2$OR and AcPAz with EDC/NHS was attempted to improve crystal quality. Since Asp and Glu on AcN$_2$OR were expected to interact with Lys on AcPAz during electron transfer (92; 87), this method was thought to be suitable for crosslink between AcN$_2$OR and AcPAz as an electron transfer complex. In a preliminary experiment, both AcN$_2$OR and AcPAz were activated and crosslinked at the same time in the presence of different concentrations of EDC and NHS (Figure 5.7). The crosslinked protein was observed on the SDS-PAGE gel as a 79.5 kDa band (AcN$_2$OR 66.5 kDa + AcPAz 13.0 kDa) and the band intensity did not increase after the ratio between [total protein] and [EDC] was 1 to 50, which indicated that 50-fold amount of EDC was sufficient to maximize the crosslink reaction.

Figure 5.7. SDS-PAGE and band quantification for AcN$_2$OR-AcPAz crosslink

Either AcN$_2$OR or AcPAz was activated separately and crosslinked with the other protein (Figure 5.8). “Neg” in Figure 5.8 indicates non-crosslinked sample as a negative control, and “Pos” indicates the crosslinked sample from Figure 5.7 as a positive control.
The SDS-PAGE showed that the crosslink reaction between AcN2OR and AcPAz occurred only when AcN2OR was activated. The incubation time (1 ~ 4 hours) did not affect the crosslinked protein yield since the band intensities among the samples did not show any difference (Figure 5.8, right, arrow). It suggested that the crosslink reaction finished within an hour.

![Image of SDS-PAGE](image)

Figure 5.8. AcN2OR-AcPAz crosslink with activated AcPAz (left) or AcN2OR (right)

The crosslink efficiency was compared in the presence and absence of NaCl in the coupling buffer, and in terms of incubation times (2, 3, and 4 hours) (Figure 5.9). The crosslink efficiency was calculated with the band intensity of crosslinked protein at 79.5 kDa (“Crosslinked (C)” in Table 5.2) over the band intensities of the other AcN2OR related bands (“Total N2OR (T)” in Table 5.2) including AcN2OR monomer (66.5 kDa), AcN2OR+AcPAz (79.5 kDa), AcN2OR dimer (133 kDa), and the other unassigned bands between 66.5 and 170 kDa (Figure 5.9). The crosslink efficiencies of both conditions were calculated as ~ 33 % (Table 5.2). The result showed that NaCl (137 mM) in the coupling buffer had no affect on the crosslink reaction between AcN2OR and AcPAz.
Also, elongated incubation time did not improve the efficiency as observed in the previous experiments.

Figure 5.9. AcN2OR-AcPAz crosslink in the coupling buffer with or without NaCl

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<td></td>
<td>4h</td>
<td>54</td>
<td>80</td>
<td>162</td>
<td>33%</td>
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Table 5.2. Band intensities on the SDS-PAGE gel in Figure 5.9

Discussion

There is a lot of progress in co-crystallization of AcN2OR and AcPAz although further optimizations will be required to obtain crystals suitable for structural
determination. To date, the best crystals were obtained from condition #4 (100 mM Bis-Tris (pH 6.5), and 20 % PEG monomethylether 5000). The first crystals in condition #4 appeared after more than 50 days following the initial screening plate preparation. Also, the micro-seeding method significantly improved the crystal formation. These results indicate that nucleation is the rate-limiting step for the crystallization (169).

The crosslink between AcN2OR and AcPAz was observed only when AcN2OR was activated (Figure 5.8). The result indicated that Glu and/or Asp on AcN2OR were crosslinked to Lys on AcPAz. These amino acids were expected to be involved in the formation of AcN2OR-AcPAz complex for electron transfer (92; 87). Possible interaction sites of AcN2OR and AcPAz for the electron transfer complex were shown in Figure 5.10 (91). AcN2OR has a hydrophobic patch close to the CuA site, which is surrounded with negatively charged amino acids (Asp and Glu), whereas AcPAz has a hydrophobic patch around the Cu center and positively charged amino acids (Lys) are located around the patch (Figure 5.10). The colors on the AcPAz and AcN2OR structures in Figure 5.10 indicate the Cu centers (in purple), the hydrophobic amino acids (in blue), the lysines on AcPAz (in red), and the aspartic acids and glutamic acids on AcN2OR (in red). These hydrophobic patches (shown with orange circles in Figure 5.10) and the charged amino acids (red) were well conserved among species, suggesting that these sites were involved in the electron transfer complex formation (93). Since these Glu and/or Asp on AcN2OR (Glu331, 333, 588, Asp356, 384, 416 and 536) and Lys on AcPAz (Lys38, 46, 57, 77 and 109) were expected to interact with one another in solution, one or several of these amino acids are candidates for crosslinking.
Figure 5.10. Expected crosslink sites of AcPAz (left) and AcN₂OR (right)

NaCl did not affect the crosslink reaction between AcN₂OR and AcPAz (Figure 5.9, Table 5.2). Since chloride ion was found to inhibit the N₂O reduction by AcN₂OR using reduced AcPAz as the electron source (Chapter 4), it was possible that NaCl interrupted formation of the AcN₂OR-AcPAz complex for electron transfer. If so, there would be a difference on the crosslink efficiency with or without NaCl. However, no
such difference was observed regardless of NaCl (Figure 5.9). The result suggested that the interaction between $AcN_2OR$ and $AcPAz$ in solution was not significantly perturbed by NaCl.

Conclusions

The objective of the studies on this chapter was to elucidate the docking structure of $AcN_2OR$ and $AcPAz$. The $AcN_2OR$-$AcPAz$ complex structure was investigated with co-crystallization and crosslink of two proteins. The conditions that grew co-crystals including $AcN_2OR$ and $AcPAz$ were successfully identified, although further optimizations for the crystal growth conditions and the freezing process were necessary for the structural analysis. The crosslink experiments suggested that Glu and/or Asp on $AcN_2OR$ interacted with Lys on $AcPAz$ in solution since the crosslink reaction was observed only when $AcN_2OR$ was activated. This observation was consistent with the results from the DFT calculation by Mattila et al (92).
The main objective of this project was to investigate the structure and function of AcNosX. The results from this project form the basis for the study on NosX and the other Nos proteins, and provided some insights into the mechanism of the N₂OR activation. This research contributes to a field of the electron transfer reactions within the denitrification system.

Prior to the investigations, it was essential to increase the NosX yield since the expression level of NosX was very low and the following experiments required a substantial amount of AcNosX protein. In chapter 2, the optimization of the culture conditions resulted in a great improvement in the NosX yield. It was found that the LB media that was purchased from Fisher brand significantly increased the NosX yield when pMLClustXst plasmid was used. The new expression systems did not show such effects with the LB media. The factor(s) in the LB media and the mechanism of the NosX yield enhancement need to be identified. Also, it would be interesting to monitor if the expression level of the other Nos proteins (NosR, NosD, NosF, NosY, and NosL) are enhanced by the LB media or not. These studies will reveal the regulation system for the nos gene expression. Another intriguing result in chapter 2 was that the NosX yield was constant under both aerobic and micro-aerobic conditions. The precise level of oxygen in the media during cultures and the effect of oxygen to the NosX expression should be further investigated.
It is still unclear why the new expression system did not enhance the NosX yield. Northern blot analyses for nosX transcripts in Ac (wild type), Ac/p5-(Np/Pp)-nosX and Ac/pMLClustXst will be required. Also, it would be a good idea to make a p5-Pp-paz plasmid to check if AcPAz was overexpressed in Ac/p5-Pp-paz as a positive control.

The structure of AcNosX was investigated in chapter 3. The calculated AcNosX model showed several insights for the AcNosX structure. Tyr46 was expected to be important for the FAD binding. This hypothesis needs to be investigated by a site directed mutagenesis. Also, a potential binding site with AcN2OR for electron transfer was suggested. To confirm if this site interacts with AcN2OR, a precise structure of AcNosX with X-ray crystallography is required to calculate docking structures with AcN2OR. With respect to the crystallization trials for AcNosX, further optimization of the conditions is required. Removing the strep-tag of AcNosX with Factor Xa protease might facilitate the crystal growth of AcNosX.

The interaction between AcNosX and AcN2OR was investigated in chapter 4. The results indicated that AcNosX activated AcN2OR by donating electrons, which supports the hypothesis by Wunsch et al (83). The next question is the function of NosX on the CuZ* state. Most importantly, it is essential to define the character and structure of the CuZ* state. As recently reported (63), it is required to prove whether the [4Cu:1S] state corresponds to the CuZ* state. It was reported that deletion of nosX gene resulted in increase of the population of the CuZ* state (83). Currently, there are two hypotheses on the function of NosX to explain this observation. First, NosX may be involved in the
regeneration of the Cu$Z^*$ state. If this is the case, the conditions that NosX requires for the regeneration reaction should be identified. In our experiment in vitro, it failed (chapter 4). Second, NosX may prevent generation of the Cu$Z^*$ state. In this case, it will be required to identify the process of generation of the Cu$Z^*$ state. If we could monitor the generation process of the Cu$Z^*$ state with or without reduced AcNosX, it would show the effect of AcNosX on the Cu$Z^*$ state generation.

Stabilization of AcNosX in vitro is another matter of concern. We could not find any reagent that substituted chloride to stabilize AcNosX in solution. Since chloride inhibited the N$_2$OR catalytic reaction with AcPAz, the catalytic reaction with AcNosX also could not be measured. It is essential to find how to stabilize AcNosX in solution without using chloride ion for further investigations on the electron transfer from AcNosX to AcN$_2$OR during the catalytic cycle.

Co-crystallization of AcN$_2$OR and AcPAz was attempted in chapter 5. To solve the structure, the freezing condition of the crystals from condition #4 needs to be optimized to lower the mosaicity. Another option is the crystals from conditions #2 and 3. Since the optimization has been focused on the crystals from condition #4, there is room for improvement on the crystals from conditions #2 and 3 to solve the structure. Also, the crosslinked AcN$_2$OR-AcPAz complex needs to be crystallized under these conditions to see if the crosslink technique has some effects on the crystal quality. Alternatively, the crosslink reagents can be added to the crystals to crosslink the proteins in the crystals.
APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 1
Figure S1.1. Multiple sequence alignment for NorB with CLUSTAL W 2.1 and BoxShade 3.21

Ps, Pseudomonas stutzeri (GenBank: CAA82229.1); Pa, Pseudomonas aeruginosa (GenBank: BAA07330.1); Mh, Marinobacter hydrocarbonoclasticus (GenBank:
CCG96517.1; Ac, *Achromobacter cycloclastes* (GenBank: CAC27381.1); Pd, *Paracoccus denitrificans* (GenBank: BAA32546.1)

Figure S1.2. Multiple sequence alignment for NorC with CLUSTAL W 2.11 and BoxShade 3.21

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MhNorC 53 NCIIGCHSIMGEGAYFAPELGNVQGRGQ----EETKPEFLAWMINDPPEGRAMPQFN
AcNorC 44 SCINCHTLLGEGAYFAPELGNVQGRGQ----EETKPEFLAWMINDPPEGRAMPQFN
PdNorC 61 ACNCHTLLGEGAYFAPELGNVQGRGQ----EETKPEFLAWMINDPPEGRAMPQFN

PsNorC 117 LSQCVDPASPFSIFTSKIDTNWPPNKG
PaNorC 117 LSQCVDPLAPFSIFTSKIDTNWPPNKG
MhNorC 112 LSQCVDPASPFSIFTSKIDTNWPPNKG
AcNorC 104 LSQCVDPLAPFSIFTSKIDTNWPPNKG
PdNorC 121 LSQCVDPLAPFSIFTSKIDTNWPPNKG
APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 2
Table S2.1. List of AcNosX culture (Ac/pMLClustXst) and yield

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<th>mg/10g</th>
<th>LB</th>
<th>Speed (rpm)</th>
<th>24 or 48 h</th>
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APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 3
Figure S3.1. Surface electrostatic potential of ApbE protein from *Salmonella enterica*.
Figure S3.2. Phylogenetic tree based on NosX amino acid sequences.
APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER 4
Figure S4.1. Cu standard curves for spin quantification at 14, 20, and 30 K.
N₂OR Oxidation with Ferricyanide and Cu Content Check with AA.

N₂OR oxidations with ferricyanide and buffer exchange were carried out in a glove box (O₂ < 5 ppm). The “As purified” form of N₂OR (300 µL) was aliquoted into two tubes (150 µL each). To oxidize N₂OR, 10, 45, or 100-fold excess amount of ferricyanide powder (ferri) was added to one sample (labeled as “oxidized (OX)”). The other sample was labeled as “as purified (AP)”. 1 mL of 20mM Tris (pH8.0, bubbled with Ar for 1 hour) was added to both tubes. Both samples were centrifuged for 5 – 10 min at 4500 rpm with Amicon centrifuge tubes (YM-50 membrane). 1 mL of buffer was added and again centrifuged. This process was repeated for seven times. Cu amounts of concentrated N₂OR samples (AP and OX) and its corresponding flow-through (FT) were measured with AA and compared in terms of the ratio between [ferricyanide] and [N₂OR].

Analysis of Removed Cu amount from N₂OR.

The measured values of Cu concentrations are listed in Table S4.1. The removed Cu from N₂OR was calculated with a following equation;

\[
\text{Removed Cu (\%)} = \frac{[\text{Cu} \text{ in FT}]}{([\text{Cu} \text{ in N₂OR}]+[\text{Cu} \text{ in FT}])}
\]

A positive correlation was observed between the removed Cu from N₂OR and the ferricyanide amount (Figure S4.2). No Cu was detected in the ferricyanide solution (Table S4.1, Exp #4), indicating that the detected Cu in FT was derived from N₂OR.
Table S4.1. Cu concentrations in N$_2$OR (AP and OX) and flow-through

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Figure S4.2. Correlation between removed [Cu] from N$_2$OR and [ferricyanide].
Figure S4.3. UV/Vis spectra of AcN₂OR in Buffer PE
Table S4.2. Concentrations of Cu, sulfide, and activity of N$_2$OR from different purification batches

<table>
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<tr>
<th>Prep #</th>
<th>[Cu]/monomer</th>
<th>[S]/monomer</th>
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(Data set from AcN$_2$OR prep by Dr. Koyu Fujita)
Figure S4.3. Correlations between Cu or sulfide contents and activity of AcN$_2$OR (data points are from Table S4.1).


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