To my Grandfather
Molecular Enzymology of the Copper-Containing Enzymes Involved in Denitrification

A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

by

Miguel Prudêncio

March 2000

© This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis, nor any information derived therefrom, may be published without the author’s prior written consent.
This PhD was sponsored by grant BD / 5471 / 95 of the PRAXIS XXI programme of the Ministry of Science and Technology, Foundation for Science and Technology

PORTUGAL
ACKNOWLEDGEMENTS

Four years in one’s life are likely to represent a unique experience both at the professional and at the personal levels. The last four years of my life have been very rich in both and they would not have happened in the way they did if it weren’t for a few people who, in one way or the other, made them possible. These people, who I would now like to acknowledge, helped shape my life and, to some extent, my own personality. This thesis would not be the same without them. And quite possibly, neither would I.

I would like to start by thanking my supervisors, Prof. Isabel Moura and Dr. Robert Eady, for accepting me as their student and allowing me to learn so much with them. Isabel’s commitment to the lab and the way she’d never miss an opportunity to put a lab coat on and load a column impressed me from the beginning and made me realise what it is to love working in science. Bob’s permanent availability to plan and discuss my project and the constant support I got from him made my work so much more enjoyable and taught me a lot about what a PhD is all about. To both of them, my most sincere thanks.

To Dr. Gary Sawers, “the Boss”, I owe more than I can write in a few lines. I thank him for being my adviser and for having known how to do it in such a brilliant manner; I thank him for teaching me all I know about molecular biology; I thank him for always being ready to discuss my “crap”. Above all, I thank him for his friendship.

I’d like to thank Prof. José Moura for the way he knows how to share his enthusiasm about science and about life. Rarely, if ever, have I met anyone with such a contagiously excited attitude towards the science they know about and yet so capable of talking about so many other things with equally contagious excitement. I’d like to thank him for various long conversations we had, especially in the early years, and for what I learned from them.

Often the people that you learn the most from in a lab are those who are there to help you and teach you what they know whenever you need them. And when they know as much as Dr. Pedro Tavares and Dr. Alice Pereira do, there is a lot one can learn. Despite later misunderstandings, I will never forget that I possibly learnt more about science from Pedro and Alice than from anybody else I’ve ever met. I’d like to thank them both for that and for the friendship we once shared.

My project at the John Innes Centre had the invaluable help of a few people that provided essential contributions to my work. I’d like to thank Dr. Shirley Fairhurst for her help with all the EPR measurements performed, Mr. Mike Chan for the large-scale growths of overproducing \textit{E. coli} cells, Dr. Mike Naldrett for the sequencing and mass spectrometry analysis, Dr. Chris Pickett and Dr. Saad Ibrahim for their help with the electrochemistry studies and Dr. Gillian Ashby and Mr. Tom Clarke for their help with the stopped-flow measurements.

Also my project at the Universidade Nova counted with the participation of M. Inês Cabrito, who I would like to acknowledge for her work in the cloning and sequencing of the gene coding for nitrous oxide reductase, and of Dr. Stephane Besson, who helped me with protein purification and activity measurements, especially at the earlier stages of the project.

I would like to thank Dr. Kieron Brown, Dr. Mariella Tegoni and Prof. Christian Cambillau for their work on the determination of the structure of the first nitrous oxide reductase. This major achievement is owed to them above any others and I am happy to have had the opportunity to modestly contribute towards it.
I would also like to express my gratitude to Dr. Fraser Dodd and Mr. Mark Ellis at Prof. Samar Hasnain’s laboratory, for their excellent work on structure determination of nitrite reductase and its derived mutants. I would also like to thank them for several very useful discussions and for the various pictures that they kindly produced and provided for this thesis.

I would like to thank all the people that I shared the lab with in Lisbon over the last six years. I will not forget the time we spent together and the way in which we could, sometimes, be so many and yet feel so close. I would like to thank especially Ad, Inês, Nini, Rikk and Susana for their friendship and constant support. A special word for Cristina, who maybe does not know it, but changed my life. I would also like to thank the rest of my friends at home, especially Alves, Carla, Catarina, João, Luis, Mourão, Pedro and Tópê for remaining so special despite the distance and for being there for my birthday party every year, when I feared that people might have forgotten about me.

Also in Norwich I met a few people that I will never forget. I would like to thank all my lab mates for their companionship and for teaching me about “the english way”. Dan, Dave, Jason, Rachael and Paqui certainly made my time in the lab a lot more enjoyable since the early days. I would like to thank many other people in the rest of the John Innes Centre (and not only) for so many great moments. For their friendship and/or their support during the stressful times of writing-up I would like to thank especially Ajay, Clarisse, Cristina, Elena, Maria, Silvana, Pampa and Paul. I would also like to thank Suzy for offering to proof-read my thesis.

I would like to thank my flatmates in Norwich for making me feel that I could call home to the place where I’d go everyday after work. Patrice and Tania literally rescued me during one of the most difficult periods of my time in Norwich and I will never forget that. Katy and Laure are simply the best flatmates one can wish for and I will miss them more than I can say.

I would like to thank the Security guards at the John Innes Centre, Charlie, Dave, George, Graham, Ray and Stewart for always being ready for a chat. Thank you also to Rachel in the library, who always got me the references I needed, even when I needed more than would be sensible.

I would like to thank Lisa and Guida for being such good friends and spoiling me each and every time I am at home. I would also like to thank Tete, Aida, Rosa and Tomás for caring so much about me.

I would like to thank Manuela for knowing me so well and, so often, being the only person that can understand me. I owe her the experience of living in Norwich but, more importantly, I owe her much of what I am and what I’ve lived. My friendship for her is beyond what can be put into words and I can only wish that it will last forever.

I would like to thank the Portuguese Ministry of Science and Technology and the Praxis XXI programme for financing my PhD and agreeing to change the initial terms of my grant.

Finally, but by no means less importantly, I would like to thank my brother, my mother, my father and my grandmother for the way they always believed in me and supported me throughout my PhD and throughout my life. I thank them for always caring about me and, despite having to cope with my absence, always making me feel loved. Whatever my achievements may be, they are owed to them.
ABSTRACT

Microbial denitrification is the stepwise reduction of nitrate to nitrogen in a process coupled to energy generation. Nitrite reductase (NiR) and nitrous oxide reductase (N2OR) are the two copper-containing enzymes that form part of the denitrification pathway. Each of these enzymes possesses two types of Cu centres, specialised in electron transfer and catalysis.

The gene encoding the NiR from *Alcaligenes xylosoxidans* was cloned and sequenced. The overproduced recombinant protein was purified and characterised biochemically and spectroscopically and shown to be indistinguishable from the wild-type enzyme. A site-directed mutagenesis programme was undertaken aimed at putative key residues involved in Cu centre assembly, intra-molecular electron transfer and catalysis. The His139, Met144 and Cys130 ligands of the electron transfer type 1 Cu centre were independently replaced by Ala residues and the purified mutant proteins were analysed in terms of their Cu-binding ability as well as their kinetic and spectroscopic properties. The Asp92 residue in the neighbourhood of the catalytic type 2 Cu centre of the enzyme was replaced by a Glu residue and an Asn residue and the effects of the mutations on the activity and spectroscopic properties of the purified mutant proteins were studied. The results support the notion that the type 2 Cu site is responsible for binding and reducing nitrite. Furthermore, they indicate redox coupling between the two Cu centres of NiR and suggest that the protein undergoes a conformational change upon interaction with the physiological electron donor. The determination of the X-ray structure of the Met144Ala mutant form of NiR was undertaken in collaboration with the group of Prof. Samar Hasnain. Preliminary electrochemical studies were carried out both with the recombinant NiR and with selected mutant forms of the protein in order to understand the role of both Cu centres in the electrochemical response of the protein.

The N2OR from *Pseudomonas nautica* was purified in two different forms, A and B, shown to correspond to different oxidation states of the protein. Both forms of the enzyme were shown to possess a Cu content higher than previously reported for other N2ORs and to co-purify with a chaperonin-like peptide. Forms A and B of the enzyme were characterised biochemically and spectroscopically in different redox states. The results support a model in which the two Cu centres of N2OR contribute independently to the overall spectroscopic properties of the protein. The amino acid sequence of the protein and part of the DNA sequence of the gene coding for N2OR from *Pseudomonas nautica* were determined. In collaboration with the group of Prof. Christian Cambillau, the X-ray structure of the protein was determined and confirmed the presence of an electron transfer CuA centre. Moreover, these studies revealed the presence of a novel type of tetranuclear Cu cluster, which is suggested to be involved in catalysis. The studies carried out with NiR and N2OR provided an insight into the mechanism of both these enzymes and revealed previously unknown features of their respective Cu centres.
ABBREVIATIONS

Abs – absorbance
amu – atomic mass units
AP – nitrate/nitrite antiporter
Bicine – N,N-bis[s-hydroxyethyl]glycine
bp – base pair
BSA – bovine serum albumin
CA – carbonic anhydrase
CD – circular dichroism
CMC – carboxymethyl cellulose
COX – cytochrome c oxidase
CT – charge transfer
CTAB – cetyl trimethyl ammonium bromide
CuNiR – copper-containing nitrite reductase
CV – cyclic voltammetry
DNase I – deoxyribonuclease I
ds – double-stranded
dNTP – dATP, dCTP, dGTP, dUTP
ECL – enhanced chemiluminescence
EDTA – ethylenediamine tetraacetic acid
ENDOR – electron nuclear double resonance
EPR – electron paramagnetic resonance
ESE – electron spin echo
Esr – electron spin resonance
EXAFS – extended X-ray absorption fine structure
Fc – ferroene
FTIR – Fourier transform infra-red
HPLC – high performance liquid chromatography
I – nuclear spin
iPCR – inverse polymerase chain reaction
ICP – inductively coupled plasma emission
IPTG – isopropyl-β-D-thiogalactopyranoside
LB – Luria Broth
LMCT – ligand to metal charge transfer
LT-MCD – low temperature magnetic circular dichroism
MALDI-TOF – matrix assisted laser desorption/ionisation – time-of-flight
Abbreviations

MES – 2-[N-morpholino]ethanesulphonic acid
MCD – magnetic circular dichroism
MV – methyl viologen
N₂OR – N₂O reductase; nitrous oxide reductase
NADH – α-nicotinamide adenine dinucleotide, reduced form
NADPH – α-nicotinamide adenine dinucleotide phosphate, reduced form
Nap – nitrate reductase (periplasmic)
Nar – nitrate reductase (membrane-bound)
Nas – cytoplasmic nitrate reductase (assimilatory)
NC – nitrocellulose
NEDA – naphthylethylenediamine
NiR – nitrite reductase
Nor – nitric oxide reductase
OD – optical density
PAGE – polyacrylamide gel electrophoresis
PCR – polymerase chain reaction
PEG – polyethylene glycol
PITC – phenylisothiocyanate
PTH – phenylthiohydantoin
PVDF – polyvinylidene difluoride
4-Pyds – di-4-pyridyl disulfide
Pyranine – 8-hydroxy-pyrene-1,3,6-trisulfonate
RR – Resonance Raman
SDS – sodium dodecyl sulphate
SOD – superoxide dismutase
SSC – sodium chloride-sodium citrate
T1D – type 1 Cu-depleted
T2D – type 2 Cu-depleted
TBE – Tris-borate-EDTA buffer
TE – Tris-EDTA buffer
TEMED – N,N,N′,N′-tetramethylglylenediamine
TH – thiohydantoin
UV – ultra-violet
Vis – visible
wt – wild-type
X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS  iv

ABSTRACT  vi

ABBREVIATIONS  vii

TABLE OF CONTENTS  ix

FOREWORD: THESIS STRUCTURE  xv

## CHAPTER I. GENERAL INTRODUCTION  1

### I.1. DENITRIFICATION  2
- I.1.1. The nitrogen cycle  2
- I.1.2. Nitrogen fixation and nitrification  2
- I.1.3. Three pathways of nitrate reduction  4
- I.1.4. The denitrification pathway  5
- I.1.5. Ecological aspects of denitrification  7

### I.2. COPPER CENTRES IN BIOLOGY  9
- I.2.1. Copper in biological systems  9
- I.2.2. Copper centres in proteins  10
  - I.2.2.1. The type 1 Cu sites  10
  - I.2.2.2. The type 2 Cu sites  12
  - I.2.2.3. The type 3 Cu sites  13
  - I.2.2.4. The Cuₐ sites  13
  - I.2.2.5. The multi-copper oxidases: Trinuclear Cu sites  14

## CHAPTER II. MATERIALS AND METHODS  16

### II.1. MATERIALS  17
- II.1.1. Denitrifying Bacteria  17
- II.1.2. *Escherichia coli* strains  18
- II.1.3. Plasmids  18
II.1.4. _E. coli_ growth media 18

II.2. METHODS 19

II.2.1. Molecular biology methods 19

II.2.1.1. Bacterial DNA extraction 20

II.2.1.2. Phenol/Chloroform extraction of DNA 20

II.2.1.3. Ethanol precipitation of DNA 20

II.2.1.4. Transformation of _E. coli_ 21

II.2.1.4.1. TSS transformation 21

II.2.1.4.2. CaCl₂ transformation 21

II.2.1.4.3. Electroporation 22

II.2.1.5. Agarose gel electrophoresis 22

II.2.1.6. Restriction digestions and ligations 23

II.2.1.7. DNA modification 24

II.2.1.8. Polymerase Chain Reaction (PCR) 24

II.2.1.9. Plasmid purification, DNA extraction from agarose gels and purification of PCR products 25

II.2.1.10. DNA labelling, (pre-)hybridisation and detection 25

II.2.1.11. Southern blotting 26

II.2.1.12. Colony hybridisation 27

II.2.1.13. DNA sequencing 27

II.2.1.14. Autoradiography 28

II.2.1.15. Analysis of DNA and amino acid sequences 28

II.2.1.16. Site-directed mutagenesis 28

II.2.2. Protein methods 29

II.2.2.1. Protein determination 29

II.2.2.2. Concentration, dialysis and reconstitution 29

II.2.2.3. Metal determination 30

II.2.2.4. Enzyme activity assays 30

II.2.2.4.1. Nitrite reductase 31

II.2.2.4.1.1. Discontinuous assay 31

II.2.2.4.1.2. Continuous spectrophotometric assay 32

II.2.2.4.1.3. Electron donation by reduced azurin 32

II.2.2.4.2. Nitrous oxide reductase 33

II.2.2.4.3. Carbonic anhydrase 33

II.2.2.4.4. Superoxide dismutase 34

II.2.2.5. Chromatographic techniques 35

II.2.2.6. Electrophoresis 35
## Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.2.2.7. Crystallisation</td>
<td>36</td>
</tr>
<tr>
<td>II.2.2.8. Redox titrations</td>
<td>36</td>
</tr>
<tr>
<td>II.2.2.9. Protein sequencing</td>
<td>37</td>
</tr>
<tr>
<td>II.2.2.10. Mass spectrometry</td>
<td>38</td>
</tr>
<tr>
<td>II.2.2.11. Spectroscopic methods</td>
<td>39</td>
</tr>
<tr>
<td>II.2.2.11.1. UV/Vis</td>
<td>39</td>
</tr>
<tr>
<td>II.2.2.11.2. EPR</td>
<td>40</td>
</tr>
<tr>
<td>II.2.3. Other methods</td>
<td>42</td>
</tr>
<tr>
<td>II.2.3.1. Cell fractionation and disruption</td>
<td>42</td>
</tr>
<tr>
<td>II.2.3.2. Methionine-labelling</td>
<td>43</td>
</tr>
<tr>
<td>II.2.3.3. Electrochemistry</td>
<td>43</td>
</tr>
</tbody>
</table>

# III. NITRITE REDUCTASE 45

## III.1. INTRODUCTION 46

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.1.1. Two types of dissimilatory NiR</td>
<td>46</td>
</tr>
<tr>
<td>III.1.2. Historical background</td>
<td>47</td>
</tr>
<tr>
<td>III.1.3. Solubility and subcellular localisation of NiR</td>
<td>48</td>
</tr>
<tr>
<td>III.1.4. Molecular properties of NiR</td>
<td>48</td>
</tr>
<tr>
<td>III.1.5. The Cu centres</td>
<td>51</td>
</tr>
<tr>
<td>III.1.5.1. The type 1 Cu centre</td>
<td>51</td>
</tr>
<tr>
<td>III.1.5.2. The type 2 Cu centre</td>
<td>52</td>
</tr>
<tr>
<td>III.1.6. X-ray structure of NiR</td>
<td>53</td>
</tr>
<tr>
<td>III.1.6.1. Overall structure</td>
<td>53</td>
</tr>
<tr>
<td>III.1.6.2. Structure of the type 1 Cu site</td>
<td>55</td>
</tr>
<tr>
<td>III.1.6.3. Structure of the type 2 Cu site</td>
<td>58</td>
</tr>
<tr>
<td>III.1.6.4. Structure of the active site pocket: Implications for catalysis</td>
<td>58</td>
</tr>
<tr>
<td>III.1.7. Electron donation to NiR</td>
<td>61</td>
</tr>
<tr>
<td>III.1.8. <em>A. xylosoxidans</em> NiR</td>
<td>62</td>
</tr>
<tr>
<td>III.1.9. Project aims</td>
<td>63</td>
</tr>
</tbody>
</table>

## III.2. RESULTS 65

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.2.1. Recombinant NiR</td>
<td>65</td>
</tr>
<tr>
<td>III.2.1.1. Cloning and sequencing the <em>nirA</em> gene</td>
<td>65</td>
</tr>
<tr>
<td>III.2.1.2. Overexpression of <em>nirA</em> and overproduction of recombinant NiR</td>
<td>78</td>
</tr>
<tr>
<td>III.2.1.3. Subcellular localisation and purification of recombinant NiR</td>
<td>82</td>
</tr>
</tbody>
</table>
### Table of Contents

III.2.1.4. Characterisation of recombinant NiR and comparison with the wild-type enzyme  
  III.2.1.4.1. NiR activity  
  III.2.1.4.2. Spectroscopy and metal content  
  III.2.1.4.3. Molecular mass and thermostability  
  III.2.1.4.4. Carbonic anhydrase and superoxide dismutase activities  
  III.2.1.4.5. Crystallisation of recombinant NiR

III.2.2. Site-directed mutagenesis  
  III.2.2.1. Selection of mutations  
    III.2.2.1.1. Mutations of the type 1 Cu ligands  
    III.2.2.1.2. Mutations in the environment of the type 2 Cu centre  
  III.2.2.2. Site-directed mutagenesis programme  
  III.2.2.3. Characterisation of mutant NiR proteins  
    III.2.2.3.1. His139Ala mutant form of NiR  
      III.2.2.3.1.1. UV/Vis and EPR spectroscopies  
      III.2.2.3.1.2. Is type 1 Cu present in His139Ala?  
      III.2.2.3.1.3. Activity and substrate binding  
    III.2.2.3.2. Met144Ala mutant form of NiR  
      III.2.2.3.2.1. Spectroscopy and metal content  
      III.2.2.3.2.2. Activity and substrate binding  
      III.2.2.3.2.3. X-ray structure  
    III.2.2.3.3. Cys130Ala mutant form of NiR  
      III.2.2.3.3.1. Spectroscopy and metal content  
      III.2.2.3.3.2. Activity  
    III.2.2.3.4. Asp92Glu and Asp92Asn mutant forms of NiR  
      III.2.2.3.4.1. Spectroscopy and metal content  
      III.2.2.3.4.2. Activity and substrate binding  
    III.2.2.3.5. His249Phe mutant form of NiR  
  III.2.3. Electrochemistry  
    III.2.3.1. Cyclic voltammetry: Principles and instrumentation  
    III.2.3.2. Direct electrochemistry of proteins  
    III.2.3.3. Cyclic voltammetry of NiR and mutant forms of NiR

III.3. DISCUSSION
CHAPTER IV. NITROUS OXIDE REDUCTASE 165

IV.1. INTRODUCTION 166
   IV.1.1. Identification of N$_2$OR: Historical background 166
   IV.1.2. Solubility and subcellular localisation of N$_2$OR 167
   IV.1.3. Molecular properties of N$_2$OR: Different forms of the enzyme 169
   IV.1.4. The Cu centres in N$_2$OR 174
      IV.1.4.1. The Cu$_A$ centre 174
         IV.1.4.1.1. Cu$_A$ is present in COX and N$_2$OR 174
         IV.1.4.1.2. Probing the structure of Cu$_A$ 176
         IV.1.4.1.3. The X-ray structure of the Cu$_A$ site in COX 176
         IV.1.4.1.4. Further studies on the Cu$_A$ site of N$_2$OR 178
      IV.1.4.2. The catalytic centre 179
      IV.1.4.3. Two models for N$_2$OR 180
         IV.1.4.3.1. The Farrar model – 1991 181
         IV.1.4.3.2. The Farrar model – 1998 183
   IV.1.5. Project aims 186

IV.2. RESULTS 187
   IV.2.1. Purification of N$_2$OR: Molecular properties 187
      IV.2.1.1. Two forms of the enzyme 187
      IV.2.1.2. Co-purification of a 10.3 kDa putative chaperone with N$_2$OR 189
      IV.2.1.3. Metal content and activity 190
      IV.2.1.4. DNA and deduced amino acid sequence 191
   IV.2.2. X-ray structure of N$_2$OR 193
      IV.2.2.1. Overall structure 193
      IV.2.2.2. The Cu$_{cat}$ centre 194
      IV.2.2.3. The Cu$_A$ centre 196
      IV.2.2.4. Implications for the mechanism of N$_2$OR 199
   IV.2.3. Spectroscopic characterisation 200
      IV.2.3.1. UV/Vis spectroscopy 200
      IV.2.3.2. EPR spectroscopy 203
      IV.2.3.3. Redox titration 207

IV.3. DISCUSSION 213

CHAPTER V. REFERENCES 217
This is the way … step inside

“Atrocity exhibition”
Ian Curtis
1980
Foreword: Thesis structure

The work presented in this thesis concerns two projects that were carried out in two different Institutions as part of a dual-site PhD programme. The two projects are closely related. As reflected in the title “Molecular Enzymology of the Copper-Containing Enzymes Involved in Denitrification”, each of the projects concerned the study of one of the two copper-containing enzymes directly involved in the bacterial denitrification pathway. These enzymes are the nitrite reductase from *Alcaligenes xylosoxidans* and the nitrous oxide reductase from *Pseudomonas nautica*, two proteins that use copper as their sole metal cofactor. They were studied at the Departments of Molecular Microbiology and Biological Chemistry of the John Innes Centre in Norwich, UK and at the Faculty of Sciences and Technology of the New University of Lisbon, Portugal, respectively. Both projects aimed at investigating biochemical, kinetic, spectroscopic and structural aspects of those proteins and, in particular, of their copper centres. Various techniques of molecular biology, biochemistry and spectroscopy were employed at both sites to achieve these goals. Thus, the two projects are linked both in terms of their aims and of the methodologies employed to tackle them.

This thesis is structured in the following way: Chapter I provides a general overview both of denitrification and of copper centres in biological systems, highlighting those aspects that are more relevant for the work undertaken in the two projects. In Chapter II, the materials and methodologies used are described, emphasising when and why they were employed. Chapters III and IV constitute the bulk of this thesis. They deal with the studies performed on nitrite reductase and with those carried out with regard to nitrous oxide reductase, respectively. Each one of these two chapters comprises an Introduction section, intended to provide all the relevant information on the system under study, a Results section, where the results obtained are presented and briefly explained and a Discussion section, where those results are discussed as a whole and in relation to the published literature. Finally, the bibliographic material cited throughout the thesis is listed in Chapter V.
CHAPTER I

GENERAL INTRODUCTION
I.1. DENITRIFICATION

I.1.1. The nitrogen cycle

In its most inert form, nitrogen makes up most of the Earth’s atmosphere. Nitrogen is an essential component of many of the compounds which are necessary for life, such as amino acids and DNA. However, only a few, highly specialised microorganisms are able to extract nitrogen from the air (nitrogen fixation) and turn it into biologically available compounds. Conversely, nitrogen is lost to the atmosphere by the activities of bacteria that reduce oxidised nitrogen compounds to nitrogen gas and various other volatile compounds, while using them as electron acceptors (denitrification). Between these two extremes, a host of reactions take place including the oxidation of ammonia, produced by nitrogen fixation, to nitrite and nitrate (nitrification), the reduction of these oxidised nitrogen compounds to ammonia (dissimilatory nitrate reduction) and the incorporation of the various compounds in organic molecules (assimilation / biosynthesis) (Kuenen and Robertson, 1988). This is referred to as the nitrogen cycle, which is depicted in Fig. I.1. The nitrogen cycle includes a rich variety of oxidation and reduction reactions. These can be divided into two categories: dissimilatory reactions, that are found principally amongst prokaryotes and assimilatory reactions, that occur both in prokaryotes and in eukaryotes (Ferguson, 1988). The vast majority of the enzymes involved in the nitrogen cycle contain metal cofactors which include Cu, Fe and Mo.

I.1.2. Nitrogen fixation and nitrification

Biological nitrogen fixation is the conversion of N\textsubscript{2} to a form utilisable by plants. This process is catalysed by nitrogenase, a nitrogen-fixing enzyme found in several groups of bacteria (Eady et al., 1988; Eady, 1992). Nitrogenase comprises two proteins. One, a homodimer called the Fe protein, has one Fe-S cluster and is regarded as the transmitter of electrons from a donor protein to the second protein. This is the MoFe protein, which contains the site for binding and reduction of nitrogen, and has two novel Fe-S clusters. One is the P cluster whilst the other also contains molybdenum (the so-called “conventional nitrogenases”), or, alternatively, V or additional Fe (the so called “alternative nitrogenases”). A review on the various forms of this enzyme can be found in Ferguson (1998).
Chapter I. General Introduction

GASEOUS NITROGEN (N₂)

NITROGEN FIXATION
nitrogen-fixing bacteria convert N₂ to NH₃
Clostridium sp., Rhizobium sp.

ASSIMILATION / BIOSYNTHESIS
Organic nitrogen formed in plants, then animals

EXCRETION (e.g. urea)

DECOMPOSITION (bacteria, fungi)

AMMONIFICATION
cconversion of nitrogenous residues to NH₃ by bacteria and fungi;
this dissolves to form ammonium (NH₄⁺)

NITROGEN FIXATION
nitrogen-fixing bacteria convert N₂ to NH₃
Clostridium sp., Rhizobium sp.

DENITRIFICATION
denitrifying bacteria convert NO₃⁻ → NO₂⁻ → NO → N₂O → N₂

Nitrogen loss via leaching

Nitrates in soil

Soil

Atmosphere

Figure I.1 – The Nitrogen Cycle. Atmospheric nitrogen gas (N₂) can be reduced to ammonia (NH₃) either by chemical processes or by bacteria that possess the nitrogenase system. Ammonia can be incorporated into biological material but, especially in soils, it is sequentially oxidised to nitrite (NO₂⁻) and nitrate (NO₃⁻). Nitrate in the soil can be used as the source of nitrogen for plant cell growth. Decaying biological material can return ammonia into the cycle. Nitrate can be sequentially reduced via nitrite, nitric oxide (NO) and nitrous oxide (N₂O) to nitrogen in an overall process known as denitrification.
Nitrification may be defined as the oxidation of reduced nitrogen compounds. This can be carried out by autotrophic bacteria, in which ammonia serves as an energy source for chemolithotrophic growth. Heterotrophic nitrification is carried out by a heterogeneous group of prokaryotes and eukaryotes. The apparent rates of nitrification by heterotrophs appear to be low compared with those of autotrophs. However, some of the heterotrophic nitrifiers denitrify simultaneously, and therefore accumulate little or no nitrite (Kuenen and Robertson, 1988; Richardson et al., 1998).

The key enzymes involved in nitrification are ammonia monooxygenase and hydroxylamine oxidoreductase. Ammonia monooxygenase has been notoriously difficult to study owing to a lability during purification, but the protein has been shown to resemble a membrane-bound methane monooxygenase that is believed to contain Cu and possibly also Fe. Hydroxylamine oxidoreductase is a multiheme periplasmic protein. The molecular properties of both these proteins are reviewed in Ferguson (1998) and Richardson et al. (1998).

### I.1.3. Three pathways of nitrate reduction

As pointed out by Cole (1988), nitrate is both a strong oxidising agent and a potential source of nitrogen for growth. Consequently, different groups of bacteria exploit it in different ways (Fig. I.2). The *dissimilatory reduction* of nitrate leads to the formation of nitrite, in a process also known as respiratory nitrate reduction or nitrate respiration. The dissimilatory process can proceed from nitrite either by reduction to ammonia for purposes other than N autotrophy (*ammonification*) or by transformation to an oxyanionic gas species concomitant with energy conservation (*denitrification*). The inverse process to denitrification is the *assimilatory reduction* of those same oxyanions to ammonia for biosynthetic purposes (*assimilation*). The ammonifying pathway is mostly not electrogenic. It is used by prokaryotes mostly for nitrite detoxification and serves as an electron sink. On the other hand, the denitrifying pathway is used to generate energy and may equally serve as an electron sink. Finally, the assimilatory pathway is used by the cell for the biosynthesis of nitrogen-containing compounds (Zumft, 1997). Whilst dissimilatory nitrate reduction is a rapid process which can result in a correspondingly rapid accumulation of nitrite, nitrate assimilation is a tightly regulated process which normally proceeds slowly at the rate that ammonia is required for growth (Cole, 1988).
Chapter I. General Introduction

Figure I.2 – Dissimilatory and assimilatory branches of nitrate reduction in the prokaryotic nitrogen cycle. Adapted from Zumft (1997).

I.1.4. The denitrification pathway

As became clear throughout sections I.1.1 and I.1.3, the denitrification pathway (Fig. I.3) constitutes one of the main branches of the global nitrogen cycle. It is the sole process by which large quantities of gaseous nitrogen are returned to the atmosphere (see Fig. I.1). Denitrification may be defined as the reduction of nitrate via nitrite, nitric oxide and nitrous oxide to nitrogen gas by bacteria using the pathway as a respiratory alternative to oxygen (Kuenen and Robertson, 1988). Indeed, denitrification is used by the bacterial cell to obtain energy under anaerobic conditions, in a process where these N oxyanions and gaseous N oxides serve instead of dioxygen as terminal acceptors for electron transport phosphorylation (Zumft, 1997).
Chapter I. General Introduction

Each of the reduction steps that make up a complete denitrification pathway is catalysed by a different enzyme. Bacterial reduction of nitrate to nitrite can be carried out by three different types of nitrate reductase. One of these, often referred to as Nas, is a soluble cytoplasmic enzyme that has a typical assimilatory function, allowing the utilisation of nitrate as a nitrogen source (Moreno-Vivián and Ferguson, 1998). The other two are associated with the cytoplasmic membrane electron transport systems of bacteria (Berks et al., 1995a). One is a membrane-bound complex, often known as Nar, that contains Fe-S centres (reviewed in Zumft, 1997 and Ferguson, 1998). The other, known as Nap, is a multimeric periplasmic enzyme that contains both a Fe-S centre and Mo in the active site (Berks et al., 1995b; Reyes et al., 1996).

The reduction of nitrite to nitric oxide is carried out by the dissimilatory nitrite reductase, NiR. This is regarded as the key enzyme in denitrification in that it catalyses the first committed step of this pathway (see Fig. I.2). Two types of NiR are known; those containing Cu as cofactor and those containing heme. These two types of NiR are described in section III.1.1 and special emphasis is given to the former throughout sections III.1.2 to III.1.8.

For several years, there has been some controversy regarding whether or not NO is as an obligatory intermediate in denitrification (see, for example, Zumft et al., 1988). Evidence that this is indeed the case is now convincing, and has been reviewed by Averill (1996) and Zumft (1997). The enzyme that reduces NO, bacterial nitric oxide reductase (NoR), is a heterodimeric membrane-bound enzyme. The protein contains heme and, possibly, non-heme Fe cofactors and its activity has been

Figure I.3 – The denitrification pathway, including the enzymes that catalyse each reaction and their metal cofactors.
Chapter I. General Introduction

suggested to be strongly coupled to that of NiR. Recent reviews on the properties of this enzyme can be found in Averill (1996), Zumft (1997) and Ferguson (1998).

The last step of a complete denitrification pathway is the reduction of nitrous oxide to nitrogen by the periplasmic enzyme nitrous oxide reductase (N_{2}OR). This is a Cu-containing protein and its properties are discussed in detail throughout sections IV.1.1 to IV.1.4.

The organisation of denitrification components in a gram-negative bacterial cell is shown in Fig. I.4. As discussed earlier in this section, the membrane-bound enzymes of the pathway are the respiratory Nar and NoR. NiR and N_{2}OR are periplasmic enzymes, together with the soluble variant of nitrate reductase, Nap. Although both Nap and Nar enzymes may exist in the same organism Nar is the one that is produced under denitrifying conditions. As is clear from Fig. I.4, the oxyanionic and gaseous intermediates of denitrification may enter or leave the bacterial cell at any stage of the pathway.

Nitrite is chemically reactive and therefore potentially toxic to the bacterium (Cole, 1988). Thus, when nitrate reduction is carried out by the membrane-bound Nar, the resulting nitrite is readily exported into the periplasmic space where the subsequent reactions of the pathway take place. Nitrate and nitrite flow to and from the cytoplasmic compartment, respectively, has been postulated to occur via a membranous nitrate/nitrite antiporter, which is also depicted in Fig. I.4 (see Zumft, 1997).

I.1.5. Ecological aspects of denitrification

The nitrogen cycle, like the other element cycles, is normally in balance. However, the cycle can be deliberately or inadvertently boosted by agricultural or industrial activities or by natural causes. This results in the undesirable accumulation of intermediates of the cycle, many of which are toxic. When the nitrogen cycle becomes unbalanced, it almost always results in ecological problems (Kuenen and Robertson, 1988). As will be discussed below, the intermediates of the denitrification pathway play a key role in the delicate ecological balance of soil, water and atmospheric environments.

When it was discovered in the last century, denitrification was considered a threat to soil fertility (Kuenen and Robertson, 1988). However, it can be considered to
be beneficial since nitrate, irrespective of its role as an essential plant nutrient, has become a pollutant both of groundwater and of surface water, due to its extensive use in agricultural fertilisers (reviewed in Conrad, 1996). On the other hand, the knowledge of the process of dissimilatory nitrate reduction has allowed methods to be developed that use immobilised enzymes to reduce the levels of nitrate and nitrite in water (Mellor et al., 1992).

$N_2O$ is the fourth most important contributor to the so-called ‘greenhouse effect’, which is responsible for the phenomenon of global warming (Dickinson and Cicerone, 1986; Leggett, 1990). Together with NO, $N_2O$ is also implicated in the depletion of the ozone layer (Rasmussen and Khalil, 1986). Fertiliser denitrification (see above) and treatment plants for nitrogen removal from wastewater (Otte et al., 1996) are among the causes for the increase in the atmospheric levels of these gases (see also Conrad, 1996).

It seems clear that the study of denitrification is of great ecological and economical importance. A detailed knowledge of the molecular species that actively participate in this pathway may help understand and control the detrimental effects of widespread human activities.
Chapter I. General Introduction

1.2. COPPER CENTRES IN BIOLOGY

1.2.1. Copper in biological systems

Copper is among the 25 most abundant elements in the Earth’s crust. Its presence in plants and animals was noted in the first half of the 19th century but only relatively recently has Cu been shown to be required for the normal growth and development of living organisms. It is now a well established fact that trace amounts of Cu are present in every life form on Earth (Conry and Karlin, 1994). In humans, it is the third most abundant trace element, after Fe and Zn (see Adman, 1991).

The bioinorganic chemistry of Cu has developed rapidly over the last two decades, mainly due to the body of information obtained from trace element analysis, protein crystallography, spectroscopy and molecular biology. In addition, advances in bioinorganic chemistry have stimulated a highly successful interaction between the chemistry of model complexes and metalloprotein biochemistry (Kaim and Rall, 1996).

Several classes of Cu-containing metalloproteins have been identified in animals, plants and microorganisms, where they have a great variety of functions. For example, Cu centres are involved in electron transport (cytochrome c oxidase, plastocyanin, azurin, stellacyanin); hemocyanin, a Cu-containing protein, acts as oxygen carrier in arthropods and molluscs, replacing haemoglobin, myoglobin and hemerythrin (iron-containing proteins) present in other species; several Cu proteins can act as oxidases, for example in the reduction of O₂ to H₂O (laccase, ascorbate oxidase, cytochrome c oxidase), in the reduction of O₂ to H₂O₂ (amine oxidase, galactose oxidase) or acting as monooxygenases (tyrosinase, methane monooxygenase, dopamine β-hydroxylase); finally, there are several Cu-containing reductases, such as nitrite reductase and nitrous oxide reductase (key enzymes in bacterial denitrification) or superoxide dismutase (a protein that contains both Cu and zinc) (Adman, 1991; Conry and Karlin, 1994; Kaim and Rall, 1996). The wide variety of metabolic functions of Cu-containing proteins is illustrated in the representation shown in Fig. I.5.
Figure I.5 - Essential metabolic functions of Cu-containing proteins. Not included are transport, regulatory, and storage proteins for Cu. Adapted from Kaim and Rall (1996).

1.2.2. Copper centres in proteins

Initially, Cu centres in proteins were subdivided into three classes (types 1, 2 and 3) on the basis of their spectral features (Malkin and Malmström, 1970; Solomon, 1981; Adman, 1991; Solomon and Lowery, 1993). However, this division does not accommodate all the Cu centres known today and additional types of Cu centres need to be considered when looking at the diversity of Cu sites in proteins. The sections below provide a brief description of the properties of the different types of Cu sites known to date. In addition to these a novel type of tetranuclear cluster has recently been identified in nitrous oxide reductase and this is discussed in section IV.2.2.2.

1.2.2.1. The type 1 Cu sites

Type 1 Cu sites are mononuclear Cu centres. In the oxidised state, they are characterised by an intense absorption (ε~3000-5000 M⁻¹cm⁻¹) at around 600 nm. This absorption is responsible for the intense blue colour of proteins containing (at least)
one of these centres, which are often referred to as “blue copper proteins” (Adman, 1991; Sykes, 1991; Solomon and Lowery, 1993; Solomon et al., 1998). Type 1 Cu centres exhibit an unusually small parallel hyperfine splitting and a low g factor (Gray and Solomon, 1981; Solomon and Lowery, 1993; Kaim and Rall, 1996; Solomon et al., 1998). The EPR spectra of these centres can be predominantly axial, with \( g_\perp \approx 2.05 \), or predominantly rhombic, with \( g_x \approx 2.02 \) and \( g_y \approx 2.08 \) (Sanders-Loehr, 1993). Type 1 Cu centres have relatively high redox potentials [generally in the range of +184 to +680 mV vs +115 mV for \( \text{Cu}^{2+}(\text{aq}) \)] and exhibit high rates of long-range, outer-sphere electron transfer (Solomon and Lowery, 1993).

Type 1 Cu centres may exist in a protein in conjunction with other types of Cu sites (for example, ascorbate oxidase, laccase and nitrite reductase) or be the sole metal component of a range of so-called small blue Cu proteins including plastocyanins, azurins and pseudoazurins. These are relatively simple proteins which are invariably involved in electron transfer. They have been collectively termed cupredoxins, to emphasise their common origin and their role as electron mediators, in analogy to the Fe-containing ferredoxins (see Adman, 1991).

Cupredoxins fold into a single domain consisting mainly of a \( \beta \)-sandwich or \( \beta \)-barrel. This \( \beta \)-sandwich may comprise 6 to 13 \( \beta \)-strands in an antiparallel arrangement showing the Greek-key motif. This structural feature, known as the cupredoxin fold (Fig. I.6), is common to all the type 1 Cu-containing proteins (Messerschmidt, 1998) and is also seen in proteins containing a binuclear CuA site (see sections IV.1.4.1.3 and IV.2.2.3).

The type 1 Cu centre has four protein side-chain ligands and in some cases (for example, azurin) a weak main-chain carbonyl oxygen as a fifth ligand. The four canonical type 1 Cu ligands are His, Cys, His, Met arranged in this sequence on the polypeptide chain. The Met ligand may be replaced by a Gln in the subgroup of phytocyananinins (Messerschmidt, 1998). The most abundant geometry of type 1 Cu sites is between trigonal bipyramidal and distorted tetrahedral depending on the presence or absence of the fifth ligand (Sanders-Loehr, 1993; Messerschmidt, 1998).

Further descriptions of the functional, spectroscopic and structural features of the type 1 Cu site are provided in sections III.1.5.1 and III.1.6.2. For recent reviews on these properties see, for example, Messerschmidt (1998) and Solomon et al. (1998).
Figure IV.6 – The cupredoxin fold illustrated by a schematic diagram of the folding of azurin from *A. denitrificans*. The β-sandwich consists of eight β-strands, represented as arrows. The picture was taken from Baker (1988).

1.2.2.2. The type 2 Cu sites

Type 2 Cu sites are mononuclear Cu centres that do not exhibit the unique spectroscopic and magnetic characteristics presented by the other types of Cu centres. In fact, the basis for classifying a Cu centre as belonging to type 2 is that the EPR spectrum of its oxidised form is similar to the one of “normal” tetragonal Cu(II) complexes, with $g_\parallel > g_\perp > 2$ and $A_\parallel \geq 120$ G (Dooley, 1994). The “normal” Cu proteins exhibit absorption spectra in the visible region that reveal weak ligand-field transitions (Solomon and Lowery, 1993). Examples of proteins containing type 2 Cu centres are Cu, Zn superoxide dismutase, dopamine β-monooxygenase and nitrite reductase.

The basic spectroscopic properties of these centres indicate that they contain isolated Cu(II) S=1/2 ions in a tetragonal geometry (Dooley, 1994). However, the determination of the structures of ascorbate oxidase (see Messerschmidt, 1998 and references therein) and nitrite reductase (see section III.1.6 and references therein) has shown that the geometry of the type 2 Cu site is better described as either square-planar or tetrahedral. The Cu atom is ligated by three N(His) atoms with one or two
additional exogenous ligands. It is usually involved in catalysis and redox reactivity (Kaim and Rall, 1996).

Further descriptions of the functional, spectroscopic and structural features of the type 2 Cu site are presented in sections III.1.5.2 and III.1.6.3. The properties of the type 2 Cu sites were reviewed, for example, in Adman (1991) and Kaim and Rall (1996).

I.2.2.3. The type 3 Cu sites

Type 3 Cu sites are binuclear Cu centres, formed by a pair of coupled Cu atoms. The absence of an EPR signal in these centres results from the strong antiferromagnetic coupling (spin-pairing) between the electrons in each Cu, which is due to the presence of a bridging ligand (Solomon, 1981; Solomon and Lowery, 1993; Solomon et al., 1993). This type of centre shows unique spectroscopic features, which arise from its binuclear nature. Several derivatives of the binuclear Cu centres of tyrosinase and hemocyanin have been produced and characterised by EPR spectroscopy which has allowed the difficulties that arise from the fact that this is an EPR-silent centre in its native state to be overcome (Himmelwright et al., 1980; Solomon, 1981).

Binuclear Cu proteins include tyrosinase and hemocyanin. Both these proteins reversibly bind dioxygen, and in the case of tyrosinase activate it for hydroxylation of phenol to ortHO-diphenol and further oxidise this to ortHO-quinone (Solomon et al., 1993).

The type 3 binuclear Cu centres have a trigonal planar geometry with each Cu ligated by three N(His) atoms (Gaykema et al., 1984; Kaim and Rall, 1996). Their spectroscopic properties are reviewed, for example, in Solomon (1981), Adman (1991), Solomon et al. (1993) and Kaim and Rall (1996).

I.2.2.4. The Cuₐ sites

The Cuₐ sites are binuclear Cu centres. In the oxidised state they exhibit a seven-line pattern in their EPR spectra and an absorption at around 540 nm, which is why they are often referred to as “purple centres” (Kelly et al., 1993; Messerschmidt, 1998).
This site was initially described as a mononuclear Cu site with “unusual and enigmatic spectroscopic signatures” (Li et al., 1989; Chan and Li, 1990). However, multifrequency EPR spectroscopy, g-factor analysis and computer simulation of the EPR spectra (Kroneck et al., 1990; Kelly et al., 1993; Zumft and Kroneck, 1996) were used to establish the presence of a binuclear, mixed-valence $S = \frac{1}{2}$ site, $[\text{Cu}^{1.5}...\text{Cu}^{1.5}]$ for CuA as opposed to the “classical” mononuclear site found in type-1 Cu proteins.

So far, the CuA sites have been identified in the enzymes cytochrome $c$ oxidase and nitrous oxide reductase. In both cases, the CuA centre plays an electron transfer role, accepting electrons from the enzyme’s physiological donor and transferring them to the catalytic site (Messerschmidt, 1998). See also section IV.1.4.1).

Each of the two Cu atoms on the CuA site is ligated by two S(Cys), two N(His) and one S(Met) atoms and a peptide carbonyl of a Glu residue. The ligands for each Cu atom form a distorted tetrahedron and the two Cys thiolates bridge the Cu atoms (Iwata et al., 1995; Zumft and Kroneck, 1996; Messerschmidt et al., 1998).

The functional, spectroscopic and structural features of the CuA site are discussed in great detail in sections IV.1.4.1 and IV.2.2.3. For recent reviews on the properties of this centre see Kaim and Rall (1996), Beinert (1997), Zumft (1997) and Messerschmidt (1998).

### 1.2.2.5. The multi-copper oxidases: Trinuclear Cu sites

Blue multi-copper oxidases contain, in addition to a type 1 Cu centre, a trinuclear Cu site. On the basis of their spectroscopic properties, the three Cu atoms in this site were initially described as one type 2 Cu centre and one type 3 Cu centre (see Kaim and Rall, 1996). This notion was somewhat changed by the determination of the crystal structure of ascorbate oxidase which indicated that the three non-type 1 Cu atoms in the protein behave as a trinuclear centre (Messerschmidt et al., 1993).

Multi-copper oxidases include ascorbate oxidase, laccase and ceruloplasmin. These enzymes catalyse the four electron reduction of dioxygen to water with concomitant one-electron oxidation of the reducing substrate. The trinuclear Cu centre is the catalytic site of these enzymes (Kaim and Rall, 1996; Messerschmidt, 1998).

The trinuclear Cu cluster has eight His ligands symmetrically provided by the N- and C- terminal domains of the protein. The putative spectroscopic type 3 Cu
atoms are coordinated by N(His) atoms that exhibit a trigonal prismatic arrangement. The remaining Cu atom has two ligands and is the putative spectroscopic type 2 Cu. In addition, two oxygens are bound to the trinuclear species; as OH- or O\(^{2-}\), bridging the putative type 3 Cu pair, and as OH- or H\(_2\)O to the putative type 2 Cu atom. The Cu atoms of the pair are both tetrahedrally coordinated, whereas the spectroscopic type 2 Cu atom exhibits a square-planar coordination (Messerschmidt et al., 1993; Messerschmidt, 1998).

For reviews on the Cu centres in multi-copper oxidases see, for example, Solomon et al. (1993), Solomon and Lowery (1993) Kaim and Rall (1996) and Messerschmidt (1998).

Cu atoms in proteins are found in a wide range of conformations and can assume very diverse biological functions. The properties of these Cu sites provide extremely interesting challenges in terms of their spectroscopic and structural properties. A detailed knowledge of these properties is essential if we are to understand the role of the Cu atoms in biological Cu centres and, ultimately, fully comprehend the functions and mechanisms of the proteins that contain them.
CHAPTER II

MATERIALS AND METHODS
Chapter II. Materials and Methods

During the dual-site work presented in this thesis, a range of materials and techniques were used. Some of these were common to both projects and others were specific to a particular one. Therefore, throughout this chapter, the materials and methods presented will, where relevant, be accompanied by a note relating them to the project where they were applied. Typically, work done on nitrite reductase (NiR) at the Departments of Molecular Microbiology and Biological Chemistry (formerly known as Nitrogen Fixation Laboratory) of the John Innes Centre will be denoted by “NiR” or “NFL-JIC”. Similarly, work done on nitrous oxide reductase (N₂OR) at the Faculty of Sciences and Technology of the New University of Lisbon will be denoted by “N₂OR” or “FCT-UNL”.

II.1. MATERIALS

II.1.1. Denitrifying bacteria

*Alcaligenes xylosoxidans* cells used in the work at NFL-JIC were grown in the Department of Biological Chemistry of the John Innes Centre, as described in Abraham *et al.* (1993).

Cells of *Pseudomonas nautica* strain 617 used in the work at FCT-UNL were grown by Dr. René Toci at the C.N.R.S., Marseille, France. The reclassification of the type strain of *Pseudomonas nautica* as *Marinobacter hydrocarbonoclasticus* has been proposed (Spröer *et al.*, 1998). However, since strain 617 awaits a formal classification it will be referred to throughout this thesis as *Ps. nautica* 617. *Ps. nautica* 617 (Pasteur Institute Collection, ref. *Ps. nautica* No. 617 / 1.85) was grown under denitrifying conditions in artificial seawater at 30 °C with 10 mM nitrate as electron acceptor (Baumann and Baumann, 1986). Yeast extract (0.1% w/v) and lactate (1% w/v) were used as carbon and energy sources. The medium was supplemented with a separately sterilised Starkey oligoelement solution (Starkey, 1938) at 0.2 ml / litre of culture. Cells were harvested in the late exponential phase of growth by centrifugation. Cells were disrupted in a French press after resuspension in 100 mM Tris-HCl, pH 7.0.
II.1.2. *Escherichia coli* strains

The *Escherichia* (*E.*) *coli* strains used in the molecular biology studies carried out during the work presented in this thesis were:

JM109 - $\text{F}^+ \text{traD36 lacI^q } \Delta(lacZ)M15 \text{ proA}^+\text{B}^+\text{e14}^-\text{McrA}^-\Delta(\text{lac-proAB}) \text{ thi} \text{ gyrA}96(\text{Nal}^r) \text{ endA1} \text{ hsdR17} \text{ (rK}^-\text{mK}^+\text{)} \text{ relA1 supE44 recA1} \text{ (Yanisch-Perron et al., 1985).}$

BL21(DE3) - $\text{F}^- \text{ompT gal [dcm] [lon] hsdS_B (r_B^-m_B^-); an E. coli B strain} \text{ with DE3, a } \lambda \text{ prophage carrying the T7 RNA polymerase gene} \text{ (Studier and Moffatt, 1986; Studier et al., 1990).}$

Epicurian coli XL1-Blue (Stratagene)

II.1.3. Plasmids

The plasmids used during the work presented in this thesis were as follows:

- pUC18 (ampicillin) Yanisch–Perron et al., 1985
- pUC19 (ampicillin) Yanisch–Perron et al., 1985
- pBR322 (ampicillin) Bolivar et al., 1977
- pET28a (kanamycin) Novagen
- pGEM-Teasy (ampicillin) Promega

Antibiotics were used at a final concentration of 50 – 100 µg/ml.

II.1.4. *E. coli* growth media

The following liquid media were used for growing *E. coli* cultures. In all cases, small-scale (up to two-litre) growths of bacteria were performed aerobically at 30 °C or 37 °C in a shaking incubator (250 rpm). Large-scale (20- or 200-litre) growths of bacteria were performed by Mr. Mike Chan in New Brunswick fermentors under the control of Bio-Comand software, with 20% air saturation. Media were supplemented
with the appropriate antibiotic solution. Antibiotics were purchased from Sigma and stock solutions were made up in water and filter-sterilised through sterile 0.2 µm syringe filters (Sartorius).

Luria Broth (LB) - 1% (w/v) Bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl.
YT - 0.8% (w/v) Bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, pH 7.0.
M9 – 0.6% (w/v) NaH2PO4, 0.3% (w/v) KH2PO4, 0.05% (w/v) NaCl, 0.1% (w/v) NH4Cl (pH 7.4), 2 mM MgSO4, 0.2% (w/v) glucose, 0.1 mM CaCl2.

Bacteria were also grown at 30 °C or 37 °C as colonies on LB-agar plates [as for LB but including 1.5% (w/v) Bactoagar (Difco)] supplemented with the appropriate antibiotic solution. For “blue/white” screening of colonies carrying the appropriate plasmids, LB-agar was further supplemented with 20 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For plasmid amplification during colony hybridisation, LB-agar was supplemented with 150 µg/ml chloramphenicol (Sigma. Stock solution -10 mg/ml in ethanol).

II.2. METHODS

II.2.1. Molecular biology methods

The work presented in this thesis included the cloning, overexpression and site-directed mutagenesis of NiR, as well as the determination of the sequence of the gene encoding N2OR. All the methods presented in this section were used in the study of the NiR system. Those required for the determination of the sequence of the gene encoding N2OR were also used in this part of the work. In general, methods described in this section are described in Ausubel et al. (1987). Where that is not the case, the appropriate reference is given. Oligonucleotides were purchased from MWG Biotech (JIC-NFL) and Pharmacia (FCT-UNL). Except where noted, all centrifugation steps were performed at approximately 18000 g at room temperature.
II.2.1.1. Bacterial DNA extraction

In the extraction of chromosomal DNA from *Alcaligenes xylosoxidans* (NFL-JIC) and *Pseudomonas nautica* (FCT-UNL) a cetyl trimethyl ammonium bromide (CTAB)-extraction method was used. This method is based on the lysis of the bacterial cells, followed by the removal of proteins by digestion with proteinase K. Cell wall debris, polysaccharides and residual proteins and peptides were removed by selective precipitation with CTAB and high molecular weight DNA was recovered from the resulting supernatant by isopropanol precipitation. The method used was exactly as described by Ausubel *et al.* (1987), except that lysozyme was added after resuspension of the cell pellet to facilitate lysis of the bacterial cell wall.

II.2.1.2. Phenol/Chloroform extraction of DNA

This procedure was used to remove proteins from DNA preparations, after bacterial DNA extraction or treatment with restriction or modifying enzymes. One volume of a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol equilibrated with TE (10 mM Tris, 1 mM EDTA) pH 8.0 was added to the DNA solution. The aqueous and organic phases were mixed by vortexing after which the samples were centrifuged for 5 min at room temperature and the upper (aqueous) phase, containing the DNA, removed without disturbing the interphase. If necessary, this procedure was repeated before a final extraction with 1 volume of 24:1 chloroform:isoamyl alcohol to remove traces of phenol.

II.2.1.3. Ethanol precipitation of DNA

DNA samples were concentrated by precipitation using ethanol, high salt and low temperature conditions. LiCl at 0.4 M final concentration and 3 volumes of ice-cold ethanol were added to the DNA solution and this was left to precipitate at –20 ºC for at least 30 min. DNA fragments under 500 bp were precipitated for 1 h to overnight at the same temperature. The sample was then centrifuged at 4 ºC for 15 min, the supernatant discarded and the pellet washed with ice-cold 80% (v/v) ethanol to remove traces of salt. Following centrifugation at 4 ºC for 5 min, the supernatant
was discarded and the pellet dried in a Speed-Vac for approximately 10 min. The dry pellet was then resuspended in the appropriate volume of water or TE buffer.

II.2.1.4. Transformation of *E. coli*

*E. coli* cells were made competent and transformed using one of three alternative methods, depending on the transformation efficiency required.

II.2.1.4.1. TSS transformation

For each transformation, 1.5 ml of an exponentially growing culture of the appropriate *E. coli* strain were centrifuged for 5 min at room temperature. The cell pellet was incubated on ice for 5 min and the plasmid DNA added to it and incubated on ice for a further 30 min. The cells were then resuspended in cold TSS solution (filter-sterilised solution containing 10% (w/v) PEG 6000, 5% (v/v) DMSO, 50 mM MgSO₄, 1% (w/v) peptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl at pH 6.5) and incubated on ice for 40 min. LB (900 µl) was then added to the mixture and this was incubated for 1 h at 37 °C, to allow for phenotypic expression of the plasmid gene(s) conferring resistance to the specific antibiotic(s) used. Typically, 50 – 200 µl of the cell suspension were plated on selective LB-agar plates containing the appropriate antibiotic(s).

II.2.1.4.2. CaCl₂ transformation

A 5 ml aliquot of an exponentially growing culture of the appropriate *E. coli* strain was centrifuged for 5 min at room temperature and the cell pellet resuspended in 0.5 – 1.0 ml ice-cold 50 mM CaCl₂. Cells were then transferred into Eppendorf tubes (100 – 200 µl per tube, one tube per transformation) and the appropriate volume of plasmid DNA was added and mixed by inversion. The mixture was incubated on ice for 20 min after which the tubes were frozen by immersing them in liquid nitrogen for 1 – 2 min. The tubes were then thawed at 37 °C and the cells were transferred to 5 ml Universal bottles and incubated with shaking at 37 °C for 2 – 4 h to allow for phenotypic expression of the plasmid gene(s) conferring resistance to the antibiotic(s)
used. In those cases where a high transformation efficiency was expected, 100 – 200 µl of the cell suspension were plated on selective LB-agar plates containing the appropriate antibiotic. When low transformation efficiency was expected, cells were centrifuged, resuspended in 0.5 ml LB and 100 µl were plated in selective LB-agar plates.

II.2.1.4.3. Electroporation

A 100 ml aliquot of an exponentially growing culture of the appropriate strain of *E. coli* was centrifuged for 5 min at room temperature and the cell pellet resuspended in 100 ml of ice-cold sterile, distilled water. Cells were then re-centrifuged and resuspended in 50 ml ice-cold sterile distilled water. After a third centrifugation, the cell pellet was resuspended in 2 ml of a 10% (v/v) ice-cold sterile glycerol solution and the cells were aliquoted into ice-cold Eppendorf tubes (1 ml per tube). Cells were harvested by centrifugation and the pellets resuspended in 200-300 µl 10% (v/v) ice-cold sterile glycerol solution (100 – 150 µl per tube). The cell suspension was distributed in 65 µl aliquots, ‘flash’-frozen in liquid nitrogen and stored at –70 ºC. One aliquot was used per transformation. Cells were thawed at room temperature and mixed with the appropriate volume of desalted plasmid DNA solution. The mixture was placed in electroporation cuvettes and electroporated at 1500 V for 5 ms. The electroporated sample was recovered by flushing the cuvette with 1 ml of 2 x YT, which was then transferred into 4 ml of 2 x YT in a conical flask and the culture was shaken at 37 ºC for at least 2 h to allow for phenotypic expression of the plasmid gene(s) conferring resistance to the antibiotic(s) used. Cells were centrifuged, resuspended in 1 ml of 2 x YT and 100 - 200 µl were plated onto selective LB-agar plates containing the appropriate antibiotic.

II.2.1.5. Agarose gel electrophoresis

Separation of DNA fragments according to size was performed by electrophoresis through horizontal submerged agarose gels. This was done routinely as a confirmatory technique during the cloning of the genes encoding NiR and N₂OR, to separate restriction-digested bacterial chromosomal DNA and to visualise the
results of PCR reactions. The gels used were typically 0.8 – 1 % (w/v) in agarose, depending on the size of the fragments to be separated. All gels were made and run in 1 x TBE buffer (10 x TBE: 0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA, pH 8.0) and were electrophoresed at 60 – 120 V for 1 – 4 h. A 1 kb ladder was used as a size marker (Gibco BRL Life Technologies at NFL-JIC; Promega at FCT-UNL) and was electrophoresed in parallel with the samples. To visualise the DNA, 0.1 µg/ml ethidium bromide was added to the cooled molten agarose before casting the gel. Gels were examined on a UV transilluminator.

II.2.1.6. Restriction digestions and ligations

All restriction digestions were carried out for 1 – 4 h at the recommended optimum temperature (usually 37 °C) for the restriction enzyme(s) used. All digestions contained the appropriate restriction enzyme buffer supplied by the manufacturers and 1 – 20 units of enzyme. For double digests, the buffer was chosen so that the highest possible efficiency was achieved for both enzymes. Plasmid digestions were typically carried out in small volumes of 20 – 30 µl. Bacterial chromosomal DNA digestions were carried out in larger volumes, typically between 300 and 400 µl, and digestion efficiency was monitored by analysing aliquots of the digestion mixture at 1 h intervals by agarose gel electrophoresis. All the restriction enzymes used were obtained from Pharmacia Ltd., Boehringer Mannheim GmbH, Gibco BRL Life Technologies, Promega or New England Biolabs.

DNA ligations were carried out either at 16 ºC for 8 – 16 h or following the quick ligation method described below. In either case, ligations were carried out in volumes of 10 – 20 µl in 1x ligation buffer supplied by the manufacturers of T4 DNA ligase (Boehringer Mannheim GmbH at NFL-JIC; Promega at FCT-UNL). Typically, 1 – 2 Weiss units of this enzyme and 1:1 or 1:3 ratios of vector:insert (with 50 – 100 ng linearised vector DNA) were used in a ligation reaction. When a quick ligation method was used, the ligation mixture was incubated on ice for 20 min, followed by 20 min sonication in a sonicating bath containing ice-cold water. The sample was then incubated for a further 20 min on ice.
II.2.1.7. DNA modification

When plasmid DNA was linearised by use of a single restriction enzyme, the 5’ phosphate group was removed from both ends of linear DNA to minimise recircularisation of the plasmid. This dephosphorylation reaction was carried out by use of alkaline phosphatase (Boehringer Mannheim GmbH at NFL-JIC; Sigma at FCT-UNL), typically in a total volume of 60 µl. The reaction proceeded for 1 h at 37 °C using 5 units alkaline phosphatase; 2.5 units were added at the start of the reaction and a further 2.5 units were added after 30 min.

When oligonucleotide primers used in PCR experiments are not phosphorylated, PCR products cannot be cloned without prior phosphorylation. This was done by use of polynucleotide kinase, typically in a total volume of 60 µl and in 1 x kinase buffer (supplied by the manufacturer of the enzyme). The reaction was carried out for 1 h at 37 °C in the presence of 1 µl of 100 mM ATP (phosphate source) and 10 units T4 polynucleotide kinase (Boehringer Mannheim GmbH at NFL-JIC; Sigma at FCT-UNL).

For the ligation of “blunt-end” phosphorylated PCR products, a fill-in reaction was carried out to ensure that the DNA fragments to be ligated did not lack any bases. This could arise from a decrease in the efficiency of the DNA polymerase used in the PCR experiment, especially in the final cycles of the reaction. The fill-in reaction was done using Klenow polymerase (Gibco BRL Life Technologies at NFL-JIC; Amersham at FCT-UNL), typically in a total volume of 75 µl and 1 x nick-translation buffer [10 x nick-translation buffer: 0.5M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 1mM dithiothreitol, 0.5 mg/ml BSA]. The reaction was carried out for 20 min at room temperature in the presence of 5 µl of 2mM dNTPs and 5 units Klenow polymerase. After each of the reactions described above the enzymes were removed prior to ligation, either by DNA precipitation with ethanol, by means of a phenol/chloroform extraction, or by purification from an agarose gel.

II.2.1.8. Polymerase chain reaction (PCR)

PCR was used to amplify genomic DNA sequences for cloning and sequencing as well as to amplify the complete nirA gene and to add appropriate
restriction sites that enabled its cloning in the appropriate overexpression vector. An inverse PCR (iPCR) approach was also used to identify a portion of the sequence of the \textit{nirA} gene and is described in detail in section III.2.1.1. Typically, in the NiR work 1 - 10 ng of template DNA were used in each reaction with 1 µl (2.5 units / µl) \textit{Pfu} polymerase (Stratagene), 0.2 mM dNTPs and 20 – 100 pmoles of each oligonucleotide in 1 x \textit{Pfu} buffer (supplied by the manufacturer of the enzyme) in a total volume of 100 µl. In the N\textsubscript{2}OR work, \textit{Taq} DNA polymerase (Amersham Pharmacia Biotech) and the PCR buffer supplied by the same manufacturer were used in a total volume of 50 µl. The rest of the conditions were the same as above. In both cases, amplification usually involved 35 cycles, each consisting of 1 min at 94 ºC (denaturation), 1.5 min at 55 – 60 ºC (annealing) and 1.5 min at 72 ºC (extension). The amplification cycles were usually preceded by 4 min at 94 ºC to denature the DNA and were followed by 5 min at 72 ºC to complete synthesis. PCR products were analysed by running 10% by volume of the reaction on an agarose gel. The most frequently used thermal cyclers were manufactured by MJ Research Inc. or Hybaid (NFL-JIC) or Biometra (FCT-UNL).

\textbf{II.2.1.9. Plasmid purification, DNA extraction from agarose gels and purification of PCR products}

Plasmid purification (minipreps), DNA extraction from agarose gels and purification of PCR products were all performed with QIAprep and QIAquick kits (Qiagen), following the instructions of the manufacturer. The DNA obtained by these methods is of high purity and can be used directly in cloning and sequencing experiments. Plasmid purification during the N\textsubscript{2}OR work was also performed with Promega’s “Wizard Plus” Minipreps DNA Purification System, following the manufacturer’s instructions.

\textbf{II.2.1.10. DNA labelling, (pre-)hybridisation and detection}

Fluorescence-labelling and detection of DNA fragments used as probes in Southern blotting and colony hybridisation experiments were performed by the enhanced chemiluminescence (ECL) random prime system (Amersham LifeScience).
This is a nucleic acid labelling, hybridisation and detection system based on a combination of enhanced chemiluminescence detection and random primer labelling of DNA. In this method, the DNA fragment is fluorescein-labelled by the technique of random priming and fluorescein residues within the labelled DNA probe are detected as haptens by an enzyme-linked anti-fluorescein antibody. All labelling, (pre-)hybridisation and detection procedures were performed following the instructions of the manufacturer of the ECL system. Radioactive labelling with $[^\alpha^\text{-}^{32}\text{P}]$-dCTP (NEN) was carried out following the Amersham LifeScience protocol. Rapid-Hyb hybridisation buffer and protocol from Amersham LifeScience were used for the hybridisation.

II.2.1.11. Southern blotting

Subsequent to electrophoresis, the agarose gel was washed for 2 x 20 min with 0.25 M HCl (depurination solution), 2 x 20 min with 0.5 M NaOH, 1.5 M NaCl (denaturation solution) and 2 x 20 min with 3 M NaCl, 0.5 M Tris-HCl, pH 7.0 (neutralisation solution) and then equilibrated for 5 min in 10 x SSC prior to transfer (20 x SSC – 3 M NaCl, 3 M sodium citrate, pH 7.0). DNA fragments separated by electrophoresis on agarose gels were transferred onto nitrocellulose (NC) membranes (Hybond C) by vacuum blotting. On a gel drier apparatus, a set-up was mounted that consisted of 3 sheets of Whatman 3MM paper soaked in 2 x SSC on top of which the NC membrane, the agarose gel and a further 3 sheets of Whatman 3MM paper soaked in 10 x SSC were placed. The set-up was covered by a piece of strong plastic sheet, and vacuum was applied for 10 min. The vacuum was then released, the plastic sheet removed and the top three sheets of paper carefully replaced by a further 3 sheets, freshly soaked in 10 x SSC. The blotting procedure was repeated for a further 10 min. Transfer was then complete and its efficiency checked by visualising the agarose gel on a UV transilluminator. Prior to pre-hybridisation, the NC membrane was baked for 2 h at 80 ºC to link the DNA covalently to the membrane.
II.2.1.12. Colony hybridisation

Colony hybridisation was used to check for colonies carrying plasmids containing DNA fragments of interest. Colonies growing on LB-agar plates containing the appropriate antibiotic (masterplates) were replica-plated onto NC discs (Millipore) by covering the plate with the disc for a few seconds and then carefully lifting it. The masterplates were then left overnight at room temperature to allow for bacteria to grow at the same spots where the colonies originally were located. The NC discs containing the colonies were placed on LB-agar plates with 150 µM chloramphenicol and left overnight at 37 ºC to allow for plasmid amplification. Following this, the discs were consecutively placed on filter paper soaked in 0.5 M NaOH (5 – 7 min) for colony lysis; 1 M Tris, pH 7.4 (2 x 1 min) for neutralisation; 1.5 M NaCl in 0.5 M Tris, pH 7.4 (1 min) to increase salt concentration and finally ethanol-soaked filter paper (2 – 3 min) to precipitate the DNA. Between each of these steps and after the last one, the filters were placed for 1 min on filter paper soaked with water to remove any excess of the previous solution. Prior to pre-hybridisation, excess water was removed and the NC discs were baked for 2 h at 80 ºC.

II.2.1.13. DNA sequencing

DNA sequencing was performed on both plasmid and PCR-amplified DNA of the genes coding for NiR and N\textsubscript{2}OR, as well as on mutated forms of the nir\textsubscript{A} gene, obtained by site-directed mutagenesis. The dideoxy sequencing method used is based on the method originally described by Sanger et al. (1977) and depends upon base-specific termination of enzyme-catalysed primer-extension reactions. For manual sequencing, the T\textsuperscript{7} sequencing Kit (Pharmacia Biotech) was used, according to the instructions of the manufacturer, with [\textalpha-\textsuperscript{35}S]dATP (NEN) as the radioactive isotope. Automatic sequencing was performed either at the Sainsbury Laboratory of the John Innes Centre (NiR) or at the Mayo Clinic Molecular Biology Facility, Mayo Clinic Foundation, Rochester, MN, USA (N\textsubscript{2}OR) and made use of the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science). In either case, double-stranded DNA templates of recombinant plasmid DNA, prepared using Qiagen or Promega kits, were used.
II.2.1.14. Autoradiography

Sequencing gels and NC membranes were exposed to Kodak X-OMAT AR films for a period ranging from 2 h to one week, depending on the intensity of the signal. Films were developed in an Agfa-Gevaert Gevamatic 60 film developer (NFL-JIC) or manually with processing chemicals from Kodak/Sigma and following the manufacturer’s protocol. Alternatively, gels and membranes were exposed to phospho-imager screens (Fuji BAS-MP) and visualised by means of a Fujix BAS 1000 phosphorimager apparatus and Fujix MacBAS 2.0 software.

II.2.1.15. Analysis of DNA and amino acid sequences

DNA sequence analysis at NFL-JIC was performed using the programs available in the Wisconsin Package: Genetics Computer Group (GCG) sequence analysis software package, version 7.2 (1992). Alignments of amino acid sequences were generated with the CLUSTAL W package (Thompson et al., 1994). DNA and amino acid sequence analysis at FCT-UNL was kindly performed by Dr. Alice Pereira, using Macaw and CLUSTAL W.

II.2.1.16. Site-directed mutagenesis

Site-directed mutagenesis of the nirA gene was performed in order to obtain mutant forms of NiR. Mutagenesis was done with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), following the instructions of the manufacturer. This method utilises a supercoiled, double-stranded (ds) DNA template with the appropriate insert and two complementary synthetic oligonucleotide primers both of which include the desired mutation. The oligonucleotide primers are extended during temperature cycling by means of PfuTurbo DNA polymerase (Stratagene). Upon incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with DpnI. This endonuclease is specific for methylated and hemimethylated DNA (DNA isolated from almost all E. coli strains is dam methylated and therefore susceptible to DpnI digestion) and is used to digest the parental DNA template and to select for synthetic DNA containing the desired mutation. The nicked vector DNA
incorporating the mutation is then transformed into Epicurian Coli XL1-Blue Supercompetent Cells (Strategene).

II.2.2. Protein methods

The main objectives of the work presented in this thesis relate to the characterisation of the Cu-containing proteins NiR and N2OR. In this section the most relevant biochemical techniques used in the study of these two proteins will be described. Except where noted, all centrifugation steps were carried out at 4 °C.

II.2.2.1. Protein determination

Protein determinations were done by the method of Lowry et al. (1951), which is based on the use of Folin-Ciocalteau’s reagent. In this method, there are two distinct steps which lead to the final colour with protein. These are (a) the reaction with copper in an alkaline solution and (b) the reduction of the phosphomolybdic-phosphotungstic Folin-Ciocalteau’s reagent by the copper-treated protein. As a result, a protein-dependent absorption in the 650-750 nm range is generated and is measured spectrophotometrically.

For pure preparations of NiR and mutant forms of NiR, the extinction coefficient of this protein at 280 nm ($\varepsilon_{280} = 1.54 \text{ mg}^{-1}\cdot\text{ml}^{-1}\cdot\text{cm}^{-1}$) (Dr. Robert Eady, personal communication) was used to estimate protein concentration.

II.2.2.2. Concentration, dialysis and reconstitution

Concentration of large volumes of protein samples (above 50 ml) was performed at 4 °C in a diaflow apparatus equipped with appropriate cut-off membranes (Amicon), usually 10 or 30 kDa, under N$_2$ pressure. For small volumes of sample, usually pure protein preparations, centricons or centripreps (Amicon) within the same range of cut-offs were used. Protein samples were dialysed in dialysis tubes (Medicell International) of 12 - 14 kDa cut-off. As a general rule, dialysis was carried out at 4 °C for at least 10 h, with 2 to 3 buffer exchanges, in minimally a 50-fold excess of buffer volume over that of the sample.
Chapter II. Materials and Methods

Reconstitution of type 2 Cu centres in NiR was performed by dialysing the periplasmic fraction or the purified protein solution in the same conditions as described above, against 100 µM CuSO₄ in 10 mM Tris-HCl, pH 7.1 followed by removal of excess Cu by dialysis against 10 mM Tris-HCl, pH 7.1.

II.2.2.3. Metal determination

Both NiR and N₂OR are copper-containing proteins. To estimate the copper content in preparations of both enzymes and mutant forms of NiR, as well as to investigate the presence of other metals (Zn in particular), inductively coupled plasma emission (ICP) was used. NiR samples were ICP-analysed at Southern Water laboratories (Sussex). In some cases, protein samples were digested by wetashing with concentrated sulphuric acid and hydrogen peroxide (as described in Abraham et al., 1993) prior to analysis. N₂OR samples were ICP-analysed at the University of Georgia, USA. Routine determinations of copper in N₂OR samples were performed by the spectrophotometric method described in Poillon and Dawson (1963). This method uses a solution of 2,2’-biquinoline (cuproine) in glacial acetic acid in combination with hydroxylamine hydrochloride to determine the amount of copper in protein samples. The cuproine reagent gives a deep purple colour (λₘᵞₓ= 540 nm) with Cu(I) in glacial acetic acid. Denaturing the enzyme by acidification (below pH 3) in the presence of NH₂OH.HCl and cuproine completely reduces the liberated copper ions. Therefore, the Cu(I) complex is determined colourimetrically by comparison with the absorbance per µg of Cu per ml obtained with a suitable aliquot of an identically treated standard Cu solution.

II.2.2.4. Enzyme activity assays

The determination of the enzymatic activities of NiR (and mutant forms of NiR) and N₂OR, as well as other putative activities that might be associated with NiR was performed both on a routine basis and in particular instances to address specific questions. The methods used for the different activity determinations will be described in the following sections.
II.2.2.4.1. Nitrite reductase

For reasons that will become apparent in the sections dealing with results and discussion of the NiR project, nitrite reductase activity was determined by three different methods, which are outlined below.

II.2.2.4.1.1. Discontinuous assay

The discontinuous assay for nitrite reductase activity (MacGregor, 1978; Abraham et al., 1993) measures the enzymatic consumption of a limiting amount of nitrite in a defined period of time at a specific temperature. The reaction was performed anaerobically in a total volume of 2 ml, in the presence of 250 mM potassium phosphate buffer, pH 7.1, and 0.1 mM sodium nitrite. Methyl viologen (MV) at a concentration of 0.5 mg/ml was used as the electron donor and the reaction was initiated by the addition of dithionite to a final concentration of 0.4 mg/ml followed by gentle mixing. As a result of the addition of dithionite, a blue colour appeared that corresponds to reduced MV. The mixture was then incubated at 25 ºC for the time required (typically 5 min) and the reaction was stopped by vortexing until the blue colour disappeared, which corresponded to the complete oxidation of the MV in solution. The amount of nitrite left in the reaction mixture was then determined in a colourimetric assay that uses sulfanilic acid [2 ml of a 1% (w/v) solution in 20% (w/v) HCl] and naphthylethylenediamine (NEDA) [2 ml of a 0.129% (w/v) solution] which react with the nitrite ion and produce a red colour. The absorbance was measured at 540 nm after 10 min. Enzymatic consumption of nitrite was determined by subtracting the amount of nitrite present in the reaction mixture from that in a control reaction mixture lacking enzyme. Using a calibration curve that relates the amount of nitrite (0 - 200 nmol) to $\text{Abs}_{540\text{ nm}}$ (usually 0 – 1.4), measured under the same conditions as described above, the total nitrite reduced was determined. One unit of enzyme activity is defined as the reduction of 1 µmol of nitrite per min at 25 ºC. The specific activity of the enzyme is expressed as units per milligram of protein.
II.2.2.4.1.2. Continuous spectrophotometric assay

The continuous spectrophotometric assay for nitrite reductase activity (Abraham et al., 1997) uses dithionite as the electron donor to enable nitrite reduction. Oxidation of dithionite was followed spectrophotometrically at 315 nm ($\varepsilon = 8000$ mM$^{-1}$cm$^{-1}$). The reaction mixture contained, in a final volume of 300 µl in a 1 cm-path-length cuvette, 100 mM potassium phosphate buffer, pH 7.1, 3 mM sodium dithionite and 1 mM sodium nitrite. The reaction was carried out under argon. Once a constant baseline had been attained, the reaction was initiated by the addition of enzyme through the rubber closure of the cuvette. Linear decomposition of dithionite in the absence of enzyme was determined in a blank assay using water instead of protein solution and this slope was subtracted from the one obtained for the protein assays. One unit of enzyme activity is defined as the oxidation of 1 µmol of dithionite per min.

II.2.2.4.1.3. Electron donation by reduced azurin

To study electron donation by azurin to NiR and mutant forms of NiR, azurin I from Alcaligenes xylosoxidans was reduced and used as the electron donor in a spectrophotometric assay. This was done according to a method previously used in the laboratory at NFL-JIC (Dr. Robert Eady and Prof. Samar Hasnain, personal communication). Reduction of azurin I ($\varepsilon_{619\text{ nm}} = 6.27$ mM$^{-1}$cm$^{-1}$) was performed in a glove box (Miller Howe Ltd. – O$_2$ at ~5 ppm). Sodium dithionite at a final concentration of 1 mM was added to a ~0.7 mM solution of azurin I in 1 ml 50 mM MES buffer, pH 6.0. Excess reductant was removed by passage of the protein through a Bio-Gel P-6 Desalting Gel (Bio-Rad) column (14 cm x 1 cm) equilibrated with degassed 100 mM Tris-HCl, pH 7.1 at a flow rate of about 0.75 ml/min. Since the reduced azurin solution is colourless, the column was previously calibrated with 1 ml of coloured protein (flavodoxin was used for this purpose), in order to determine the volume of buffer eluted prior to elution of the loaded protein, as well as its total elution volume. After reduction and removal of excess dithionite, azurin I was ~0.45 mM in a total volume of ~1.5 ml. The activity assay measured the reoxidation of reduced azurin by measuring the increase in absorption at 619 nm. The degassed
reaction mixture contained 22.5 µM reduced azurin I and 20 mM sodium nitrite in 100 mM Tris-HCl, pH 7.1. The reaction occurred under argon and was started by the addition of enzyme through the rubber closure of the cuvette. Oxidation of azurin I in the absence of enzyme was determined in a blank assay using water instead of protein solution and this slope was subtracted from the one obtained for the protein assays. One unit of enzyme activity is defined as the oxidation of 1 µmol of azurin per min.

### II.2.2.4.2. Nitrous oxide reductase

Nitrous oxide reductase activity was measured spectrophotometrically by following the oxidation of reduced methyl viologen at 600 nm (ε = 11 400 M⁻¹.cm⁻¹), as described in Kristjansson and Hollocher (1980). The degassed reaction mixture, in 3-ml cuvettes, contained 2.5 ml of 2.5 mM methyl viologen (MV) in 50 mM potassium phosphate buffer (pH 7.1), to which the protein sample was added. Dithionite was present at a final concentration of 0.06 mg/ml (Abs 600 nm = 1 – 1.2). When the baseline had stabilised (usually 1 – 2 min), the reaction was started by the addition of 50 µl of N₂O-saturated water (25 mM at 1 atm and 25 ºC). N₂OR activity is expressed as µmol N₂O reduced.min⁻¹.(mg enzyme)⁻¹.

### II.2.2.4.3. Carbonic anhydrase

As will be further discussed in this thesis, the substrate-binding site in NiR is similar to the Zn site in carbonic anhydrase (CA) (Strange et al., 1995). In order to assay NiR for CA activity, a stopped-flow spectrofluorimetric method was used, which was based on that described by Shingles and Moroney (1997). This method uses the fluorescent pH indicator, 8-hydroxy-pyrene-1,3,6-trisulfonate (pyranine) in combination with stopped-flow spectrofluorimetry to measure pH changes that occur when HCO₃⁻ is enzymatically converted to CO₂. Mixing KHCO₃ in pH 8.0 buffer with a pH 6.0 buffer causes a pH shift converting HCO₃⁻ to CO₂, a process which consumes one proton. The noncatalysed reaction proceeds slowly, eventually reaching an equilibrium determined by the final pH after mixing the two solutions. The catalysed reaction quickly reaches the same equilibrium. In this stopped-flow rapid
mixing experiment, pyranine is added as a fluorescent indicator to follow pH changes as the reaction occurs. At the pH-sensitive wavelength, pyranine fluorescence increases as the conversion of HCO$_3^-$ to CO$_2$ proceeds. This rate appears to be linear in the non-catalysed reaction but is greatly accelerated by the addition of CA and the catalysed reaction follows first-order kinetics. Fluorescence measurements were collected with a Hi-Tech Scientific spectrofluorometer equipped with a stopped-flow apparatus manufactured by the same company. Chamber A contained 5 mM KHCO$_3$ in 0.5 mM bicine-KOH, at pH 8.0. Chamber B contained the protein (or blank) solution and 100 nM pyranine in 0.5 mM Hepes-KOH at pH 6.0. Mixing of the samples was achieved by a nitrogen-driven piston at ~0.5 MPa. A cut-off filter at 455 nm was used at the entrance of the emission monochromator and excitation was set at 437 nm.

II.2.2.4.4. Superoxide dismutase

As will be further discussed in this thesis, a similarity between the catalytic sites of NiR and superoxide dismutase (SOD) has recently been reported (Strange et al., 1999). In order to assay NiR for SOD activity, a method based on the oxidation of NAD(P)H was used, as described by Paoletti and Mocali (1990). The method consists of a purely chemical reaction sequence which generates superoxide from molecular oxygen in the presence of EDTA, MnCl$_2$ and mercaptoethanol. NAD(P)H oxidation is linked to the availability of superoxide anions in the medium. As soon as SOD is added to the assay mixture, it brings about the inhibition of nucleotide oxidation. Therefore, at high concentrations of the enzyme the absorbance at 340 nm remains unchanged while in the control (no SOD added) absorbance decreases according to predictable kinetics. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NAD(P)H oxidation of the control by 50%. The reaction mixture (965 µl total volume) contained triethanolamine-diethanolamine (80 mM each)-HCl (pH 7.4), 0.3 mM NADPH, EDTA (2.5 mM), MnCl$_2$ (1.25 mM) and the protein (or protein buffer for the control). This was mixed thoroughly and Abs (340 nm) was read for a stable baseline recorded over a 5 min period. 100 µl of 10 mM mercaptoethanol was then added to the mixture and the decrease in absorbance was monitored for about 20 min. Due to a certain variability in the control measurements
II.2.2.5. Chromatographic techniques

Chromatography was used both for protein purification and for the determination or comparison of protein molecular masses. The purification of NiR and mutant forms of NiR was performed by conventional chromatography on a carboxymethyl cellulose (CMC) matrix. CM52 (Whatman) was the cation exchange resin used for this purpose.

The purification of N2OR involved different resins and was done by conventional chromatography as well as by HPLC. The resins used in the purification of N2OR were Bio-Rad’s DEAE-Biogel (anion exchange), Pharmacia’s Superdex 75 (gel filtration) and Pharmacia’s Source 15Q (anion exchange). Details on these purification procedures are given in section IV.2.1.

The molecular masses of recombinant and wild-type NiR were determined by comparing their retention times upon HPLC chromatography on a Superdex 200 HR 10/30 (Pharmacia) gel filtration column. The elution buffer used was 50 mM potassium phosphate, pH 7.0, containing 150 mM NaCl and the flow rate was 0.3 ml/min. The molecular mass of native N2OR was determined by HPLC on a calibrated Superdex 200 HR 10/30 (Pharmacia) gel filtration column. Elution buffer was 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl, at a flow rate of 0.5 ml/min. The molecular mass standards used to calibrate the column were ferritin (440 kDa, Pharmacia), catalase (232 kDa, Pharmacia), aldolase (158 kDa, Pharmacia), bovine serum albumin (67 kDa, Sigma), ovalbumin (43 kDa, Sigma), chymotrypsinogen A (25 kDa, Pharmacia) and ribonuclease A (13.7 kDa, Pharmacia).

II.2.2.6. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS-PAGE) was used routinely to check the purity of protein samples throughout the purification of NiR and N2OR, as well as to estimate the molecular mass of protein subunits and/or precursors and to check overproduction levels of recombinant NiR and NiR mutant proteins. SDS-PAGE was performed according to...
Laemmli (1970). Typically, 12.5% (w/v) or 15% (w/v) acrylamide gels were run, at 150 – 200 V for 40 – 60 min. Molecular mass markers (Pharmacia, Sigma) were separated alongside the samples for comparison and molecular mass determinations. For protein solutions (usually 5 to 50 µg depending on purity), ½ volume of 3 x sample buffer [6% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) mercaptoethanol and 0.001% (w/v) bromophenol blue in 0.2 M Tris-HCl, pH 6.8] was added to the sample followed by boiling for 2 min to denature the proteins. For whole cell extracts, OD600nm was measured and a volume of 1.2 / OD600nm (in ml) of cell suspension was centrifuged and the cell pellet resuspended in 80 µl of 3 x sample buffer. After boiling for 5 min, 8 µl of the sample were loaded on the gel. Gels were stained in Coomassie Blue solution [0.2% (w/v) Coomassie Blue, 50% (v/v) methanol and 10% (v/v) acetic acid] for a minimum of 1 h and destained in a solution containing 40% (v/v) methanol and 10 % (v/v) acetic acid.

II.2.2.7. Crystallisation

Crystallisation trials for recombinant NiR were prepared by the hanging-drop, vapour-diffusion method with PEG 4000 as the precipitant at 4°C. An ~5 mg/ml solution of protein in 100 mM Tris-HCl, pH 8.5 was used. Four microlitre droplets (2 µl protein solution + 2 µl reservoir solution) were set against 1 ml of reservoir solution. The composition of the reservoir solutions used are described in further detail in the Results section of this thesis. Crystallisation experiments for NiR mutant proteins were performed by Mr. Mark Ellis at Daresbury Laboratory, Daresbury, UK. X-ray diffraction data for NiR and NiR mutant proteins were collected at the Daresbury Synchrotron, Daresbury, UK. Crystallisation experiments for N2OR were performed by Dr. Kieron Brown at Architecture et Fonction des Macromolécules Biologiques, Marseille, France. X-ray diffraction data for N2OR were collected at the ESRF Synchrotron, Grenoble, France.

II.2.2.8. Redox titrations

Redox titrations of N2OR were performed anaerobically at 20 °C and monitored by EPR spectroscopy. A mixture of 16 mediators covering redox potentials
Chapter II. Materials and Methods

ranging from –500 mV to + 430 mV, each at a final concentration of 2 µM, was used to facilitate electron transfer during the oxidation and reduction cycles. The complete list of mediators and their midpoint redox potential is presented in section IV.2.3.3. The protein solution was reduced by the addition of aliquots of sodium dithionite (stock solution 1 to 10 mM in 100 mM Tris-HCl, pH 9.0) and oxidised with potassium ferricyanide (stock solution 1 to 10 mM in 100 mM Tris-HCl, pH 7.6). The potential was measured using a platinum electrode as the measuring electrode and a Ag/AgCl electrode as the reference electrode. Calibration was performed with a saturated solution of quinidrone at pH 7.0. Samples at different potentials were anaerobically collected and frozen. The initial volume of the protein solution was such that it allowed collection of 10 experimental points without the need to reintroduce any sample into the redox cell.

II.2.2.9. Protein sequencing

Sequencing of wild-type NiR was performed by Dr. Jozef Van Beeumen at the Laboratory of Protein Biochemistry and Protein Engineering of the University of Gent, Belgium, using a Model 476 pulsed-liquid sequenator as well as a Model 477 instrument (Perkin Elmer, Applied Biosystems). Peptides were generated using proteolytic digestions with Lys-C, Glu-C and N-Asp endoproteinases on separate samples of the protein and separated on a µC2C18 3.2/3 reversed phase column (Pharmacia Biotech) (Vanderberghe et al., 1998).

N-terminal sequencing of recombinant NiR and NiR mutant proteins was performed by Dr. Mike Naldrett at the Nitrogen Fixation Laboratory of the John Innes Centre, Norwich, UK, using a Model 491 Precise sequencer (Perkin Elmer, Applied Biosystems). Sequencing of N2OR was performed by Dr. Jozef Van Beeumen at the Laboratory of Protein Biochemistry and Protein Engineering of the University of Gent, Belgium. N-terminal sequencing was performed on a Model 476 pulsed-liquid sequenator (Perkin Elmer, Applied Biosystems). N-terminal sequencing of NiR and N2OR followed the method of Edman (Edman, 1950). This is a cyclic process that requires an unmodified α-amino group at the N-terminal end of the molecule. After modification with phenylisothiocyanate (PITC), the derivatised terminal amino acid is removed by acid cleavage as its phenylthiohydantoin (PTH) derivative and a new α-
amino group on the next amino acid is now available to react with PITC. Prior to sequencing proteins separated by SDS-PAGE, samples were blotted onto Immobilon P or P<sup>SQ</sup> polyvinylidene difluoride (PVDF) protein sequencing membranes (Millipore). Samples in solution were concentrated onto PVDF membranes using ProSorb cartridges (Perkin Elmer, Applied Biosystems) according to the instructions of the manufacturer and washed to remove salts prior to sequencing.

C-terminal sequence analysis of N<sub>2</sub>OR was performed on a Procise 494C sequencer (Perkin Elmer, Applied Biosystems). C-terminal sequencing of N<sub>2</sub>OR followed a chemical procedure similar to the one described by Boyd <i>et al.</i> (1992). This sequencing procedure consists of converting the C-terminal amino acid into a thiohydantoin (TH) derivative, followed by transformation of the TH into a good leaving group by alkylation. Next, the alkylated TH is cleaved mildly and efficiently with [N=C=S]⁻ (isothiocyanate) anion, which simultaneously forms a TH on the newly truncated protein or peptide. Thus, after the initial TH derivatisation, there is no return to a free carboxyl group at the C-terminus. In the present work, the alkylated THs were identified on-line by reversed-phase analysis on a 140C microgradient system (Perkin Elmer, Applied Biosystems) using a linear gradient from 3.5% (v/v) tetrahydrofuran in 35 mM sodium acetate, pH 3.8 to 100% acetonitrile. Prior to sequence analysis, the protein was adsorbed onto a ProSorb sample preparation cartridge (Perkin Elmer, Applied Biosystems) and, after several subsequent washes with water, treated with phenylisothiocyanate to modify the lysine chains, as described in Bozzini <i>et al.</i> (1995).

**II.2.2.10. Mass spectrometry**

Matrix assisted laser desorption/ionisation – time-of-flight (MALDI-TOF) analysis of NiR and NiR mutants was performed by Dr. Mike Naldrett at the Nitrogen Fixation Laboratory of the John Innes Centre, Norwich, UK, on a Bruker Reflex III mass spectrometer following a procedure similar to that described below.

Electrospray mass spectrometry analysis of N<sub>2</sub>OR samples was performed by Dr. Jozef Van Beeumen at the Laboratory of Protein Biochemistry and Protein Engineering of the University of Gent, Belgium, on a Bio-Q quadropole mass spectrometer equipped with an electrospray ionisation source (Micromass, Altrincham, UK) as used in (Hu <i>et al.</i>, 1997). In this procedure, 10 µl of sample
solution in 50% (v/v) acetonitrile and 1% (v/v) acetic acid were injected manually in the 10 µl loop of the Rheodyne injector and pumped to the source at a flow rate of 5 µl/min. The solvent [50% (v/v) acetonitrile, 1% (v/v) acetic acid] was delivered by a 140A solvent delivery system. Scans of 12 s over the mass range of 400 – 1600 atomic mass units (amu) were collected over a period of 2 min. Calibration of the scans was performed with 50 pmol of horse heart myoglobin.

II.2.2.11. Spectroscopic methods

Spectroscopic characterisation of NiR and mutants forms of NiR, as well as of N₂OR constitutes an important aspect of the work presented in this thesis. Two spectroscopic techniques, UV/Vis and electron paramagnetic resonance (EPR), were used in both systems under study. An exhaustive description of these spectroscopies does not fall within the scope of this thesis and, therefore, will not be attempted. However, given the relevance that they assumed in the work presented here, their main principles and applications will be briefly outlined in the following sections.

II.2.2.11.1. UV/Vis

UV/Vis spectroscopy, also called spectrophotometry, was used routinely as a tool to follow protein purification as well as to study the spectroscopic properties of purified protein samples. It was also thoroughly used in the study of the redox behaviour of NiR, NiR mutant proteins and N₂OR as well as in the measurement of enzyme activities, the determination of protein concentrations and the quantification of copper in protein samples.

UV/Vis spectra are, in principle, easy to collect and can provide important information about the systems under study. Redox or chemical processes that affect the absorption properties of any component of the system (such as a purified protein sample undergoing reduction or oxidation or the production or consumption of a specific metabolite in an enzymatic or chemical reaction) are likely to be analysed by this technique and the information obtained often provides a valuable insight into the system’s properties.
UV/Vis spectroscopy measures the absorption of ultraviolet and visible light (200 to 800 nm region of the electromagnetic spectrum) by molecules or ions. As light passes through a substance, certain quantised energies may be transferred to the sample, raising its electrons to higher energy states. Visible and ultraviolet portions of the electromagnetic spectrum are associated only with transitions of valence electrons. The various quantities of energy (wavelengths of light) absorbed by a given molecule can be used to detect and identify it quantitatively and qualitatively. Spectrophotometry measures the absorbance (Abs) (or optical density - OD) of a sample, which is a measure of its absorption of radiation at a given wavelength (Cooper, 1977; Freifelder, 1982).

During the work presented in this thesis, the most frequently used spectrophotometers were Hewlett-Packard 8452A diode array and Perkin Elmer Lambda 18 (NFL-JIC) or Shimadzu UV-160A or Shimadzu UV-265FS (FCT-UNL).

II.2.11.2. EPR

Electron paramagnetic resonance (EPR), also known as electron spin resonance (Esr), is a spectroscopic technique that requires samples with unpaired electrons which are thus paramagnetic. In biological systems there are two major categories of entities which have unpaired electrons: organic free radicals (produced by homolytic bond cleavage or other processes such as one-electron reductions or oxidations) and atoms or ions which have partially filled inner shells (notably those of the transition elements, which contain unpaired d-orbital electrons). This technique, therefore, is ideally suited for studying many metalloproteins, including those containing Cu(II), Co(II), Fe(II), Fe(III), Mn(II), Mn(III), Mo(V), and metal clusters such as [Fe₂O]³⁺ and [Fe₄S₄]³⁺.

EPR is a valuable technique in establishing electronic structures and their dependence on metal coordination-sphere composition and geometry. The detection of an EPR spectrum indicates the presence of unpaired electrons, while complexities within the spectrum (such as hyperfine splitting, resonance position and linewidth) reflect the environment of the orbital which contains the unpaired electron. Copper(II) is especially amenable to EPR investigation because it has only one unpaired electron. In addition, the ⁶³Cu and ⁶⁵Cu (nuclear spin, I = 3/2) nuclei give rise to a characteristic four-line pattern because of electron-nuclear-spin hyperfine interactions. Thus, EPR
can provide valuable information not only for the identification of the metal ion in a metalloprotein, its coordinating ligands and the geometry of the site, but also on redox changes that may occur at the metal site as a result of a chemical or enzymatic process and on substrate and/or inhibitor binding to a metal catalytic site.

According to electromagnetic theory, a spinning, charged particle (in this case, an electron) will generate a magnetic dipole, oriented along its spin axis. This electron is in a degenerate energy level. If an external field is applied, this will remove that degenerancy and thereby generate two energy levels that are accessible to the spinning electron (Zeeman effect). The energy gap between these energy levels is field-dependent and quantum rules require that the spin of the particle be either parallel (in the low-energy state) or antiparallel (in the high-energy state) to the applied field vector. For the electron, this quantised energy gap is determined by $g \beta H$, where $H$ is the external field strength, $\beta$ the Bohr magneton for electrons and $g$ the Landé splitting factor which has a value of 2.0023 for a free electron, i.e., an electron not associated with an atom. If a radiation of the appropriate frequency is applied, the electron can be promoted to the higher energy level and then decay back to the original energy state. In other words, the resonance condition is attained. The resonance condition for electrons, $h \nu = g \beta H$, occurs in the gigahertz (microwave) frequency range when the applied field is of the order of 1 – 10 kilogauss. The proportionality between field and resonance frequency is such that at any applied field, $H_0$, a unique resonance frequency, $\nu_0$, must exist. Under these conditions, the sample will absorb microwave energy. This will generate a line in the EPR spectrum. In an EPR experiment, the sample is irradiated with microwave radiation of constant frequency and the magnetic field is swept in the 1000 – 10000 Gauss region. When resonance condition is attained, the sample absorbs microwave energy, generating a line in the EPR spectrum. The interactions between the electron and nuclear dipoles will cause the hyperfine splitting of this line, depending on the nuclear spin, $I$, of the nucleus (or nuclei) in question. As a general rule, the line will be split in $2I + 1$ hyperfine lines of equal intensity as a result of the interaction of the electron dipole with that of a nucleus with nuclear spin $I$.

Two main parameters, $g$ and $A$, are obtainable from an EPR spectrum. The position of the line in the spectrum is given by the parameter $g$; $A$ is the hyperfine coupling constant which measures the splitting between hyperfine lines in a spectrum.
and is dependent upon the interaction between electron and nuclear spins (Wright et al., 1986; Lippard and Berg, 1994).

At NFL-JIC, EPR data were collected with a Bruker ER ER200 D-SRC spectrometer fitted with an ER042 MRH microwave bridge with an ER033C field frequency lock and an Oxford Instruments Ite$^{503}$ temperature controller. At FCT-UNL, a Bruker EMX spectrometer equipped with dual mode cavity ER4116DM and an Oxford Instruments continuous flow cryostat were used. EPR spectra were simulated using the program WINEPR – SimFonia (Bruker). Spin quantitations were made according to the method of Aasa and Vänngård (1975), by comparing the area of the experimental curve under nonsaturating conditions to that obtained, under the same conditions, for a sample of Cu(II)-EDTA.

II.2.3. Other methods

II.2.3.1. Cell fractionation and disruption

Gram-negative bacteria are surrounded by a peptidoglycan cell wall and outer membrane, in addition to the conventional cytoplasmic membrane. Between the outer and plasma membranes is the periplasm, containing a distinct set of proteins. When the outer membrane and cell wall are removed, these periplasmic proteins are released. If the removal is carried out in the presence of osmotic support, the cytoplasmic membrane and cytoplasmic compartment remain intact but the cell shape can no longer be maintained and spheroplasts are formed.

In order to determine the subcellular localisation and solubility of overproduced NiR, extraction of the periplasmic fraction of E. coli cells was performed according to the method of Osborn et al. (1972). Pelleted cells were resuspended in 10 mM potassium phosphate buffer, pH 7.0 at a concentration of 1 ml/g (wet weight) and added to a well-stirred solution containing 625 mM sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM neutralised EDTA and 0.75 mg/ml lysozyme. The mixture was stirred for 2 min and then 1 ml of 100 mM MgCl$_2$ and 10 ml of water were added per gram of cells. In some cases, due to a small amount of cells bursting, deoxyribonuclease I (DNase I - Sigma) was added in order to reduce viscosity of the solution. This was then incubated at 30 ºC for 30 min. The periplasmic fraction was separated from the spheroplasts by centrifuging the suspension at 12 000 x g for 30 –
60 min. Soluble periplasmic material is found in the supernatant and the pellet contains the intact spheroplasts. To analyse the solubility of the cytoplasmic material, spheroplasts were resuspended in 10 mM potassium phosphate buffer, pH 7.0 (5 ml buffer / 1 g cells) and disrupted by two passages through a French press (American Instrument Company) at 16 000 lb/in² (1.03 x 10² MPa). Soluble material was separated from cell debris and insoluble cytoplasmic material by centrifugation at 10 000 x g for 30 min.

II.2.3.2. Methionine-labelling

Pulse-chase experiments were performed in order to study the precursors involved in generating the mature NiR protein. This was done by radioactive labelling of polypeptides with \(^{35}\)S methionine as described in Tabor and Richardson (1985) and in Leinfelder et al. (1988). E. coli cells containing the appropriate vectors were grown in LB at 37 °C. For each 5 time points required, 1 ml of cells were centrifuged when OD\(_{590}\)nm= 0.5. The pelleted cells were then washed with 1.5 ml M9 medium, after re-centrifugation, resuspended in 5 ml of M9 medium supplemented with 20 \(\mu\)g/ml thiamine and 18 amino acids (0.01% (w/v), minus cysteine and methionine). Cells were then grown with shaking at 37 °C for 60 min after which time they were induced with 0.25 mM IPTG. After 15 min, rifampicin (20 mg/ml stock in methanol) was added to a final concentration of 200 \(\mu\)g/ml and cells were left to grow at 37 °C for an additional 30 min. For each time point, 1 ml of cells were then taken and pulsed with 10 \(\mu\)Ci of \(^{35}\)S Met (NEN). After incubation at 37 °C for the appropriate time(s), cells were centrifuged for 20 sec and the pellet resuspended directly in 60 \(\mu\)l sample buffer. The polypeptides in 20 \(\mu\)l of each sample were separated by SDS-PAGE and, after staining and destaining of the gel, this was dried and exposed overnight to an autoradiography film.

II.2.3.3. Electrochemistry

Bioelectrochemical studies of NiR and NiR mutants were performed as described by Kohzuma et al. (1994). Cyclic voltammetry was carried out by using a Hi-Tek Instruments’ PP RI waveform generator and a type DT2101 potentiostat
manufactured by the same company and the voltammograms were recorded on a Philips PM 8043 X-Y recorder. Prior to each experiment, the gold working electrode was polished with a 3-μm particle size of aluminium oxide-coated film, sonicated for 1 min and then rinsed thoroughly with deionised water. Modification of the gold electrode with di-4-pyridyl disulfide (4-Pyds) was performed by dipping the freshly polished electrode in a saturated solution of this compound for 1 min. A calomel reference electrode (Radiometer) and a platinum wire (secondary electrode) were used. The experiments were carried out at room temperature in a glove box (Alvic Scientific) with O₂ at 1 – 2 ppm. Typically, ~500 μl degassed protein solution were used in each experiment.
CHAPTER III

NITRITE REDUCTASE
II.1. INTRODUCTION

The study of nitrite reductase (NiR) from *Alcaligenes (A.) xylosoxidans* was carried out at the Molecular Microbiology and Biological Chemistry Departments (formerly joined as the Nitrogen Fixation Laboratory) of the John Innes Centre, Norwich, UK, under the supervision of Dr. Robert Eady and the co-supervision of Dr. Gary Sawers. The cloning and overexpression of the gene encoding NiR opened the doors to the characterisation of the recombinant protein (Prudêncio et al., 1999) and to a programme of site-directed mutagenesis that enabled an insight into interesting and previously unknown features of this enzyme to be gained (two manuscripts in preparation). In collaboration with the group of Prof. Samar Hasnain, Daresbury, UK, the X-ray structures of the recombinant NiR, as well as that of several mutant proteins, is currently being undertaken.

The findings regarding the recombinant NiR and those regarding the mutant forms of this enzyme are presented in sections III.2.1 and III.2.2, respectively. Section III.1 outlines the most important background information on this protein while showing its relevance in the wider context of this thesis.

II.1.1. Two types of dissimilatory NiR

Nitrite reductase (EC 1.7.99.3) catalyses the one-electron reduction of nitrite to nitric oxide and water:

\[
\text{NO}_2^- + 2\text{H}^+ + e^- \rightarrow \text{NO} + \text{H}_2\text{O} \quad [\text{E}'0 (\text{pH 7.0}) = +0.37 \text{ V}; \Delta G_0' = -76.2 \text{ kJ/mol}] 
\]

Two types of dissimilatory nitrite reductase have been identified in denitrifying bacteria. They are the tetraheme cytochrome *cd*$_1$ and the Cu-containing NiR (CuNiR) enzymes. These two proteins are never found in the same cell and there seems to be no correlation between the type of enzyme present in one organism and its genus or species (reviewed in Averill, 1996).

Cytochrome *cd*$_1$ is a homodimeric protein of ~120 kDa with one heme *c* (electron-transfer site) and one heme *d*$_1$ (catalytic site) in each subunit. The properties
of cytochrome \( cd_1 \) were reviewed in Hochstein and Tomlinson (1988), Averill (1996) and Zumft (1997).

CuNiRs have been conveniently subdivided in green and blue, on the basis of their absorbance properties. In \( A. \ xylosoxidans \), nitrite respiration is carried out by a blue CuNiR, which is the subject of the present work. In addition to containing two types of Cu centre, involved in electron transfer and catalysis, CuNiRs share a number of molecular and structural properties. These will be presented and discussed throughout the following sections. To aid clarity, unless otherwise noted, NiR will be used hereafter to mean CuNiR.

III.1.2. Historical background

In 1963, Iwasaki et al. reported the isolation of the first NiR from the denitrifying bacterium \( Pseudomonas \) (\( Ps. \) \( denitrificans \)). These authors described the purification of what they referred to as the “denitrifying enzyme” and suggested that Cu was its “metal constituent”. The report includes the first UV/Vis spectrum of a NiR, with its characteristic absorption maximum at \(~600\) nm and an estimation of \( 149 \) kDa for the molecular mass of the protein.

Since the report of Iwasaki et al. (1963), NiRs have been purified and characterised from a variety of bacterial sources. These include \( Archobacter \) (\( Ach. \) \( cycloclastes \) (Iwasaki and Matsubara, 1972; Liu et al., 1986; Fenderson et al., 1991); \( Rhodobacter \) (\( Rh. \) \( sphaeroides \) (formerly \( Rhodopseudomonas \) \( sphaeroides \) f. sp. \( denitrificans \)) (Sawada et al., 1978); \( A. \ faecalis \) S-6 (Kakutani et al., 1981); \( Nitrosomonas \) \( europaea \) (Miller and Wood, 1983); \( A. \ xylosoxidans \) subsp. \( xylosoxidans \) (formerly \( Alcaligenes \) sp. N.C.I.M.B. 11015, \( Archobacter \) \( xylosoxidans \) and \( Pseudomonas \) \( denitrificans \)) (Masuko et al., 1984; Abraham et al., 1993); \( Ps. \) \( aureofaciens \) (Zumft et al., 1987); \( Bacillus \) \( halodenitrificans \) (Denariaz et al., 1991); and \( Haloferax \) \( denitrificans \) (Inatomi and Hochstein, 1996).

Initial reports on these enzymes seemed to indicate a considerable degree of heterogeneity among NiRs (see Averill, 1996 and Zumft, 1997 for reviews on those reports). However, evidence from X-ray structure determination and comparison of gene-derived amino acid sequences clearly showed that NiR species are members of the same protein family (Zumft, 1997). The enzymes from \( Arch. \) \( cycloclastes \), \( Rh. \) \( sphaeroides \), \( A. \ faecalis \), \( Bacillus \) \( halodenitrificans \) and \( Haloferax \) \( denitrificans \) are
green in colour whereas those from *Nitrosomonas europaea*, *A. xylosoxidans* and *Ps. aureofaciens* are blue.

**III.1.3. Solubility and subcellular localisation of NiR**

With the exception of the protein from the Gram-positive bacterium *Bacillus halodenitrificans*, which has been reported to be strongly associated with the cytoplasmic membrane (Denariaz *et al.*, 1991), all NiRs described to date are soluble enzymes. The first indication that NiRs are periplasmically located proteins came from the fractionation studies performed by Sawada and Satoh (1980) with cells of *Rh. sphaeroides* IL106. These findings were later confirmed by direct immunocytochemical localisation studies done on the proteins from *Ach. cycloclastes* and *A. xylosoxidans* (Coyne *et al.*, 1990) using immunogold labelling techniques.

The deduced amino acid sequences of several NiRs (see Fig. III.16, section III.2.1.1) show that these proteins are synthesised as precursors with N-terminal signal sequences. An inspection of these sequences shows that two out of seven of these proteins have signal peptides of ~24 amino acids that exhibit the standard structural features for Sec-dependent protein translocation (see Pugsley, 1993; Schatz and Dobberstein, 1996 and Pohlschröder *et al.*, 1997 for reviews on this subject). However, the remaining five proteins listed in Fig. III.16 have longer signal peptides, composed of ~45 amino acid residues, which include a double-arginine motif close to the N-terminus (indicated by the arrows in Fig. III.16). This is a type of signal sequence that is recognised by a Sec-independent export pathway and which has been proposed to export folded, redox-active proteins across the cytoplasmic membrane (Berks, 1996; Santini *et al.*, 1998; Sargent *et al.*, 1998; Weiner *et al.*, 1998. See also sections III.2.1.1 and IV.1.2). Interestingly, there seems to be a correlation between the type of NiR and the type of signal peptide that it bears, in that the two proteins that have shorter signal peptides are blue whereas the remaining five are green.

**III.1.4. Molecular properties of NiR**

NiRs share a number of common biochemical, spectroscopical and structural features. For those enzymes where the sequence is known, NiRs have highly homologous primary structures (61% to 81% positional amino acid identity) and the
X-ray structures determined thus far (see section III.1.6 and references therein) also reveal a great number of similarities between these proteins.

NiRs are homotrimeric proteins of ~109 kDa total mass (reviewed in Zumft, 1997). These enzymes usually contain ~6 Cu atoms per protein molecule, which constitute three mononuclear type 1 Cu sites, with an electron-transfer role, and three mononuclear type 2 Cu sites, where catalysis occurs (see section III.1.5). The UV/Vis spectrum of blue NiRs exhibits a predominant absorption maximum at ~590 nm and other, less intense, absorption bands at ~460 and 700 – 750 nm (Fig. III.1-A). In the case of green NiRs, the absorption at ~460 nm is predominant and that at ~590 nm is reduced. These features arise from the type 1 Cu site in the protein, as shown by the spectroscopic features of type 2 Cu-depleted (T2D) forms of the enzymes from *Ach. cycloclastes* (Libby and Averill, 1992; Adman *et al*., 1995) and *A. xylosoxidans* (Howes *et al*., 1994; Abraham *et al*., 1997, Suzuki *et al*., 1997; Prudêncio *et al*., 1999). The differences between the absorption features of the blue and the green enzymes are likely to be a reflection of slight differences in the geometry of the type 1 Cu site, as will be discussed in section III.1.6.2.

The EPR spectrum of all oxidised NiR species exhibit the characteristic features of type 1 Cu and type 2 Cu, manifest in the small and large hyperfine splitting in the $g_{\parallel}$ region of the spectrum (Fig. III.1-B). The type 2 Cu content of the purified enzymes seems to be somewhat variable and there are indications that the enzyme may become at least partially type 2 Cu-depleted (T2D) during purification (Libby and Averill, 1992). Selective depletion and reconstitution of type 2 Cu sites in NiR has been shown to be possible and to lead to a fully active protein with a full complement of Cu (Libby and Averill, 1992. See also Suzuki *et al*., 1997 and Prudêncio *et al*., 1999). The findings of Libby and Averill (1992) were particularly important in that they demonstrated a correlation between the type 2 Cu content and the activity of the enzyme and suggested that previous reports of NiRs containing type 1 Cu only might in fact correspond to largely T2D forms of the enzymes. It has also been suggested that a lower than stoichiometric content of Cu in NiR may be due to partial occupancy of the type 2 Cu sites by Zn (Abraham *et al*., 1993).
Chapter III. Nitrite Reductase

Figure III.1 – UV/Vis (A) and EPR (B) spectra of the blue NiR from *A. xylosoxidans*. The inset in A shows an expansion of the visible region of the UV/Vis spectrum. The stick diagrams above the EPR spectra show the positions of the features corresponding to type 1 Cu and type 2 Cu. The EPR spectrum was simulated with $g_{//} = 2.208$ ($A_{//} = 6.3$ mT) for type 1 Cu and $g_{//} = 2.298$ ($A_{//} = 14.2$ mT) for type 2 Cu, comprising 60% type 1 Cu and 40% type 2 Cu. The pictures were taken from Abraham *et al.* (1993) with permission.
III.1.5. The Cu centres

III.1.5.1. The type 1 Cu centre

As mentioned above, NiR contains type 1 Cu sites which are presumably involved in electron transfer from the protein’s physiological donor (see section III.1.7) to the catalytic sites in the enzyme (see following section). Initial reports on the enzymes from *A. xylosoxidans* (Masuko *et al.*, 1984) and from *Ps. aureofaciens* (Zumft *et al.*, 1987) suggested that the type 1 Cu site of those enzymes might be the catalytic site of the enzyme. However, evidence from a number of different sources has shown that catalysis occurs at the type 2 Cu site (see following section) and the previous suggestions that type 1 Cu might be catalytically active are now believed to be due to preparations that were largely depleted in the type 2 Cu centre (Averill, 1996).

Further evidence that type 1 Cu sites are electron transfer centres in NiR came from the studies of Suzuki *et al.* (1994, 1997) and Farver *et al.* (1998). These authors have used pulse radiolysis to observe the intramolecular electron transfer process from the type 1 Cu to the type 2 Cu as the reoxidation of type 1 Cu(I) centre in native NiR. Suzuki *et al.* (1994) concluded from their experiments that the intramolecular electron transfer rate from type 1 Cu to type 2 Cu is slower than the reduction of type 2 Cu. Suzuki *et al.* (1997) note that the reoxidation of type 1 Cu(I) was absent from T2D enzyme (see also following section).

The two Cu sites in NiR are connected by two alternative routes (see Fig. III.31, section III.2.2.1). Dodd *et al.* (1998) suggest that the internal electron transfer may take place via Cys129-His130 (*A. xylosoxidans* numbering) while the nitrite ligation to the type 2 Cu site may be communicated to the type 1 Cu site via the Asp92-His89-His94 link triggering the electron transfer from the type 1 Cu site.

The midpoint potential for type 1 Cu(II)/Cu(I) in NiR from *A. xylosoxidans* was reported to be +260 mV (Masuko *et al.*, 1984; Kohzuma *et al.*, 1993), somewhat lower than the value of +305 mV reported for the physiological electron donor to this enzyme [Dodd *et al.* (1995a) and see section III.1.7]. This is an uphill electron-transfer which might suggest a modulation of the potential of type 1 Cu in NiR upon formation of the complex with the electron donor. The structural features and proposed electron entry path to this site are described in section III.1.6.2.
III.1.5.2. The type 2 Cu centre

As mentioned in the previous section, evidence that type 2 Cu centres are the catalytic sites in NiR originates from several sources. The studies of selective depletion and reconstitution of the type 2 Cu site of NiR from *Ach. cycloclastes*, already mentioned in section III.1.4, performed by Libby and Averill (1992) provided the first evidence that type 2 Cu centres are the sites of nitrite reduction in this enzyme. Electron nuclear double resonance (ENDOR) studies carried out by Howes et al. (1994) on the enzyme from *A. xylosoxidans* showed that both $^1$H and $^{14}$N ENDOR of the type 2 Cu site undergo considerable changes on addition of nitrite whereas the type 1 Cu site ENDOR is unaffected. Similarly, EPR studies of *A. xylosoxidans* NiR showed that large changes occur in the $g_\parallel$ and $A_\parallel$ values of the type 2 Cu site upon incubation with nitrite, whilst the type 1 site EPR is not affected (Howes et al., 1994, Abraham et al., 1997). Difference EXAFS of native and T2D NiR from *A. xylosoxidans* was used to probe the changes in the type 2 Cu site of the native enzyme associated with the binding of nitrite (Strange et al., 1995; Eady et al., 1997). These studies showed that the phase and amplitude of the EXAFS spectrum changed upon addition of nitrite, consistent with an expansion of the inner shell, as the average His-Cu distance increased by 0.08 Å. Fourier transform infra-red (FTIR) spectroscopy studies on the NiR from *Ach. cycloclastes* have shown that CO, a strong inhibitor of this enzyme, selectively binds the type 2 Cu (Averill et al., 1998). Finally, the structures of the nitrite-bound NiRs from *Ach. cycloclastes* (Adman et al., 1995), *A. faecalis* (Murphy et al., 1997) and *A. xylosoxidans* Dodd et al. (1997, 1998) have all been determined and show that nitrite binds to the type 2 Cu site of NiR.

The midpoint redox potential of type 2 Cu(II)/Cu(I) of NiR from *A. xylosoxidans* was estimated on the basis of pulse radiolysis studies. Farver et al. (1998) determined a value of +230 mV for that potential, which is in good accord with the value of +240 mV determined by Suzuki et al. (1997) using the same method. This value is, if anything, lower than the estimated potential of +260 mV for the type 1 Cu of the same protein (see previous section), showing that the type 1 Cu - type 2 Cu electron transfer is an uphill process, which may help explain its slow rate (Farver et al., 1998). The structural features of type 2 Cu in NiR and the proposed mechanism of catalysis at this site are detailed in sections III.1.6.2 and III.1.6.3, respectively.
It should be mentioned that there are reports that a Cu-depleted type 2 site may still be able to reduce nitrite. This was shown by the pulse radiolysis studies of Suzuki et al. (1997) who, as mentioned in the previous section, report that the reoxidation of type 1 Cu(I) was absent from the T2D enzyme. However, they also showed that for T2D NiRs in the presence of nitrite, the type 1 Cu(I) is slowly oxidised, suggesting that nitrite bound to the protein accepts an electron from the type 1 Cu. This finding suggests that T2D NiRs have enzymatic activities, although they are lower than those of the native enzyme. Active or nitrite-binding T2D NiRs have also been reported by Masuko et al. (1984), Abraham et al. (1997) and Prudêncio et al. (1999). However, in all these cases, it is possible that the enzymes reported as T2D may have a low complement of type 2 Cu, which would be responsible for the activity observed.

III.1.6. X-ray structure of NiR

The first X-ray structure of a NiR to be determined was that of the green protein from Ach. cycloclastes (Godden et al., 1991; Adman et al., 1995). Since then, the structures of the green enzyme from A. faecalis S-6 (Kukimoto et al., 1994; Murphy et al., 1995; Murphy et al., 1997) and that of the blue enzyme from A. xylos oxidans (Dodd et al., 1997, 1998; Inoue et al., 1998) have also been reported. The structures of these three proteins are quite similar and reveal potential explanations for the difference in colour between the green and blue enzymes. The information in sections III.1.6.1, III.1.6.2, III.1.6.3 and III.1.6.4 is, except where noted, taken from the references cited above.

III.1.6.1. Overall structure

As mentioned above, NiRs are trimeric proteins composed of three identical subunits. Each monomer consists of two domains that contain the eight-stranded β-barrel core topology typical of cupredoxins (see section I.2.2.1). The two domains are connected by 12 amino acid residues which are rather loosely suspended in a loop. Each domain I from one monomer interacts with domain II of the adjacent monomer providing the bulk of the intermonomer contacts. Domains I of the three monomers are positioned at the corners of the trimer whereas domains II form the core of the
molecule. The C-terminal peptide of domain II forms an arm which runs along the outer edge of the adjacent monomer. The interior of both domains is largely hydrophobic and so is the interdomain surface. Smaller residues are found at the centre of the interdomain interaction, while larger residues fill in as the sheets diverge.

The structure of a monomer of NiR from *Ach. cycloclastes* is depicted in Fig. III.2. As can be seen in this picture, each monomer contains two Cu atoms, one of which forms a type 1 Cu site and the other a type 2 Cu site. The type 1 Cu atom is buried in domain I of the monomer, while the type 2 Cu atom resides in the intermonomer cleft. The two Cu atoms are situated ~13 Å apart. The structures of these sites will be described in sections III.1.6.2 and III.1.6.3.

The structure contains three nearly helical regions. One of these connects β-sheets β10 and β11 in domain II and provides interdomain interactions with the topologically analogous loop connecting β-sheets β1 and β2 in domain I. The other helical regions are an α-helix which provides three ligands to the type 1 Cu in domain I and its topological counterpart in domain II, which provides some of the residues lining the active site.

Figure III.2 – Representation of a monomer of NiR from *Ach. cycloclastes*. The residues defining secondary structures are labelled. The type 1 Cu atom (above) and the type 2 Cu atom (below) are represented by dark spheres. The picture was taken from Adman *et al.* (1995).
III.1.6.2. Structure of the type 1 Cu site

The type 1 Cu atom in NiR lies ~4 Å from the protein surface and is ligated by two His residues, one Met residue and one Cys residue. In the case of the green enzymes from *Ach. cycloclastes* and *A. faecalis* S-6, these residues are His95, His145, Met150 and Cys136 and in the case of the blue enzyme from *A. xylosoxidans* they are His89, His139, Met144 and Cys130. The ligation to the Cu atom is provided by the His $\text{N}^{\delta+}$ atoms, by the Met $\text{S}^{\delta-}$ atom and by the Cys $\text{S}^{\gamma}$ atom in a distorted trigonal planar geometry (see also section III.2.2.1.1). The two His residues and the Cys residue form the strong planar ligands, while the Met residue forms a weaker axial ligand. The second axial ligand found in some type 1 Cu sites, namely a carbonyl oxygen atom is absent from this structure (see Canters and Gilardi, 1993 for a review on this subject).

The Cu ligand His139 of the blue enzyme (His145 for the green) is oriented such that the $\text{N}^{\varepsilon_2}$ atom is exposed to the solvent at the bottom of a small depression in the protein surface. Drawing an analogy with the solvent-exposed His117 in azurins (Nar *et al.*, 1991; Dodd *et al.*, 1995a), Dodd *et al.* (1998) suggest that this residue might be involved in mediating electron-transfer to NiR.

Adman *et al.* (1995) suggested that the difference in colour between green and blue NiRs might be related to the Cu-S(Cys) distances in these two subclasses of the enzyme. However, the determination of the structure of the blue NiR from of *A. xylosoxidans* showed that this distance is the same as that found for the *Ach. cycloclastes* and *A. faecalis* enzymes. This indicates that this is not the explanation for the difference in the colour of those proteins.

Using low-temperature optical absorption, CD, MCD and sulphur K edge X-ray absorption measurements, LaCroix *et al.* (1996), propose that a coupled angular movement of the Met residue towards the NNS plane and a rotation of the Cys residue within the NNS plane S would be the predominant effect controlling the colour of the type 1 Cu site.

Inoue *et al.* (1998) have noted that the orientation of the Met ligand side-chain for the enzymes from *Ach. cycloclastes* and *A. faecalis* appears to be different from that observed for the enzyme from *A. xylosoxidans* (Fig. III.3-A). Moreover, the main
Figure III.3 – Superimposed structures of the NiR from *A. faecalis* (shown in green) and that from *A. xylosoxidans* (shown in blue). A. The type 1 Cu sites. The C\(^\gamma\) and S\(^\delta\) atoms in the Met residue in *A. faecalis* NiR are shown in orange and the corresponding atoms in the *A. xylosoxidans* protein are shown in red. B. The region around the Met Cu-ligand, where the amino acid residues are common to both enzymes, the numbering is that of the green protein from *A. faecalis*. Where those amino acid residues are different, that of *A. faecalis* NiR is shown on top and that of the *A. xylosoxidans* protein is shown below. The asterisks denote amino acid residues which are conserved in green or blue NiRs. The pictures were adapted from Inoue *et al.* (1998).
Chapter III. Nitrite Reductase

chain from Met150 to Gly152 in the green enzymes is shifted compared to the blue protein.

Inoue et al. (1998) suggest that this displacement of the main chain may be due to the difference in the amino acid residue in position 177 (Ach. cycloclastes and A. faecalis numbering), which is a conserved Tyr residue in all green enzymes and a conserved Thr residue (at position 171 in the A. xylosoxidans protein) in the blue enzymes (Fig. III.3-B).

An alternative suggestion comes from the report of Dodd et al. (1997). These authors note significant differences in the position of the Cu atom with respect to the plane formed by the three strong ligands for the green and the blue enzymes. In the protein from Ach. cycloclastes and A. faecalis the Cu atom is out of this plane by ~0.5 Å and 0.6 Å, respectively, whereas in the protein from A. xylosoxidans the Cu atom is displaced by ≤ 0.2 Å (which at 2.8 – 3.0 Å resolution is not significantly different from the Cu being in the plane). Dodd et al. (1997) suggest that this difference in the displacement may partly be due to the length of the Cu-Met150 distance which, in itself, may result from a weak (or absent) carbonyl interaction. Thus, Dodd et al. (1997) propose that, among other factors, the position of the Cu atom with respect to the N2S plane may result from a subtle balance between the two axial interactions and that the extent of the ‘out-of-plane’ movement of Cu controls the green or blue colour of this centre. However, this contradicts the findings of LaCroix et al. (1996) who proposed that the Cu-Met length and the Cu displacement would have only minor effects on the electronic structure of the site.

The latest suggestion to explain the differences observed between blue and green NiRs comes from the report of Dodd et al. (1998). These authors showed that the structures of the blue and green NiRs show no differences with regard to the Cys-Cu-Met angle and note that the His-Cu-Met angle in the blue NiR is 115° whereas it is 132° in the blue enzyme. Thus, these authors proposed this, together with other subtle factors accounts for the differences in colour for the two groups of NiRs.
III.1.6.3. Structure of the type 2 Cu site

The type 2 Cu atom in NiR lies at the bottom of a cleft between the monomers, at a distance of ~12 Å from the protein exterior. It is ligated by two His residues from domain I of one monomer and one His residue of domain II of the adjacent monomer. In the case of the green enzymes from *Ach. cycloclastes* and *A. faecalis* S-6, these residues are His100, His135 and His 206, respectively, whereas in the case of the blue enzyme from *A. xylosoxidans* they are His94, His129 and His300, respectively. The ligation to the Cu atom is provided by the His N$_{\varepsilon2}$ atoms. The structures also show the presence of an exogenous ligand to type 2 Cu. This was reported to be a water molecule for the enzyme from *Ach. cycloclastes* and either a water molecule or a chloride ion for the enzyme from *A. xylosoxidans*. The geometry of the site is nearly tetrahedral.

Two other residues present in the type 2 Cu site are suggested to be of potential importance in substrate binding and reduction. These are Asp92 and His249 (*A. xylosoxidans* NiR numbering; Asp98 and His255 for the enzymes from *Ach. cycloclastes* and *A. faecalis*). The Asp residue may form a hydrogen bond to the Cu-ligating water molecule (Dodd *et al.*, 1998). The side-chain of this residue is suggested to be hydrogen-bonded to another water molecule close to the Cu atom and to a second water molecule bound between this residue and the His residue (see Fig. III.32, section III.2.2.3). The possible implications of these residues in substrate binding and catalysis will be addressed in the following section.

III.1.6.4. Structure of the active site pocket: Implications for catalysis

As mentioned above, the type 2 Cu atom and its ligands are situated in a hydrophobic pocket formed by apposition of domain II of one monomer and domain I of another. The Cu atom is directly accessible from the solvent via a channel of ~12 Å of depth. The pocket is formed because residues protruding from the helical loop do not allow residues just under the loop to get very close to one another. The residues under the loop provide ligands to the Cu atom. In addition there are residues which line the pocket that play a role in binding and releasing substrate and product. The
access to the catalytic site is suggested to be via the hydrophobic pocket since solvent access to the rear of the molecule is blocked by a His residue.

The structures of substrate-bound NiR from *Ach. cycloclastes* (Adman *et al.*, 1995) and *A. xylosoxidans* (Dodd *et al.*, 1997, 1998) have shown that nitrite displaces the exogenous Cu-ligand (water or chloride) and binds in an asymmetric bidentate fashion, with the oxygens toward the Cu atom and with the Asp92 residue (see previous section) in hydrogen-bonding distance to the oxygen closest to the Cu. Hydrogen bonding to His249 (see previous section) is not possible. These findings are common to both the proteins from *Ach. cycloclastes* and from *A. xylosoxidans* except that the nitrite molecule in the former is rotated longitudinally by 90° relative to its position in the latter. Also, the results obtained for the *A. faecalis* enzyme are similar to those obtained for the one from *Ach. cycloclastes* except that in the former the nitrogen points away from the Asp residue instead of pointing towards it. The bidentate mode of NO$_2^-$-Cu binding is in accord with the findings of Tolman *et al.* (1993) using a model compound to model the NiR-substrate adduct as well as with the findings of Howes *et al.* (1994) in which ENDOR was used to probe nitrite binding to NiR from *A. xylosoxidans* (see also section III.1.5.2).

For the one-electron reduction of NO$_2^-$ to NO and H$_2$O, two protons must be taken up. Adman *et al.* (1995) suggest that the most likely residues for providing these protons are the Asp residue and the His residue mentioned in the previous section and above, and the water molecule bound between them. Adman *et al.* (1995) and Murphy *et al.* (1997) suggest that when nitrite displaces the water ligand, one of the protons remains on the Asp residue so that effectively only hydroxide is actually displaced, leaving the remaining proton required for the reaction to come from elsewhere. The role of the His residue might then be to provide a hydrogen bond to the water molecule, which also hydrogen bonds the Asp residue, in order to allow this residue to temporarily hold a hydrogen on the other carboxylate oxygen during the displacement reaction. Thus, the authors propose that NO$_2^-$ displaces the bound water, which leaves as OH$^-$, leaving a proton on the Asp residue, and picking up a proton to regenerate H$_2$O as it exits along the hydrophilic side of the active site. The Cu-ONO would then have a redox potential appropriate for electron transfer to it from the type 1 Cu site. Reduction and the protonated Asp residue would facilitate cleavage of the NO bond, leaving one of the NO$_2^-$ oxygens bound to the Cu. Cu$^{2+}$ would then be regenerated, and NO would leave along the hydrophobic side of the pocket.
Hulse et al. (1989) and Jackson et al. (1991) have presented evidence for a NO-rebound mechanism for production of N₂O from nitrite by NiR. The results obtained by those authors are consistent with the formation of a labile Cu-nitrosyl (Cu⁺-NO⁺) species which normally decomposes to NO. This can rebind to the enzyme to combine with a second nitrite ion or a species derived therefrom to produce N₂O. In order to accommodate the finding that N₂O can be produced by NiR from nitrite, Adman et al. (1995) propose that the Cu⁺-NO⁺ species must have a long enough lifetime to permit formation of N₂O. Murphy et al. (1997) also address this issue in their report. Their proposed scheme for production of N₂O does not include an N-coordinated Cu⁺-NO⁺ intermediate. However, these authors suggest that such a species may be formed by a rebound mechanism, as proposed by Jackson et al. (1991), whereby released NO displaces the solvent ligand. Alternatively, Murphy et al. (1997) suggest that NO may remain near the active site so that when another nitrite is reduced that species reacts with it to produce N₂O.

Dodd et al. (1997) argue in favour of a Cu-nitrosyl intermediate which is formed after cleavage of the O-N bond in nitrite and which, in turn, is facilitated by reduction of the type 2 Cu and donation of a proton from the Asp residue. Contrary to the proposal of Adman et al. (1995), Dodd et al. (1997) suggest that, following the cleavage of an O-N bond of nitrite, the release of NO occurs only after the release of the second O atom thus allowing a longer lifetime of the Cu⁺-NO⁺ species. As noted by Dodd et al. (1998), although this intermediate has not been directly observed in enzyme turnover of CuNiRs, a Fe²⁺-NO⁺ species has been detected in heme cd₁-containing NiR from Ps. stutzeri (Wang and Averill, 1996).

As stated by Ferguson (1998), it will be difficult to distinguish between the possibilities suggested but NO rebinding may lead to trapping of an apparent nitrogen-bound nitrosyl intermediate. In any case, it seems clear that the combined structural data suggest a mechanism where nitrite binds to an oxidised type 2 Cu site that is then reduced by electron transfer from the type 1 Cu site (Murphy et al., 1997). Such a notion is supported by the findings of Strange et al. (1999) who, on the basis of EXAFS, structural and kinetic data, also propose that turnover of NiR from A. xylosoxidans proceeds by an ordered mechanism in which nitrite binds to the oxidised type 2 Cu centres before electron transfer from the reduced type 1 Cu centre occurs.
III.1.7. Electron donation to NiR

The main electron donors to NiR are azurin and pseudoazurin. These two proteins are members of the same family of small Cu-containing, electron transfer proteins, known as cupredoxins (see section I.2.2.1). Azurins and pseudoazurins occur in organisms where NiRs are blue and green, respectively. *A. xylosoxidans* has been found to contain two different azurins with similar redox potentials (~305 mV), both equally effective electron donors *in vitro* to the NiR from *A. xylosoxidans* (Dodd et al., 1995a). Electron donation experiments using three NiRs, one green and two blue, and five cupredoxins, four azurins and one pseudoazurin, have shown that pseudoazurin can readily donate electrons to both blue and green NiRs whereas all of the azurins react very sluggishly with the green NiR (Murphy, L.M., Dodd, F.E., Yousafzai, F. K., Eady, R.R., Hasnain, S.S., unpublished results).

The X-ray structures of the pseudoazurin from *A. faecalis* S-6 (Petratos et al., 1987, 1995; Adman et al., 1989; Vakoufari et al., 1994) and the azurins from *Ps. aeruginosa* (Nar et al., 1991) and *A. xylosoxidans* (Dodd et al., 1995b) have been determined. Together with site-directed mutagenesis studies (Kukimoto et al., 1996a, 1996b; van Pouderoyen et al., 1997), the structural data have provided evidence that the interaction between NiR and its electron donor involves a strong electrostatic element. A comparison of the surface charges of NiRs from *A. xylosoxidans* (blue) and *A. faecalis* (green) shows that the former has an overall charge of +6e whereas the latter has an overall charge of −30e (Dodd et al., 1998). This results in a surface charge distribution for *A. faecalis* NiR that is almost exclusively negative. The most negatively charged areas are those in the neighbourhood of the hydrophobic patch above the type 1 Cu site. This region is surrounded by a ring of eight Asp and Glu residues which have been proposed as the docking site for a ring of positively charged Lys residues on pseudoazurin (Murphy et al., 1995). The report of Dodd et al. (1998) shows that some positively charged areas can be seen in the inter-monomer cleft. The surface charge of the *A. xylosoxidans* enzyme is much more neutral. The intermonomer cleft is of largely positive charge with a negative region, again, observed about the hydrophobic patch above the type 1 Cu site. Four of the eight Asp and Glu residues show charge conservation in *A. xylosoxidans* NiR, the other four being neutral. Dodd et al. (1998) suggest that these differences may help explain the differing specificities of the blue and green NiR species for azurin as electron donor.
The charge distribution on the surfaces of pseudoazurin and azurin are such that they complement those of the respective redox partners. Pseudoazurin has a highly positive hydrophobic face. This face is surrounded by a ring of Lys residues that may interact with a ring of negatively charged residues on the green NiR, as suggested by Kukimoto et al. (1995). In contrast, azurin shows a much reduced charge on the hydrophobic surface, with only two Lys residues present in close proximity. The number of charged residues present on the putative docking surface of the blue NiR is also much reduced and may result in the docking of azurin in a more specific orientation (Dodd et al., 1998).

III.1.8. *A. xylosoxidans* NiR

As mentioned before, the blue NiR from *A. xylosoxidans* shares a number of properties with the other CuNiRs studied so far. These properties were discussed throughout the previous sections where this protein was described and compared to those from other sources. This section is aimed at providing a brief outline of the historical background to *A. xylosoxidans* NiR while highlighting some of its more relevant molecular and structural properties.

The first description of the purification and characterisation of this protein was that of Masuko et al. (1984). These authors report a homodimeric enzyme containing only one type 1 Cu centre per monomer. These findings were disputed in a later report by Abraham et al. (1993) which showed that the enzyme contains two types of Cu centres (see Fig. III.1 in section III.1.4) and suggested that the protein is a trimer rather than a dimer. The trimeric composition of *A. xylosoxidans* NiR was confirmed by the X-ray scattering studies of Grossman et al. (1993). Since then, the protein has been analysed by a number of spectroscopic techniques, including EPR (Howes et al., 1994; Abraham et al., 1997), ENDOR (Howes et al., 1994) and EAXFS (Strange et al., 1995; Eady et al., 1997). The structure of NiR was determined in 1997 by Dodd et al. (see also Dodd et al., 1998) and confirmed the trimeric arrangement of the subunits of the protein and the presence of one type 1 Cu and one type 2 Cu centres per protein monomer (see section III.1.6). Fig. III.4 shows a representation of the overall structure of the *A. xylosoxidans* NiR (see Fig.III.31, section III.2.2.1 for a detailed picture of the Cu centres in the enzyme).
Chapter III. Nitrite Reductase

Figure III.4 – Overall structure of the NiR from *A. xylosoxidans*. The subunits are represented in orange, green and red and the Cu atoms are represented as blue spheres. Type 1 Cu centres are buried within each of the subunits whereas type 2 Cu centres are located at the interfaces between two adjacent subunits. The picture was kindly provided by Dr. Robert Eady.

III.1.9. Project aims

The findings presented and discussed throughout the previous sections of this chapter represent important steps towards understanding the mechanism of nitrite reduction by NiR. The availability of the crystal structure of this enzyme, as well as the knowledge about the role of each of its two types of Cu atoms provide the basis for further studies that will elucidate some aspects of the way in which this enzyme functions. Thus, this project was aimed at providing an insight into the role of specific amino acid residues in the mechanism of the enzyme. The first step in this
process was the cloning and overexpression of the NiR structural gene, followed by a site-directed mutagenesis programme aimed at key residues involved in intramolecular electron transfer and catalysis. The effect of the mutations introduced in the protein was monitored by various spectroscopic techniques such as UV/Vis and EPR, whilst relating those findings with the effects on the enzyme’s activity with physiological and artificial electron donors. The study of these effects on carefully chosen single-point mutants of NiR was aimed at allowing a better understanding of this enzyme and the roles of some of its crucial amino acid residues. In collaboration with Prof. Samar Hasnain’s group, the determination of the structures of these mutant proteins was undertaken, in order to fully understand the changes caused by the mutations introduced. The following sections deal with the strategies followed in the pursuit of these objectives and the results thus obtained.
III.2. RESULTS

III.2.1. Recombinant NiR

III.2.1.1. Cloning and sequencing the nirA gene

As a first step towards the objectives outlined above, the gene encoding NiR from *A. xylosoxidans* was cloned and overexpressed in *E. coli*. Initially, degenerate oligonucleotides based on the amino acid sequences of conserved regions of NiRs from different organisms were designed and used in PCR experiments, to try to amplify a portion of the *nirA* gene from *A. xylosoxidans*. Several oligonucleotides were used in different combinations, however they failed to generate a DNA fragment of the appropriate size and sequence. At this stage, part of the internal amino acid sequence of the protein from *A. xylosoxidans* became available, allowing new, more defined oligonucleotides to be designed. By sequence comparison with other nitrite reductases, the available internal sequences of *A. xylosoxidans* NiR were located. A forward primer corresponding to amino acids at positions 36 to 44 (Nit-7: 5’-AAG GAR TTC CAN ATG AC-3’) and a reverse primer corresponding to amino acids at positions 265 to 260 (Nit-8: 5’-CCA NAC CCA RTC NCC RTG-3’) were designed and used in a PCR experiment with *A. xylosoxidans* chromosomal DNA as template. The letters R and N in the oligonucleotide sequences above signify the redundancies A/G and A/C/G/T, respectively. The amplified DNA fragment (Fig. III.5) was estimated to be ~680 bp long, closely matching the predicted size of 672 bp. In order to facilitate DNA sequence determination, the amplified fragment was first cloned in the *Sma*I site of vector pUC19. The resulting plasmid was called pUAx1. The insert in pUAx1 was sequenced on both DNA strands, initially using Universal and Reverse sequencing primers and then with specific oligonucleotides. The amino acid sequence thus derived was compared to the available portions of the internal sequence of *A. xylosoxidans* NiR, as well as to known sequences of NiRs form other organisms. This confirmed that the amplified DNA fragment corresponded to a portion of the gene coding for nitrite reductase (see Fig. III.15).
Chapter III. Nitrite Reductase

Figure III.5 – PCR-amplification of DNA fragments analysed by 0.8 % (w/v) agarose gel. Nit-7 and Nit-8 were used as forward and reverse primers, respectively. Lane 1: Negative control (no template DNA); lanes 2 and 3 – 10 ng *A. xylosoxidans* chromosomal DNA used as template. The arrows indicate bands corresponding to DNA fragments of estimated size ~680 bp.

The ~680 bp DNA fragment was specifically fluorescence-labelled to allow it to be used as a probe to isolate the complete, native *nirA* gene. The DNA required for this purpose was obtained from the digestion of plasmid pUAx1 with *Eco*RI and *Hind*III (Fig. III.6).

*A. xylosoxidans* chromosomal DNA was extracted and digested with different restriction enzymes. Initially, *Bam*HI, *Sal*I, *Sph*I and *Pst*I digestions were performed. The fragments resulting from these digestions were separated by agarose gel electrophoresis (Fig. III.7-A) and subsequently blotted onto nitrocellulose (NC) membranes and hybridised with the fluorescent probe (Fig. III.7-B).
Chapter III. Nitrite Reductase

Figure III.7 – Hybridisation of digested *A. xylosoxidans* chromosomal DNA with the fluorescently-labelled ~680 bp probe isolated from pUAx1. A. Separation of the digested fragments on a 0.8 % (w/v) agarose gel. B. Nitrocellulose membrane following hybridisation with the probe. Lane a- 1 kb ladder DNA size markers; 1- *A. xylosoxidans* chromosomal DNA digested with *Bam*HI; 2- *A. xylosoxidans* chromosomal DNA digested with *Sal*I; 3- *A. xylosoxidans* chromosomal DNA digested with *Sph*I; 4- *A. xylosoxidans* chromosomal DNA digested with *Pst*I; 5- Positive control (plasmid pUAx1 digested with *Eco*RI and *Sal*I). The arrows indicate the position of the bands that were excised in subsequent experiments in preparation for cloning. B1 and B2 indicate fragments obtained from *Bam*HI digestion; S1 and S2 indicate fragments obtained from *Sal*I digestion.

As can be observed in Fig. III.7-B, each of the *Bam*HI and *Sal*I digestions of chromosomal DNA generated two fragments that hybridised with the fluorescent probe. These bands were labelled B1, B2, S1 and S2 and the sizes of the fragments were estimated to be ~2.9 kb, ~2.0 kb, ~1.3 kb and ~1.1 kb, respectively. Bands located at these positions were subsequently excised from a gel containing the same samples and run under the same conditions as the gel used for Southern transfer.

After extraction from the gel, the DNA fragments in B1 and B2 were cloned in the *Bam*HI site of pUC19, generating plasmids pUB1 and pUB2, respectively. The DNA fragments in S1 and S2 were cloned in the *Sal*I site of the same vector, generating plasmids pUS1 and pUS2, respectively. These plasmids were then used to transform *E. coli* JM109 and the transformants were screened by colony hybridisation (Fig. III.8).
Chapter III. Nitrite Reductase

Figure III.8 – Examples of colony hybridisations of *E. coli* JM109 cells transformed with plasmids: A. pUB1; B. pUB2; C. pUS1; D. pUS2. The arrows indicate colonies that hybridised with the probe (positive colonies). ⊕ denotes the positive controls (*E. coli* JM109 cells transformed with plasmid pUAx1).

Approximately between three and five positive colonies were identified per 100 colonies tested. These were picked and their plasmids were extracted. In order to determine the size of the inserts, plasmids pUB1 and pUB2 were digested with *BamHI* and plasmids pUS1 and pUS2 were digested with *SalI*. The digestion products were analysed on an agarose gel (Fig. III.9).

![Table: Size of inserts (bp)]

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3054</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2036</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1635</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure III.9 – Estimation of the size of the inserts in plasmids pUB1 (lane 1), pUB2 (lane 2), pUS2 (lane 3) and pUS1 (lane 4). A 0.8% (w/v) agarose gel showing the results of the digestions of plasmids pUB1, pUB2 with *BamHI* and pUS1, pUS2 with *SalI* is depicted.
Chapter III. Nitrite Reductase

Plasmid pUB2 contained, in all colonies assayed, a single insert of ~2.0 kb. Similarly, plasmid pUS2 contained a single insert of ~1.1 kb. The size of the fragment in each of these plasmids corresponded, respectively, to the size of the smaller BamHI- and SalI-restricted DNA fragments detected in the Southern blot analysis. Sequencing of the inserts in pUB2 and pUS2 showed that they contained part of the A. xylosoxidans nirA gene. In both cases, the fragment cloned corresponded to the 5’ portion of the gene. On the other hand, plasmids pUB1 and pUS1 showed multiple bands upon digestion. The reasons for this are not clear, but it is possible that they correspond to multiple inserts in the plasmids. Another possibility is that contamination from colonies containing different inserts had occurred. Whatever the reason, sequencing of the inserts in these plasmids did not reveal the presence of a portion of the nirA gene. Despite repeated attempts, it has not been possible to clone the B1 and S1 fragments. These results indicate that the DNA fragments including the 3’ portion of the nirA gene could not be cloned by the method used. This suggests that the DNA downstream of nirA may code for a product which is toxic to the transformed cells, therefore preventing its successful cloning under these conditions. Fig. III.10 summarises the hybridisation results discussed above.

![Diagram](image-url)

Figure III.10 – Hybridisation of SalI- and BamHI-digested DNA fragments of A. xylosoxidans with fluorescence-labelled probe. The blue line represents the nirA gene, the red line represents the probe, the green line represents the SalI-digested DNA fragments, the brown line represents the BamHI-digested DNA fragments and the yellow line represents the insert in plasmid pUS6 (see below). The dashed lines denote the regions which have been cloned in pUC19.
At this stage, approximately 70% of the total nirA gene sequence could be determined on both strands. In order to determine the remaining sequence of the gene, a new probe was prepared by radioactively labelling the fragment resulting from the digestion of plasmid pUAx1 with SalI and XbaI (Fig. III.11).

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3054</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1635</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>394</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure III.11 – Purification of ~500 bp DNA fragment for subsequent labelling. A 0.8 % (w/v) agarose gel showing: lane 1- 1 kb ladder DNA size markers; lane 2- SalI / XbaI-digested pUAx1. The arrow indicates the position of the band extracted from the gel.

The ~500 bp fragment thus obtained did not contain the region upstream the SalI restriction site which was present in the ~680 bp probe being, therefore, more specific to the 3’ end of the nirA gene. Freshly extracted A. xylosoxidans chromosomal DNA was digested with SalI and PstI restriction enzymes. DNA sequencing analysis (see Fig. III.15) showed that the nirA gene contains a PstI restriction site approximately 175 bp upstream from the SalI restriction site. Therefore, for both the digestions performed, the fragments hybridising with the ~500 bp probe did not contain the 5’ end of the nirA gene. Following digestion, the DNA fragments obtained were separated on a 0.8% (w/v) agarose gel, blotted onto a NC membrane and hybridised with the ~500 bp radioactive probe (Fig. III.12). Each of the SalI and PstI digestions of chromosomal DNA generated one fragment that hybridised with the radioactive probe (bands S and P). The size of these fragments was estimated to be ~1.4 kb and ~2.1 kb, respectively. Following a procedure similar to the one described above, they were extracted from a gel run under the same conditions as the one used for the Southern blot and cloned into the SalI and PstI restriction sites of pBR322. Following colony hybridisation, plasmids containing putative positive inserts were picked and their inserts sequenced. The sequences did not correspond to the nirA gene, further supporting the notion that the DNA downstream of the nirA gene may code for a product which is toxic to the cells.
Figure III.12 – Hybridisation of digested *A. xylosoxidans* chromosomal DNA with the radioactive ~500 bp probe. A. Separation of the digested fragments on a 0.8 % (w/v) agarose gel. B. Nitrocellulose membrane following hybridisation with the probe. Lane 1- 1 kb ladder DNA size markers; 2- *A. xylosoxidans* chromosomal DNA digested with *SalI*; 3- *A. xylosoxidans* chromosomal DNA digested with *PstI*; 4- Positive control (plasmid pUAx1 digested with *SalI* and *XbaI*). The arrows indicate the position of the bands excised from a gel of the same samples run under the same conditions as the gel shown. S indicates the fragment obtained from *SalI* digestion; P indicates the fragment obtained from *PstI* digestion.

As an alternative approach to obtain the sequence of the 3’ end of *nirA*, an inverse PCR (iPCR) strategy was followed, as schematised in Fig. III.13-A. The fragments extracted from bands S and P (see above) were self-ligated, generating “mini-circles” that contained the target sequence of the *nirA* gene. These “mini-circles” were then used as a template in a PCR experiment, using primers intended to amplify a DNA fragment containing the 3’ end sequence of that gene.
Chapter III. Nitrite Reductase

A

\[ \text{SalI} \quad \text{SalI} \quad \text{PstI} \quad \text{SalI} \quad \text{PstI} \]

\[ \text{nirA} \]

\[ \sim 1.0 \text{ kb} \]

\[ \text{SalI} \]

\[ \sim 1.3 \text{ kb} \]

\[ \text{SalI} \]

\[ \sim 2.1 \text{ kb} \]

\[ \text{SalI} \quad \text{SalI} \quad \text{SalI} \quad \text{SalI} \]

\[ \text{Ligation} \]

\[ \text{SalI} \]

\[ \text{“mini-circle”} \]

\[ \text{SalI} \quad \text{SalI} \quad \text{SalI} \quad \text{SalI} \]

\[ \text{iPCR} \]

\[ \sim 900 \text{ bp fragment} \]

\[ \text{PstI} \]

\[ \text{“mini-circle”} \]

\[ \sim 1.7 \text{ kb fragment} \]

unknown sequence of the \textit{nirA} gene
Chapter III. Nitrite Reductase

**B Size**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bp)</td>
<td>2036</td>
<td>1635</td>
<td>1018</td>
<td>516</td>
<td>2036</td>
<td>1635</td>
<td>1018</td>
<td>516</td>
</tr>
</tbody>
</table>

Figure III.13 - Inverse PCR experiment. A (previous page). Schematic representation of the inverse-PCR experiment, showing the positions of the oligonucleotides used as primers and the approximate expected size of the products. The red arrows represent primer Nit-15 and the blue arrows represent primer Nit-11. B. A 0.8% (w/v) agarose gel of the PCR products is shown. Lanes 1 and 5 contain the DNA size markers (1 kb ladder). Lanes 3 and 4 contain the PCR products of the \( \text{SalI} \) “mini-circles” using 1 and 10 ng template DNA, respectively. Lanes 7 and 8 contain the PCR products of the \( \text{PstI} \) “mini-circles” using 1 and 10 ng template DNA, respectively. Lanes 2 and 6 contain the negative controls for the \( \text{SalI} \) and \( \text{PstI} \) reactions, respectively. The arrows indicate the fragments showing the expected size of \(~900\) bp, obtained with the \( \text{SalI} \) “mini-circles”.

Synthetic oligonucleotides Nit-15 (5’-CCG CAC CTG ATC GGC GGC-3’) and Nit-11 (5’-CCT GCT CGC CAG GGT T GA-3’) were designed to be used, respectively, as forward and reverse primers, in this PCR reaction. The expected size of the PCR-amplified fragments resulting from the \( \text{SalI} \) and \( \text{PstI} \) “mini-circles” was estimated to be \(~900\) bp and \(~1.7\) kb, respectively. Agarose gel analysis of the PCR products showed that the PCR amplification of the \( \text{SalI} \) “mini-circles” yielded a fragment of the expected size (Fig. III.13-B). This fragment was extracted from an agarose gel and cloned into the \( \text{SmaI} \) restriction site of pUC19 (blunt-end ligation), generating plasmid pUS6. Sequencing of the insert in this plasmid revealed that it included the 3’ end of the \( \text{nirA} \) gene (see Fig. III.15). As can be seen in Fig. III.13-B, the PCR-amplification of the \( \text{PstI} \) “mini-circles” did not yield a fragment of the expected size. The reason why no fragments of the correct size were obtained was not
investigated further. However, it may have been due to incomplete ligatong of the \textit{PstI}-
digested fragments to create “mini-circles”.

Once the sequences for the 5’ and the 3’ ends of the gene had been
determined, oligonucleotides based on these sequences were designed and used as
primers for the PCR-amplification of the complete nitrite reductase gene, using \textit{A. xylosoxidans}
chromosomal DNA as template. The forward and reverse primers used
in this experiment were, respectively, Nit-UP2: 5’-GGG GAG CTC GAT AAG GAG
CTG GAC ATG-3’ and Nit-DOWN: 5’-GGA AGC TTC CAG TGC CAA TCT GAT
TGC-3’. These primers include synthetic recognition sequences for the restriction
enzymes \textit{SacI} and \textit{HindIII}, to allow subsequent cloning in both the sequencing and the
overexpression vectors. The forward primer also included the wild-type ribosome
binding site of the \textit{nirA} gene. Due to its extremely high fidelity, \textit{Pfu} polymerase from
Stratagene was used in this PCR experiment to amplify the \textit{nirA} gene. The PCR-
amplified \textit{nirA} gene was extracted from an agarose gel (Fig. III.14-A) and cloned into
\textit{pUC19} using the \textit{SacI} and \textit{HindIII} restriction sites, generating plasmid pUnirsp1 (Fig.
III.14-B).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
A & Size (bp) & 1 & 2 & 3 & 4 \\
\hline
3054 & 2036 & 1635 & 1018 & 516 & \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
B & Size (bp) & 5 & 6 \\
\hline
3054 & 2036 & 1635 & 1018 & 516 & \\
\hline
\end{tabular}
\end{table}

Figure III.14 – Cloning of the \textit{nirA} gene into \textit{pUC19}. A. A 0.8 (w/v) agarose gel showing the results of
the PCR amplification of the \textit{nirA} gene is depicted. The arrows indicate the bands that were extracted
from the gel. B. A 0.8 % (w/v) agarose gel showing the digestion of plasmid pUnirsp1 with \textit{SacI} and
\textit{HindIII}. The arrow indicates the band corresponding to the insert in this plasmid. Lanes 1 and 5 contain
the DNA size markers (1kb ladder); lanes 2, 3 and 4 contain the results of the PCR reaction using 0, 1
and 10 ng of template DNA, respectively; lane 6 contains the digestion products of plasmid pUnirsp1
with \textit{SacI} and \textit{HindIII}.
The insert in plasmid pUnirsp1 was sequenced completely and the sequence matched exactly that previously obtained for the inserts in plasmids pUAx1, pUB2, pUS2 and pUS6. The complete *nirA* sequence was thus obtained and its derived amino acid sequence deduced. The *nirA* gene encodes a 360-amino acid protein, the first 24 amino acids of which constitute the signal peptide (Berks, 1996 and see below). The complete sequence of the *nirA* gene and its derived amino acid sequence are shown in Fig. III.15 (see also Prudêncio *et al.*, 1999). The DNA sequence of *A. xylosoxidans* NiR was published later by Suzuki *et al.* (1999) and appeared in the NCBI Entrez database under the accession number AF051831. Their sequence matched that obtained in the present work. Also, the amino acid sequence of *A. xylosoxidans* NiR, determined by Edman degradation, was published by Vanderberghe *et al.* (1998) and matched the one presented here except that the gene sequence predicts the first codon of the mature enzyme to be a glutaminyl residue, whereas the N-terminal amino acid of the native NiR enzyme isolated from *A. xylosoxidans* has been shown to be pyroglutamate.

The amino acid sequence of the enzyme from *A. xylosoxidans* was compared to those of other copper-containing nitrite reductases (Fig. III.16). The comparison revealed a high degree of similarity between all of the sequences analysed. The percentage identity to *A. xylosoxidans* nirA ranged between 62% and 67% for the green NiRs from *Ach. cycloclastes* (accession number Z48635), *A. faecalis* (accession number D13155), *P. sp.* strain G-179 (accession number M97294), *Rhizobium hedysari* (accession number U65658) and *Rh. sphaeroides* (accession number U62291) and was 77% for the blue NiR from *Ps. aureofaciens* (accession number Z21945).

Of the seven proteins shown in Fig. III.16, only the NiRs from *A. xylosoxidans* and *Ps. aureofaciens* have short signal peptides with the characteristics typical of proteins secreted by the Sec-dependent export pathway (Berks, 1996). Interestingly, these are the only two blue NiRs depicted in Fig. III.16 and those that share the highest degree of similarity. The remaining five NiRs have long signal peptides of approximately 43 to 45 amino acids with a double-arginine motif close to the N-terminus (indicated by the arrows in Fig. III.16). As will be mentioned again in section IV.1.2., this type of signal sequence has been shown to be recognised by a novel export pathway that is Sec-independent and which has been proposed to secrete folded, redox-active proteins (Berks, 1996; Santini *et al.*, 1998; Sargent *et al.*, 1998; Weiner *et al.*, 1998). The possible physiological significance of this difference remains to be established.
Figure III.15 – Sequence of the *A. xylosoxidans* nirA gene and deduced amino acid sequence of the NiR protein. The red box indicates the ribosome-binding sequence. The blue box encompasses the signal peptide of the protein. The location of relevant restriction sites and primers is indicated.
Chapter III. Nitrite Reductase

**Figure III.16** – Alignments of the deduced amino acid sequences of copper-containing NiRs from different bacterial sources. Asterisks represent amino acid identity and dots indicate similar amino acids. Groups of similar amino acids are: aromatic amino acids, basic amino acids, acidic amino acids, and hydrophobic amino acids. The numerals 1 and 2 above the sequence alignment signify the type 1 and 2 Cu ligands, respectively. ALCXY, and hydrophobic amino acids. The numerals 1 and 2 above the sequence alignment signify the type 1 and 2 Cu ligands, respectively. ALCXY, and hydrophobic amino acids. The numerals 1 and 2 above the sequence alignment signify the type 1 and 2 Cu ligands, respectively.
III.2.1.2. Overexpression of nirA and overproduction of recombinant NiR

In order to overexpress the nirA gene and overproduce NiR, the insert in plasmid pUnirsp1 was subcloned into pET28a, using the SacI and HindIII restriction sites. This generated plasmid pEnirsp1 (Fig. III.17) which was used to transform E. coli BL21(DE3) cells.

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>6108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5090</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3054</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1635</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1018</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure III.17 – Cloning the nirA gene into pET28a. A 0.8% (w/v) agarose gel is shown: lane 1- DNA size markers (1kb ladder); lane 2- digestion products of plasmid pEnirsp1 with SacI and HindIII.

The nirA gene could be overexpressed in exponentially-growing E. coli BL21(DE3) cells containing vector pEnirsp1 by inducing the T7 RNA polymerase-directed transcription of this gene with IPTG. Different growth and induction conditions were studied using small-scale (5 to 10 ml) cultures in order to optimise overproduction (Fig. III.18). Overproduction at growth temperatures of 20°C, 30°C and 37 °C and IPTG concentrations ranging from 0.01mM to 1 mM was analysed. The use of lactose to induce overexpression was also tested. In these experiments induction was started when OD_{600} of exponentially growing cultures was 0.5 and cells were harvested 90 min after induction.
Figure III.18 – Analysis by SDS-PAGE containing 12.5% (w/v) acrylamide of total cellular polypeptides from cultures of *E. coli* overexpressing *nirA*, grown or induced under different conditions. A. Lane 1- Molecular mass markers; lane 2- 0.5 mM IPTG, 37°C; lane 3- 0.1 mM IPTG, 37°C; lane 4- 0.01 mM IPTG, 37°C; lane 5- 0.1% lactose, 37°C; lane 6- 0.5 mM IPTG, 30°C; lane 7- 0.5 mM IPTG, 20°C; lane 8- negative control, BL21(DE3). B. Lane 1- Molecular mass markers; lane 2- 1 mM IPTG, 30°C; lane 3- 0.5 mM IPTG, 30°C; lane 4- 0.25 mM IPTG, 30°C; lane 5- 0.1 mM IPTG, 30°C; lane 6- 0.01 mM IPTG, 30°C; lane 7- 1 mM IPTG, 37°C; lane 8- negative control, BL21(DE3). Induction was started at an OD₆₀₀ of 0.5. Cells were harvested 90 min after induction.

The extent of cell growth prior to induction and the time that cells were left to grow after induction was also assayed on a one-litre scale in a batch-fed fermentor. Cells were grown at 30 °C and induction with 0.25 mM IPTG was carried out when OD₆₀₀ was 0.5 or 1.0. Samples were then collected at 30 min intervals for up to 4 h and 30 min. The results are shown in Fig. III.19.

As can be observed in Figs. III.18 and III.19, the overproduced protein is present in two different forms. These correspond to an unprocessed form of the protein, of higher apparent molecular mass (~43 kDa), and a mature form of the protein, of lower apparent molecular mass (~35 kDa).
Chapter III. Nitrite Reductase

Figure III.19 – SDS-PAGE containing 12.5% (w/v) acrylamide of whole cell extracts of BL21 (DE3) containing pEnirsp1 grown at 30°C in a 1-litre fermenter. A. Induction at OD$_{600}$ ~0.5; B. Induction at OD$_{600}$ ~1.0. 1- Molecular mass markers; 2- negative control, BL21(DE3); 3- samples taken immediately after addition of 0.25 mM IPTG; 4 to 13 (A) or 4 to 11 (B)- samples taken at 30 min intervals after induction.

As can be observed in Fig. III.19, overproduction of NiR worked well for a number of conditions assayed. Growing the cells at 30°C, initiating induction with 0.25 mM IPTG when OD$_{600}$ was 0.5-0.6 and incubating the cultures at 30 ºC for a further 90 to 180 min with subsequent harvesting by centrifugation yielded the highest ratio of mature : unprocessed forms of the protein. Thus, these conditions were chosen to be used in subsequent experiments.

The N-terminal amino acid sequence of the two overproduced polypeptides detected by SDS-PAGE was determined. The N-terminus of the smaller polypeptide had the sequence Q-D-A-D-K-L, which is in perfect agreement with that predicted for the mature NiR protein lacking the signal sequence (see Figs. III.15 and III.16) and is identical to that determined for native NiR isolated from *A. xylosoxidans* (Vandenberghe et al., 1998). Thus, mature recombinant NiR (35 kDa) migrated with a molecular mass that was in close agreement with the DNA-deduced molecular mass of 36.5 kDa. Clearly, *E. coli*, unlike *A. xylosoxidans*, is unable to modify the N-terminal glutamine residue to pyroglutamate (Vandenberghe et al., 1998). The N-terminal sequence of the larger polypeptide was determined to be G-S-S-H-H-H-H, which corresponds to the first eight amino acids (minus the initiator Met residue) of the His tag peptide of pET28a. DNA sequence analysis of pEnirsp1 confirmed that the
Chapter III. Nitrite Reductase

*nirA* gene was fortuitously cloned in frame with the coding region of the polyhistidine tag of pET28a, generating a hybrid His-tagged NiR protein. This explains why the unprocessed 43-kDa peptide (His6-NiR) detected by SDS-PAGE had a molecular mass ~4 kDa greater than that predicted for the precursor form of NiR (~39 kDa). His6-Nir is, therefore, a fusion protein that includes the sequence of NiR with its signal peptide, plus a sequence of 43 amino acid residues originating from the pET28a vector.

To determine whether the mature NiR protein resulted from cleavage of the native precursor protein or the larger polypeptide, a pulse-chase experiment with $[^{35}\text{S}]\text{Met}$ was carried out (Fig. III.20). In this experiment, the production of a protein by *E. coli* can be monitored at specific time intervals. Samples were collected 30 s, 2 min, 10 min and 60 min after pulsing the cells with radioactive $[^{35}\text{S}]$-labelled methionine. When the experiment was performed with BL21(DE3) transformed with pEnirsp1 (Fig. III.20-A), two radioactive polypeptides with molecular masses of 43 kDa and 35 kDa were detected. Both polypeptides accumulated over the time period of the experiment and did not show a typical precursor-product relationship. Surprisingly, the expected ~39-kDa precursor of NiR was not detected. The reasons for this are unclear. A possible explanation would be that processing of the precursor into mature NiR would occur at a much faster rate in this strain than in the one containing the pEnirsp2 plasmid (see below), but there is no obvious reason why that would happen. At present, the possibility that a proportion of the His6-NiR fusion protein produced in the pEnirsp1-containing strain is processed into mature NiR cannot be ruled out.

Plasmid pEnirsp1 was digested with *Nde* I, the resulting 5’ overhanging ends were filled-in using Klenow enzyme and the plasmid was re-ligated. This procedure inserted two bases in the pEnirsp1 plasmid, thereby generating plasmid pEnirsp2 which carries a frameshift in the coding region for the larger polypeptide carrying the polyhistidine tag. When the previous experiment was repeated with BL21(DE3) transformed with pEnirsp2 (Fig. III.20-B), the large 43-kDa protein was not synthesised. However, the processed, mature 35-kDa NiR polypeptide was still produced at a level similar to that observed when the experiment was performed with pEnirsp1. This result strongly suggests that the precursor of mature NiR was not the 43-kDa polypeptide. This finding was further supported by the appearance of a polypeptide of ~41 kDa, which had a size slightly larger than that expected for the
wild-type precursor. Nevertheless, this polypeptide exhibited a precursor-product relationship with the 35 kDa protein, suggesting that it is indeed the precursor of mature NiR.

![Image](image_url)

Figure III.20 – [35S]Met-labelling of induced *E. coli* BL21(DE3) containing the pEnirsp1 (A) and pEnirsp2 (B) plasmids. Samples were taken 30′′, 2′, 10′ and 60′ after addition of radioactive methionine. Whole-cell extracts of the samples were separated by SDS-PAGE containing 12.5% (w/v) acrylamide.

**III.2.1.3. Subcellular localisation and purification of recombinant NiR**

In *A. xylosoxidans*, NiR is a periplasmic enzyme (See section III.1.3). In order to determine the subcellular localisation of the overproduced forms of NiR in the heterologous host *E. coli*, periplasmic and cytoplasmic fractions of induced overproducing cells and non-overproducing cells (control) were separated and analysed by SDS-PAGE containing 12.5% (w/v) acrylamide (Fig. III.21).

The results obtained showed that approximately 50% of mature NiR is found in the periplasm (Fig. III.21, lane 3+), whereas the uncleaved, His-tagged NiR fusion polypeptide was exclusively located in the cytoplasm (Fig. III.21, lane 2+), in the form of insoluble inclusion bodies. This is a clear indication that the native NiR signal peptide is recognised and efficiently cleaved by the *E. coli* export apparatus. These results also provide further support for the contention that mature NiR does not result
from cleavage of the hybrid His-tagged fusion protein, since this is in inclusion bodies and, therefore, inaccessible for export to the periplasm.

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure III.21 - Subcellular localisation of recombinant NiR in the heterologous host *E. coli* BL21(DE3). Polypeptides (50 µg of total protein per lane) were separated by SDS-PAGE in a gel containing 12.5 % (w/v) acrylamide. Lanes: 1- whole cell extract; 2- insoluble material from the cytoplasmic fraction; 3- periplasmic fraction. The signs - and + denote the BL21(DE3) control and BL21(DE3) containing pEnirsp1, respectively.

Mature, recombinant NiR was purified to homogeneity following the scheme outlined in Fig. III.22-A which was a modification of that described in Abraham *et al.* (1993). Purification of NiR from the periplasmic fraction was achieved in a single chromatographic step on a carboxymethyl cellulose (CMC) column. For small-scale purifications (up to 5 g wet weight of cells) the freshly-extracted periplasmic fraction was dialysed and subsequently loaded onto the CMC column (15 cm x 1.5 cm). For larger-scale purifications, the proteins in the periplasmic fraction were precipitated with 65% (w/v) ammonium sulphate and resuspended in 10 mM Tris-HCl buffer, pH 7.0 (~0.5 ml / g cells) prior to dialysis. In either case, the CMC column was equilibrated with H₂O and the unbound fraction was removed with H₂O. Nitrite reductase was eluted from the column with 20 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl. Fig. III.22-B shows the electrophoretic pattern of the purified sample.
Recombinant NiR was purified from growths of pEnirsp1-containing *E. coli* BL21(DE3) done at various scales: two-litre growths were performed in flasks with vigorous shaking and yielded ~1.8 g wet weight of cells per litre of culture; twenty- and two hundred-litre growths typically yielded ~2.5 and ~4.5 g wet weight of cells per litre of culture, respectively. Approximately 0.5 mg of purified NiR were obtained from 1 g of cells.

Figure III.22 - Purification of mature, recombinant nitrite reductase. A. Purification scheme. The carboxymethyl cellulose column was equilibrated with H$_2$O. Eluted material was monitored by following the absorbance at 280 nm. B. Analysis of NiR by SDS-PAGE containing 12.5% (w/v) acrylamide during the different purification stages. Lane 1- Molecular mass markers; lane 2- Whole cell extract of BL21(DE3) (control; 60 µg of protein); lane 3- Whole cell extract of induced BL21(DE3) containing pEnirsp1 vector (50 µg of protein); lane 4- Periplasmic fraction from induced BL21(DE3) containing pEnirsp1 vector (20 µg of protein); lane 5- Purified NiR after carboxymethyl cellulose chromatography (5.5 µg of protein).
III.2.1.4. Characterisation of recombinant NiR and comparison with the wild-type enzyme

Recombinant NiR purified from various growths of *E. coli* cells was characterised in terms of its catalytic, spectroscopic and biochemical properties. These were compared to those of the wild-type NiR (wt-NiR) from *A. xylosoxidans* and the two proteins were shown to be indistinguishable with regard to the parameters studied.

### III.2.1.4.1. NiR activity

The specific activity of the as-purified enzyme, as determined by the methyl viologen-linked assay, was 10.8 units/mg of protein, where 1 unit is defined as 1 µmol of nitrite reduced-min⁻¹. This is a rather low specific activity which corresponds to a type 2 Cu-depleted (T2D) form of the enzyme (Howes *et al.*, 1994; Abraham *et al.*, 1997; Eady *et al.*, 1997; Suzuki *et al.*, 1997; Prudêncio *et al.*, 1999 and see below). The activity of the protein increased significantly upon incubation with CuSO₄ (see section II.2.2.2 for details). An interesting difference in the extent of reactivation was observed dependent upon the stage of purification at which the incubation with CuSO₄ was performed. Incubation of the purified protein with CuSO₄ resulted in an enzyme with a specific activity of 47.4 units/mg. This corresponds to a ~4-fold increase in activity when compared with the as-purified, non-activated enzyme. However, incubation of the periplasmic fraction with CuSO₄, prior to purification, resulted in an enzyme with a specific activity of 167.7 units/mg, a ~15-fold increase in activity compared with the non-activated enzyme. This activation was found to be even higher when incubation of the periplasmic fraction with CuSO₄ was performed after precipitation of the periplasmic proteins with ammonium sulphate. One batch of protein purified under these conditions had a specific activity of 345.3 units/mg, which corresponds to a ~30-fold increase in activity relative to that of the non-activated enzyme. However, this result could not be reproduced and cannot be explained at present. Values of 150 units/mg, 240 units/mg and 300 units/mg have been found for the specific activity of wt-NiR (Abraham *et al.*, 1993).
This increase in activity following incubation with CuSO₄ corresponds to a reconstitution of the type-2 Cu centres in the protein (Prudêncio et al., 1999 and see below). The results above suggest that the incorporation of Cu in the type 2 sites of NiR is facilitated by the activity of a periplasmic protein or peptide. When the purified enzyme is incubated with CuSO₄, only partial reconstitution of the type 2 Cu sites occurs. This finding is in accord with previous observations that suggested the presence of an “insertase” in A. xylosoxidans, involved in the insertion of type 2 Cu in the wt-NiR (Dr. Robert Eady, personal communication). The fact that activation of the enzyme is more effective on a concentrated periplasmic protein extract than on a diluted periplasmic fraction is in accord with the presence of an “insertase” in the periplasm of the cells.

Nitrite reductase activity determined using either reduced azurin or dithionite as electron donors (see sections II.2.2.4.1.2 and II.2.2.4.1.3) gave similar results for the reconstituted recombinant NiR and the wild-type enzyme. For the dithionite assay these values were 10.7 ± 1.1 µmol of dithionite oxidised-min⁻¹·mg⁻¹ and 9.3 ± 0.7 µmol of dithionite oxidised-min⁻¹·mg⁻¹, respectively; for the azurin assay, the activities determined were, respectively, 83.8 ± 18.0 µmol of azurin oxidised-min⁻¹·mg⁻¹ and 77.35 ± 15.0 µmol of azurin oxidised-min⁻¹·mg⁻¹.

III.2.1.4.2. Spectroscopy and metal content

Purified, concentrated (~10 mg/ml) recombinant NiR was dark blue in colour. The UV-Vis spectrum of the as-purified, recombinant NiR (Fig. III.23) is very similar to that of wt-NiR with an absorption maximum at 592 nm, and a ~10-fold smaller absorption maximum at ~470 nm. The absorption maximum at ~590 nm is characteristic of oxidised type 1 Cu centres of NiR. The Abs₂₈₀nm / Abs₅₉₂nm ratio provides an indication of the type 1 Cu content of the protein as well as information regarding the oxidation state of the Cu.

For the wt-NiR, this ratio ranges between 12.0 and 18.0, for reasons that are unclear (Abraham et al., 1993; Dr. Robert Eady, personal communication). The ratios found for the recombinant NiR ranged between 11.6 and 16.4, which is in good agreement with the ratios observed for the wt-NiR. A higher value for this ratio is indicative of only partial occupancy of type 1 Cu centres.
Chapter III. Nitrite Reductase

Figure III.23 – UV-Vis absorption spectrum of recombinant NiR. The spectrum was recorded at room temperature in 20 mM MES buffer, pH 6.0 at 1.1 mg/ml protein. The inset shows an expansion of the visible region of the spectrum.

The electron paramagnetic resonance (EPR) spectrum of the as-purified, non-activated NiR shows the typical features of type 1 Cu and a complete absence of a type 2 Cu EPR signal (Fig. III.24-A). Metal analysis indicated that this protein contains ~2.1 Cu atoms / trimer and the Abs$_{280nm}$ / Abs$_{592nm}$ was ~20. This shows that this form of the enzyme is T2D and also does not have a full type 1 Cu complement. These results are in accord with the low specific activity found for this protein.

Upon incubation of the purified protein with CuSO$_4$, type 2 Cu EPR features appear in the spectrum of NiR (Fig. III.24-B). The intensity of these features is lower than that of the type 1 signal, the ratio Abs$_{280nm}$ / Abs$_{592nm}$ is ~16 and the Cu content is increased to ~3.8 Cu atoms / trimer, based on metal analysis. This indicates that the type 2 Cu sites in the protein have been only partially reconstituted. Again, this result is consistent with the value obtained for the activity of this form of the enzyme.

The EPR spectrum of the NiR purified after incubation of the periplasmic fraction with CuSO$_4$ shows type 1 Cu and type 2 Cu features of about equal intensities (Fig. III.24-C). The ratio Abs$_{280nm}$ / Abs$_{592nm}$ was ~13 and the protein was estimated to have ~6.3 Cu atoms / trimer based on metal analysis. These results show that the protein has a full complement of both type 1 Cu and type 2 Cu centres. As expected, this form of the enzyme has maximal activity. This correlation between the type 2 Cu
Figure III.24 – EPR spectra of recombinant NiR in 20 mM MES buffer, pH 6.0. A. As-purified protein (T2D form) at 9.4 mg/ml (255 µM monomer); B. Protein incubated with CuSO₄ after purification (partially reconstituted form) at 6.1 mg/ml (165 µM monomer); C. Protein purified after incubation of the periplasmic fraction with CuSO₄ (fully reconstituted form) at 4.8 mg/ml (135 µM monomer). The spectra were recorded at 60 K at a microwave frequency of 9.312 GHz and normalised for ease of comparison. The inset shows the low-field region of the spectrum in greater detail.
content and the activity of NiR is consistent with the notion that the type 2 Cu centres in this enzyme are the sites of catalysis (Libby and Averill, 1992; Howes et al., 1994; Strange et al., 1995; Abraham et al., 1997; Eady et al., 1997; Suzuki et al., 1997; Dodd et al., 1997; Murphy et al., 1997; Dodd et al., 1998; Farver et al., 1998; Veselov et al., 1998). The low activity found for the T2D form of the enzyme is in accord with the suggestion that T2D NiRs are able to catalyse the reduction of nitrite (Masuko et al., 1994; Kohzuma et al., 1994; Suzuki et al., 1997). However, as previously mentioned, this activity may also be accounted for by the presence of a low complement of type 2 Cu in proteins reported as T2D.

Simulation of the experimental EPR spectra measured at pH 6.0 determined the type 1 Cu and type 2 Cu EPR parameters of the fully reconstituted enzyme (Fig. III.25). The type 1 Cu centre has an axial EPR spectrum with $g_\perp = 2.11$ ($A_\perp = 0$ mT) and $g_\parallel = 2.29$ ($A_\parallel = 6.4$ mT). The EPR spectrum of the type 2 Cu centre has a small degree of rhombicity with $g_x = 2.12$ ($A_x = 0$ mT), $g_y = 2.13$ ($A_y = 4.6$ mT) and $g_z = 2.41$ ($A_z = 12.5$ mT). Although the signal of type 2 Cu in wt-NiR is referred to as axial, the values obtained for the EPR parameters of recombinant NiR are in reasonably good agreement with those reported for the wild-type enzyme from *A. xylosoxidans*: $g_\parallel = 2.21$, $A_\parallel = 6.3$ mT for type 1 Cu; $g_\parallel = 2.30$, $A_\parallel = 12.5$ mT for type 2 Cu (Abraham et al., 1993).

**III.2.1.4.3. Molecular mass and thermostability**

Recombinant NiR was shown to be indistinguishable from the wild-type, native enzyme from *A. xylosoxidans*, both in terms of electrophoretic pattern under denaturing conditions (Fig. III.26) and when chromatographed on a Superdex-200 HR 10/30 gel filtration column (data not shown). This indicates that the recombinant NiR is, like its native counterpart, a homotrimer of ~109 kDa global mass (~36 kDa per monomer) (Abraham et al., 1993; Grossman et al., 1993).
Figure III.25 – Simulation of the type 1 Cu and type 2 Cu signals in the EPR spectrum of fully reconstituted recombinant NiR in 20 mM MES buffer, pH 6.0. A. Experimental spectrum collected under the conditions described above and in the legend of Fig. III.24; B. Simulation of the type 1 Cu signal; C. Simulation of the type 2 Cu signal; D. Addition of the simulations of the type 1 Cu and the type 2 Cu signals.
III.2.1.4.4. Carbonic anhydrase and superoxide dismutase activities

The similarity between the substrate-binding site in Cu-containing NiR and the Zn site in carbonic anhydrase (CA) has been noted in reports by Strange et al. (1995) and Dodd et al. (1998). Strange et al. (1999) have shown that T2D-NiR from A. xylosoxidans does not have CA activity. In order to address this issue further and examine whether or not other forms of NiR exhibit CA activity, this was determined for different forms of the enzyme. Particular attention was given to a form of recombinant NiR (hereafter called ZnNiR) which appeared to be nearly Cu-depleted (0.5 Cu atoms / trimer) but had, instead, an unusually high Zn content (2.8 Zn atoms / trimer). ZnNiR was obtained from a 200-litre growth of pEnirsp1-containing E. coli
Chapter III. Nitrite Reductase

BL21(DE3). The reasons why this protein had such an unusual metal content are unclear, but it is possible that the copper available during the growth of the cells in the fermentor was limiting for incorporation in NiR. ZnNiR had extremely low nitrite reductase activity (<0.1 units/mg) and the presence of ~3 Zn atoms per trimer suggested that it might have CA activity. Recombinant NiR and wt-NiR were also assayed together with commercial CA as a control in these experiments. CA activity was measured using a stopped-flow spectrofluorimetric method which measures the initial rates of pH change as a result of bicarbonate dehydration (Shingles and Moroney, 1997; see section II.2.2.4.3 for details).

The catalysed reaction has a higher initial rate (and, therefore, reaches the equilibrium faster) than the non-catalysed reaction. As can be observed in Fig. III.27, none of the forms of NiR studied had CA activity. This was also true for ZnNiR, even when it was assayed at 100 times the concentration of the CA used as control. This clearly shows that, despite the similarities between the catalytic centres in NiR and CA, the former cannot catalyse the reaction catalysed by the latter. One possible explanation for this is that the Zn detected in the ZnNiR protein was adventitious and the protein does not contain, as was assumed, Zn in the type 2 Cu site. This has already been noted for A. xylosoxidans T2D-NiR, and it has been suggested that NiR has controls in place to ensure that Zn does not enter the active site (Strange et al., 1999). Another possibility is that, although Zn is present at that site, differences in the ligand geometry between the Zn sites of the two proteins are responsible for the absence of activity in ZnNiR. Although no structure of Zn-containing NiR is available, that of CA where Zn has been replaced by Cu has been determined (Håkansson et al., 1994) and indicates that the ligand geometry around the Cu atom is different between the two enzymes, possibly supporting the latter hypothesis. The availability of an X-ray structure of Zn-containing NiR could provide further insight into this issue.

Similarities have also been noted between the type 2 Cu site of NiR and the Cu site in reduced superoxide dismutase (Cu2Zn2 SOD or SOD) in reports by Strange et al. (1995), Dodd et al. (1998) and Strange et al. (1999). Strange et al. (1999) have reported that SOD activity was absent from A. xylosoxidans T2D-NiR but was present at a significant level in A. xylosoxidans NiR with a full complement of type 1 Cu and
type 2 Cu. These experiments have been repeated and extended to different forms of the enzyme.

Figure III.27 – Carbonic anhydrase activity followed by stopped-flow spectrofluorimetry as described by Shingles and Moroney (1997). Activity was determined as a function of the initial rates, given by the increase in fluorescence emission. Only the commercial carbonic anhydrase (CA - pink line) was shown to have carbonic anhydrase activity. The blank reaction was performed in the absence of any enzyme. CA, recombinant NiR and wt-NiR were present at 1 µg/ml in the assay. ZnNiR x 100 denotes ZnNiR used at 100 times the concentration of CA, i.e., at 100µg/ml. Activity assays were performed at 30ºC.

In particular, the SOD activity of recombinant NiR and wt-NiR from *A. xylosoxidans* was measured by the method of Paoletti and Mocali (1990) (see section II.2.2.4.4 for details). This method is based on the oxidation of NAD(P)H, which is followed by measuring the decrease in absorbance at 340 nm. This nucleotide oxidation is linked to the availability of superoxide anions in the medium and is, therefore, inhibited by SOD. As shown in Fig. III.28, neither of the forms of NiR studied exhibited SOD activity, even when as much as ~30 µg of protein were used in
Chapter III. Nitrite Reductase

the assay. This was also true for wt-NiR, in contrast to the report of Strange et al. (1999), where 700 ng of this enzyme inhibited NADPH oxidation by 50%. Approximately 105 ng of the commercial SOD used as a positive control in these experiments caused 50% inhibition of NADPH oxidation, in good agreement with the value obtained for the control experiment of Strange et al. (1999) (50% inhibition of NADPH oxidation by 80 ng of enzyme). The difference between the results obtained in these experiments and those of Strange et al. (1999) can only be explained by assuming a very high difference in SOD activities in two different batches of A. xylosoxidans wt-NiR. The results obtained in this work clearly indicate that NiR does not have superoxide dismutase activity suggesting that, despite the similarities found between the catalytic site in NiR and the Cu site in SOD, differences in geometry and oxidation state of the site may play a determinant role in catalysis.

III.2.1.4.5. Crystallisation of recombinant NiR

In order to obtain crystals of recombinant NiR that would allow the determination of the X-ray structure of this protein, fully reconstituted recombinant NiR was crystallised using conditions similar to those used for the crystallisation of wt-NiR, in the Daresbury laboratory, Daresbury, UK. The crystallisation buffer used in those studies was 0.1 M Tris-HCl, pH 8.5 containing 0.1 M MgCl₂ and 30% (w/v) polyethylene glycol (PEG) 4000 as precipitant (Mr. Mark Ellis, personal communication). Fully reconstituted recombinant enzyme was exchanged into 0.1 M Tris-HCl, pH 8.5 and its concentration was adjusted to 5.3 mg/ml. Crystallisation trays were prepared by the hanging-drop, vapour-diffusion method (Fig. III.29, see also Materials and Methods) with PEG 4000 as the precipitant at 4 ºC. Crystallisation experiments were performed where: (1) the concentration of PEG 4000 was varied between 20% (w/v) and 30% (w/v) in steps of 2% (w/v) at a constant concentration of 100 mM MgCl₂; (2) the concentration of MgCl₂ was varied between 0 mM and 200 mM in steps of 40 mM at a constant concentration of 30% (w/v) PEG 4000; (3) the concentration of PEG 4000 was varied between 20% (w/v) and 30% (w/v) in steps of 2% (w/v) at a constant concentration of 0 mM MgCl₂; (4) the concentration of MgCl₂ was varied between 0 mM and 60 mM in steps of 20 mM at a constant concentration of 30% (w/v) PEG 4000.
Figure III.28 – Superoxide dismutase activity measured as a function of the degree of inhibition of NADPH oxidation. To account for changes in the rate of NADPH oxidation in two sets of experiments, a blank reaction (enzyme solution replaced by H₂O) was performed for each sample assayed. The difference in the slopes of the sample and its respective blank is proportional to SOD activity. Only the commercial SOD used as a control in this experiment (SOD - red line) was shown to have superoxide dismutase activity. The data shown for SOD were obtained with 105 ng of protein and correspond to 50% inhibition of NADPH oxidation. The data obtained for recombinant NiR (Rec. NiR) and *A. xylosoxidans* wild-type NiR (wt-NiR) were both obtained with >30 µg protein and show no inhibition of NADPH oxidation. The origin of the lines was corrected for clarity. Activity determinations were carried out at room temperature.
Needle-shaped crystals of NiR (Fig. III.30) were obtained after 2-3 weeks at 30% (w/v) PEG 4000 with both 40 mM and 80 mM MgCl₂.

Figure III.29 - Setup for crystallisation of recombinant NiR by the hanging-drop, vapour-diffusion method. Four microlitre droplets (2 µl protein solution + 2 µl reservoir solution) were set against 1 ml of reservoir solution.

Figure III.30 – Needle-shaped crystals of recombinant NiR obtained at 30% (w/v) PEG with 40 mM MgCl₂. Fully reconstituted recombinant NiR protein had a concentration of 5.3 mg/ml in 0.1 M Tris-HCl, pH 8.5. Crystals were formed after 2-3 weeks at 4°C.
III.2.2. Site-directed mutagenesis

III.2.2.1. Selection of mutations

As previously mentioned, site-directed mutagenesis was used in this work as an important tool to probe relationships between structure and function of nitrite reductase. With this objective in mind, several mutations were designed aiming at further understanding the role of the metal ligands in the enzyme, as well as that of residues in the vicinity of the copper centres. The selected mutations can be divided into two main groups based on the type 1 Cu and type 2 Cu centres, and the rationale behind their selection is explained below:

III.2.2.1.1. Mutations of the type 1 Cu ligands

The type 1 Cu in NiR is ligated by two histidine nitrogen atoms, one cysteine sulphur and one methionine sulphur. In the enzyme from *A. xylosoxidans*, the type 1 Cu exhibits a distorted trigonal planar geometry, and is ligated by His89N$^{δ}$, His139N$^{δ}$, Cys130S$^{γ}$ (three strong planar ligands) and Met144S$^{δ}$ (axial ligand) [Dodd et al. (1997, 1998). See also section III.1.6.2 and Fig. III.31].

The His139 residue was mutated into an alanine residue. This mutation should perturb type 1 Cu ligation without, however, directly disturbing the peptide bridges for electron flow from the type 1 Cu to the type 2 Cu centre. An effect on the redox potential of type 1 Cu is also expected as a result of breaking the Cu-His bond, which thereby reduces the coordination geometry of Cu to three-fold (Canters and Gilardi, 1993).

The Cys130 residue was mutated into an alanine residue. The Cys residue is believed to be the only type 1 Cu ligand which is essential for maintaining the type 1 character of the site (Mizoguchi *et al.*, 1992; Canters and Gilardi, 1993). Cys130 provides a peptide bridge linking both Cu centres via His129. Replacement of this residue should perturb this bridge and may have an effect on the type 2 Cu site. The accessible surface area for the two amino acids is similar, which should prevent steric constraints in the immediate environment of the replaced residue.
Finally, the Met144 residue was mutated into an alanine residue. The Met type 1 Cu ligand has been mutated in other type 1 Cu-containing proteins and shown to have an effect on the optical properties of the centre as well as on its redox potential (Murphy et al., 1993; Pascher et al., 1993; Romero et al., 1993; Bonander et al., 1996; Olesen et al., 1998). The Ala residue is unable to coordinate the Cu atom in the type 1 site and the difference in the accessible surface areas of the two residues is likely to affect the geometry of the site. The effects of this mutation on the properties of the type 1 Cu centre in NiR and on the electron transfer between the two Cu centres in the enzyme are interesting issues that will be addressed with this mutant.

III.2.2.1.2. Mutations in the environment of the type 2 Cu centre

The type 2 Cu in *A. xylosoxidans* NiR is ligated by His94 and His129 from one monomer and His300 from another monomer. All three His residues ligate the copper via their N\textsubscript{ε} atoms (Dodd et al., 1997, 1998. See also section III.1.6.3 and Figs. III.31 and III.32). Two other residues of potential importance are Asp92 and His249. These two residues hydrogen-bond a water molecule which is close to the copper atom (not the coordinated water) and are likely to play an important role in the active-site pocket. This is suggested by the studies of Adman et al. (1995) on the enzyme from *A. cycloclastes*, and is in agreement with the results of Murphy et al. (1997) for the enzyme from *A. faecalis* and those of Dodd et al. (1997, 1998) for the enzyme from *A. xylosoxidans* (see section III.1.6.4).

The Asp92 residue is suggested by the above-mentioned authors to hydrogen-bond nitrite upon its binding to the Cu active site. A mutation in the Asp92 residue is, therefore, likely to perturb the binding of the nitrite ion to the Cu atom. Aspartate has a negatively charged side-chain, capable of hydrogen-bonding to the water molecule. This residue was replaced by glutamic acid and asparagine.

The same authors suggested the His249 residue to be of potential importance in the binding of nitrite to the type 2 Cu. Although it is known that hydrogen-bonding between nitrite and His249 is not possible, this residue is hydrogen-bonded to a water found near the type 2 Cu, which also hydrogen bonds to Asp92 (Adman et al., 1995; Dodd et al., 1998). The His249 residue was mutated into a phenylalanine residue in order to try to understand the role of this residue in substrate binding and catalysis.
Fig. III.31 – The type 1 (dark blue sphere) and type 2 (light blue sphere) sites of *A. xylosoxidans* NiR showing the peptide bridges (His129 – Cys130 and His89 – Asp92 – His94) between the Cu atoms. The type 2 exogenous ligand (Cu/Water) is shown in green and the water hydrogen-bonded to Asp92 in brown. Sulphur atoms from the Met and Cys residues are shown in yellow. Residues from monomer 1 and monomer 2 are coloured in green and red, respectively. The arrows indicate the mutated residues. The picture was kindly provided by Dr. Robert Eady.
III.2.2.2. Site-directed mutagenesis programme

Site-directed mutagenesis of NiR residues was performed using the QuikChange site-directed mutagenesis method, from Stratagene. This method is described in detail in section II.2.1.16. It utilises a supercoiled, double-stranded DNA vector with an insert of interest and two complementary synthetic oligonucleotide primers, each containing the desired mutation. Plasmid pEnirsp1, which carries the nirA gene, was used as a template to obtain the mutations outlined.

The synthetic oligonucleotide primers used for mutagenesis were between 24 and 30 bases in length and are listed in Table III.1. The plasmids containing the mutagenised inserts were isolated from E. coli XL1-Blue cells and, following sequencing over the mutated region to confirm the mutation, cloned into E. coli BL21(DE3) for subsequent overproduction of the mutant proteins.

III.2.2.3. Characterisation of mutant NiR proteins

E. coli BL21(DE3) cells carrying the plasmids containing the mutagenised nirA genes were grown using the optimum growth and inducing conditions determined for overexpression of nirA (see section III.2.1.2). All the mutant proteins were exported to the periplasmic space of E. coli and N-terminal sequencing of the first five amino acid residues of the mature mutant proteins gave, in all cases, the sequence Q-D-A-D-K, confirming that cleavage occurred at the expected amino acid residue.
Chapter III. Nitrite Reductase

Table III.1 – Synthetic oligonucleotides used as primers to generate mutants of NiR. Underlining denotes the bases that were changed relative to the wild-type gene sequence. F- Forward primer; R- Reverse Primer.

<table>
<thead>
<tr>
<th>Mutant Proteins</th>
<th>Synthetic Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>His139Ala</td>
<td>5’- G GTG CCC TGG <strong>GCC</strong> GTG GTG TCG GG -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- CC CGA CAC CAC <strong>GCC</strong> CCA GGG CAC C -3’  R</td>
</tr>
<tr>
<td>Met144Ala</td>
<td>5’- GTG GTG TCG GGC <strong>GCG</strong> AGC GGC ACG CTG -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- CAG CGT GCC GCT <strong>GCC</strong> GCC CGA CAC CAC -3’  R</td>
</tr>
<tr>
<td>Cys130Ala</td>
<td>5’- CC TTC GTC TAC CAC <strong>GCC</strong> GCC CCC GAA GG -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- CC TTC GGG CGC <strong>GCG</strong> GTG GTA GAC GAA GG -3’  R</td>
</tr>
<tr>
<td>Asp92Glu</td>
<td>5’- CG CAC AAC GTC <strong>GAG</strong> TTC CAC GGC GC -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- GC GCC GTG GAA <strong>CTC</strong> GAC GTT GTG CG -3’  R</td>
</tr>
<tr>
<td>Asp92Asn</td>
<td>5’- CG CAC AAC GTC <strong>AAC</strong> TTC CAC GGC GC -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- GC GCC GTG GAA <strong>GTT</strong> GAC GTT GTG CG -3’  R</td>
</tr>
<tr>
<td>His249Phe</td>
<td>5’- GC GAC ACC CGC <strong>CCG</strong> TTC CTG ATC GCC G -3’  F</td>
</tr>
<tr>
<td></td>
<td>5’- C GCC GAT CAG GAA <strong>CGG</strong> GCG GGT GTC GC -3’  R</td>
</tr>
</tbody>
</table>

Overproduced mutant proteins were purified from *E. coli* BL21(DE3) cells following a procedure similar to the one described for recombinant NiR. All the mutant proteins showed a chromatographic behaviour very similar to that of the recombinant NiR protein and were purified from the periplasmic fraction in a single step.

Initially, all the mutant proteins were purified from small-scale (usually two-litre) growths (up to 5 g wet weight of cells) of *E. coli* BL21(DE3) cells containing the appropriate plasmids. Typical yields from these growths were 1.5 to 2.0 mg purified mutant protein / litre of culture. Basic biochemical and spectroscopic characterisation of these proteins was performed and, based on these results, large-scale (two hundred-litre) growths of cells overproducing selected mutant proteins were carried out. Yields of these growths were up to 7.5 mg purified mutant protein / litre of culture. The following sections deal with the characterisation of these mutants.
In all cases, the data presented were obtained from proteins purified from 200-litre growths of cells grown in the presence of a 1 mM CuSO\textsubscript{4} supplement. Two forms of each mutant were purified, one from the untreated periplasmic fraction of these cells and one from periplasm incubated overnight with 100 µM CuSO\textsubscript{4}. Throughout the following sections these two forms of each mutant protein will be called UxyzW and UxyzW\textsuperscript{Cu}, respectively, where U stands for the one-letter code of the original residue, W stands for the one-letter code of the replacement residue and xyz stands for the residue number. The superscript \textsuperscript{Cu} denotes the protein purified from the Cu-incubated periplasmic fraction.

Also, throughout the following sections, the activity of the mutant proteins will be given relative to the activity of the recombinant NiR. As described previously, activity was measured using methyl viologen, sodium dithionite and reduced azurin I as electron donors. The activity of recombinant NiR with these donors, to which the activities of the mutants were reported, was as follows: 170 µmol of nitrite reduced-min\textsuperscript{-1}·mg\textsuperscript{-1} for the viologen assay; 10 µmol of dithionite oxidised-min\textsuperscript{-1}·mg\textsuperscript{-1} for the dithionite assay; 83 µmol of azurin oxidised-min\textsuperscript{-1}·mg\textsuperscript{-1} for the azurin assay (see section III.2.1.4.1).

For all the mutant proteins studied, a computer simulation of the mutated sites has been performed by Dr. Fraser Dodd at the Daresbury Laboratory, Daresbury, UK and will also be presented.

III.2.2.3.1. His139Ala mutant form of NiR

In the His139Ala mutant form of the NiR protein a basic amino acid residue, positively charged at pH 6.0 to 7.0, was replaced with one that is essentially nonpolar and, therefore, hydrophobic at this pH. Furthermore, the two amino acid residues have quite different sizes, as indicated by the difference in their molecular masses (155.2 g/mol for His and 89.1 g/mol for Ala) and accessible surface areas (195 Å\textsuperscript{2} for His and 115 Å\textsuperscript{2} for Ala). The side-chain of His contains an imidazole group whereas Ala contains an aliphatic hydrocarbon side-chain.

Taking into account that His139 is one of the strong planar ligands of type 1 Cu in NiR, it is expected that this mutation will cause a significant perturbation of this site. Fig. III.33 shows the type 1 Cu site in NiR and a computer simulation of the replacement of His by Ala in the H139A mutant form of the protein.
Figure III.33 – The His139Ala mutation in NiR. The figure shows the type 1 Cu site found in the native protein and a simulation of the same site where His139 has been replaced by Ala. Carbon atoms are represented in grey (α-carbon atoms in black), sulphur atoms are represented in yellow and nitrogen atoms are represented in dark blue (positive charges). The Cu atom is represented in light blue. The carboxyl group in the Cys residue is represented in red (negative charge). The pictures were kindly produced and provided by Dr. Fraser Dodd.
III.2.2.3.1. UV/Vis and EPR spectroscopies

The UV/Vis spectrum of the H139A and the H139A\textsuperscript{Cu} proteins, purified from 200 g of cells, did not have an absorbance maximum in the visible region (Fig. III.34) and the protein solutions were colourless even at concentrations above 40 mg/ml.

The EPR spectrum of the H139A protein, purified from the untreated periplasm of cells grown in medium supplemented with 1 mM CuSO\textsubscript{4}, clearly showed a type 2 Cu signal (Fig. III.35-B) with $g_\perp = 2.16$ ($A_\perp = 3.2$ mT) and $g_\parallel = 2.44$ ($A_\parallel = 10.0$ mT), designated Signal 1. In contrast, the H139A\textsuperscript{Cu} protein, purified after incubation of the periplasmic fraction with CuSO\textsubscript{4}, displayed a more complex EPR spectrum (Fig. III.35-A) which was composed of two different type 2 Cu signals. By simulation of the former spectrum and subtraction from the latter (Fig. III.35-C), the two signals could be deconvoluted and this additional type 2 signal in the spectrum, designated Signal 2, could be simulated. The EPR parameters of this signal were determined to be $g_\perp = 2.13$ ($A_\perp = 1.6$ mT $A_\parallel = 0$ mT) and $g_\parallel = 2.31$ ($A_\parallel = 18.0$ mT). In neither case did the EPR spectrum of H139A show a type 1 Cu signal.

The results obtained show that the type 2 Cu EPR signal in His139Ala is different depending on whether or not the Cu atoms in this centre are incorporated during the growth of the cells. The data obtained for this protein indicates that the EPR signal of the type 2 Cu incorporated during growth exhibits EPR Signal 1, whereas the protein isolated from the periplasmic fraction incubated with CuSO\textsubscript{4} has an additional type 2 Cu species which exhibits EPR Signal 2. A comparison of the integrated areas corresponding to each one of these two signals showed that they contribute 59\% and 41\%, respectively, to the spectrum in Fig. III.35-A. Since the type 2 Cu signal is dependent upon the environment of the Cu atom in this site and shows some variability for different preparations of NiR (Abraham \textit{et al.}, 1993; Howes \textit{et al.}, 1994; Dr. Robert Eady, personal communication), these two signals may correspond to two different forms of type 2 Cu centre.
Figure III.34 – UV/Vis spectra of the His139Ala mutant form of NiR. The green line corresponds to the spectrum of the H139A<sup>Cu</sup> protein (protein purified after reconstitution of the periplasmic fraction with CuSO<sub>4</sub>) at a concentration of 0.55 mg/ml; the blue line represents the spectrum of the H139A protein (protein purified from the same batch of cells and where no reconstitution of the periplasmic fraction was performed) at a concentration of 1.1 mg/ml. The inset shows an expansion of the visible region of the spectra. The spectra were recorded at room temperature in 100 mM Tris-HCl, pH 7.1.

### III.2.2.3.1.2. Is type 1 Cu present in His139Ala?

The UV/Vis and EPR data presented so far show a complete absence of type 1 Cu spectroscopic features. This would indicate that either H139A is type 1 Cu-depleted (T1D) or that the type 1 Cu in the protein is reduced and, therefore spectroscopically silent, when analysed by the techniques used. Previous reports of mutations performed on His ligands to the type 1 Cu in azurin have shown that this residue was not essential to maintain a type 1 Cu site (reviewed in Canters and Gilardi, 1993) and would thus suggest that H139A was not T1D. The amount of EPR-detectable Cu in different samples of H139A was estimated by integration and their total Cu content was determined by metal analysis. The results obtained are summarised in Table III.2.
Figure III.35 - EPR spectra of the His139Ala mutant purified from cells grown in medium supplemented with 1 mM CuSO₄. A. Spectrum of the H139A⁸Cu protein (protein purified after reconstitution of the periplasmic fraction with CuSO₄) (22.1 mg/ml in protein). B. Spectrum of the H139A protein (purified from the untreated periplasmic fraction of the same batch of cells) (43.6 mg/ml in protein). C. Difference spectrum between spectra A and B. A’, B’ and C’ are the theoretical simulations of spectra A, B and C, respectively. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.40 GHz. The spectra were normalised to the same protein concentration to facilitate comparison.
Table III.2 – Estimation of the copper content of the His139Ala mutant. EPR-detectable copper was determined by comparison of double integrals with a Cu-EDTA standard. Total copper was determined by ICP analysis. The numbers correspond to the number of copper atoms per protein trimer. The H139A\textsuperscript{Cu} protein was purified from cells grown on medium supplemented with 1 mM CuSO\textsubscript{4}, after reconstitution of the periplasmic fraction with CuSO\textsubscript{4}. The H139A protein was purified from the untreated periplasmic fraction of the same batch of cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EPR-detectable copper</th>
<th>Total copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>H139A\textsuperscript{Cu}</td>
<td>2.71 (Signal 1 + Signal 2)</td>
<td>5.67</td>
</tr>
<tr>
<td>H139A</td>
<td>1.65 (Signal 1)</td>
<td>4.41</td>
</tr>
</tbody>
</table>

The presence of ~6 Cu atoms in sample H139A\textsuperscript{Cu} clearly indicates that this protein possesses a full complement of both type 1 Cu and type 2 Cu. Furthermore, the results of metal analysis are in perfect agreement with the conclusions drawn from the quantification of the independent contributions of both forms of type 2 Cu to the spectrum of the fully loaded, H139A\textsuperscript{Cu}, sample. Thus, both samples of the His139Ala mutant have a full complement of type 1 Cu which is spectroscopically silent and must, therefore, be reduced.

Since only oxidised type 1 Cu centres are detectable by UV/Vis and EPR spectroscopies, attempts were made to oxidise the H139A protein with a 5-fold molar excess of potassium ferricyanide (\(E_0^\text{\textsuperscript{Fe(CN)}\textsubscript{6}^{3-}} \approx +430\text{ mV}\)) relative to the type 1 Cu content. However, the UV/Vis spectrum of H139A remained unchanged even after 90 min incubation with this oxidant (data not shown), indicating that the potential of the type 1 Cu site in His139Ala must be higher than the potential of potassium ferricyanide.

In order to oxidise the Cu in the type 1 centres, the strong oxidant potassium hexachloroiridate (IV) (K\textsubscript{2}IrCl\textsubscript{6}, \(E_0^\text{\textsuperscript{IrCl\textsubscript{6}^{2+}}} \approx -800\text{ mV}\)) was used. A 5 mg/ml solution of H139A in 100 mM Tris-HCl, pH 7.1 was titrated with K\textsubscript{2}IrCl\textsubscript{6} and UV/Vis spectra were recorded after 2 min incubation (Fig. III.36).
Figure III.36 – UV/Vis spectra of the His139Ala mutant (5 mg/ml in protein) upon addition of increasing amounts of K$_2$IrCl$_6$. Spectra of H139A were recorded at room temperature in a 500 µl cuvette. Spectra were normalised for absorption at 820 nm to account for baseline shifts and/or effects of protein precipitation.

An absorption band in the 600 nm region of the spectrum started to appear when the concentration of K$_2$IrCl$_6$ was ~1 mM, which corresponds to an approximately 7.5-fold molar excess of K$_2$IrCl$_6$ over the estimated type 1 Cu content of the protein. The absorption in this region continued to increase upon addition of up to ~4.5 mM (~30-fold molar excess) of oxidant and did not change further for concentrations of K$_2$IrCl$_6$ of up to 6.5 mM (~50-fold molar excess).
The fully oxidised H139A had an absorption maximum at 618 nm, which corresponds to a shift of ~26 nm with regard to NiR. The final ratio of \( \text{Abs}_{280\text{nm}} / \text{Abs}_{618\text{nm}} \) was ~30, significantly higher than that found for NiR (Abraham et al., 1993). It should be noted, however, that for \( \text{K}_2\text{IrCl}_6 \) concentrations above ~1 mM some protein precipitation was observed which cannot be quantified but may help explain the high value obtained for that ratio.

Although the redox potential of the type 1 Cu in His139Ala was not determined, the high excess of oxidant used in this experiment suggests that this potential is remarkably high, possibly in the vicinity of the potential of \( \text{K}_2\text{IrCl}_6 \), (~+ 800 mV). This would correspond to a ~560 mV increase with regard to the redox potential reported for type 1 Cu in NiR from *A. xylosoxidans*, which is + 240 mV (Suzuki et al., 1994; see also Farver et al., 1998). The consequence of this would be the uncoupling of the two Cu redox centres. An increase in the redox potential as a result of the replacement of one of the His ligands in the type 1 Cu centre is not unexpected, since a 3-fold coordination of the Cu atom favours the Cu(I) over the Cu(II) state (reviewed in Canters and Gilardi, 1993). However, the likely extent of the increase observed in the present case would constitute the highest redox shift ever reported for a type 1 Cu centre as a result of a single point mutation.

**III.2.2.3.1.3. Activity and substrate binding**

Nitrite reductase activity of His139Ala was determined by three different methods and compared to that of recombinant NiR. These methods used two artificial electron donors, methyl viologen and dithionite, and a physiological electron donor, reduced azurin I (see Materials and Methods for details). These studies provide information on electron flow between type 1 Cu and type 2 Cu, as well as on accessibility to the Cu atoms in the catalytic sites. The results obtained are summarised in Table III.3.
Several conclusions can be drawn from the results obtained. The data of Table 3 show a clear difference in the activity of His139Ala depending upon the type of electron donor. Dithionite was clearly the most effective electron donor whereas methyl viologen functioned only very poorly and no activity was observed with reduced azurin I as donor. Assuming that the type 1 Cu and the type 2 Cu redox centres in the protein are uncoupled as a consequence of the shift in the redox potential of the type 1 Cu site and therefore electron donation from type 1 Cu to type 2 Cu does not occur, these data show that direct electron transfer by artificial donors to the catalytic type 2 Cu site is possible. The difference in the effectiveness of dithionite and methyl viologen as electron donors for catalysis could be explained on the basis of their different accessibilities to the type 2 Cu site. The lack of activity when reduced azurin I, the physiological electron donor to type 1 Cu in NiR, was used provides further confirmation for the uncoupling of the two Cu centres thus preventing electron transfer from type 1 Cu to type 2 Cu in the His139Ala mutant.

The data presented in Table III.3 also provide useful information on the catalytic ability of the different type 2 Cu species detected by EPR spectroscopy, giving rise to Signal 1 and Signal 2. As previously shown, Signal 1 is formed when Cu is incorporated in vivo and Signal 2 when Cu is incorporated in vitro, by addition of CuSO₄ to the periplasmic fraction. There is little difference in the activity of His139Ala regardless of whether the sample was purified after incubation of the periplasmic fraction with CuSO₄ or not. This indicates that the type 2 Cu incorporated
following activation of the periplasmic extract is not catalytically functional. Thus, it is possible that the type 2 Cu atoms that generate this EPR signal are in an environment which renders them unable to bind and reduce nitrite, possibly due to the presence of an extra ligand. The catalytically active species corresponds to a form of type 2 Cu that displays an EPR signal atypical for NiR, with $g_\parallel = 2.44$ and $A_\parallel = 10.0$ mT (see section III.2.2.3.1.1).

III.2.2.3.1.3.2. Substrate binding to His139Ala monitored by EPR

When nitrite binds to NiR, the EPR spectrum of the type 2 Cu shows a decrease in $g_\parallel$ and an increase in $A_\parallel$ (Howes et al., 1994; Abraham et al., 1997). Nitrite binding to the type 2 site Cu in His139Ala was monitored by EPR spectroscopy. The H139A protein was used in this experiment. This protein has an estimated type 2 Cu content of 1.65 atoms / trimer (see Table III.2) and was titrated with various amounts of NaNO$_2$ up to a final concentration of 45 mM and the EPR spectra thus obtained were recorded. Fig. III.37 shows the EPR spectra corresponding to the stages in the transition from H139A without nitrite to H139A in the presence of excess nitrite. The bottom panel of Fig. III.37 shows an expansion of the low-field region of these spectra, demonstrating the changes in the $g_\parallel$ and $A_\parallel$ parameters. As occurs with NiR, the $g_\parallel$ value of H139A decreases with increasing concentrations of nitrite whilst $A_\parallel$ follows the opposite trend. Simulation of the EPR spectrum of H139A in the presence of excess nitrite (not shown) determined $g_\parallel = 2.37$ with $A_\parallel = 11.0$ mT (hereafter called Signal 1’. See below, Fig. III.39), as opposed to the original values of $g_\parallel = 2.44$ and $A_\parallel = 10.0$ mT.

The presence of the isosbestic points in the spectra shows that only two species of this centre are involved in this process, thus allowing the corresponding changes to be measured (Fig. III.38). The intensity of the EPR spectra at a given point (see Fig. III.37) was plotted against the ratio of nitrite and type 2 Cu molar ratios and the data were fitted assuming an equilibrium between the bound and unbound species. The analysis of the binding curves for the NO$_2^-$-induced change in type 2 Cu EPR gave a best-fit binding constant for H139A at pH 7.1 of ~1000 μM, approximately 3-fold higher than that determined for nitrite binding to NiR at pH 7.5 (Abraham et al., 1997).
Figure III.37 – EPR spectra of His139Ala mutant form of NiR during titration with nitrite. Sodium nitrite up to a concentration of 45 mM was added to the protein in 100 mM Tris-HCl, pH 7.6. The spectra were normalised to the same protein concentration to account for the dilution occurring as the NaNO₂ solution was added. The top panel shows the complete EPR spectra of H139A (22.1 mg/ml protein concentration) and the bottom panel shows an expansion of their low-field region. The spectra were recorded at 60 K, at a microwave frequency of 9.40 GHz. The arrow in the bottom panel shows where the measurements plotted in Fig. III.38 were taken.
Figure III.38 – Titration of the His139Ala mutant with nitrite, monitored by EPR. The points are the intensities of the EPR signal of H139A at the position indicated by the arrow in the bottom panel of Fig. III.37. The curve shows a fit to the data using a binding constant of ~1.0 mM.

The EPR data in Fig. III.37 clearly show that the form of the type 2 Cu site which generates the EPR Signal 1 (see Fig. III.35), corresponding to the Cu incorporated during the growth of the cells, is catalytically active and changes its geometry upon binding nitrite. The question arises as to whether the form of type 2 Cu centre that generates Signal 2 (see Fig. III.35), corresponding to the Cu incorporated upon reconstitution of the periplasmic fraction with CuSO₄, is capable of binding nitrite. To answer this question, the H139A⁰Cu sample containing both forms of type 2 Cu centre was incubated with a 20-fold excess of nitrite and the resulting EPR spectrum was compared with that of the H139A sample containing only one form of type 2 Cu centre, also in the presence of excess nitrite (Fig. III.39). The results clearly show that when the EPR signal of sample H139A (Fig. III.39-B) was subtracted from the EPR signal of sample H139A⁰Cu (Fig. III.39-A) (both in the presence of excess nitrite), the resulting spectrum (Fig III.39-C) is dominated by a type 2 Cu signal with $g_\parallel = 2.32$ and $A_\parallel = 17.8$ mT as determined from its simulation (Fig. III.39-C'). These parameters are virtually identical to the values of Signal 2,
showing that this form of type 2 Cu site does not bind nitrite. Conversely, when the simulation of this signal (Fig. III.39-C’) was subtracted from the spectrum of sample H139A\textsuperscript{Cu} in the presence of excess nitrite (Fig. III.39-A), the resulting spectrum (not shown) had \( g_v = 2.37 \) and \( A_v = 11.0 \) mT, which are the same values as those found for Signal 1’. This is completely consistent with the finding that H139A\textsuperscript{Cu} and H139A have similar activities (see Table III.3).

These results are summarised in Table III.4 and clearly show that only one form of type 2 Cu centre in H139A is capable of binding nitrite and is catalytically active.

Table III.4 – Summary of activity and nitrite-binding results for His139Ala mutant form of NiR. A. H139A\textsuperscript{Cu} protein, purified from cells grown on medium supplemented with 1 mM CuSO\textsubscript{4} after reconstitution of the periplasmic fraction with CuSO\textsubscript{4}. B. H139A protein, purified from the same batch of cells as in A and where no reconstitution of the periplasmic fraction was performed.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>(two forms of type 2 Cu)</td>
<td>(one form of type 2 Cu)</td>
<td></td>
</tr>
<tr>
<td>Signal 1</td>
<td>Signal 2</td>
<td>Signal 1</td>
</tr>
<tr>
<td>((g_v = 2.44; A_v = 10.0 \text{ mT}))</td>
<td>((g_v = 2.31; A_v = 18.0 \text{ mT}))</td>
<td>((g_v = 2.44; A_v = 10.0 \text{ mT}))</td>
</tr>
<tr>
<td><strong>Incubation with 20-fold molar excess of nitrite over type 2 Cu</strong></td>
<td><strong>Incubation with 20-fold molar excess of nitrite over type 2 Cu</strong></td>
<td></td>
</tr>
<tr>
<td>Signal 1’</td>
<td>Signal 2</td>
<td>Signal 1’</td>
</tr>
<tr>
<td>((g_v = 2.37; A_v = 11.0 \text{ mT}))</td>
<td>((g_v = 2.32; A_v = 17.8 \text{ mT}))</td>
<td>((g_v = 2.37; A_v = 11.0 \text{ mT}))</td>
</tr>
<tr>
<td>catalytically active</td>
<td>catalytically inactive</td>
<td>catalytically active</td>
</tr>
</tbody>
</table>
Figure III.39– EPR spectra of samples of the His139Ala mutant in the presence of a 20-fold molar excess of nitrite over the type 2 Cu. A. Spectrum of the H139A\(^{\text{Cu}}\) protein (protein purified from cells grown on medium supplemented with 1 mM CuSO\(_4\), after reconstitution of the periplasmic fraction with CuSO\(_4\)) (~22 mg/ml in protein). B. Spectrum of the H139A protein (purified from the untreated periplasmic fraction of the same batch of cells) (~43 mg/ml in protein). C. Difference spectrum between spectra A and B. C’. Theoretical simulation of spectrum C. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.40 GHz. The spectra were normalised to the same protein concentration to facilitate comparison.
III.2.2.3.2. Met144Ala mutant form of NiR

In the Met144Ala mutant form of the NiR protein, an essentially nonpolar amino acid replaces one that is also nonpolar and hydrophobic at pH 6.0 to pH 7.0. The two amino acid residues have quite different sizes, as indicated by the difference in their molecular masses (149.2 g/mol for Met and 89.1 g/mol for Ala) and accessible surface areas (185 Å$^2$ for Met and 115 Å$^2$ for Ala). The side-chain of Met contains a sulphur atom which axially ligation the Cu atom in the type 1 centre of NiR, whereas Ala contains an aliphatic hydrocarbon side-chain and is unable to provide this ligation to Cu.

It is expected that this mutation will not prevent assembly of the type 1 Cu site, as has been found in various studies of type 1 Cu-containing proteins such as azurin (Karlsson et al., 1991; Murphy et al., 1993; Pascher et al., 1993; Romero et al., 1993; Bonander et al., 1996; Strange et al., 1996), rusticyanin (Hall et al., 1999) or nitrite reductase (Kukimoto et al., 1994; Murphy et al., 1995; Averill et al., 1998; Olesen et al., 1998; Veselov et al., 1998). Fig. III.40 shows the type 1 Cu site in NiR and a computer simulation of the replacement of Met by Ala in the Met144Ala mutant.

III.2.2.3.2.1. Spectroscopy and metal content

The M144A and M144ACu proteins were purified from 200 g of cells. Both proteins were blue in colour and their UV/Vis spectra are shown in Fig. III.41.

The absorption bands in the 600 nm region of the spectra indicate that both proteins contain type 1 Cu centres. The ratio $\text{Abs}_{280\text{nm}} / \text{Abs}_{592\text{nm}}$ is 18.8 for M144A and 15.2 for M144ACu. Both these values are within the range of the values found for NiR (Abraham et al., 1993) and are indicative of a full, or nearly full, complement of type 1 Cu in both forms of the protein. The difference between the values found for M144A and M144ACu suggests that some incorporation of type 1 Cu occurred upon incubation of the periplasmic fraction with CuSO$_4$. 

Figure III.40 – The Met144Ala mutation in NiR. The figure shows the type 1 Cu site found in the native protein (top) and a simulation of the same site where Met144 has been replaced by Ala (bottom). Carbon atoms are represented in grey (α-carbon atoms in black), sulphur atoms are represented in yellow and nitrogen atoms are represented in blue (positive charges). The carboxyl group in the Cys residue is represented in red (negative charge). The pictures were kindly produced and provided by Dr. Fraser Dodd.
Figure III.41 – UV/Vis spectra of the His139Ala mutant form of NiR. The green line corresponds to the spectrum of the M144A\textsuperscript{Cu} protein (purified from cells grown on medium supplemented with 1 mM CuSO\textsubscript{4}, after reconstitution of the periplasmic fraction with CuSO\textsubscript{4}) at a concentration of 0.55 mg/ml; the blue line represents the spectrum of the M144A protein (purified from the same batch of cells but without reconstitution of the periplasmic fraction) at a concentration of 1.1 mg/ml. The inset shows an expansion of the visible region of the spectra. Both spectra were recorded at room temperature in 100 mM Tris-HCl, pH 7.1.

The EPR spectra of M144A and M144A\textsuperscript{Cu} are shown in Fig. III.42. The two spectra are quite similar, suggesting that there was little incorporation of Cu upon incubation of the periplasmic fraction with CuSO\textsubscript{4}.

The EPR spectra obtained proved to be quite complex and their simulation was not successfully accomplished. It is possible that they are the result of a composition of the signals arising from a type 1 Cu centre and more than one form of type 2 Cu centre.
Figure III.42 – EPR spectra of Met144Ala mutant forms of NiR. A. Spectrum of the M144A<sup>Cu</sup> protein, purified from cells grown on medium supplemented with 1 mM CuSO<sub>4</sub>, after reconstitution of the periplasmic fraction with CuSO<sub>4</sub> (6.9 mg/ml protein concentration)  B. Spectrum of the M144A protein, purified from the same batch of cells, where no reconstitution of the periplasmic fraction was performed (14.9 mg/ml protein concentration). The arrows indicate the position of type 2 Cu features similar to those found in the H139A protein. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.41 GHz. The spectra were normalised to the same protein concentration to facilitate comparison.
In any case, it seems clear that a type 2 Cu signal similar to Signal 1 found in H139A (with $g_\parallel = 2.44$ and $A_\parallel = 10.0$ mT) is present in the spectra (indicated by the arrows in Fig. III.42. Compare with Fig. III.35). This is consistent with the previous finding that the form of type 2 Cu centre that gives rise to this signal is incorporated during growth of the cells.

Somewhat surprisingly, neither M144A nor M144A$^{Cu}$ contains a full complement of Cu, as shown by integration of the EPR spectra and metal analysis (Table III.5).

Table III.5 – Estimation of the copper and zinc contents of the Met144Ala mutant. EPR-detectable copper was determined by comparison of double integrals with a Cu-EDTA standard. Total copper and total zinc were determined by ICP analysis. The numbers correspond to the number of metal atoms per protein trimer. The M144A$^{Cu}$ protein was purified from cells grown on medium supplemented with 1 mM CuSO$_4$ after reconstitution of the periplasmic fraction with CuSO$_4$. The M144A protein was purified from the same batch of cells but no reconstitution of the periplasmic fraction was performed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EPR-detectable copper</th>
<th>Total copper</th>
<th>Total zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>M144A$^{Cu}$</td>
<td>3.77</td>
<td>4.0</td>
<td>1.86</td>
</tr>
<tr>
<td>M144A</td>
<td>3.08</td>
<td>4.12</td>
<td>3.02</td>
</tr>
</tbody>
</table>

Assuming that both proteins contain a full, or nearly full, complement of type 1 Cu, as suggested by the UV/Vis data presented above, this lower-than-expected copper content must be due to a partial depletion of the type 2 Cu centres. As can be observed in Table III.5, both the samples contain a considerable amount of Zn. Although the hypothesis that this may be adventitious Zn cannot be excluded, it is also possible that at least some of the Zn atoms found in the Met144Ala samples are occupying the type 2 Cu sites, thereby preventing the assembly of a full complement of type 2 Cu centres.

**III.2.2.3.2.2. Activity and substrate binding**

Nitrite reductase activity of Met144Ala was determined by three different methods, using methyl viologen, dithionite and azurin I as electron donors (see
section II.2.2.4.1 for details) and compared to that of recombinant NiR. The results obtained are summarised in Table III.6.

Table III.6 – Activity of the Met144Ala mutant with three different electron donors. The M144A\textsuperscript{Cu} protein was purified from cells grown on medium supplemented with 1 mM CuSO\textsubscript{4} after reconstitution of the periplasmic fraction with CuSO\textsubscript{4}. The M144A protein was purified from the same batch of cells, where no reconstitution of the periplasmic fraction with CuSO\textsubscript{4} was performed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methyl viologen</th>
<th>Dithionite</th>
<th>Azurin I</th>
</tr>
</thead>
<tbody>
<tr>
<td>M144A\textsuperscript{Cu}</td>
<td>31.5 ± 4.5</td>
<td>48.9 ± 8.0</td>
<td>38.6 ± 5.5</td>
</tr>
<tr>
<td>M144A</td>
<td>24.3 ± 3.5</td>
<td>26.6 ± 3.5</td>
<td>39.5 ± 9.0</td>
</tr>
</tbody>
</table>

The first conclusion that can be drawn from the results above is that there seems to be little difference between the activities of M144A\textsuperscript{Cu} and M144A when methyl viologen or the physiological donor, azurin I, are used as electron donors in the assay. This is in accord with the results found for the Cu content of the two forms of the protein (See Table III.5), which suggest a type 2 Cu content of ~1.0 Cu atoms / trimer and ~1.1 Cu atoms / trimer, respectively, for the two forms of the protein (assuming a full complement of type 1 Cu for both). This corresponds approximately to 35% of a full complement of type 2 Cu centres and is in agreement with the activity values obtained for the two electron donors mentioned.

The results obtained when dithionite was used as an electron donor in the assay are somewhat more difficult to interpret since the two proteins exhibit quite different activities. These results are difficult to rationalise since the Cu contents of M144A\textsuperscript{Cu} and M144A do not differ significantly. Furthermore, the relative activity of M144A\textsuperscript{Cu} is higher than what would be expected from its estimated type 2 Cu content. A tentative explanation for the data taking into account the Zn content of the two proteins and the possibility that catalysis may occur at a metal-depleted type 2 Cu site can be proposed. Reduced catalytic activity of T2D NiR compared to the fully loaded enzyme has been reported (Masuko \textit{et al.}, 1984; Suzuki \textit{et al.}, 1997; Prudêncio \textit{et al.}, 1999), suggesting that catalysis may indeed occur at a T2D site. Thus, considering
that the M144A has ~3.0 Zn atoms / trimer whereas M144ACu has only ~1.9 Zn atoms / trimer, the following explanation can be put forward: if the Zn found in the M144ACu protein is adventitious, the type 2 Cu sites in this form of the protein are metal-depleted and are therefore able to perform the reduction of nitrite when dithionite is used as the electron donor. The extra Zn found in the M144A protein, relative to M144ACu (~1.1 Zn atoms) may occupy the type 2 Cu sites and thus prevent them from being catalytically functional, which would explain the difference in the activities of the two proteins with dithionite as electron donor. The lower relative activity of M144ACu when methyl viologen is used to donate electrons may be explained assuming that only dithionite is effective in donating electrons to a metal-depleted type 2 Cu site whilst methyl viologen donates electrons mainly via the type 1 Cu site. Thus, the extra activity found for M144ACu with dithionite may be accounted for by direct electron donation to the type 2 site. Evidently, when azurin is used as the electron donor, electrons must flow from the type 1 Cu atoms to the type 2 Cu atoms and a metal-depleted type 2 Cu centre is therefore unable to reduce nitrite.

The substrate-binding ability of Met144Ala was analysed by EPR. The M144A form of the protein was incubated with a 20-fold molar excess of nitrite over the estimated type 2 Cu content and the EPR spectrum thus obtained was recorded (Fig. III.43).

It is clear from the spectra in Fig. III.43 that nitrite binds the M144A protein and thereby promotes a change in its EPR spectrum. Even in the absence of the simulations of both the spectra before and after addition of substrate, it seems clear that the features at low-field, tentatively assigned to a signal similar to Signal 1 found in H139ACu and in H139A (see Fig. III.35), have changed (signalled by the arrows in Fig. III.43). Furthermore, when the spectrum of M144A in the absence of nitrite (Fig. III.43-A) is subtracted from the spectrum of the same protein in the presence of excess nitrite (Fig. III.43-B), the resulting spectrum (Fig. III.43-C) has a signal with $g_\parallel = 2.37$ and $A_\parallel = 11.2$ mT. This signal is similar to the Signal 1’ found both in H139A Cu and in H139A in the presence of excess nitrite (See Fig. III.39). This constitutes a clear indication that the M144A protein contains a form of type 2 Cu centre similar to the one found in His139Ala, which is capable of binding nitrite and is probably responsible for at least part of the catalytic activity displayed by the Met144Ala mutant protein.
Figure III.43 – Substrate binding to Met144Ala monitored by EPR. A. Spectrum of the M144A protein (protein purified from the untreated periplasmic fraction of cells grown on medium supplemented with 1 mM CuSO₄), (9.4 mg/ml in protein). B. Spectrum of the same protein after incubation with a 20-fold molar excess of nitrite relative to the estimated type 2 Cu content. C. Spectrum B after subtraction of spectrum A. The arrows indicate the position of the type 2 Cu features that change upon incubation with nitrite, which is similar to what was found for the His139Ala mutant (see text). The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.41 GHz.
III.2.2.3.2.3. X-ray structure

The X-ray structure of Met144Ala was determined to 2.2 Å resolution by Dr. Fraser Dodd, Mr. Mark Ellis and Prof. Samar Hasnain at the Daresbury Laboratory, Daresbury, UK. Crystals of M144A\textsubscript{Cu}, obtained by Mr. Mark Ellis at the same laboratory, were used to collect a 99.4 % complete data set on this protein. The trimeric structure of Met144Ala is shown in Fig. III.44. A more detailed picture of the copper centres in this protein and a comparison with NiR is shown in Fig. III.45.

Figure III.44 – X-ray structure of the trimeric Met144Ala protein. Monomers are represented in blue, green and orange, with the two shadings of each colour representing the two domains in each subunit. Copper atoms are represented as grey spheres, with the type 1 Cu centres buried in the polypeptide chain of each monomer and the type 2 Cu sites located at the interface between two adjacent subunits. Crystals of Met144Ala grew in 25% (w/v) PEG 4000, 0.1 M MgCl\textsubscript{2} and 0.1 M Tris-HCl, pH 8.5. The picture was kindly produced and provided by Mr. Mark Ellis and Dr. Fraser Dodd.
Figure III.45 – The copper sites in Met144Ala. The figure shows a superposition of the structures of the Cu centres in Met144Ala (red) and in wild-type NiR from *A. xylosidans* (yellow). Both the structures were determined from crystals grown at pH 8.5. The letters A and B represent two different monomers of the protein. The Wat600 water molecule resides in the same position for both structures. The water molecule represented by Wat504 is exclusive of the structure of NiR. The long chain His89-His94 was used as the marker to align the two structures. The picture was kindly produced and provided by Mr. Mark Ellis and Dr. Fraser Dodd.
Chapter III. Nitrite Reductase

The trimeric structure is identical to that of the previous models used for the structure of NiR (Dodd et al., 1997, 1998). The model of the Met144Ala structure contains 336 residues, 1 magnesium atom and 162 water molecules. In addition, the difference density map of M144A$^{Cu}$ shows the presence of a Cu atom in the type 1 Cu site and 85 % - 100 % occupancy of Cu in the type 2 Cu site. However, the model does not exclude the possibility that some of the metal in this site may be Zn (Mr. Mark Ellis, personal communication). A full complement of type 2 Cu in the structure of the M144A$^{Cu}$ protein would be surprising, considering the results obtained from the determination of the metal content of this protein. Thus, it is possible that the Cu in the type 2 sites of Met144Ala may have been partially incorporated during the crystallisation of the protein or that, indeed, the site is partially occupied by Zn. It should also be noted that the type 1 Cu in the crystallised Met144Ala appeared to be in the reduced state, as suggested by the fact that the crystals were colourless (Mr. Mark. Ellis, personal communication). The reasons for this are not clear but are likely to be related to the crystallisation conditions used. The type 1 Cu site in Met144Ala shows a slight change in geometry with regard to NiR as a result of the loss of the axial ligand.

III.2.2.3.3. Cys130Ala mutant form of NiR

In the Cys130Ala mutant form of the NiR protein an essentially nonpolar amino acid replaces one that has an uncharged, polar side-chain. Polar amino acid residues are relatively more soluble in water than those with nonpolar side-chains and contain functional groups that are capable of forming hydrogen-bonds. The two amino acid residues have different sizes, as indicated by the difference in their molecular masses (121.2 g/mol for Cys and 89.1 g/mol for Ala) and accessible surface areas (135 Å$^2$ for Cys and 115 Å$^2$ for Ala). The side-chain of the Cys residue contains a sulfhydryl (-SH) group. It is also the sulphur atom in this residue that ligates the Cu in type 1 Cu centres (see Fig. III.31). The short Cu-S bond has a significantly covalent character and the absorption band in the 600 nm region, characteristic of the blue copper proteins, primarily results from ligand-to-metal-charge-transfer (LMCT) transitions that occur between the S and the Cu atoms (Han et al., 1993; Shadle et al., 1993; Kaim and Rall, 1996).
The Cys residue is believed to be the only ligand which is essential for maintaining the type 1 character of the site as shown by studies in which the replacement of this residue has led to a complete loss of Cu binding or the conversion of the site into a type 2 Cu site (Mizoguchi et al., 1992; Canters and Gilardi, 1993). It is, therefore, anticipated that this mutation will cause a major perturbation of the type 1 Cu site. The effects of this mutation on the type 2 Cu site of NiR will also be monitored closely. Fig. III.46 shows the type 1 Cu site in NiR and a computer simulation of the replacement of Cys by Ala in the Cys130Ala mutant.

### III.2.2.3.3.1. Spectroscopy and metal content

The C130A and the C130A\(^{\text{Cu}}\) proteins were purified from 60 g of cells. Both the proteins were colourless even at concentrations above 30 mg/ml. Their UV/Vis spectra are shown in Fig. III.47.

The absence of any absorption features in the visible region of the spectra are indicative of a perturbation of the type 1 Cu site, which either led to the loss of Cu-binding ability or rendered this centre UV/Vis-silent.

The EPR spectra of both the C130A\(^{\text{Cu}}\) and the C130A forms of the protein (Fig. III.48) do not show any type-1 Cu features and only an extremely weak type 2 Cu signal. This signal has \(g_\|= 2.44\) and \(A_\|= 10.0\) mT, the same parameters as Signal 1 previously observed in the His139Ala mutant (see Fig. III.35). The Cu generating this signal was shown to be incorporated during growth of the cells and to correspond to a catalytically active form of the type 2 Cu centre (see section III.2.2.3.1.3). It should be noted that in the case of Cys130Ala, this signal is common to both the C130A\(^{\text{Cu}}\) and the C130A proteins but is only present at very low intensities.

By comparison of double integrals with a Cu-EDTA standard, the signal intensities for the C130A\(^{\text{Cu}}\) and the C130A samples were determined to be 0.46 and 0.23 spins per protein trimer, respectively, suggesting that some incorporation of Cu may have occurred upon incubation of the periplasmic fraction of the cells with CuSO\(_4\).
Figure III.46 – The Cys130Ala mutation in NiR. The figure shows the type 1 Cu site found in the native protein (top) and a simulation of the same site where Cys130 has been replaced by Ala (bottom). Carbon atoms are represented in grey (α-carbon atoms in black), sulphur atoms are represented in yellow and nitrogen atoms are represented in blue (positive charges). The carboxyl group in the Cys residue is represented in red (negative charge). The pictures were kindly produced and provided by Dr. Fraser Dodd.
Chapter III. Nitrite Reductase

Figure III.47 – UV/Vis spectra of the Cys130Ala mutant form of NiR. The green line corresponds to the spectrum of the C130ACu protein (protein purified from cells grown on medium supplemented with 1 mM CuSO₄, after reconstitution of the periplasmic fraction with CuSO₄) at a concentration of 0.77 mg/ml; the blue line represents the spectrum of the C130A protein (protein purified from the same batch of cells and where no reconstitution of the periplasmic fraction was performed) at a concentration of 0.75 mg/ml. The inset shows an expansion of the visible region of the spectra. The spectra were recorded at room temperature in 100 mM Tris-HCl, pH 7.1.

The determination of the metal content of the same samples by ICP indicated that the Cu content of both proteins was below a detectable level, showing that the Cu sites in the Cys130Ala mutant have a very low complement of Cu. Assuming a total depletion of type 1 Cu, as suggested by the UV/Vis and EPR data, and considering the results obtained for the quantification of the EPR-active species in the two samples, C130ACu may have up to 15 % occupancy of the type 2 Cu sites whereas C130A may have up to 7.5 % occupancy of those sites.

These results clearly show that, not surprisingly, the Cys130Ala mutant lost the type 1 Cu-binding ability altogether. Perhaps more interestingly, these results also show that the replacement of the Cys residue by an Ala residue perturbs the binding of Cu to the type 2 Cu sites. This finding is somewhat unexpected and implies a major distortion of the protein backbone as a result of this mutation.
Figure III.48 – EPR spectra of the Cys130Ala mutant form of NiR. A. Spectrum of the C130A\textsuperscript{Cu} protein, purified from cells grown on medium supplemented with 1 mM CuSO\textsubscript{4}, after reconstitution of the periplasmic fraction with CuSO\textsubscript{4} (29.4 mg/ml protein concentration). B. Spectrum of the C130A protein, purified from the same batch of cells and where no reconstitution of the periplasmic fraction was performed (46.7 mg/ml protein concentration). H139A denotes the spectrum of the His139Ala protein purified from the untreated periplasmic fraction of cells grown on medium supplemented with 1 mM CuSO\textsubscript{4} (see Fig. III.35) and is included to facilitate comparison of signal intensities. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.41 GHz and were normalised to the same protein concentration.
It should be noted that Cys130 hydrogen-bonds with His129, one of the type 2 Cu ligands (Dodd et al., 1997, 1998; see Fig. III.31). Thus, it is likely that in the Cys130Ala mutant this hydrogen-bond is no longer formed, thus causing a change in the type 2 Cu site geometry rendering this site unable to bind Cu effectively.

### III.2.2.3.3.2. Activity

Nitrite reductase activity of Cys130Ala was determined using methyl viologen, dithionite and azurin I as electron donors (see Materials and Methods for details) and compared to that of recombinant NiR. The results obtained are summarised in Table III.7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methyl viologen</th>
<th>Dithionite</th>
<th>Azurin I</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.11 ± 0.05</td>
<td>15.1 ± 1.5</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0.04 ± 0.01</td>
<td>5.2 ± 0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

The complete absence of activity when azurin I was used as the electron donor was expected and provides further support for the proposal that physiological electron donors transfer electrons to the Cu atoms in the type 1 Cu centres which are subsequently transferred to the catalytic type 2 Cu centres. Since the Cys130A mutant does not contain type 1 Cu this route of electron transfer is abolished and the protein does not have any activity when the physiological donor is used.

Both forms of the Cys130Ala mutant displayed nitrite reductase activity when the artificial electron donors, methyl viologen and dithionite, were used, indicating
that direct electron donation to the type 2 Cu sites is possible. This is in accord with the results obtained for the His139Ala mutant (see section III.2.2.3.1.3) where the two Cu centres are uncoupled, yet the protein was active when those electron donors were used. Furthermore, there seems to be a correlation between the type 2 Cu content of the two forms of the Cys130Ala protein and their activity. In fact, both with methyl viologen and dithionite, the activity of the C130A\textsuperscript{Cu} protein is two to three times higher than that of the C130A protein, which correlates well with the estimated type 2 Cu content of the two proteins. It should be noted that, unlike the His139Ala mutant, both the C130A\textsuperscript{Cu} and the C130A proteins contain the same form of type 2 Cu centre (which has \(g_c=2.44\) and \(A_c=10.0\) mT) previously shown to correspond to a catalytically active form of this centre (see section III.2.2.3.1.3). Remarkably, the activity of the proteins when methyl viologen is used as the electron donor is significantly lower than that obtained when dithionite is used. This suggests that there is a difference in the accessibility of these two electron donors to the type 2 Cu site of the Cys130Ala mutant or that, more likely, methyl viologen donates electrons mainly via the type 1 Cu site. On the other hand, a comparison of the activities of the His139Ala and the Cys130Ala mutants with that of the Met144Ala mutant (see section III.2.2.3.2) indicates that part of the activity observed with dithionite as the electron donor arises from electron donation to the type 1 Cu and from this site to the catalytic type 2 site. In fact, even the His139Ala mutant, with an estimated full complement of type 2 Cu, has a maximum of \(\sim20\%\) activity relative to recombinant NiR whereas in the Met144Ala, with an estimated \(\sim35\%\) complement of type 2 Cu, this relative activity may be as high as \(\sim50\%\). This can be explained by taking into account that in this latter mutant, contrary to the His139Ala and the Cys130Ala mutants, the coupling between the two types of Cu centres has not been disrupted and electron flow between the type 1 Cu and the type 2 site (which may be either fully or only partially loaded with Cu) is possible. This issue will be further discussed in section III.3.
Aspartate is an acidic amino acid residue with a molecular mass of 133.1 g/mol and an accessible surface area of 150 Å². It has a carboxyl side-chain, which is negatively charged at pH 6.0 to 7.0, and is capable of forming hydrogen-bonds. The Asp92 residue in NiR has been replaced by two different amino acid residues, Glu and Asn. Glutamate is also an acidic amino acid that differs from Asp in that its carboxyl side-chain contains an extra methylene group. Thus, it is a “bulkier” amino acid residue than Asp, as indicated by its molecular mass of 147.1 g/mol and its accessible surface area of 190 Å². Asparagine is the amide of Asp. Its side-chain is polar but it lacks the negative charge of Asp. Its molecular mass of 132.1 g/mol and its accessible surface area of 160 Å² are quite similar to those of Asp.

As previously mentioned, Asp92 has been shown to form an hydrogen-bond with a water ligand to the type 2 Cu (Dodd et al., 1997, 1998; see also Fig III.31), in agreement with previous findings of Adman et al. (1995) for the A. cycloclastes enzyme. This residue has been suggested by those authors to be important for catalysis in that it also forms an hydrogen-bond with the nitrite substrate when it binds to the type 2 Cu atom. The mutations introduced in this residue are likely to influence substrate-binding and catalysis.

The type 2 Cu site in NiR and a computer simulation of the replacement of Asp by Glu and Asn in the Asp92Glu and Asp92Asn mutants are shown in Fig. III.49.

### III.2.2.3.4.1. Spectroscopy and metal content

The D92E and D92E^Cu and the D92N and D92N^Cu mutant proteins were purified from 200 g of cells. Both forms of each of the two mutants were blue in colour. Their UV/Vis spectra are presented in Fig. III.50 and show an absorption maximum in the 600 nm region, consistent with the presence of oxidised type 1 Cu centres in the proteins. The Abs\_280nm / Abs\_592nm ratios of the purified proteins are listed in Table III.8 and provide an indication of their type 1 Cu content.

The EPR spectra of the Asp92Glu mutant are shown in Fig. III.51. The two forms of the Asp92Glu mutant differ only in their type 1 Cu content, as shown by the difference between the spectra of the D92E^Cu and the D92E proteins (spectra A and B, respectively, in Fig. III.51).
Figure III.49 – The Asp92Glu and Asp92Asn mutations in NiR. The figure shows the type 2 Cu site found in the native protein (top) and computer simulations of the same site where Asp92 has been replaced by Glu and Asn (bottom). Carbon atoms are represented in grey (α-carbon atoms in black), nitrogen atoms are represented in blue (positive charges), negatively charged carboxyl groups are represented in red and the fourth Cu ligand (H₂O or Cl⁻) is represented in purple. The pictures were kindly produced and provided by Dr. Fraser Dodd.
Figure III.50 – UV/Vis spectra of the Asp92Glu (A) and Asp92Asn (B) mutants. The insets show an expansion of the visible region of the spectra. The spectra were recorded at room temperature in 100 mM Tris-HCl, pH 7.1. Protein concentrations are 0.80 mg/ml (D92E), 0.31 mg/ml (D92E\textsuperscript{Cu}), 0.97 mg/ml (D92N) and 0.87 mg/ml (D92N\textsuperscript{Cu}).
Figure III.51 – EPR spectra of the Asp92Glu mutant of NiR. A. Spectrum of the D92ECu protein, purified from cells grown on medium supplemented with 1 mM CuSO₄, after reconstitution of the periplasmic fraction with CuSO₄ (12.5 mg/ml protein concentration). B. Spectrum of the D92E protein, purified from the same batch of cells and where no reconstitution of the periplasmic fraction was performed (16.0 mg/ml protein concentration). C. Subtraction of spectrum B from spectrum A. D. Spectrum A after subtraction of the type 1 Cu signal (see text). A’, B’, C’, and D’ are the theoretical simulations of spectra A, B, C and D, respectively. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.41 GHz and were normalised to the same protein concentration.
This difference spectrum (spectrum C in Fig. III.51) has a ‘clean’ type 1 Cu signal, which was simulated with $g_{//} = 2.12$ ($A_{//} = 0$ mT) and $g_{⊥} = 2.30$ ($A_{⊥} = 6.4$ mT) (spectrum C’ in Fig. III.51). By double integration of the spectra, this signal was shown to contribute with 25.6% to the total area of the spectrum of D92E$^{Cu}$. Assuming that, as indicated by the ratio of $Abs_{280nm} / Abs_{592nm}$ of the D92E$^{Cu}$ protein (see Table III.8), this protein has a full complement of type 1 Cu, and taking into account that its total Cu content was determined by ICP metal analysis to be $\sim$5 Cu atoms per trimer (see Table III.8) then it can be estimated that $\sim$1.25 type 1 Cu atoms were incorporated during incubation of the periplasmic fraction with CuSO$_4$. This means that the D92E protein contains an estimated $\sim$1.75 type 1 Cu atoms contributing to its EPR spectrum (Fig. III.51-B). Therefore, by subtracting this contribution ($1.75 / 1.25 = 1.4$ times the intensity of the type 1 Cu signal in spectrum C) from the spectrum of the D92E protein, the resulting spectrum (Fig. III.51-D) represents the type 2 Cu contribution to the overall signal. The theoretical simulation of this difference spectrum (Fig. III.51-D’) showed the type 2 signal in the Asp92Glu mutant to be rhombic, with $g_{x} = 2.10$ ($A_{x} = 0$ mT), $g_{y} = 2.18$ ($A_{y} = 2.1$ mT) and $g_{z} = 2.40$ ($A_{z} = 13.3$ mT). The spectra of the D92E$^{Cu}$ and the D92E proteins were simulated (spectra A’ and B’ in Fig. III.51, respectively) by addition of the appropriate contributions of each one of the simulated type 1 Cu and type 2 Cu EPR signals.

The EPR spectra of the Asp92Asn mutant are shown in Fig. III.52. The spectra of the two forms of the Asp92Asn mutant are virtually identical, as indicated by the absence of a signal in the spectrum of the difference between the spectra of the D92N$^{Cu}$ and the D92N (not shown). In their as-purified forms, both proteins display a type 1 Cu signal only. The theoretical simulation of this signal (Fig. III.52-C) showed that it has $g_{//} = 2.12$ ($A_{//} = 0$ mT) and $g_{⊥} = 2.30$ ($A_{⊥} = 6.4$ mT). The absence of type 2 Cu features in the EPR spectra of D92N$^{Cu}$ and D92N is rather surprising considering the values obtained for the total Cu content of these proteins (see Table III.8), which indicate that both forms of the Asp92Asn mutant contain $\sim$4.5 Cu atoms per trimer.
Figure III.52 – EPR spectra of the Asp92Asn mutant of NiR. A. Spectrum of the D92NCu protein, purified from cells grown on medium supplemented with 1 mM CuSO₄, after reconstitution of the periplasmic fraction with CuSO₄ (11.1 mg/ml protein concentration). B. Spectrum of the D92N protein, purified from the same batch of cells and where no reconstitution of the periplasmic fraction was performed (8.5 mg/ml protein concentration). C. Simulation of the type 1 Cu signal in spectra A and B. D. Spectrum of the D92N protein after oxidation with K₂IrCl₆. E. Subtraction of spectrum B from spectrum D. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.40 GHz and were normalised to the same protein concentration.
Assuming that the proteins have a full complement of type 1 Cu, as indicated by their ratio of Abs$_{280nm}$/Abs$_{592nm}$, this suggests that in this protein type 2 Cu might have a partial complement of reduced (therefore, EPR-silent) type 2 Cu. In order to test this hypothesis, the D92N sample was oxidised with a 20-fold molar excess of K$_2$IrCl$_6$ over the estimated type 2 Cu. The results are not conclusive, in that the difference between the spectrum of the oxidised D92N protein (Fig. III.52-D) and that of the as-purified protein (Fig. III.52-B), shown in Fig. III.52-E, does not have a signal with clear type 2 Cu features (in fact there seems to be at least some type 1 Cu character in this additional signal). Nevertheless, it should be noted that the double integration of the spectra showed that the intensity of the EPR signal in the oxidised sample is $\sim$1.15 times higher than that of the as-purified sample, suggesting that indeed part of the Cu in the D92N protein was in the reduced state. It is, however, difficult to determine unequivocally the nature of this additional Cu.

The metal content of the Asp92 mutant proteins was determined by ICP analysis. These results, as well as the Abs$_{280nm}$/Abs$_{592nm}$ ratios of both forms of each of the mutant proteins are shown in Table III.8. Together with the EPR data presented (Figs. III.51 and III.52), the data in Table III.8 allow several conclusions to be drawn with regard to the metal content of these mutants as well as to the incorporation of the Cu atoms in the protein and their spectroscopic properties.

Table III.8 – Metal content of the Asp92Glu and Asp92Asn mutant proteins. The ratios Abs$_{280nm}$/Abs$_{592nm}$ were determined from the spectra in Fig. III.50. Total copper and total zinc were determined by ICP analysis. The numbers correspond to the number of metal atoms per protein trimer.

<table>
<thead>
<tr>
<th>Asp92 mutant protein</th>
<th>D92E</th>
<th>D92E$^{Cu}$</th>
<th>D92N</th>
<th>D92N$^{Cu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Ab$<em>{280nm}$/Ab$</em>{592nm}$</td>
<td>29.0</td>
<td>12.1</td>
<td>11.4</td>
<td>11.2</td>
</tr>
<tr>
<td>Total Cu</td>
<td>4.55</td>
<td>5.18</td>
<td>4.56</td>
<td>4.52</td>
</tr>
<tr>
<td>Total Zn</td>
<td>2.15</td>
<td>1.79</td>
<td>0.78</td>
<td>0.96</td>
</tr>
</tbody>
</table>
Chapter III. Nitrite Reductase

When purified from an untreated periplasmic fraction, the Asp92Glu mutant protein appears to have only partially occupied type 1 Cu centres, as shown both by the $\frac{\text{Abs}_{280nm}}{\text{Abs}_{592nm}}$ ratios and the EPR spectra of the D92E$^{\text{Cu}}$ and the D92E proteins. An alternative explanation for these results is that the D92E protein may have partially reduced type 1 Cu sites. UV/Vis data shows that the protein purified from the reconstituted periplasmic fraction has a full complement of oxidised type 1 Cu, suggesting that the type 1 Cu was partially incorporated (or oxidised) upon incubation of the periplasmic fraction with CuSO$_4$. The EPR data obtained shows that no incorporation of type 2 Cu occurred as a result of this procedure, which indicates that type 2 Cu incorporation in the Asp92Glu mutant took place exclusively in vivo. While the type 1 Cu in this mutant has a typical axial EPR signal similar to that found in the wild-type native protein, the type 2 Cu in this mutant has an unusually rhombic signal which may reflect changes in the geometry of the site as a consequence of the introduction of a bulkier amino acid residue. ICP metal analysis shows that the protein does not have a full complement of type 2 Cu, which may result from partial occupancy of the type 2 sites by Zn.

Both forms of the Asp92Asn mutant were shown by UV/Vis spectroscopy to contain a full complement of type 1 Cu, which is consistent with the notion that the copper in these centres was incorporated in vivo. The EPR spectra of D92N$^{\text{Cu}}$ and D92N do not show obvious type 2 Cu features but ICP metal analysis indicated that the two forms of the protein contain $\sim$4.5 Cu atoms per trimer, suggesting that they may contain $\sim$1.5 type 2 Cu atoms. Attempts at the reconstitution of the type 2 Cu signals by oxidation of the D92N protein failed to give a conclusive result so the possibility remains that the extra Cu detected by chemical analysis may in fact be, at least partially, adventitious copper. As in the case of Asp92Glu, the type 1 Cu in the Asp92Asn mutant has a typical axial EPR signal, similar to that found in the wild-type native protein.

Taken together, the results above provide insights into the effect of each of the two Asp92 mutations upon the assembly of the type 2 Cu centres. It appears that the Asp92Glu mutant retains an almost complete ability to incorporate Cu in the type 2 sites, although these may have a somewhat different conformation to that found in the wild-type native NiR enzyme. On the other hand, incorporation of the type 2 Cu in the Asp92Asn mutant seems to be markedly reduced with regard to NiR and the
possibility that some type 2 Cu atoms in this mutant are in a reduced state cannot be excluded.

Thus, the presence of an acidic amino acid residue at position 92 of NiR seems to facilitate the assembly of the type 2 Cu centres. The size constraints imposed by the Glu residue at that position on substrate binding and catalysis will be analysed in the following section.

III.2.2.3.4.2. Activity and substrate binding

Nitrite reductase activity of the Asp92 mutants was determined by three different methods, using methyl viologen, dithionite and azurin I as electron donors (see section II.2.2.4.1 for details) and compared to that of recombinant NiR. The results obtained are summarised in Table III.9.

Table III.9 – Activity of the Asp92Glu and Asp92Asn mutants with three different electron donors. The numbers represent the percentage of activity relative to recombinant NiR. Nd – Not determined.

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Asp92 mutant protein</th>
<th>D92E</th>
<th>D92E&lt;sup&gt;Cu&lt;/sup&gt;</th>
<th>D92N</th>
<th>D92N&lt;sup&gt;Cu&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl viologen</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Dithionite</td>
<td>2.7 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>5.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Azurin I</td>
<td>14.2 ± 3.5</td>
<td>Nd</td>
<td>58.5 ± 12.5</td>
<td>Nd</td>
<td></td>
</tr>
</tbody>
</table>

The data shown in Table III.9 reveals some interesting and quite unexpected results. There seems to be a remarkable difference in the activity of the Asp92Glu and the Asp92Asn mutants when either the artificial electron donors (methyl viologen and dithionite) or the physiological electron donor (reduced azurin I) is used in the assay. This is particularly interesting if combined with the information on substrate binding to Asp92Glu, monitored by EPR (Fig. III.53), which reveals that no significant changes in the spectrum of the D92E mutant protein occur upon incubation with nitrite. This implies that the type 2 Cu site in Asp92Glu is not accessible to the nitrite
substrate and explains why the activity of this protein is so low when methyl viologen or dithionite is used as electron donor in the activity assay.

Figure III.53– Substrate binding to Asp92Glu monitored by EPR. A. Spectrum of the D92E protein (protein purified from the untreated periplasmic fraction of cells grown on medium supplemented with 1 mM CuSO₄), (16.0 mg/ml in protein). B. Spectrum of the same protein after incubation with a 20-fold molar excess of nitrite relative to the estimated type 2 Cu content. The experimental spectra were recorded in 100 mM Tris-HCl, pH 7.1 at 60 K and at a microwave frequency of 9.41 GHz.
The significant increase in activity observed when azurin I is used as electron donor strongly suggests that the accessibility of that site has changed as a result of the interaction of the protein with its physiological electron donor. In the case of Asp92Asn, this increase is even more pronounced, and the activity found for D92N with azurin I as the electron donor is higher than would be expected from the spectroscopic data of this protein. This suggests either that catalysis is possible at a metal-depleted type 2 site or that Cu is indeed present in a spectroscopically silent form in the protein. In either case, it seems clear that these data support the idea that upon interaction with its physiological electron donor, the Asp92Glu and the Asp92Asn proteins undergo a significant conformational change which facilitates catalysis, possibly by enhancing substrate accessibility to the catalytic site.

III.2.2.3.5. His249Phe mutant form of NiR

In the His249Phe mutant form of the NiR protein a basic amino acid residue, positively charged at pH 6.0 to 7.0, was replaced with one that is essentially nonpolar and, therefore, hydrophobic. Phenylalanine has a molecular mass of 165.2 g/mol and an accessible surface area of 210 Å². It is a bulkier amino acid residue than His (155.2 g/mol molecular mass and 195 Å² accessible surface area) and it has an aromatic side-chain whereas the side-chain of His contains an imidazole group.

Dodd et al. (1997, 1998) suggested that His249 is of potential importance for catalysis due to its position at the type 2 Cu site (see also Fig. III.31). It is expected that the replacement of this residue by one with a bulky side-chain will perturb the protein environment of the catalytic site. Fig. III.54 shows the type 2 Cu site in NiR and a computer simulation of the replacement of His by Phe in the His249Phe mutant.

The His249Phe mutant was found to precipitate during or immediately after purification and, therefore, could not be characterised to the same extent as the other mutants. The protein was purified from 65 g of *E. coli* cells from a 20-litre growth done in the absence of a copper supplement. The protein was purified both from untreated periplasmic fraction of those cells and from periplasmic fraction previously incubated with CuSO₄ and the same notation as that used for the other mutants will be used hereafter. Thus, the proteins will be called H249F and H249F⁺, respectively.
Figure III.54 – The His249Phe mutation in NiR. The figures show two different perspectives of the type 2 Cu site found in the native protein and the respective computer simulations of the same site where His249 has been replaced by Phe. Residues from monomers A and B (see text) are represented in yellow and green, respectively. Carbon atoms are represented in grey (α-carbon atoms in black), nitrogen atoms are represented in blue (positive charges), negatively charged carboxyl groups are represented in red, water molecules are represented in orange and the fourth Cu ligand (H$_2$O or Cl) is represented in purple. Only two of the three His ligands of the type 2 Cu are represented in the pictures on the right panel. The pictures were kindly produced and provided by Dr. Fraser Dodd.
Chapter III. Nitrite Reductase

Following centrifugation of the concentrated material eluted from the CMC column, a blue pellet was noticeable which corresponded to the precipitated protein. Attempts to resolubilise the precipitated material by changing the pH of the solution failed for the H249F and the H249F$_{Cu}$ proteins. Considering the yield of protein obtained for other mutants grown and purified under the same conditions, it is possible to estimate that the soluble fraction of both forms of the His249Phe mutant corresponded to less than 10% of the expected amount of overproduced protein.

The reasons for this are likely to be related to the fact that a bulky amino acid residue, such as phenylalanine, at position 249 will hamper the assembly of the trimer and cause the protein to precipitate. In fact, the computer simulation of this mutation suggests that the Phe residue at position 249 would have close contacts with residues Glu273 and Ile251 of monomer B (residues from monomer B are represented in green in Fig. III.54). This would, most likely, disrupt the interface between the monomers or even produce some misfolding (Dr. Fraser Dodd, personal communication). The Phe residue at that position would also result in the exclusion of three waters. These effects are depicted in the right panel of Fig. III.54.

III.2.3. Electrochemistry

III.2.3.1. Cyclic voltammetry: Principles and instrumentation

Electrochemical techniques in which a potential is imposed upon an electrochemical cell and the resulting current is measured are generally categorised as voltammetric methods (Sawyer et al., 1984). Voltammetric techniques are, thus, based on controlling the electrode potential and measuring the resulting current. A key feature in voltammetry is the relationship between the potential applied to an electrode and the concentration of redox species at the electrode surface. Let us consider two species, O and R, in which O is capable of being reduced to R at the electrode by the reversible electrochemical reaction $O + n\cdot e^- \leftrightarrow R$, where $n$ stands for the number of electrons, $e^-$, involved in the reduction process. The fundamental equation that relates the potential, E, which is applied to the electrode, and the concentrations of the species O and R, at the electrode surface, is the Nernst equation,
Chapter III. Nitrite Reductase

\[
E = E_0' + \frac{0.0591}{n} \cdot \log \left( \frac{C_S^O}{C_S^R} \right)
\]

where

- \(E\) = potential applied to electrode
- \(E_0'\) = formal reduction potential of the couple vs the reference electrode
- \(n\) = number of electrons in reaction
- \(C_S^O\) = surface concentration of species O
- \(C_S^R\) = surface concentration of species R

A voltammogram (current-potential curve) is typically obtained by scanning the potential of an electrode from positive to negative, or vice-versa, and recording the current. Potentiostats utilise a three-electrode configuration. The potentiostat applies the desired potential between a working electrode and a reference electrode. The working electrode is the electrode at which the electrolysis of interest takes place. The current required to sustain the electrolysis at the working electrode is provided by the auxiliary electrode. This arrangement prevents the reference electrode from being subjected to large currents that could change its potential (Sawyer et al., 1984).

Cyclic voltammetry (CV) is a versatile electroanalytical technique which has been used in the study of metalloproteins and redox enzymes over the past 25 years. Its effectiveness results from its capability of allowing rapid observation of redox behaviour over a wide potential range (Guo and Hill, 1991). The resulting voltammogram is analogous to a conventional spectrum in that it conveys information as a function of an energy scan. CV consists of cycling the potential of a stationary microelectrode which is in contact with an electrolyte solution containing the species of interest, and measuring the resulting current. The potential of this working electrode is controlled vs a reference electrode such as a calomel electrode. The controlling potential that is applied across these two electrodes can be considered an excitation signal. The excitation signal for CV is a linear potential scan with a triangular waveform. This triangular potential excitation signal sweeps the potential of the electrode between two values, sometimes called the switching potentials. A cyclic voltammogram is obtained by measuring the current at the working electrode during
the potential scan. The current can be considered the response signal to the potential excitation signal. The voltammogram is a display of current (vertical axis) vs potential (horizontal axis).

The important parameters of a cyclic voltammogram are the magnitudes of the reduction peak current ($i_{p\text{Red}}$), the oxidation peak current ($i_{p\text{Ox}}$), the reduction peak potential ($E_{p\text{Red}}$) and the oxidation peak potential ($E_{p\text{Ox}}$) (Sawyer et al., 1984; Ibrahim, 1992). These parameters are labelled in Fig. III.55, which shows a typical cyclic voltammogram.

Figure III.55 – Cyclic voltammogram of a solution of ferrocene in 0.2 M CH$_3$CN / [NBu$_4$][BF$_4$] at a platinum wire electrode. Scan rate, $\nu = 0.25$ V/s. Fc – ferrocene; E, E$^1$ – switching potentials. Reprinted with permission from Ibrahim (1992).
Chapter III. Nitrite Reductase

A redox couple in which both species rapidly exchange electrons with the working electrode is termed an electrochemically reversible couple. The formal reduction potential, $E_0^*$, for a reversible couple is centred between $E_{p\text{Red}}^*$ and $E_{p\text{Ox}}^*$:

$$E_0^* = E_{1/2} = \frac{E_{p\text{Red}}^* + E_{p\text{Ox}}^*}{2}$$

The number of electrons, $n$, transferred in the electron reaction for a reversible couple can be determined from the separation between the peak potentials:

$$\Delta E_p = E_{p\text{Ox}}^* - E_{p\text{Red}}^* = \frac{0.059}{n}$$

The peak current for a reversible system is described by the Randles-Sevcik equation:

$$i_p = 2.69 \times 10^5 \cdot n^{3/2} \cdot A \cdot D^{1/2} \cdot C \cdot \nu^{1/2}$$

where $i_p = \text{peak current (A)}$
$n = \text{electron stoichiometry}$
$A = \text{electrode area (cm}^2\text{)}$
$D = \text{diffusion coefficient (cm}^2/\text{s}\text{)}$
$C = \text{concentration (ml/cm}^3\text{)}$
$\nu = \text{scan rate (V/s)}$

Cyclic voltammetry can thus be used as a means to investigate the reversibility of a redox system and the number of electrons involved in the electron-transfer reaction. Furthermore, cyclic voltammetric measurements on reversible or quasi-reversible systems can, of course, provide a rapid and convenient method for determining $E_{1/2}$ values. These are usually within a few millivolts of the thermodynamic $E^0$ potentials (Ibrahim, 1992). Voltammetric studies offer information not only on redox potentials but also on dynamic aspects such as kinetic analysis of electrode reactions and inter-protein electron-transfer reactions (Guo and Hill, 1991).
III.2.3.2. Direct electrochemistry of proteins

In order to enhance the electrochemical response of a protein interacting with an electrode, promoters such as di-4-pyridyl disulfide (4-pyds) have been used to coat the surface of the electrode. The organic adsorbate allows electron transfer to occur directly by providing the electrode surface with chemical functionalities capable of interacting specifically and reversibly with the protein surface (Guo and Hill, 1991). One of the first reports of the use of a modified gold electrode to study the quasireversible electrochemistry of redox proteins is that of Eddowes and Hill (1977) on horse heart cytochrome c (see also Eddowes and Hill, 1979). Since then, modified gold electrodes have been used in the electrochemical study of various proteins, such as sulphide : cytochrome c oxidoreductase (flavocytochrome c552) (Guo et al., 1990), methylamine dehydrogenase (Burrows et al., 1991) and pseudoazurin (Kohzuma et al., 1995).

Kohzuma et al. (1993) described the first voltammetric studies on NiR from Achromobacter xylosoxidans. These authors report on the observation of direct electrochemical response of this enzyme at a 4-pyds-modified gold electrode and determined the half-wave peak potential to be 260 mV. Based on the similarity of this potential to that of other blue Cu electron-transfer proteins, the authors assumed that the potential observed for NiR corresponds to the type 1 Cu and suggest that it might be impossible to observe the direct electron-transfer of type 2 Cu because this site is too far away from the protein surface. A later study by Kohzuma et al. (1994) utilised the NiR from Achromobacter cycloclastes. The authors describe a complete absence of electrochemical response of the protein at a 4-pyds-modified gold electrode and report that a cyclic voltammetric response was observed upon addition of apopseudoazurin to the reductase solution. Under these conditions, Kohzuma et al. (1994) determined a midpoint potential of +240 mV which the authors attribute to the type 1 Cu in the protein on the basis that T2D NiR also exhibited a well-defined electrochemical response, with a redox potential of +204 mV.
III.2.3.3. Cyclic voltammetry of NiR and mutant forms of NiR

Cyclic voltammetry studies were performed with recombinant NiR and on the wild-type, native enzyme. The two proteins exhibited a similar response at the 4-pyds-modified gold electrode (Fig. III.56).

![Cyclic voltammograms of recombinant (top) and wild-type (bottom) NiR.](image)

Figure III.56 – Cyclic voltammograms of recombinant (top) and wild-type (bottom) NiR. The proteins were in 100 mM Tris-HCl, pH 7.1 at a concentration of 7.2 mg/ml and 15.3 mg/ml, respectively. Scan rate was 20 mV/sec.

Both the recombinant and the wild-type enzymes have a full complement of type 1 Cu and type 2 Cu and exhibit a quasi-reversible redox behaviour at the 4-pyds-modified gold electrode. The half-wave potentials, $E_{1/2}$, determined from the cyclic voltammograms of the two proteins were +210 mV and +235 mV, respectively. No enhanced catalytic current was observed upon addition of nitrite to the electrochemical cell (data not shown).
When the His139Ala mutant, in which the two types of Cu centres are uncoupled (see section III.2.2.3.1) was scanned over the same range of potentials as that used for NiR, no electrochemical response was observed. The same result was obtained for the Asp92Asn mutant of NiR, which has a full complement of type 1 Cu and a very low complement of type 2 Cu (see Fig. III.52, section III.2.2.3.4), and for the wild-type T2D enzyme. Fig. III.57 shows the cyclic voltammograms for these three proteins. These results indicate that only the protein with a complement of both types of Cu is capable of exhibiting an electrochemical response at a 4-pyds-modified gold-electrode. Furthermore, the results obtained with the His139Ala mutant form of NiR suggest that the two types of Cu centres in the protein must be coupled in order for a voltammetric response to be observed.

Figure III.57 – Cyclic voltammograms of the H139A protein (top), the D92N protein (centre) and the wild-type T2D NiR (bottom). The proteins were in 100 mM Tris-HCl, pH 7.1 at a concentration of 20.0 mg/ml, 31.9 mg/ml and 14.8 mg/ml, respectively. Scan rate was 20 mV/sec.
These results contradict those of Kohzuma et al. (1994) with the NiR from *A. cycloclastes*, who report a similar electrochemical response for both the fully loaded and the T2D proteins. These differences may be explained if the T2D enzyme used in the studies of Kohzuma et al. (1994) was not totally depleted in type 2 Cu. This is supported by the fact that these authors report an activity for the T2D NiR which is more than 50% that of the native enzyme, which is considerably higher than what would be expected for a T2D protein (Howes et al., 1994; Abraham et al., 1997; Eady et al., 1997; Suzuki et al., 1997; Prudêncio et al., 1999). Another possibility is that the use of pseudoazurin as a promoter may significantly enhance the contact between the protein and the electrode, thus allowing an otherwise silent response to be observed. However, it should be noted that Kohzuma et al. (1993) reported a direct electrochemical response of NiR from *A. xylosoxidans* in the absence of apopseudoazurin, in apparent contradiction with the report of Kohzuma et al. (1994) for the *A. cycloclastes* enzyme.
III.3. DISCUSSION

The study of NiR and selected mutant forms of this protein has generated new and interesting information providing insight into the biochemical, spectroscopic and kinetic properties of this enzyme.

The A. xylosoxidans nirA gene was cloned and sequenced (section III.2.1.1). The deduced amino acid sequence of the enzyme showed a high degree of similarity with other CuNiRs, as anticipated for proteins belonging to the same family (Zumft, 1997). An interesting and unexpected correlation between the blue or green type of NiR and the type of signal peptide that preceded the enzyme sequence was noted (Prudêncio et al., 1999). Out of the seven sequences compared, the two blue NiRs carry signal peptides characteristic of the Sec-dependent export pathway (Pugsley, 1993; Schatz and Dobberstein, 1996; Pohlschröder et al., 1997), whereas five green proteins exhibit longer signal peptides carrying a twin-arginine motif, recently shown to correspond to a novel type of specialised export pathway (Berks, 1996; Santini et al., 1998; Sargent et al., 1998; Weiner et al., 1998). Whether this corresponds to a general rule in the family of CuNiRs or is no more than a coincidence cannot be established at present but it is possible that it is a reflection of different evolutionary origins. Also, it is difficult to envisage the physiological significance of this difference in terms of the molecular properties of the enzymes since all CuNiRs share very similar characteristics. However, it should be noted the double-arginine machinery is proposed to export fully folded proteins in which cofactor attachment occurs in the cytoplasm (Berks, 1996). On the other hand, proteins are exported by the Sec pathway in a substantially unfolded state, leaving processing and metal assembly to occur in the periplasm (Pugsley, 1993; Wülfing and Plückthun, 1994).

At this point, a few observations regarding previous studies on the overexpression of the genes encoding NiRs from different sources should be made. The first report of the overproduction of a CuNiR was that of Nishiyama et al. (1993) on the green enzyme from A. faecalis. These authors constructed a plasmid carrying the sequence of the gene encoding NiR and that of its original, twin arginine-containing, signal peptide. Nishiyama et al. (1993) report that when E. coli containing this plasmid was grown at 37 ºC, the protein was found to be unprocessed and located in inclusion bodies in the cytoplasm. When the cells were grown at 26.5 ºC, some
processing of the protein occurred and only when the cells were grown at 20 °C was some processed NiR protein found in the periplasm. Later studies with enzyme from the same source (Kukimoto et al., 1994) showed that correct expression and secretion of the enzyme into the periplasm only occurred when the signal peptide was modified in relation to that found in the protein from *A. faecalis*. The second NiR reported to be overexpressed in *E. coli* is that of the green enzyme *Ach. cycloclastes* (Chen et al., 1996). These authors used a His-tagged peptide fused to the mature NiR protein for overexpression in *E. coli* and they purified the fusion protein from the cytoplasmic fraction of the cells. Finally, Olesen et al. (1998) describe the overexpression of the gene encoding the green NiR from *Rh. sphaeroides* 2.4.3 and report that the overproduced protein contained no Cu, even though the cells were grown in medium supplemented with Cu. They expressed the gene including the original signal peptide of the protein from *Rh. sphaeroides* and reported that the recombinant protein was not processed. The signal peptide of NiR from *Rh. sphaeroides* contains a twin-arginine motif. Thus, it is possible that the recombinant protein described in the report of Olesen et al. (1998) is indeed a trimer of the precursor form of NiR which *E. coli* is not able to export to the periplasm. The absence of Cu in this protein can be explained if Cu insertion is concomitant with the adequate export of the protein and cleavage of its signal peptide. Thus, the results obtained for the twin arginine-containing green enzymes from *A. faecalis*, *Ach. cycloclastes* and *Rh. sphaeroides* seem to indicate that *E. coli* cannot cope with the correct export of the protein to the periplasm, unlike what happens with the Sec-dependent translocated blue enzyme from *A. xylosoxidans* (section III.2.1.3 and see below). This suggests that NiR protein export via the twin arginine-dependent pathway may be subject to more strict control than the Sec-dependent export pathway.

The overproduced recombinant *A. xylosoxidans* NiR was characterised in terms of its activity, spectroscopy and molecular properties (section III.2.1.4) and was shown to be essentially the same as from the wild-type enzyme. This shows that overproduction of *A. xylosoxidans* NiR in the heterologous host *E. coli* is possible (section III.2.1.2) and that, as stated above, the latter contains the machinery for correct folding, export and Cu centre assembly into NiR.

The recombinant *A. xylosoxidans* NiR was purified in a simple two-step procedure which was found to work equally well for the mutant forms of the enzyme.
This fast purification method involves a single chromatographic step and is likely to be at least partly responsible for the “cleaner” T2D form of the enzyme obtained, when compared to that purified from *A. xylosoxidans* (Abraham *et al.*, 1993; Abraham *et al.*, 1997).

The similarities between the active site in NiR and the Zn site in carbonic anhydrase (CA) have been noted (Strange *et al.*, 1995; Dodd *et al.*, 1998). A presumptive Zn-enriched form of NiR was assayed for CA activity (section III.2.1.4.4) but was found to be inactive, suggesting that differences in the ligand geometry between the Zn sites of the two proteins are responsible for the absence of CA activity in NiR. The structure of CA where Zn has been replaced by Cu has been determined (Håkansson *et al.*, 1994) and indicates that the ligand geometry around the Cu atom is different between the two enzymes, possibly supporting this hypothesis. On the other hand, despite the striking similarities between the active sites of NiR and superoxide dismutase (SOD) (Strange *et al.*, 1995; Dodd *et al.*, 1998; Strange *et al.*, 1999), when recombinant and wild-type forms of NiR were assayed for SOD activity both were found to be inactive (section III.2.1.4.4), suggesting that the geometry of the type 2 site plays a crucial role in this respect. These results contradict those of Strange *et al.* (1999), who reported a considerable SOD activity for NiR. This can only be explained by assuming a very high variability in the SOD activities of different batches of NiR which may be related to metal occupancy of the type 2 sites. Taken together, the results obtained for the CA and SOD activities of NiR support the notion that the activity of a protein depends both on the nature of the metal atoms at the active site and on the geometry of this site. Quite possibly, the overall geometry of the protein also plays a role in the catalytic specificity of an enzyme, in that it may control factors such as, for example, substrate accessibility to the active site.

A site-directed mutagenesis programme was undertaken in order to generate mutants of NiR that would elucidate the role of specific amino acid residues in the assembly of Cu centres, intra-molecular electron transfer and catalysis (section III.2.2.1). The characterisation of the purified mutants provided useful information and allowed several conclusions to be drawn regarding those aspects of the protein’s organisation and mechanism.
The His139Ala mutation (section III.2.2.3.1) replaced one of the ligands of the type 1 Cu site by an amino acid residue which is unable to ligate the Cu atom. The His139 residue of NiR is oriented similarly to the corresponding His117 in azurin (Dodd et al., 1997), a residue suggested to be involved in exit and/or entry of electrons (van de Kamp et al., 1990). Previous studies on a His117Gly mutant of the azurin from *Ps. aeruginosa* indicated that removal of a His ligand from a type 1 Cu site leads to a surprising flexibility in the coordination geometry of this site, which changes from trigonal to tetrahedral upon loss of the His ligand. However, geometrical changes in the site can be induced by the addition of exogenous ligands and the UV/Vis and EPR properties of wt-azurin can thus be restored. This implies that the structure of the mutant protein and the geometry of the metal site are maintained despite the lack of the His ligand (den Blaauwen and Canters, 1991, 1993; den Blaauwen et al., 1991, 1993; Danielsen et al., 1995).

The His139Ala mutant of *A. xylosoxidans* NiR contained reduced type 1 Cu centres which could only be oxidised by a large excess of a strong oxidant (section III.2.2.3.1.2). An increase in the potential of the type 1 Cu site as a result of a three-fold rather than a four-fold coordination when the Cu-His bond is broken has been reported for pseudoazurin (reviewed in Canters and Gilardi, 1993). Also, the His46 residue of *Ps. aeruginosa* azurin has been proposed to fine-tune the reduction potential of the type 1 Cu site (Germanas et al., 1993). However, the likely magnitude of the change observed in the case of His139Ala is bigger than any change previously reported as a result of a single point mutation of a type 1 Cu ligand. Clearly, this mutant did not lose its ability to bind type 1 Cu, in agreement with the studies of den Blaauwen and Canters (1991, 1993), den Blaauwen et al. (1991, 1993), and Danielsen et al. (1995) (see also Canters and Gilardi, 1993 for a review on this subject). However, it also seems likely that the mutation caused a considerable perturbation of the redox potential of the site, suggesting that the His139 residue plays a crucial role in controlling that potential.

The His139Ala mutant constitutes an ideal system to study the properties of the type 2 Cu sites of NiR by EPR spectroscopy without the interference of the signal arising from the type 1 Cu which, when reduced, is EPR-silent (section III.2.2.3.1.1). The analysis of the EPR spectra of this mutant revealed that the type 2 Cu sites assembled in vivo (responsible for EPR Signal 1) and in vitro (responsible for EPR Signal 2) correspond to two different forms of this centre. In the absence of the X-ray
structures of the H139A and the H139A\textsuperscript{Cu} proteins, the nature of this difference can only be speculated upon. However, it is likely that it is related to the presence or absence of a fourth, exogenous, ligand of the Cu atom, possibly a water molecule or, as suggested by the studies of Dodd et al. (1998), a chloride ion.

An interesting correlation was found between the form of the type 2 Cu site in the His139Ala mutant and its catalytic ability (section III.2.2.3.1.3). Activity measurements and substrate-binding studies carried out indicate that only the type 2 Cu incorporated during growth of the cells is catalytically competent. This is a surprising finding in that there is evidence that \textit{in vitro}-reconstituted type 2 Cu sites of wt-NiR are catalytically active (Libby and Averill, 1992; Prudêncio et al., 1999). In order to understand these results, the uncoupling of the type 1 Cu and type 2 Cu centres as a result of the shift in the potential of the type 1 site (and the perturbation of the putative pathway of electron entry into type 1 Cu) must be taken into account. It seems clear that the increase in the midpoint redox potential of the type 1 site results in the loss of redox equilibrium between the type 1 Cu and the type 2 Cu in the His139Ala mutant. Therefore, the activity observed when dithionite is used as electron donor, must result from direct electron donation to the type 2 site. Thus, it is possible that only one form of the type 2 Cu site is capable of binding and reducing nitrite, and still accept electrons from dithionite. In other words: when electrons are able to flow from the type 1 Cu to the type 2 Cu site, any form of this site is able to bind and reduce nitrite. However, when direct electron donation is required, only a form of type 2 Cu site (presumably one that has a coordination position available to receive electrons from an external donor) is able to perform catalysis. In His139Ala, this corresponds to the type 2 Cu incorporated \textit{in vivo}, a species which generates EPR Signal 1 (see section III.2.2.3.1.1). Methyl viologen functions only very poorly as an electron donor to His139Ala, presumably because its accessibility to the type 2 site for direct electron donation is very limited. As expected, azurin is unable to function as electron donor at all, since it can only donate electrons \textit{via} the type 1 Cu site, which, in this mutant, has a significantly higher midpoint redox potential so as to prevent electron transfer to the catalytic site.

In the Met144Ala mutant (section III.2.2.3.2), the weak, axial ligand to the type 1 Cu of NiR was replaced by an amino acid residue that is unable to provide that ligation. The Met ligand has been previously replaced by various different residues in
azurin (Karlsson et al., 1991; Murphy et al., 1993; Pascher et al., 1993; Romero et al., 1993; Bonander et al., 1996; Strange et al., 1996), rusticyanin (Hall et al., 1999) and nitrite reductase (Kukimoto et al., 1994; Murphy et al., 1995; Averill et al., 1998; Olesen et al., 1998; Veselov et al., 1998). The results obtained from these studies indicate that the Met residue is not essential for maintaining a type 1 Cu site. This replacement was found to affect the redox potential of the type 1 Cu site (Murphy et al., 1993; Pascher et al., 1993; Olesen et al., 1998; Hall et al., 1999) but otherwise no significant changes in the electronic properties of the site were observed (reviewed in Canters and Gilardi, 1993). Kukimoto et al. (1994) reported a Met150Glu mutant of the nitrite reductase from A. faecalis that is active when methyl viologen is used to donate electrons to the enzyme, but shows no activity when pseudoazurin is used as the electron donor. However, the determination of the X-ray structure of this mutant (Murphy et al., 1995) showed that the type 1 site contained Zn rather than Cu, which may explain those results. Averill et al. (1998) have reported that a Met189Ala mutant of Pseudomonas G-179 NiR is enzymatically active.

The Met144Ala mutant form of A. xylosoxidans NiR exhibits an essentially full complement of type 1 Cu (section III.2.2.3.2.1), consistent with the notion that the Met residue is not required for the assembly of a type 1 Cu site. Interestingly, the purified M144A and M144A\textsuperscript{Cu} proteins do not have a full complement of type 2 Cu. This is in agreement with the findings of Olesen et al. (1998) and can be tentatively explained by the presence of Zn atoms at the type 2 site of the Met144Ala enzyme (section III.2.2.3.2.1). However, the X-ray structure of this protein (section III.2.2.3.2.3) has shown that the mutation introduced has effects that extend beyond the type 1 Cu site, which may influence type 2 Cu binding. This is interesting, and should be kept in mind when analysing other mutant forms of NiR, because it shows that the overall effects of a single point mutation cannot easily be predicted and may often not be restricted to the immediate environment of the replaced residue.

The activity and substrate-binding studies carried out with the Met144Ala protein (section III.2.2.3.2.2) have provided interesting insights regarding the electron donation and intra-molecular electron transfer in this protein. The results obtained when methyl viologen and reduced azurin were used as electron donors are consistent with the notion that both these donors donate electrons to the type 1 Cu atoms which then transfer them to the type 2 site. In fact, the percentage of activity found when both these electron donors were used shows a good correlation with the estimated type
2 Cu content of the proteins. Since, as has been noted for the His139Ala mutant, neither methyl viologen nor azurin is able to donate electrons directly to a type 2 Cu site, these electrons must proceed via the type 1 Cu atoms. An ordered mechanism of the enzyme (Strange et al., 1999) implies that during catalysis type 1 Cu does not donate electrons to the type 2 site unless substrate is bound. Thus, the activity of the protein is only limited by its type 2 Cu content, thereby explaining the results obtained.

When dithionite is used as the electron donor, the M144A\textsuperscript{Cu} protein exhibits an activity that is higher than would be expected from its estimated type 2 Cu content alone. This finding may indicate that catalysis is possible at a Cu-depleted type 2 site, as suggested by activity studies of the T2D enzyme (Kohzuma et al., 1984; Prudêncio et al., 1999), nitrite-binding studies (Abraham et al., 1997) and pulse radiolysis studies (Suzuki et al., 1997). Catalysis at the Cu-depleted type 2 site of Met144Ala would, most likely, involve direct electron donation to this site since an ordered mechanism of the enzyme would, in principle, imply communication between the type 2 Cu and the type 1 Cu atoms. Adman et al. (1995) have determined the structure of T2D NiR from \textit{Ach. cycloclastes} and shown that when Cu is not bound, a water molecule is found at the type 2 site. This water is found to be displaced 1.6 Å from the position of the Cu towards the solvent, such that reasonable hydrogen bonds can be made from two of the His residues and to the Asp98 residue (\textit{Ach. cycloclastes} numbering). Thus, it is possible to speculate that in the absence of the type 2 Cu atom, nitrite can still enter the catalytic site, displace the hydrogen-bonded water molecule and be reduced.

The Cys130Ala mutation (section III.2.2.3.3) replaces one of the strong planar ligands to type 1 Cu by a residue that is unable to provide such a ligation. Studies of \textit{Ps. aeruginosa} azurin have shown that replacement of this residue by an Asp residue converts the site into a type 2 Cu site (Mizoguchi et al., 1992) whilst replacement by a Ser leads to loss of Cu binding altogether (see Canters and Gilardi, 1993). Averill et al. (1998) also reported inactive proteins when the Cys ligand of type 1 Cu in NiR from \textit{Pseudomonas} G-179 was replaced by an Ala residue, a Ser residue or an Asp residue. Thus, this residue is usually regarded as the only ligand of a type 1 Cu site that is essential to maintain its spectroscopic properties.
Not surprisingly, the Cys130Ala mutant form of NiR from *A. xylosoxidans* does not exhibit UV/Vis or EPR type 1 Cu features (section III.2.2.3.3.1). More interestingly, both the C130A and the C130A\textsuperscript{Cu} proteins have a very low overall Cu content, showing low occupancy of the type 2 Cu sites (section III.2.2.3.3.1). This suggests that the type 2 Cu site was also perturbed by the mutation, most likely as a result of breakage of the hydrogen bond between Cys130 and the His129 ligand to type 2 Cu. A complete data set has recently been collected on crystals of this mutant protein and is currently being processed. Hopefully, the determination of this structure will provide a more clear picture of the exact extent of the perturbation of the overall structure of the protein and, more precisely, of its Cu centres as a result of the mutation introduced.

The results of the activity studies carried out with the Cys130Ala mutant form of NiR (section III.2.2.3.3.2) are in accord with those obtained for the His139Ala and Met144Ala mutants. Reduced azurin is unable to donate electrons to the protein while methyl viologen does so only extremely poorly. This is consistent with the notion that both these electron donors can only donate electrons *via* the type 1 Cu centres of NiR. As was found for the His139Ala mutant, the Cys130Ala protein exhibits activity when dithionite is used as the electron donor, indicating that dithionite is capable of donating electrons directly to the type 2 site. There seems to be a good correlation between the activities determined for the C130A and C130A\textsuperscript{Cu} proteins when dithionite was used as the electron donor and their estimated type 2 Cu content of these proteins. However, these activities are somewhat higher than would be expected considering the results obtained with the His139Ala mutant. For this protein, the estimated type 2 Cu content was significantly higher than in the Cys130Ala mutant but activity with dithionite was not proportionally higher. Thus, catalysis at Cu-depleted type 2 sites, as suggested by the results obtained with Met144Ala, cannot be excluded but equally is by no means proved by these findings.

In the Asp92Glu and Asp92Asn mutants (section III.2.2.3.4), the environment of the type 2 Cu atom is perturbed, respectively, by the introduction of a bulkier amino acid residue that retains the charge properties of the original one and by the introduction of a non-charged amino acid residue that has approximately the same size as the original one.
The Asp92 residue (*A. xylosoxidans* numbering) has been proposed to be of considerable importance for catalysis, as explained in sections III.1.6.3 and III.1.6.4 and its replacement is likely to perturb the catalytic site of the enzyme. A previous report by Murphy *et al.* (1995) concentrated on the type 2 Cu ligand His135 of the enzyme from *A. faecalis* and showed that replacement of that residue by Lys did not prevent type 2 Cu ligation but totally abolished enzyme activity. Olesen *et al.* (1998) have introduced a mutation in the His287 residue of the enzyme from *Rh. sphaeroides*, located in the active site pocket, and shown that the activity of the mutant protein was markedly diminished and substrate-binding to its type 2 Cu was abolished. A very recent report by Kataoka *et al.* (2000) has shown that mutation of the Asp92 or His255 residues in the *A. xylosoxidans* enzyme resulted in loss of activity and suggested that those residues are involved in substrate anchoring.

The spectroscopic and metal content data collected on both Asp92Glu and Asp92Asn (section III.2.2.3.4.1) indicate that the former contains a nearly full complement of both type 1 Cu and type 2 Cu, while the latter is at least partially depleted in type 2 Cu. This is an interesting finding in that it indicates that the charge of the residue at position 92 plays an important role in the assembly of the type 2 Cu sites of NiR. However, the rhombic character found for type 2 Cu in Asp92Glu also indicates that the size constraints imposed by the presence of a bulkier amino acid residue at the type 2 site perturbs the geometry of this site, with possible implications for substrate accessibility and catalysis. As mentioned in section III.2.2.3.4.1, the possibility that reduced type 2 Cu is present at the type 2 site of Asp92Asn cannot be excluded and may constitute an indication that an uncharged amino acid residue at position 92 has a marked effect on the redox potential of the Cu in the type 2 site.

The activity and substrate binding results obtained for the Asp92 mutant proteins (section III.2.2.3.4.2) provide interesting indications with regard to the catalytic ability of these mutants. For both of them, activity when artificial electron donors are used is remarkably low. If in the case of Asp92Asn this could be explained by an absence of Cu at the type 2 site (or by the presence of Cu atoms with very high redox potentials), in the case of Asp92Glu this is more difficult to comprehend given the presence of presumably oxidised type 2 Cu. The results obtained with the Asp92Glu protein give a clear indication that, although assembly of the type 2 Cu centres has not been prevented, substrate accessibility to the catalytic site has been greatly perturbed by the presence of a bulkier amino acid. This notion is confirmed by
the EPR-monitored nitrite binding studies carried out with the Asp92Glu mutant. Alternatively, it is possible that the mutation has not prevented the substrate from entering the active site but has perturbed its anchoring, as suggested by Kataoka et al. (2000). The possibility that substrate accessibility and/or anchoring to the catalytic site in the Asp92Asn protein has also been perturbed cannot be excluded although it is more difficult to monitor in the absence of type 2 Cu EPR features.

An extremely interesting result was obtained when the physiological donor, reduced azurin, was used to donate electrons to the Asp92 mutants. The activity values determined under these conditions were significantly higher than those obtained when the artificial electron donors were used. The obvious conclusion that can be drawn is that interaction of azurin with Asp92Glu and Asp92Asn has an overall effect on the protein that modifies its catalytic ability. The most likely explanation for this enhancement of catalysis is that, upon formation of a transient complex with azurin, the substrate accessibility to the catalytic site increases, possibly due to a conformational change in the Asp92 mutants. It is worth analysing in more detail the effects on each of these proteins: the Asp92Glu mutant gives an activity with azurin that is ~14% of that seen in NiR. The reason why this value is not higher is readily explained by the fact that the D92E protein assayed does not have a full complement of type 1 Cu, which then becomes a limiting factor for activity. In the case of the Asp92Asn protein, the activity determined is ~58% of that seen in NiR. The D92N protein assayed has an estimated full complement of type 1 Cu. Therefore, the limiting factor for the activity of this protein is, presumably, its type 2 Cu content. As mentioned above, it is not clear whether Asp92Asn is type 2 Cu-depleted or whether type 2 Cu atoms in this protein are in an EPR-silent (presumably reduced) state. Thus, the results obtained can be explained in two different ways: (i) upon interaction with azurin, Asp92Asn undergoes a conformational change that may facilitate substrate accessibility to a Cu-depleted type 2 site and thereby allow catalysis to occur at such a site; (ii) the Asp92Asn protein contains type 2 Cu atoms in a reduced, catalytically-inactive state. Upon interaction with azurin, changes are induced in the geometry of the type 2 Cu site that modulate the potential of the type 2 Cu atoms rendering them catalytically active. Substrate accessibility may also be enhanced by these changes. The first explanation requires that catalysis can occur at a metal-depleted site to an extent which is more than 50% of that seen in a fully loaded centre. Furthermore, it implies that electron transfer from the type 1 Cu would occur
in the absence of type 2 Cu, a notion that is incompatible with the ordered mechanism suggested by Strange et al. (1999). Thus, it seems that the second hypothesis provides a much more consistent explanation for the results. The determination of the metal content of Asp92Asn indicates that the protein may have ~50 % Cu-loaded type 2 centres (assuming that this Cu is present in a spectroscopically-silent state), in good correlation with the activity value determined when azurin was used as the electron donor. However, again, the results do not eliminate the possibility that part of the catalysis observed occurs at a Cu-depleted site.

Thus, the findings gathered for the two Asp92 mutant forms of NiR indicate that interaction of this protein with azurin triggers important conformational changes that are likely to influence substrate accessibility to the catalytic site and to modulate the redox potential of the type 2 Cu atoms. This may help provide an explanation as to why electron transfer occurs between type 1 Cu and type 2 Cu of NiR, in what appears to be an “uphill” process (Farver et al., 1998) in the absence of the protein’s physiological electron donor.

The data obtained for recombinant NiR suggest that methyl viologen is the most effective electron donor to this enzyme, followed by azurin and finally by dithionite. However, only dithionite seems to be able to donate electrons directly to the type 2 Cu site, as shown by the studies carried out with the mutant forms of the enzyme. This is in agreement with the suggestion that SO$_2^-$ may act as an analogue of the NO$_2^-$ ion and thereby its access to the catalytic pocket may be facilitated (Dr. Faridoon Yousafzai, personal communication), unlike the cationic methyl viologen species and, certainly, reduced azurin.

It seems clear that the results obtained from the studies carried out on recombinant NiR and some of its mutant forms have provided an insight into the way in which the catalytic reduction of nitrite is performed by this enzyme. The roles of both types of Cu atoms in the enzyme have been confirmed and a deeper understanding of the complexity of electron donation to the enzyme and subsequent internal electron transfer achieved. Questions remain to be answered regarding some of these aspects. For example, is there a physiological relationship between the signal peptide of NiRs and the colour of the enzyme? Is catalysis possible at a Cu-depleted type 2 site? What is the extent of the redox change in the His139Ala mutant? Are
there forms of type 2 Cu centres that are preferred for catalysis? How does direct electron donation to the type 2 Cu proceed? What is the exact nature of the presumed conformational change that occurs upon interaction of the protein with its physiological electron donor? What are the roles of other presumably key residues in trimer assembly, intramolecular electron transfer and catalysis? The studies now performed and the availability of an optimised strategy for generating and purifying site-directed mutants of NiR will hopefully facilitate the quest for the answers to these and other questions.
CHAPTER IV

NITROUS OXIDE REDUCTASE
Chapter IV. Nitrous Oxide Reductase

IV.1. INTRODUCTION

The study of nitrous oxide reductase (N2OR) from Pseudomonas (Ps.) nautica 617 was carried out at the Chemistry Department of the Faculty of Sciences and Technology, New University of Lisbon, Monte de Caparica, Portugal, under the supervision of Prof. Isabel Moura. The characterisation of the enzyme from the spectroscopic, kinetic and biochemical points of view, provided valuable insights into its properties and mechanism (Prudêncio et al., 2000). The X-ray structure of the protein, the first of any N2OR to become available, was determined in Prof. Christian Cambillau’s laboratory, Marseille, France, and clarified important aspects of the enzyme’s three-dimensional organisation, its metal centres and mechanism (Brown et al., 2000).

These findings will be presented and discussed throughout sections IV.2 and IV.3. Section IV.1 is intended to provide the relevant background information on the system under study, and to place it within the wider context of this thesis.

IV.1.1. Identification of N2OR: Historical background

Nitrous oxide reductase (EC 1.7.99.6) catalyses the two-electron reduction of nitrous oxide to nitrogen and water:

\[ \text{N}_2\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2 + \text{H}_2\text{O} \]  

\[ \left[ E^{\circ} \ (pH \ 7.0) = +1.35 \text{ V; } \Delta G^{\circ} = -339.5 \text{ kJ/mol} \right] \]

The conversion of N2O to N2 is the final step of the denitrification pathway and can be regarded as a respiratory process in its own right (Zumft, 1997). Although N2O is an obligatory intermediate in the denitrification pathway, there are reports of non-denitrifying bacteria that respire N2O in a process linked to ATP synthesis [see Hochstein and Tomlinson (1988) and references therein]. Thus, the reaction catalysed by N2OR is an energy-conserving process in which the enzyme catalyses the transfer of electrons from its physiological electron donor to the N2O substrate.

The key role of Cu in the respiration of N2O became apparent from the studies of Iwasaki et al. (1980) and Iwasaki and Terai (1982), who demonstrated the role of this metal as being essential for anaerobic growth with N2O and the biosynthesis of
N₂OR in denitrifying bacteria. The first report of a ~120 kDa Cu-containing protein isolated from denitrifying pseudomonads came from Matsubara and Zumft (1982). The same authors later reported for the first time the identification of N₂OR as a molecular species and the first assay in which the isolated protein quantitatively reduced N₂O to N₂ with hydrogen, clostridial hydrogenase and methyl viologen as the electron-donating system (Zumft and Matsubara, 1982).

Since the isolation of the N₂OR from *Ps. stutzeri* (formerly *Ps. perfectomarina*) by Zumft and Matsubara (1982), the enzyme has been purified and characterised from the same organism (Coyle *et al.*, 1985; Riester *et al.*, 1989), as well as from other bacterial sources: *Rhodobacter (Rh.) capsulatus* (formerly *Rhodopseudomonas capsulata*) (McEwan *et al.*, 1985); *Rh. sphaeroides* f. sp. *denitrificans* (formerly *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*) (Michalski *et al.*, 1986; Sato *et al.*, 1999); *Paracoccus denitrificans* (Snyder and Hollocher, 1987); *Wolinella succinogenes* (Teraguchi and Hollocher, 1989); *Achromobacter* (*Ach.*) *cycloclastes* (Hulse and Averill, 1990; Henery *et al.*, 1999); *Ps. aeruginosa* (SooHoo and Hollocher, 1991); *Thiospaera pantotropha* (Berk et al., 1993); *Thiobacillus denitrificans* (Hole *et al.*, 1996); and *Alcaligenes (A.) xylosoxidans* (formerly *Alcaligenes sp. N.C.I.M.B. 11015, Achromobacter xylosoxidans* and *Pseudomonas denitrificans*) (Matsubara and Sano, 1985; Ferretti *et al.*, 1999). Data presented below for the enzymes from *Rh. sphaeroides*, *Ach. cycloclastes* and *A. xylosoxidans* come from the reports of Sato *et al.* (1999), Hulse and Averill (1990) and Ferretti *et al.* (1999), respectively. Data for the remaining enzymes are from the references listed above.

### IV.1.2. Solubility and subcellular localisation of N₂OR

All the N₂ORs studied so far are soluble proteins, with the exception of the protein from the gliding soil bacterium *Flexibacter canadensis* (Jones *et al.*, 1992) and that from *Thiobacillus denitrificans* (Hole *et al.*, 1996) which were reported to be membrane-bound. The gene encoding the *Flexibacter* enzyme was shown by DNA hybridisation techniques to differ from those of other denitrifiers (Jones *et al.*, 1992), unlike that from *Thiobacillus* (Hole *et al.*, 1996). However, there are no reports of further studies of these N₂OR proteins and their molecular properties are, therefore, largely unknown.
The first indication for the periplasmic location of N$_2$ORs came from the studies of Boogerd et al. (1981) with *Paracoccus denitrificans*, where it was suggested that protons for N$_2$O reduction are provided from outside the cell and not from the cytoplasmic side of the membrane. A periplasmic location for N$_2$OR was also suggested by cell fractionation studies performed with this organism (Alefounder et al., 1983), with *Rh. sphaeroides* (Urata and Satoh, 1984) and with *Rh. capsulatus* (McEwan et al., 1985). These findings were later confirmed by direct immunocytochemical location studies done on the protein from *Ps. stutzeri* (Körner and Mayer, 1992).

The deduced amino acid sequences of several N$_2$ORs (see Fig. IV.8, section IV.2.1.4) indicate that the protein is synthesised as a precursor, with a signal peptide comprising between 43 (enzymes from *Ach. cycloclastes* and *Sinorhizobium meliloti*) and 57 amino acid residues (enzyme from *Ralstonia eutropha*). These are rather long signal peptides that share a common twin-arginine motif (indicated by the blue arrows in Fig. IV.8. See also Zumft et al., 1992). This type of signal sequence has recently been shown to be recognised by a novel export pathway that is Sec-independent and which has been proposed to export folded, redox-active proteins across the cytoplasmic membrane (Berks, 1996; Santini et al., 1998; Sargent et al., 1998; Weiner et al., 1998. See also section III.1.3). The twin arginine motif has been found in proteins with metal cofactors such as polynuclear Cu sites, iron-sulphur centres, a molybdenum cofactor, tryptophan tryptophylquinone or flavin adenine dinucleotide. All proteins of the family have the arginine pair within the consensus sequence (S/T)-R-R-X-F-L-K (Zumft, 1997). It was found for N$_2$OR from *Ps. stutzeri* that when the first of the two arginines is mutated, the protein precursor accumulates in the cytoplasm and the enzyme is devoid of Cu. This indicates that not only is the arginine residue essential for protein export but also that translocation of N$_2$OR occurs before, or is concomitant with, Cu insertion. Furthermore, in vitro reconstitution of the Cu$_A$ site was possible, indicating that the lack of metal was not due to a serious conformational restraint (Dreusch et al., 1997).
IV.1.3. Molecular properties of N_{2}OR: Different forms of the enzyme

N_{2}OR is a homodimeric protein, with a subunit molecular mass of ~66 kDa (reviewed in Zumft, 1997). With the exception of the N_{2}OR from *Rh. sphaeroides* f. sp. *denitrificans*, which was reported to contain Zn and Ni (Michalski *et al.*, 1986), and that from *Wolinella succinogenes*, which is a heme-Cu protein (Teraguchi and Hollocher, 1989), all other purified N_{2}ORs contain only Cu cofactors. Until recently, a copper content of ~4 Cu atoms per monomer was generally reported for this enzyme (reviewed in Zumft, 1997). However, the unequivocal determination of the exact number of Cu atoms in the protein has been prevented by the uncertainties inherent to the chemical analysis of a high molecular mass protein with several metal atoms. This issue could only be resolved with the determination of the structure of the catalytic centre, which for a long time was assumed to be binuclear (Zumft, 1997).

N_{2}ORs have been reported to exist in different forms, distinguished by their redox, spectroscopic and kinetic properties. These forms have been given trivial names and a numerical classification. Their properties were reviewed in Zumft (1997) and are summarised below.

**Form I of N_{2}OR**, also called the purple form, was obtained by anaerobic purification from *Ps. stutzeri*, *Paracoccus denitrificans*, *Ps. aeruginosa*, *A. xylosoxidans* and *Rh. sphaeroides* f. sp. *denitrificans*. This form of the enzyme is reported to have high activity (~75 µmol N_{2}O reduced · min^{-1} · mg^{-1}) with 7.3 - 8.2 Cu atoms / dimer (not reported for the *Rhodobacter* enzyme).

The UV/Vis spectrum of this species exhibits an intense absorption at ~540 nm, a poorly resolved shoulder at ~480 nm and another, less intense, absorption maximum at ~780 nm (Fig. IV.1-A, spectrum a). For the *Paracoccus* enzyme, this absorbance spectrum is only obtained after incubation of the protein with N_{2}O (the as-purified enzyme exhibits two confluent bands at 550 nm and 660 nm), whereas the *Rhodobacter* enzyme does not exhibit a shoulder at ~480 nm but a slight one is present at 630 nm. The average ratio Abs_{540nm} / Abs_{480nm} for form I of N_{2}OR is ~1.8.

The X-band EPR spectrum of N_{2}ORI (not shown in the reports for the *Rhodobacter* and *Ps. aeruginosa* enzymes) has an axial signal with g_{||} = 2.18 and g_{⊥} = 2.03 and a 7-line hyperfine splitting in the g_{||} and g_{⊥} regions (Fig. IV.1-B, spectrum...
A). This splitting is not defined in the enzyme from *Paracoccus*. This signal arises from a dinuclear Cu centre, Cu\(\mathrm{A}\), in which the two Cu atoms share an unpaired electron (see section IV.1.4.1). This centre is also responsible for several Cu-S transitions that contribute to the characteristic absorbance spectrum of this protein, as discussed extensively in Farrar *et al.* (1996) (see also section IV.1.4.1.4).

**Form II of N\(_2\)OR**, also called the pink form, was obtained by aerobic purification from *Ps. stutzeri*, *Ach. cycloclastes*, *Ps. aeruginosa*, *Thiosphaera pantotropha* and *Rh. sphaeroides* f. sp. *denitrificans*. This enzyme species is generally reported to have lower activity than form I, ranging between 3.7 and 30 \(\mu\)mol N\(_2\)O reduced \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) for different enzyme sources. A rather high activity of 86.4 \(\mu\)mol N\(_2\)O reduced \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) was reported for the *Achromobacter* enzyme, whilst the enzyme from *Ps. aeruginosa* is reported to be inactive. The Cu content of form II of N\(_2\)OR (N\(_2\)ORII) is reported to be between 6.6 and 7.2 Cu atoms per protein.

The UV/Vis spectrum of N\(_2\)ORII exhibits electronic absorptions at \(~480\) nm, \(~530\) nm, \(~630-650\) nm and \(~780\) nm (Fig. IV.1-A, spectrum b). For the enzyme from *Rhodobacter* these bands are at 485 nm, 541 nm, 641 nm and 758 nm, respectively; the N\(_2\)ORII from *Ps. aeruginosa* does not exhibit any features in the visible region. The ratio Abs\(_{530nm}\) / Abs\(_{480nm}\) is \(~1.2\), which is lower than that of form I.

The EPR spectrum of form II (not shown in the reports of the *Thiosphaera*, *Ps. aeruginosa* and *Rhodobacter* enzymes) resembles that of form I, but the hyperfine splitting in the \(g_{zz}\) region is less well-resolved in form II than in form I (Fig. IV.1-B, spectrum B). In addition to this signal, a broad, featureless signal similar to that found in N\(_2\)ORIII (see below) is observed to some extent in form II of N\(_2\)OR.

**Form III of N\(_2\)OR**, also called the blue form, is obtained by reduction of either form I or form II of the enzyme with a 2- to 10-fold excess of dithionite or ascorbate under exclusion of O\(_2\). This form of the protein has been studied for all the bacterial sources listed in section IV.1.1 and is reported to be inactive for the enzymes from *Ps. stutzeri*, *Rh. capsulatus*, *Ach. cycloclastes*, and *A. xylosoxidans*. Its activity is not reported for the enzymes from *Paracoccus* and *Ps. aeruginosa*. However, form III of the enzyme from *Thiosphaera* retained \(~70\)% of its activity upon incubation with
dithionite and, in the case of the *Rhodobacter* enzyme, similar activities are reported for forms I and III.

The UV/Vis spectrum of N\(_2\)ORIII exhibits a broad absorption maximum at ~650 nm, which gives the protein a blue colour (Fig. IV.1-A, spectra c and d). In the case of the enzyme from *Rhodobacter*, a slight shoulder is also present at 750 nm. Once formed, the blue chromophore is rather inert and can not be further reduced (Riester *et al*., 1989).

The reaction of N\(_2\)ORI with dithionite is reported to proceed in two phases: the first is a fast phase in which the shoulder at 480 nm is removed (Farrar *et al*., 1991). During this phase Abs\(_{540}\) nm decreases and Abs\(_{640}\) nm decreases simultaneously (Riester *et al*., 1989). In the second, slower phase, the broad band at ~650 nm appears until it reaches its final intensity (~10% higher than that of N\(_2\)ORI). When N\(_2\)ORII is anaerobically titrated with dithionite, no additional blue chromophore is formed (Riester *et al*., 1989). Anaerobic potentiometric titrations of both the purple and the pink forms of N\(_2\)OR from *Ps. stutzeri* with ferricyanide as a mediator and dithionite as the reductant gave a midpoint potential \(E_{0, 540}\) of \(+260\) mV, at pH 7.5 and 25 °C (Coyle *et al*., 1985). Incubation of N\(_2\)ORIII with ferricyanide in the presence of air restores a pink form of the enzyme, irrespective from which species N\(_2\)ORIII had originally been derived (Coyle *et al*., 1985). However, restoration of N\(_2\)ORI from N\(_2\)ORIII is only possible if incubation with ferricyanide is performed anaerobically (Riester *et al*., 1989).

The EPR spectrum of the N\(_2\)ORIII species, as reported for the enzymes from *Ps. stutzeri* and *A. xylosoxidans*, exhibits a broad, featureless signal with \(g\) values at ~2.18 and ~2.06 (Fig. IV.1-B, spectrum C) whereas that reported for the enzyme from *Paracoccus* exhibits ill-defined hyperfine lines in the \(g//\) region. N\(_2\)ORIII from *Ach.cycloclastes* has an EPR spectrum that resembles that of type 1 Cu, with \(g// = 2.19\) (\(A// = 7.2\) mT) and \(g\perp = 2.06\).

**Form IV of N\(_2\)OR**, also called the reconstituted form, was prepared from the *Ps. stutzeri* enzyme by removal and subsequent reconstitution of the Cu in the protein. Cu removal was achieved by anaerobic reduction of the enzyme with ascorbate, followed by incubation with KCN. Reconstitution of ~90% of the Cu was obtained by anaerobic incubation of the apoprotein with Cu(II). Details of these procedures are
given in Coyle et al. (1985). The same authors report the only studies done with this
form of N₂OR which showed that the protein is catalytically inactive. Its UV/Vis
spectrum exhibits absorption maxima at 480 nm, 530 nm and 800 nm. The 620 nm
band seen in N₂ORII is not present in the reconstituted species. Its EPR spectrum
resembles that of form V (see below).

**Form V of N₂OR**, also called the mutant form, was obtained from strain
MK402 of *Ps. stutzeri* ATCC 14405, which is defective in copper chromophore
biosynthesis. This strain is one of several transposon Tn₅-induced mutant strains of
that organism which are defective in nitrous oxide respiration (Zumft et al., 1985;
Viebrock and Zumft, 1987; Zumft et al., 1990). The nosZ gene is the structural gene
for the N₂OR protein (Viebrock and Zumft, 1988). Evidence suggests that Cu
insertion in the apo-N₂OR is enabled, assisted or catalysed by additional proteins
encoded by genes in the cluster. The proteins in this putative Cu insertion complex are
encoded by the nosD, nosF, and nosY genes, located downstream adjacent to nosZ
[see Zumft (1997) for a review on the apparatus for Cu assembly into apo-N₂OR]. In
strain MK402 these three genes involved in the process of Cu insertion into N₂OR are
not functional so that only Cu-incorporation that is independent of these proteins can
occur (Dooley et al., 1991a). The N₂ORV protein has ~2 Cu atoms per molecule and
is inactive (Coyle et al., 1985; Riester et al., 1989; Dooley et al., 1991a; Farrar et al.,
1996). Furthermore, the purified N₂ORV is unable to bind added Cu (Riester et al.,
1989; Farrar et al., 1996). It is important to recognise that, as pointed out by Dooley et
al. (1991a), the primary structure of N₂ORV is identical to that of the native enzyme.
During purification N₂ORV behaves like the wild-type enzyme. Furthermore, N₂ORV
is immunochemically and electrophoretically identical to the copper-depleted form of
N₂ORI. N₂ORV is also translocated across the cytoplasmic membrane into the
periplasmic space, where the wild-type enzyme functions. Hence, it is plausible that
the tertiary structures of N₂ORV and N₂ORI are similar (Dooley et al., 1991a).
Spectroscopic evidence suggests that the electron structures of the Cuₐ site in N₂ORI
and the paramagnetic centre in N₂ORV are similar. Thus, N₂ORV is generally
regarded as containing an occupied Cuₐ site and is possibly lacking the additional Cu
found in the wild-type enzyme (Dooley et al., 1991a; Farrar et al., 1996, Zumft, 1997.
See also section IV.1.4.1.1). Thus, the spectroscopic properties of N₂ORV are of great
importance in elucidating the electronic structure of Cuₐ and also in obtaining
information on the catalytic centre of N₂OR. These properties are presented and
discussed in more detail in the following sections.
Figure IV.1 – UV/Vis (A) and EPR (B) spectra of different forms of N\textsubscript{2}OR from \textit{Ps. stutzeri}. Spectra a and A correspond to form I; spectra b and B correspond to form II; spectra c and C correspond to form III obtained after addition of excess dithionite to form I, spectrum d also corresponds to form III obtained after addition of excess dithionite to form II. The spectra were taken from Coyle \textit{et al.} (1985).
IV.1.4. The Cu centres in N\textsubscript{2}OR

For many years, the Cu atoms in N\textsubscript{2}OR were believed to be organised in two binuclear centres per protein monomer. This model was proposed by Farrar \textit{et al.} (1991) and is based on optical, EPR and MCD spectroscopical evidence. It suggested that the two Cu dimers in N\textsubscript{2}OR are an electron transfer, mixed-valence \([\text{Cu}^{+1.5} \text{Cu}^{+1.5}]\), Cu\textsubscript{A} centre, similar to that found in cytochrome \textit{c} oxidase (COX), and a catalytic, Cu\textsubscript{Z} centre, which could cycle between oxidation states \([\text{Cu}^{2+} \text{Cu}^{2+}]\) and \([\text{Cu}^{2+} \text{Cu}^{1+}]\). The recent determination of the first X-ray structure of a N\textsubscript{2}OR (Brown \textit{et al.}, 2000), discussed in detail in section IV.2.2 has shown that, whereas the Cu\textsubscript{A} centre was anticipated accurately from spectroscopic studies, the catalytic Cu site is suprisingly different from what had been proposed and is, in fact, comprised of 4 Cu ions arranged in a novel type of tetrameric Cu cluster (see section IV.2.2.2). Nevertheless, the following sections deal with the previous models of the N\textsubscript{2}OR Cu centres with special emphasis on the correctly predicted Cu\textsubscript{A} centre.

IV.1.4.1. The Cu\textsubscript{A} electron transfer centre

N\textsubscript{2}OR is a rather promiscuous enzyme in that it can accept electrons from a variety of proteins. Parallel pathways of electron transfer and sometimes alternative electron donors exist in different denitrifiers. The electron donors to N\textsubscript{2}OR include various cytochromes, mainly of the \textit{c}- and \textit{b}- types (reviewed in Zumft, 1997). The reactivity of the same reductase with different types of electron carriers suggests a low degree of recognition specificity at the electron entry site. The Cu\textsubscript{A} centre in N\textsubscript{2}OR is believed to be involved in electron transfer from the physiological electron donor to the catalytic site of the enzyme (see Holz \textit{et al.}, 1999 for an NMR-based model of the Cu\textsubscript{A} domain of N\textsubscript{2}OR showing the putative docking site for cytochrome \textit{c}). The following sections deal with the electronic and structural properties of this site.

IV.1.4.1.1. Cu\textsubscript{A} is present in COX and N\textsubscript{2}OR

The Cu\textsubscript{A} centre has unique properties amongst the Cu sites identified in proteins so far. The first spectroscopic evidence that Cu\textsubscript{A} has a structure different from that of type I Cu centres was presented by Beinert \textit{et al.} (1962). These authors
used EPR spectroscopy to study cytochrome oxidase and reported the first X-band EPR spectrum ever recorded for this enzyme. For quite some time, the CuA centre was believed to be exclusive to COX and to have a mononuclear Cu(Cys)$_2$(His)$_2$ structure, known as the Chan model (Chan and Li, 1990). In COX, the optical properties of CuA are obscured by intense and overlapping transitions from the heme chromophores and the only absorption band which could be assigned to CuA was a peak at ~830 nm. In this context, the observation that N$_2$OR contained a Cu site with very similar properties to those of the CuA site in COX was of crucial importance.

The similarity between the CuA centres in COX and N$_2$OR was first shown by Kroneck et al. (1988) (see also Kroneck et al., 1989, 1990a) using a multifrequency EPR approach. The authors recorded the EPR spectra of the N$_2$OR from Ps. stutzeri at 2.4, 3.4, 9.31 and 35 GHZ. They concluded that their results could be explained by a mixed-valence [Cu$^{1.5+}$ Cu$^{1.5+}$], S= $\frac{1}{2}$, species and noted the striking similarity between the low frequency EPR signals of N$_2$OR and COX. Soon after this finding, Jin et al. (1989) studied the Cu sites in N$_2$OR by electron spin echo (ESE) spectroscopy and presented further evidence for the similarity between the CuA sites in the two enzymes. This similarity was also shown by magnetic circular dichroism (MCD) studies performed on N$_2$OR (Dooley et al., 1991a. See also Scott et al., 1989 and Farrar et al., 1991). It should be emphasised that the work of Dooley et al. (1991a) was also important in that, by comparing the MCD and X-ray absorption spectra [Cu K-edge and extended X-ray absorption fine structure (EXAFS)] of N$_2$ORI and N$_2$ORV, it provided strong evidence that N$_2$ORV contains an occupied CuA site and that the higher percentage of Cu in this species is indeed of the CuA type. Evidence for the presence of CuA centres in N$_2$OR was also obtained from the EXAFS studies of Scott et al. (1989) and SooHoo et al. (1991), which revealed that the Cu EXAFS curve-fitting results for N$_2$OR are strikingly similar to those for COX. Antholine et al. (1992) provided further EPR evidence for the presence of a CuA site in N$_2$OR which supported the notion that this is a mixed-valence site. Finally, the identification of a set of potential Cu ligands in the C-terminal domain of N$_2$OR that correspond to those of the CuA carrying subunit II of COX (Viebrock and Zumft, 1988; Scott et al., 1989; Zumft et al., 1992) was also of crucial importance in establishing that the CuA site, initially believed to be unique to COX, is clearly also present in N$_2$OR.
IV.1.4.1.2. Probing the structure of CuA

Even after the notion that the CuA centre was common to both COX and N\textsubscript{2}OR began to be generally accepted, the controversy about the structure of this site still persisted. The Chan model (Chan and Li, 1990 and see previous section), which assumed that CuA was a mononuclear Cu centre, was opposed by the model of Kroneck, Antholine and Zumft (Kroneck \textit{et al.}, 1988; Antholine \textit{et al.}, 1992), which suggested a binuclear nature for this site (see also previous section). It is interesting to read the letters exchanged between these two groups over the years of 1989 and 1990 (Kroneck \textit{et al.}, 1989, 1990a; Li \textit{et al.}, 1989) where both authors put forward their arguments in favor of one or the other model. It was probably the identification, as mentioned in the previous section, of a consensus sequence of two similarly spaced His residues, two Cys residues and a Met residue (Zumft \textit{et al.}, 1992) found in N\textsubscript{2}OR and COX that marked the beginning of the binuclear model being favoured over the mononuclear one. As it turns out, the CuA site is, indeed, a binuclear Cu centre (see sections IV.1.4.1.3 and IV.2.2.3).

Between 1994 and 1995 several groups used various spectroscopic and mutational studies to try to determine the arrangement of the ligands in the CuA centre (reviewed in Beinert, 1997). Basically, all these models assumed two His ligands and two Cys ligands for the Cu atoms. The main differences between them regarded the overall geometry of the site, the position of the Cys residues (terminal or bridging) and the presence or absence of a direct Cu-Cu bond. These issues would later be clarified with the determination of the crystal structure of COX (see following section).

IV.1.4.1.3. The X-ray structure of the CuA site in COX

The determination of the X-ray structure of the COXs from \textit{Paracoccus denitrificans} (Iwata \textit{et al.}, 1995) and from bovine heart (Tsukihara \textit{et al.}, 1995; Tsukihara \textit{et al.}, 1996) (both at 2.8 Å resolution), as well as that from the engineered CuA fragment from the CyoA quinol oxidase from \textit{E. coli} (Wilmanns \textit{et al.}, 1995) (at 2.3 Å resolution) and, more recently, that of the CuA domain of the \textit{ba}_3-type COX from \textit{Thermus thermophilus} (Williams \textit{et al.}, 1999) (at 1.6 Å resolution) revealed the structure of the CuA site and definitively established its binuclear nature (Fig. IV.2).
The Cu atoms in the Cu\textsubscript{A} site are ligated by two Cys residues, two His residues, one Met residue and a peptide carbonyl of a Glu residue. All these ligands, except for the peptide carbonyl, were previously proposed based on the results of mutagenesis experiments (Kelly et al., 1993. See also Fig. IV.3).

The ligands for each Cu atom form a distorted tetrahedron and the two Cys thiolates bridge the Cu atoms. The Cu-Cu distance was reported to be 2.6 Å in the case of the Paracoccus COX (Iwata et al., 1995), 2.7 Å in the case of the bovine heart enzyme (Tsukihara et al., 1995) and 2.5 Å for the Cu\textsubscript{A} domain of the enzyme from Thermus (Williams et al., 1999). Blackburn et al. (1997) used EXAFS to refine further the structural details of the site in both the mixed-valence and the fully reduced forms of COX from Thermus thermophilus and Bacillus subtilis. These authors determined a Cu-Cu distance of 2.44 Å and Cu-S-Cu angles of 65°. The C-terminal globular domain of the Cu\textsubscript{A}-carrying subunit II of COX contains a ten-stranded β-barrel, a cupredoxin fold (see section I.2.2.1) as expected from sequence comparisons (Iwata et al., 1995).

Figure IV.2 – Stereo view of the Cu\textsubscript{A} centre in subunit II of COX from Paracoccus denitrificans. The Cu atoms are represented in blue. The loops providing the ligands to the Cu atoms are shown and labelled according to the β-strands which they connect (see Iwata et al., 1995 for further details). The picture was taken from Iwata et al., 1995.
IV.1.4.1.4. Further studies on the CuA site of N$_2$OR

The electronic structure of the CuA site was analysed in great detail by Farrar et al. (1996). These authors used EPR, MCD, circular dichroism (CD) and optical absorption spectroscopies to study three different CuA-containing proteins, namely PdII (the soluble domain of subunit II of *P. denitrificans* COX expressed in *E. coli*), N$_2$ORV (see section IV.1.3) and purple CyoA (subunit II from the quinol oxidase of *E. coli* into which a CuA-binding site was genetically engineered).

The extremely small hyperfine splitting found in the EPR spectra of CuA is indicative of a high degree of delocalisation of the electron onto the Cu ligands. This is less pronounced in the case of the genetically engineered site of purple CyoA (Farrar et al., 1996). Similarly, the MCD, circular dichroism (CD) and optical absorption spectra of this protein show remarkable differences compared with those of the other two proteins studied. This attests to a very close similarity between the CuA centres in PdII and N$_2$ORV, in spite of considerable sequence differences in the protein environment of the second coordination sphere.

From their extensive spectroscopic analyses, Farrar et al. (1996) concluded that, in both PdII and N$_2$ORV, the CuA centre exists in a mixed-valence ground state in which the unpaired electron density is shared equally by the two Cu ions (whereas in the case of the purple CyoA these ions have become slightly inequivalent). This is in agreement with the findings of Neese et al. (1996) when studying $^{63}$Cu, $^{65}$Cu and $^{65}$Cu/$[^{15}$N]His-enriched N$_2$OR. By combining the results from absorption, CD and MCD spectroscopies, Farrar et al. (1996) proposed a rather complex assignment of the optical spectra of the proteins that provides an insight into the electronic structure of the site.

EPR data and ligand assignments from site-directed mutagenesis were used to propose a model for the CuA centre of N$_2$OR (Fig. IV.3). The atomic distances are based on the EXAFS properties of N$_2$ORV (see Zumft, 1997).

The determination of the first X-ray structure of a N$_2$OR, presented and discussed in section IV.2.2, constitutes the ultimate proof that the CuA centre in this enzyme is indeed binuclear and similar to that found in COX.
IV.1.4.2. The catalytic centre

For a long time, the model for the copper sites in nitrous oxide reductase considered that two binuclear sites existed. One is the CuA centre, discussed in section IV.1.4.1, the site of electron entry into the enzyme. The other is the catalytic, substrate-binding site, usually referred to in the literature as CuZ (Farrar et al., 1991; reviewed in Zumft, 1997). The structural and spectroscopic data for the CuZ site has always been much less abundant than that gathered for CuA. Furthermore, the recently determined crystal structure of N2OR (section IV.2.2) showed that this centre has quite unusual, and totally unpredicted features, which has hindered all spectroscopic attempts made thus far to characterise it. Nevertheless, this section will attempt to summarise the existing spectroscopic and structural information for the CuZ centre. In section IV.1.4.3 two models proposed for the Cu sites in N2OR will be presented.

In the resting state of N2OR only ~25% of the Cu atoms are EPR active (Coyle et al., 1985; Riester et al., 1989; Dooley et al., 1991b). Addressing this issue, Dooley et al. (1991b) suggested that all the paramagnetism in the enzyme may be accounted for by its CuA content. These authors suggested that the remaining Cu atoms in the protein, at the time believed to be two, could be present as antiferromagnetically
coupled type 3 Cu(II) dimers (see section I.2.2.3), in agreement with a previous suggestion by Jin et al. (1989). This notion for the configuration of the catalytic site became generally accepted and was used to explain the properties of the enzyme [reviewed in Zumft et al. (1997) and see following section].

Resonance Raman (RR) studies indicated that there is a highly covalent \([\text{Cu(II)}-\text{S}^\cdots\text{(Cys)} \leftrightarrow \text{Cu(I)}-\text{S}^\cdots(\text{Cys})]\) thiolate site on the reduced species, \(N_2\text{OR}^{\text{III}}\) (Dooley et al., 1987, 1991a). Optical, EPR and MCD spectroscopy studies by Farrar et al., (1991) led to the proposal that Cu\(Z\) would have thiolate coordination, similar to Cu\(A\) (Andrew et al., 1994 and see following section). However, the nature and origin of the Cys residue are not clear. As can be seen in Fig. IV.8 (section IV.2.1.4), there are no conserved Cys residues outside the C-terminal Cu\(A\)-containing domain of \(N_2\text{OR}\). Cys165 of the \(P.s.\) stutzeri enzyme (indicated in Fig. IV.8 by a red circle) is conserved in 5 out of the 7 sequences of \(N_2\text{OR}\)s known to date. Dreusch et al. (1996) have shown that mutating this residue to a glycine destabilises the protein but does not affect its activity or its Cu content, thus showing that Cys165 is not a ligand to Cu\(Z\).

Zumft (1997) noted the presence of 8 His residues conserved in the primary sequences of \(N_2\text{OR}\)s. This high number of conserved His residues led Farrar et al. (1998) to propose that the catalytic site might be a Cu-His centre with magnetic interaction between a pair of Cu ions. Farrar et al. (1998) also pointed out the fact that the total number of Cu atoms in the fully active \(N_2\text{OR}\) was (at the time) uncertain and suggested that the catalytic site in this enzyme may be more complex than a simple binuclear type 3 site. As it transpired, Farrar et al. (1998) were right in their prediction, as demonstrated by the crystal structure of the catalytic site of \(N_2\text{OR}\), discussed in section IV.2.2.2.

**IV.1.4.3. Two models for \(N_2\text{OR}\)**

The following two sections deal with two models that have been proposed to explain the properties of the Cu sites in \(N_2\text{OR}\). The first one was reported in 1991 by Farrar et al., and considers Cu\(A\) and Cu\(Z\) to be two different entities, the former being the electron transfer centre and the latter the catalytic centre of \(N_2\text{OR}\). The second model was proposed by Farrar et al. (1998) and suggests that Cu\(A\) and Cu\(Z\) are both
variants of the electron transfer centre of the enzyme. Both these models were proposed in the absence of a crystal structure of N\textsubscript{2}OR, when the tetranuclear Cu centre that it revealed was unknown (section IV.2.2.2). Nevertheless, both these models constitute important landmarks in the study of this enzyme and were by no means made redundant by the knowledge now at hand. Furthermore, they make use of the information assembled in the previous sections and their description contributes to the clarification of the notions presented throughout this chapter.

**IV.1.4.3.1. The Farrar model - 1991**

Farrar *et al.* (1991) proposed a model for the Cu centres of N\textsubscript{2}OR based on optical, EPR and MCD spectroscopy studies done with the enzyme from *Ps. stutzeri*. As previously mentioned (section IV.1.3), the reduction of N\textsubscript{2}OR by sodium dithionite proceeds in two kinetic steps: a fast phase followed by a slower phase. Thus, the enzyme can exist in an oxidised, a semi-reduced and a reduced state. As mentioned in section IV.1.3, the EPR spectrum of the oxidised form exhibits a well-defined 7-line hyperfine splitting whereas that of the reduced form has a broad, featureless signal. The integration of these spectra showed that in the oxidised form \(~22\%\) of the total Cu is paramagnetic whereas in the reduced form \(~10\%\) of the Cu is EPR-detectable. The semi-reduced form, however, shows an extremely weak EPR signal with only \(~0.45\%\) of the Cu being paramagnetic. In other words, the semi-reduced state of the enzyme is EPR-silent. On the other hand, the MCD spectra of the oxidised and reduced forms of N\textsubscript{2}OR are dominated in both oxidation states by the EPR-active paramagnets. The MCD intensities are similar for both oxidation states but the form and shape of the spectra are quite different.

Q-band EPR studies (Kroneck *et al.*, 1988) showed that two paramagnetic species are present in the oxidised form of N\textsubscript{2}OR, one with g values of 2.16 and 1.99 and the other with g= 2.02. So, the question now was how to identify the bands in the MCD spectrum of oxidised N\textsubscript{2}OR that correspond to these two species. N\textsubscript{2}OR\textsubscript{V} (see section IV.1.3) lacks this second EPR signal. Thus, the bands in the MCD spectrum of N\textsubscript{2}OR\textsubscript{V} that are also present in the MCD spectrum of the oxidised N\textsubscript{2}OR were identified as belonging to the species that generates the g values of 2.16 and 1.99. The remaining bands in the MCD spectrum of the oxidised protein were similar to those of the paramagnet observed in the dithionite-reduced state. This was interpreted by the
authors as an indication that the structures of the species that generate these two signals are closely related.

Thus, in the oxidised state, N₂OR has two paramagnetic species. One of these species, called centre A, exists also in N₂ORV, a form of the enzyme believed to lack the catalytic site. In the semi-reduced state, the enzyme is virtually EPR-silent and therefore contains centre A (as well, of course, as the second paramagnetic centre of the oxidised form) in a diamagnetic state. The absorbance spectrum of this semi-reduced state of the protein exhibits absorption bands at 540 nm and 650 nm which must, therefore, belong to a second centre, which the authors call centre Z. Further reduction of the protein reduces centre Z which assumes an EPR-active, paramagnetic state. Centre Z is thus assumed to be a type 3 binuclear Cu site that can cycle between oxidation states Cu(II)/Cu(II) and Cu(II)/Cu(I). In its oxidised state (when the protein is oxidised or semi-reduced), centre Z is diamagnetic but exhibits an optical spectrum (bands at 540 nm and 650 nm) as a result of ligand-to-metal charge-transfer (LMCT). In the reduced state (when the protein is dithionite-reduced), the centre is paramagnetic, therefore EPR-active, with a strong absorption band at 650 nm.

Returning to the two paramagnetic species observed in the oxidised state of the protein, one of these species is, as discussed above, centre A. Considering the contributions of oxidised centre Z to the visible spectrum of the (semi-reduced or oxidised) protein, centre A can be assumed to contribute with absorptions at 480 nm, 540 nm and 800 nm towards the observed UV/Vis spectrum of the oxidised enzyme. As mentioned above, the second paramagnetic species of the oxidised enzyme is assumed, on the basis of the MCD results, to be structurally similar to the reduced form of centre Z, namely a Cu(I)/Cu(II) dimer. By analogy with COX, centre A is suggested to be an electron transfer centre. The authors proposed that centre Z is the catalytic site and that it can assume different forms, with different reactivity towards ligands. Thus, the second paramagnetic species seen in the native enzyme would correspond to a different form of centre Z, which the authors call Z*. Z* would, therefore, be EPR-active [Cu(I)/Cu(II)] in the native N₂OR and EPR-silent [Cu(I)Cu(I)] in the semi-reduced or dithionite-reduced enzyme. Clearly, Z and Z* must differ in their redox potentials. Fig. IV.4 shows a schematic representation of the Farrar et al. (1991) model.
### IV.1.4.3.2. The Farrar model - 1998

In 1998 Farrar *et al.* proposed a different and controversial model to explain the spectroscopic properties of the Cu centres in N$_2$OR. The main claim of this model was that Cu$_A$ and Cu$_Z$ are variants of the electron transfer centre and hence all the observed optical features of N$_2$OR are due to this electron transfer centre. Farrar *et al.* (1998) based their claim on a detailed analysis of the low temperature MCD (LT-MCD) spectra of this protein, as explained below.

This model is based on a few premises that are worth summarising: if the Cu atoms in a dimer are strongly exchange-coupled, only the mixed-valence
[Cu(II)Cu(I)] state will be paramagnetic, and thus, EPR-active. Such a state will also exhibit optical absorption bands due to electronic transitions into the Cu(II) ion. In order to observe an intense LT-MCD signal both an optical absorption band and paramagnetism are required. If the Cu atoms are antiferromagnetically coupled, the oxidised [Cu(II)Cu(II)] state is diamagnetic but will still possess optical bands due to d-d and charge transfer (CT) transitions. The reduced [Cu(I)Cu(I)] state will be both diamagnetic and visible-silent.

In the dithionite-reduced state of N₂OR, only Cu₂ is in the mixed-valence state. It is, therefore, EPR-active and exhibits intense, oppositely signed bands in its LT-MCD spectrum. The position and intensity of these bands are indicative of thiolate ligation to the Cu₂ centre. Thiolate ligation was confirmed by RR studies of this state (Dooley et al., 1987). Farrar et al. (1998) assign the MCD spectrum of Cu₂ and propose that this centre is a bis-thiolate-bridged chromophore.

In order to deconvolute the Cu centres in N₂OR, Farrar et al. (1998) studied the native, semi-reduced and dithionite-reduced states of the enzyme by absorption, EPR and MCD spectroscopies. As mentioned for the previous model (section IV.1.4.3.1) the absorption spectrum of the native protein is dominated by the mixed-valence Cuₐ centre, with bands at 480 nm, 540 nm and 800 nm. Additional intensity at 540 nm and 650 nm is also observed in this oxidation state of N₂OR. The semi-reduced state is EPR-silent but retains absorption bands at 540 nm and 650 nm. LT-MCD data confirm that this is not a ferromagnetically coupled (S=1) [Cu(II)Cu(II)] centre. In the dithionite-reduced state a single absorption band at 650 nm is observed, due to the mixed-valence, EPR-active, Cu₂. Thus, the species responsible for the absorbance at 540 nm and 650 nm in the semi-reduced state of the enzyme must be the oxidised, antiferromagnetically coupled, [Cu(II)Cu(II)] state of Cu₂. Two oppositely signed bands are found in the MCD spectrum of the native N₂OR which are absent from that of the mutant N₂ORV. These bands were proposed to originate from Cu₂* (see section IV.1.4.3.1). The authors used Gaussian analysis to show that the MCD spectrum of the native N₂OR has contributions of a mixed-valence Cuₐ centre and of a mixed-valence Cu₂ centre, thereby showing that Cu₂* is a variant of Cu₂ which assumes a mixed-valence state in the native enzyme. Thus, the authors identified the mixed-valence Cu₂* as being the second paramagnet observed in the EPR spectrum of native N₂OR. By analogy with dithionite-reduced N₂OR, it can be anticipated that mixed-valence Cu₂* will have a single absorption band at 650 nm.
Up to this point, the model does not differ substantially from the Farrar et al. (1991) model, discussed in the previous section. However, the authors proceed with the quantification of the CuA, CuZ and CuZ* centres per monomer in the three oxidation states studied for N2OR. The quantification of centres in paramagnetic states is done based on EPR and MCD quantification. The quantification of centres in non-paramagnetic states is deduced assuming that the different forms do not interconvert as the reduction proceeds and that each centre undergoes only a one-electron redox process. Because no other chromophores are present in N2OR, the total concentration of centres (CuA + CuZ + CuZ*) at each oxidation level of the enzyme should be two centres per monomer if CuA and CuZ/CuZ* are distinct centres and only one if CuA, CuZ and CuZ* are variants of a single centre. The authors estimate the number of centres per monomer for the CuA and CuZ* centres in the native enzyme by comparing the relative intensities of the individual deconvoluted spectra with those for CuA in N2ORV and CuZ in dithionite-reduced N2OR, respectively. These values are, according to the authors, in agreement with those determined by EPR quantification. The results based on MCD suggest that the total number of centres is one per protein monomer, indicating that CuA, CuZ and CuZ* are variants of a single centre.

This model, summarised in Fig. IV.5, raised a few questions, which were discussed by the authors. These can be summarised in three questions for which, at the time, satisfactory answers were not found: (1) What are the functional roles of the three forms of the Cu-thiolate dimer, CuA, CuZ and CuZ*? (2) Why is the modified CuZ/CuZ* form of the electron transfer centre only observed in wild-type N2OR and not in either N2ORIV or N2ORV (two forms of N2OR which are inactive, presumably due to the absence of the catalytic site)? (3) If N2OR possesses only one thiolate-bridged binuclear centre per monomer, involved in electron transfer, how can the remaining Cu atoms in the enzyme be accounted for and what are their ligands?

These questions were at least partially answered by the determination of the crystal structure of N2OR (section IV.2.2) and will be discussed in section IV.3 of this thesis.
Figure IV.5 - The Farrar model (1998) of the Cu centres in N₂OR. The letter A denotes the maxima in the absorbance spectra (in nm). The model proposes that Cuₐ, Cu₂ and Cu₂* are variants of a single, thiolate-bridged, electron-transfer centre. Taken from Farrar et al. (1998).

IV.1.5. Project aims

As became apparent throughout the previous sections, N₂OR is an extremely interesting and challenging enzyme. Although a lot is known about this enzyme, there are many questions to be answered in order to obtain further insight into the structure of its metal centres and its catalytic mechanism. Thus, this project was aimed at purifying and characterising N₂OR from *Ps. nautica*, an organism from which the protein had not previously been isolated. Particular attention was given to the study of the Cu sites of the enzyme by biochemical and spectroscopic techniques, with a focus on UV/Vis and EPR spectroscopies. It was expected that these studies would provide clues regarding the validity of the proposed models for the metal centres of the enzyme. The determination of the gene sequence to allow subsequent cloning and overexpression of the nosZ gene was also of great interest. Finally, the project aimed at attempting to determine the X-ray structure of N₂OR in collaboration with the group of Prof. Cambillau. The following sections deal with the efforts undertaken to pursue these objectives and the results thus obtained.
IV.2. RESULTS

IV.2.1. Purification of N$_2$OR: Molecular properties

IV.2.1.1. Two forms of the enzyme

The periplasmically located nitrous oxide reductase was purified from the crude extract of *Ps. nautica* 617 cells, prepared as described in the Materials and Methods section. The extract obtained after cell disruption was centrifuged once at 8000 x g for 1 h to remove large particles and cell debris, and twice at 125000 x g to remove the cell membranes. The resulting supernatant was the soluble extract used for the purification of N$_2$OR. All purification steps were carried out at 4°C. Nitrous oxide reductase has been shown to have thermochromic behaviour (reviewed in Zumft, 1997). Thus, to avoid freeze-thaw cycles in intermediate purification fractions, all steps were carried out non-stop until the protein was considered to be pure. Nitrous oxide reductase purification was followed spectrophotometrically by measuring the absorbance at 540 nm.

The purification scheme for nitrous oxide reductase is outlined in Fig. IV.6. The soluble extract from 600 g cells of *Pseudomonas nautica* 617 was applied to an anion exchange DEAE-Biogel column (4.5 cm x 40 cm, Bio-Rad) equilibrated with 10 mM Tris-HCl, pH 7.6. Proteins were eluted at a flow rate of approximately 1 ml/min with a 2-litre linear gradient of Tris-HCl, pH 7.6 (10 mM – 400 mM). N$_2$OR was eluted between 100mM and 125 mM Tris-HCl. After concentration, this fraction was applied to a Superdex 75 column (2.6 cm x 56 cm, Pharmacia) equilibrated with 300 mM Tris-HCl, pH 7.6 and eluted with the same buffer at a flow rate of 2 ml/min. Following dialysis, the fraction containing N$_2$OR was applied to a Source 15 anion exchange column (1.6 cm x 25 cm, Pharmacia) equilibrated with 10 mM Tris-HCl, pH 7.6. A 10 mM – 500mM gradient of Tris-HCl, pH 7.6, at a flow rate of 2 ml/min, was applied. Forms A and B (see below) of N$_2$OR were eluted successively between 160 mM and 200 mM Tris-HCl, pH 7.6. The yield of N$_2$OR from 600 g of cells was approximately 135 mg of total protein (form A + form B).
Figure IV.6 – Purification of forms A and B of nitrous oxide reductase. A. Purification scheme. Both anion exchange columns were equilibrated with 10 mM Tris-HCl, pH 7.6. The gel filtration column was equilibrated with 300 mM Tris-HCl, pH 7.6. All purification steps were carried out at 4 °C. B. Analysis of forms A and B of N$_2$OR by SDS-PAGE containing 12.5% (w/v) acrylamide during the different purification stages. Lane 1- Molecular mass markers; lane 2- Purified form A of N$_2$OR (~6 µg protein); lane 3- Purified form B of N$_2$OR (~7.5 µg protein).
Chapter IV. Nitrous Oxide Reductase

After the first purification step, N\textsubscript{2}OR exhibited a purple colour, with an intense absorption peak at \textasciitilde540 nm. However, after the second anionic exchange step, a separation was achieved between a predominantly purple form A and a blue-coloured form B of the protein. Form B was eluted at a slightly higher ionic strength than form A. The two forms of N\textsubscript{2}OR exhibited similar electrophoretic patterns when analysed by SDS-PAGE (Fig. IV.6-B), yielding a band at \textasciitilde65 kDa and one at \textasciitilde16kDa (see below). Mass spectrometry analysis of form B yielded a mass of 65382 ± 34 Da for N\textsubscript{2}OR, in agreement with the mass of the monomer, calculated from the amino acid sequence (65373 Da). Determination of the global molecular mass of the protein by gel filtration gave a mass of \textasciitilde120 ± 1.2 kDa for N\textsubscript{2}OR. Although this is lower than that expected from the monomer mass determined by mass spectrometry, it is consistent with the enzyme being a dimer.

IV.2.1.2. Co-purification of a 10.3 kDa putative chaperone with N\textsubscript{2}OR

Both forms of N\textsubscript{2}OR consistently co-purified with a peptide of approximately 16 kDa as estimated by SDS-PAGE (See Fig. IV.6-B). Mass spectrometry analysis yielded a mass of 10289.9 ± 0.4 kDa for this peptide. Its separation from N\textsubscript{2}OR could not be achieved by anion exchange or gel filtration chromatographies (Fig. IV.7), indicating a strong association between the two proteins.

N-terminal sequencing of this peptide yielded the sequence M-K-I-R-P-L-H-D-G-V-V-I-K-R-K-N-F-V-I-K-D-A-S-I-I-G-L-P. Comparison of this sequence with the sequences in the Swiss-Prot database revealed high homology towards protein CPN10, a 10 kDa chaperonin belonging to the groES heat-shock chaperonin family (Yamamoto et al., 1993). This peptide was found to precipitate upon crystallisation of N\textsubscript{2}OR (see below). The physiological significance of its association with N\textsubscript{2}OR is presently unclear, but may be related to the stabilisation of a mature form of the reductase.
IV.2.1.3. Metal content and activity

The determination of the total copper content was performed by a colorimetric method (see section II.2.2.3 for details) on various batches of both forms of N\textsubscript{2}OR.
These determinations consistently yielded 10.7 ± 1.7 Cu atoms per protein dimer. This is a higher value than that previously reported for other N$_2$ORs (reviewed in Zumft, 1997. See also Ferretti et al., 1999) and is in accord with the structural data obtained (see below). No significant amounts of other metals were detected by ICP analysis.

Both forms of the enzyme were shown to be active and to exhibit similar specific activities. These were 55 ± 6 µmol N$_2$O reduced · min$^{-1}$ · (mg enzyme)$^{-1}$ for form A and 23 ± 14 µmol N$_2$O reduced · min$^{-1}$ · (mg enzyme)$^{-1}$ for form B. These numbers are within the range of values reported for the enzyme purified from other bacterial sources (reviewed in Zumft, 1997).

**IV.2.1.4. DNA and deduced amino acid sequence**

The N-terminal and C-terminal sequences of the mature N$_2$OR protein were determined by Dr. Jozef VanBeeumen using the methods described in the Materials and Methods section.

The determination of approximately 95% of the sequence of the gene coding for N$_2$OR (excluding the 5´ and 3´ ends of the gene) was performed by Ms. Inês Cabrito. This procedure followed an approach similar to that described for the cloning and the sequencing of the nirA gene (Prudêncio et al., 1999. See also section III.2.1.1) and was based on the PCR-amplification of DNA fragments of the gene coding for *Ps. nautica* N$_2$OR. Degenerate oligonucleotides based on the sequences of genes coding for other N$_2$ORs were designed. Southern blot analysis confirmed the presence of a single copy of the gene coding for N$_2$OR in the genome of *Ps. nautica*. DNA sequencing was performed by Dr. Frank Rusnak, at the Mayo Clinic Foundation, Rochester, MN, USA. An alignment of the complete amino acid sequence of N$_2$OR (obtained by N-terminal sequencing, C-terminal sequencing and translation of the PCR-amplified DNA fragments) with that of other N$_2$ORs is shown in Fig. IV.8.
Figure IV.8 – Comparison of the amino acid sequences of several N$_2$ORs. Labels are as follows: Ps.n. - *Pseudomonas nautica* (this work); Ps.s. – *Ps. stutzeri* (NCBI sequence I.D. number AAA25907); Ps.a. – *Ps. aeruginosa* (CAAS46381); A.c. – *Ach. cycloclastes* (CAAT5425); Pa.d. - *Paracoccus denitrificans* (CAAS52799); S.m. - *Sinorhizobium meliloti* (ACAC44023); and R.e. - *Ralstonia eutropha* (CAAS46383).

The sequence of N$_2$OR from *Ps. nautica* corresponds to the mature protein whereas the remaining 6 sequences were deduced from the complete gene sequences. The black and grey boxes denote the ligands of the tetranuclear Cu centre and the binuclear Cu centre, respectively (see below). The black arrow indicates a tryptophan residue that binds Cu in the binuclear centre via the carbonyl group (see below). The blue arrows indicate the conserved double-arginine motif in the signal peptide. The red circle indicates the Cys residue mutated in the *Ps. stutzeri* enzyme (see section IV.1.4.2). The alignment was produced by Dr. Alice Pereira.
IV.2.2. X-ray structure of N$_2$OR

The X-ray structure of the N$_2$OR from *Ps. nautica* was determined at 2.4 Å resolution by Dr. Kieron Brown, Dr. Mariella Tegoni and Prof. Christian Cambillau at Architecture et Fonction des Macromolécules Biologiques, Marseille, France. Form B of N$_2$OR was used in the crystallisation experiments. Crystals were obtained using the technique of vapour diffusion at 20 ºC with hanging drops of 3 µl of protein solution, mixed with 3 µl of reservoir solution containing 18 % PEG 4000, 0.1 M Bicine, 0.6 M NaCl, 15 % iso-propanol, 10 mM spermine-4⋅HCl at a final pH of 9.5.

The structure of N$_2$OR (Brown *et al.*, 2000) shows a dimer with two types of Cu centres per monomer. One of them is a dinuclear Cu$_{A}$ centre, which is thought to be involved in electron transfer to the catalytic centre. The catalytic centre comprises a novel, tetranuclear, Cu centre, hereafter named Cu$_{Cat}$. The structure of *Ps. nautica* N$_2$OR is the first structure to be determined for any N$_2$OR and is also the first description of this novel type of catalytic Cu centre. Credit for this achievement is mainly due to the above-mentioned authors, directly involved in the structural work. However, due to its considerable importance for the understanding of the mechanism of catalysis of this enzyme and for the interpretation of its spectroscopic properties, a more detailed description of this structure will be included in the subsequent sections of this thesis.

IV.2.2.1. Overall structure

The crystal structure of N$_2$OR is shown in Fig. IV.9. Each N$_2$OR monomer is composed of two distinct domains, formed from contiguous segments in the amino acid sequence. The N-terminal domain (residues 10-443) adopts a seven-bladed β-propeller fold (β1/1 to β4/7). Each blade is a repeat of a four-stranded, twisted, antiparallel β sheet. The Cu$_{Cat}$ centre is located at one end of the 7-fold axis of the propeller at a position similar to that of the active sites of the β-propeller structures of galactose oxidase from *Dactylium dendroides* (Ito *et al.*, 1991), cytochrome *cd1*-nitrite reductase from *Thiosphaera pantotropha* (Fülöp *et al.*, 1995) and cytochrome *cd1*-nitrite reductase from *Pseudomonas aeruginosa* (Nurizzo *et al.*, 1997).
The C-terminal domain (residues 478-581) contains the CuA centre and consists of nine \( \beta \)-strands that form an anti-parallel \( \beta \)-sandwich. This domain adopts a cupredoxin fold, as seen in other CuA-containing proteins such as the cytochrome \( c \) oxidases from *Paracoccus denitrificans* (Iwata *et al.*, 1995) and from bovine heart (Tsukuhara *et al.*, 1995, 1996) and the \( ba_3 \)-type cytochrome \( c \) oxidase from *Thermus thermophilus* (Williams *et al.*, 1999). The N\(_2\)OR N-terminal and C-terminal domains, although linked by a stretch of 33 residues (Arg444-Asn478), are physically separated by one of the insertions in the propeller motif (residues 47-80, insertion I). In the dimer, the C-terminal domain of one monomer faces the N-terminal domain of the second monomer.

**IV.2.2.2. The CuCat centre**

The CuCat centre of N\(_2\)OR (Fig. IV.10. See also Fig. IV.11) is a novel Cu centre. It comprises four copper ions (hereafter called CuI, CuII, CuIII and CuIV) ligated by seven histidine residues and three hydroxide ions. Two histidine residues (His270 and His437) of the CuCat centre belong to the loops located on the top of the propeller domain [the top of the propeller is the face where the outer \( \beta \) strand (\( \beta_4/\ast \)) of one blade connects to the inner \( \beta \) strand (\( \beta_1/\ast \)) of the next blade] and the remaining five (His79, His80, His128, His325 and His376) belong to the innermost strand of the blades.

The CuCat centre adopts the shape of a distorted tetrahedron, in what has been called a “butterfly” cluster (Rosenzweig, 2000). CuI and CuII are bound to two copper ions, while CuIII and CuIV are bound to three others. Three copper ions have two histidine ligands; the fourth, CuIV, has only one, the second histidine being replaced by an OH\(^-\) ion. A second OH\(^-\) ion is bound to CuI, and a third is located between the four copper ions. Five histidine residues bind to the copper ions through their N\(^{\varepsilon 2}\) atom (His80, His128, His270, His325 and His376), while two use their N\(^{\delta 1}\) atom (His79 and His 37).
Chapter IV. Nitrous Oxide Reductase

Figure IV.9 – Overall structure of Ps. nautica N₂OR. A. Amino-acid sequence. The amino-acids that are conserved in the sequences of Ps. aeruginosa, Ps. stutzeri, Paracoccus denitrificans, Ach. cycloclastes, Sinorhizobium melliloti and Ralstonia eutropha N₂ORs are displayed in red; the copper ligands are labelled by filled circles for the Cu₁ centre and filled triangles for the CuCat (named CuZ in the figure) centre. Secondary structure elements are displayed as arrows (β-strands) and cylinders (α-helices). Insertions and the linker region are indicated by black lines. For the propeller domain, β-strands are dark green and α-helices are light green. The linker is in purple, β-strands of the cupredoxin domain are in blue or in red according to the β-sheet they form and the α-helix in the cupredoxin domain is in blue. B. Overall view of the crystal structure of the N₂OR dimer. One monomer is uniformly coloured in grey while the other monomer has the same colour code as defined in A. The labels of the coloured monomer (N- and C- termini, copper centres, Cl⁻ ion, blade and insertion numbering) are in red. The seven blades and the seven insertions in the propeller domain are numbered by red arabic and roman numerals, respectively. Labels for the second monomer are in blue. The structure is taken from Brown et al. (2000).
An intricate network of hydrogen bonds keeps the imidazole rings of these seven histidine residues in the appropriate orientation for coordinating the copper ions. The carboxylate group of Asp77 stabilizes the imidazole ring of His79 and that of Asp188 stabilizes His128 and His270. His80 Nδ1 establishes a hydrogen bond with the carbonyl group of His128, while His376 and His437 are held by water molecules, themselves involved in a network of hydrogen bonds. The most original feature involves His325. This residue is locked in position by a chloride ion (See Fig. IV.9-B), and liganded by ionic and hydrogen bonds to the guanidinium group of Arg131, the NH main-chain group of Asn272, and the NH2 group of Asn273. Distances between CuI-CuIII and between CuI-CuIV are about 3.2 Å, while those between CuII-CuIII and between CuII-CuIV are only about 2.5 Å. This asymmetry might arise from coordination differences between the atom pair CuI-CuII (one additional OH- ion for CuI), and the atom pair CuIII-CuIV (His instead of OH- ion) or from differences in the redox state of the copper ions, since the CuCat centre in *Ps. nautica* N2OR is not completely reduced (Prudêncio *et al.*, 2000 and see below).

**IV.2.2.3. The CuA centre**

The CuA centre of N2OR (Fig. IV.11) is formed by two copper ions linked by the Cys561 and Cys565 Sγ atoms, the His526 and His569 Nε2 atoms, the Met572 Sγ atom and the Trp563 carbonyl group. The CuA centre can be readily superimposed on the CuA centres found in cytochrome c oxidases from *Paraccocus denitrificans* (Iwata *et al.*, 1995) and from bovine heart (Tsukuhara *et al.*, 1995, Tsukihara *et al.*, 1996) and the *ba*3-type cytochrome c oxidase from *Thermus thermophilus* (Williams *et al.*, 1999). The two Cys Sγ atoms bind to the copper ions in a distorted square planar fashion, each Sγ interacting with both copper ions. Each histidine residue binds externally to the copper ions, while the Met572 Sγ atom and the Trp563 carbonyl group bind on opposite faces of the plane. Given the relative orientations of the two monomers in the protein, the CuA centre of one monomer is in close proximity to the CuCat centre of the second monomer.
Figure IV.10 – The Cu_{Cu} site in *Ps. nautica* N_{2}OR.

A. Stereo representation. The Cu atoms are in purple and OH groups are in red. The Nδ1 and the Nε2 in the His rings are in dark blue and light blue, respectively. B. Schematic representation (distances are in Å). The picture was taken from Brown *et al.*, 2000.
Figure IV.11 – The CuA centre in *Ps. nautica* N_{2}OR. A. Stereo view of the interface region between the Cu_{A} centre (above) and the Cu_{Cat} centre (below). The two centres are from different monomers, coloured in grey and green, respectively. The side chains of Met570 and Phe564 (coloured in purple) may be involved in intramolecular electron transfer from Cu_{A} to Cu_{Cat}. B. Schematic representation of the Cu_{A} centre (distances are in Å). The picture was taken from Brown *et al.* (2000).
IV.2.2.4. Implications for the mechanism of N$_2$OR

The structure of the Cu$_{\text{Cat}}$ centre of N$_2$OR and the accessibility of the copper ions in the centre suggest that N$_2$O binding is to the CuIV atom in the cluster. Removal of the OH$^-$ ligand and two bound water molecules would leave enough space for N$_2$O to bind (Fig. IV.12). Furthermore, the conserved residue Lys397 and the NH group of His376 would form hydrogen bonds with the external nitrogen atom of N$_2$O, a configuration likely to stabilise the complex.

Observations made for Cu-containing nitrite reductases and their substrate-bound complexes (Adman et al., 1995; Murphy et al., 1997; Dodd et al., 1997, 1998) have shown that nitrite binds the Cu in the type 2 catalytic centre of NiR via the substrate oxygen in an asymmetric, bidentate fashion. Drawing an analogy between the NiR and N$_2$OR enzymes, Brown et al. (2000) propose a mechanism in which the Cu$_{\text{Cat}}$ centre would behave as an electron buffer, three copper ions being reduced by the Cu$_{\text{A}}$ centre prior to substrate processing. The catalytic CuIV atom would remain oxidised and, therefore, would be able to bind the substrate. Hence, this electron reservoir could favour a fast electron exchange and prevent the formation of dead-end products.

Figure IV.12 – Stereo view of a model for the binding of the N$_2$O molecule at the CuIV atom of the Cu$_{\text{Cat}}$ centre of N$_2$OR. The picture was taken from Brown et al. (2000).
Chapter IV. Nitrous Oxide Reductase

It should be noted that this is not the only model that can be proposed for the mechanism of N₂OR. In fact, the binding of nitrite to one of the reduced Cu atoms as opposed to the oxidised one may be chemically favoured and remains as a strong possibility that requires further study.

IV.2.3. Spectroscopic characterisation

IV.2.3.1. UV/Vis spectroscopy

Forms A and B of N₂OR have different UV/Vis spectra, as depicted in Fig. IV.13-A and IV.13-B. Both forms of the enzyme show absorption maxima at ~540 nm and ~640 nm, but the peak at ~540 nm has significantly higher intensity for form A than for form B. In addition, the spectrum of form A shows absorption peaks at ~480 and ~800 nm.

Reduction of either form of N₂OR by sodium dithionite resulted in a decrease in absorption at ~540 nm as well as bleaching of the absorption peaks at ~480 nm and ~800 nm, with a single peak remaining around 640 nm ($\varepsilon_{640\text{nm}} = 7.1 \pm 0.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the protein dimer) (Fig. IV.13-C. See also Fig. IV.14). This corresponds to form III of the enzyme, as defined by Riester et al. (1989) and Zumft (1997), obtained by the addition of excess reductant to native N₂OR. Oxidation of either form of N₂OR with potassium ferricyanide resulted in a significant increase in absorption at ~480 nm, ~540 nm and ~800 nm (Fig. IV.14). Thus, the UV/Vis data presented suggest that forms A and B of Ps. nautica N₂OR correspond to different oxidation states of the same protein.

As shown by the difference spectrum in Fig. IV.14 (green line), only the absorbance features at 480 nm, ~540 nm and ~800 nm change when the protein undergoes a redox cycle, whilst the ~640 nm absorption remains virtually unchanged. This spectroscopic feature is absent from form V of N₂OR (Dooley et al., 1991a; Farrar et al., 1996), a Cu- deficient form of the enzyme in which the Cuₐ centres are preferentially occupied (see section IV.1.3), and could not be detected even when this protein was reduced with a 10- to 100-fold excess of reductant (Riester et al., 1989).
Figure IV.13 – UV/Vis spectra of nitrous oxide reductase. A. Form A of the protein. B. Form B of the protein. C. Dithionite-reduced enzyme (equivalent spectra for both forms). Spectra were collected at room temperature in 0.2 mM Tris-HCl, pH 7.6.
This finding provides a clear indication that in the enzyme from *Ps. nautica* the absorbance at ~640 nm is associated with the CuCat centre and suggests that its oxidation state remains unchanged under the experimental conditions used. Thus, it is reasonable to suggest that the difference between forms A and B of N2OR is related to the degree of oxidation of the CuA centre in the two proteins.

Figure IV.14 – Optical spectra of nitrous oxide reductase in the fully oxidised (red line) and fully reduced (blue line) states. The difference spectrum between the oxidised and reduced forms of the enzyme (green line) shows absorption bands at ~480 nm, ~540 nm and ~820 nm.
IV.2.3.2. EPR spectroscopy

Both forms of N$_2$OR were studied by EPR spectroscopy in different oxidation states. In their native (as-purified) state, the two proteins exhibit different EPR spectra, shown in Fig. IV.15-A, B. In the dithionite-reduced state, the two forms of N$_2$OR have identical EPR spectra (Fig. IV.16-B); similarly, when either form of the enzyme was oxidised by ferricyanide, the resulting spectra exhibited similar features (Fig. IV.16-A).

All the experimental spectra could be simulated by adding specific proportions of two different signals, as explained throughout this section; the spectrum of the fully reduced protein was simulated by assuming a signal arising from a single species (Fig. IV.16, spectrum B’). This signal had a 4-line hyperfine splitting in the g$_z$ region with g$_x$ = 2.015 (A$_x$ = 1.5 mT), g$_y$ = 2.071 (A$_y$ = 2 mT) and g$_z$ = 2.138 (A$_z$ = 7 mT). Subtraction of the dithionite-reduced spectrum from the spectrum of the ferricyanide-oxidised sample yielded the spectrum in Fig. IV.16-C. This spectrum had a 7-line hyperfine splitting in the g$_z$ region and was simulated with g = g$_y$ = 2.021 (A$_x$ = A$_y$ = 0 mT) and g$_z$ = 2.178 (A$_z$ = 4 mT) (Fig IV.16, spectrum C’). Thus, the spectrum of the oxidised protein could be simulated by adding, in equal proportions, the two signals mentioned above (Fig IV.16, spectrum A’). The 7-line signal is similar to that found in other CuA-containing proteins (Riester et al., 1989; Kroneck et al., 1990a; Farrar et al., 1991, 1996; Antholine et al., 1992; Neese et al., 1996) and can therefore be assigned to this centre in Ps. nautica N$_2$OR. The 4-line signal was thus assigned the CuCat centre in this protein. The spectra of the native forms A and B of N$_2$OR were simulated (Fig IV.15, spectra A’ and B’) by adding different proportions of the CuA and the CuCat signals (see Table IV.1).
Figure IV.15 - EPR spectra of as-purified forms A and B of *Ps. nautica* N²OR. A. Form A (27.9 mg/ml in protein, 39 K, microwave power 1.59 mW, modulation 4.05 Gpp). B. Form B (34.7 mg/ml in protein, 35 K, microwave power 5.04 mW, modulation 1.05 Gpp). Spectra A’ and B’ are the theoretical simulations of spectra A and B, respectively. The experimental spectra were recorded in 160 mM Tris-HCl, pH 7.6 at a microwave frequency of 9.49 GHz. The spectra were normalised to the same protein concentration, temperature, microwave power and modulation to facilitate comparison.
Figure IV.16 - EPR spectra of oxidised and reduced \textit{Ps. nautica} N$_2$OR. A. Ferricyanide-oxidised enzyme (27.9 mg/ml in protein, 39 K, microwave power 1.59 mW, modulation 4.05 Gpp). B. Dithionite-reduced enzyme. C. Spectrum A after subtraction of spectrum B. Spectra A’, B’ and C’ are the theoretical simulations of spectra A, B and C, respectively. Protein concentration was 34.7 mg/ml and the experimental spectra were recorded in 160 mM Tris-HCl, pH 7.6 at a microwave frequency of 9.49 GHz. Other experimental conditions were the same as for sample B in Fig. IV.15.
The amount of EPR-detectable Cu spins was determined for both forms of \( \text{N}_2\text{OR} \) in the native, reduced and oxidised states. These results are summarised in Table IV.1 which also includes information on the contributions of each of the Cu\( \text{A} \) and Cu\( \text{Cat} \) EPR signals towards the simulated spectra shown in Fig. IV.15 and Fig. IV.16. For both forms A and B of \( \text{N}_2\text{OR} \), the EPR spectra of the oxidised proteins quantified approximately 2 spins per protein monomer, whereas both fully reduced forms yielded approximately 1 spin per monomer. The as-purified form A of the protein has \(~90\%\) of the spin intensity found for the ferricyanide-oxidised state, whilst form B shows \(~60\%\) of that spin intensity.

Table IV.1 - Spin quantification and proportions of oxidised Cu\( \text{A} \) signal (7 lines) and Cu\( \text{Z} \) signal (4 lines) for the EPR spectra of forms A and B of \( \text{Ps. nautica} \) \( \text{N}_2\text{OR} \). EPR-detectable copper was determined by comparison of double integrals with a Cu-EDTA standard.

<table>
<thead>
<tr>
<th>( \text{N}_2\text{OR} ) form</th>
<th>Oxidation state</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxidised</td>
<td>As-purified</td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td>EPR spectrum</td>
<td>0.5 Cu( \text{A} ) / 0.5 Cu( \text{Z} )</td>
<td>0.45 Cu( \text{A} ) / 0.55 Cu( \text{Z} )</td>
<td>1 Cu( \text{Z} )</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.03</td>
<td>1.89</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.2 (^a)</td>
<td>1.24</td>
<td>1.05</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Deduced from the spectral deconvolution

The EPR spectroscopy data obtained for both forms of \( \text{N}_2\text{OR} \) are in agreement with the UV/Vis data presented and support the suggestion that forms A and B of the enzyme correspond to different oxidation states of the protein, which are intermediate between those found for the dithionite-reduced and the ferricyanide-oxidised states of the enzyme. Furthermore, these results can be explained assuming that the Cu\( \text{A} \) and Cu\( \text{Cat} \) centres contribute independently to the UV/Vis and EPR spectra. In its oxidised state (Cu\( ^{1.5+} \), Cu\( ^{1.5+} \)), the binuclear Cu\( \text{A} \) centre is responsible for the absorption bands
at ~480, ~540 and ~800 nm in the UV/Vis spectrum and for the 7-line signal in the EPR spectrum of *Ps. nautica* N₂OR. This is in agreement with previous studies of N₂ORs from other bacterial sources, as well as of the Cu₅-containing cytochrome *c* oxidase (Riester *et al.*, 1989; Kroneck *et al.*, 1990a; Farrar *et al.*, 1991, 1996; Antholine *et al.*, 1992; Neese *et al.*, 1996; Zumft, 1997). The tetranuclear CuCat centre must therefore be responsible for the ~650 nm absorption and the 4-line signal found in the UV/Vis and EPR spectra, respectively. The spectral features assigned to this centre do not change for the oxidation states studied for both forms A and B of N₂OR, suggesting that the CuCat centre did not undergo any redox transition under the experimental conditions used. Under these conditions, the CuCat centre is always EPR-active and contributes with 1 spin per protein monomer towards all the EPR spectra shown, whilst the Cu₅ centre oscillates between a reduced (Cu⁺⁺ Cu⁺⁺), EPR-silent state and an oxidised, (Cu¹.⁵⁺ Cu¹.⁵⁺) EPR-active state. Thus, the native forms of N₂OR differ in the extent of the oxidation of their Cu₅ centres, which are ~89% oxidised in form A and ~24% oxidised in form B of the protein.

IV.2.3.3. Redox titration

Redox titrations of N₂OR were followed by EPR spectroscopy. The titrations were performed as described in the Materials and Methods section, using the experimental set-up depicted in Fig. IV.17 for sample collection.

A mixture of 16 mediators was added to the protein solution to facilitate electron transfer processes. These were used at a final concentration of 2 µM and are listed in Table IV.2. Both forms A and B of the enzyme were used in the experiment and yielded similar results.

Samples were collected anerobically and their EPR spectra recorded (Fig. IV.18). The spin intensity of each EPR spectrum was plotted against the redox potential measured and the data obtained were fitted to a Nernst equation for a one-electron process (Fig. IV.19). A mid-point redox potential of approximately +275 ± 20 mV was obtained. No EPR-silent state was ever detected for either form of *Ps. nautica* N₂OR.
Figure IV.17 – Experimental set-up used for the collection of samples for the EPR redox titration of N$_2$OR. A- bubbler; B- suba seal; C- working electrode; D- reference electrode; E- pontentiometer; F- cell compartment for temperature control; G- stirring plate; H- magnetic stirrer; I- EPR tube; J- gas-tight syringe with titrating solution; K- gas-tight syringe for sample collection; L- glass adaptor. Adapted with permission from Tavares, 1994.
Table IV.2 – List of mediators used in the redox titrations of N$_2$OR

<table>
<thead>
<tr>
<th>Mediator</th>
<th>$E_0$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triquat</td>
<td>-550</td>
</tr>
<tr>
<td>methyl viologen</td>
<td>-436</td>
</tr>
<tr>
<td>neutral red</td>
<td>-325</td>
</tr>
<tr>
<td>saphranine</td>
<td>-280</td>
</tr>
<tr>
<td>phenosaphranine</td>
<td>-275</td>
</tr>
<tr>
<td>anthraquinone-2-sulfonic acid</td>
<td>-225</td>
</tr>
<tr>
<td>2-hydroxy-1,4-naphthoquinone</td>
<td>-145</td>
</tr>
<tr>
<td>phenazine</td>
<td>-125</td>
</tr>
<tr>
<td>indigo carmine</td>
<td>-111</td>
</tr>
<tr>
<td>indigo tetrasulphonate</td>
<td>-46</td>
</tr>
<tr>
<td>duroquinone</td>
<td>+5</td>
</tr>
<tr>
<td>5-hydroxy-1,4-naphthoquinone</td>
<td>+30</td>
</tr>
<tr>
<td>1,4-naphthoquinone</td>
<td>+60</td>
</tr>
<tr>
<td>1,2-naphthoquinone</td>
<td>+118</td>
</tr>
<tr>
<td>2,6-dichlorophenol indophenol</td>
<td>+217</td>
</tr>
<tr>
<td>potassium ferricyanide</td>
<td>+430</td>
</tr>
</tbody>
</table>
Figure IV.18 – EPR spectra of N₂OR during redox titration. A. Form A of the enzyme (protein concentration was 10.5 mg/ml in 100 mM Tris-HCl, pH 7.6, microwave power was 1.26 mW). B. Form B of the enzyme (protein concentration was 22.5 mg/ml in 100 mM Tris-HCl, pH 7.6). Both sets of spectra were collected at 35 K, with a modulation of 1.05 Gpp and a microwave frequency of 9.65 GHz.
Figure IV.19 – Redox titration of N₂OR followed by EPR. A. Form A of the enzyme. B. Form B of the enzyme.
These results support the previous suggestions concerning the nature of the Cu centres in *Ps. nautica* N$_2$OR. The midpoint redox potential determined for the two forms of the enzyme corresponds to the Cu$_A$ centre, which is able to cycle between an oxidised and a reduced state, as mentioned above.

The spectroscopic data presented suggest that the tetranuclear Cu$_{Cat}$ centre in *Ps. nautica* N$_2$OR may have either a (3Cu$^{1+}$, 1Cu$^{2+}$) or a (1Cu$^{1+}$, 3Cu$^{2+}$) configuration. In either case, the total spin of the cluster will be equal to $\frac{1}{2}$, thus explaining the spectroscopic data shown. However, the absence of an EPR-silent state in the –500 to + 500 mV range of potentials confirms that the Cu$_{Cat}$ centre cannot be further reduced under these conditions and suggests an electron-rich nature for this tetranuclear centre, which favours the first hypothesis (Prudêncio et al., 2000). Furthermore, a (3Cu$^{1+}$, 1Cu$^{2+}$) arrangement is more likely to explain a possible enzymatic mechanism, as suggested by Brown *et al.* (2000) (see section IV.2.2.4), in which the Cu$_{Cat}$ centre would act as an electron reservoir to favour fast electron exchange.
IV.3 DISCUSSION

The studies performed on N₂OR from *Ps. nautica* have provided new insights into the structure and catalytic mechanism of this enzyme. The protein was purified in two active forms, A and B, of distinct colours, which exhibit different UV/Vis and EPR spectral properties (section IV.2.1.1). Biochemical and spectroscopic analyses of both these forms (sections IV.2.1 and IV.2.3) showed that they correspond to the same protein in different oxidation states (Prudêncio *et al.*, 2000). The proteins co-purified with a peptide bearing strong sequence homology to chaperonin proteins of the GroES family (section IV.2.1.2). Both forms of *Ps. nautica* N₂OR have an unusually high Cu content (section IV.2.1.3). This finding is of considerable importance, since until now there existed no reports of any N₂OR with a Cu content higher than 8 atoms per molecule. The results obtained from the determination of the crystal structure of the protein show that it possesses one binuclear Cuₐ centre and one novel tetranuclear Cu centre per monomer, giving a total of 12 Cu atoms per molecule of protein.

The purification procedure of *Ps. nautica* N₂OR does not differ greatly from that followed in the purification of this enzyme from other bacterial sources. Thus, it is difficult to explain this difference in the Cu content by invoking essential differences in the protocols of protein purification. One possible explanation is that the chaperonin-like peptide found to be strongly associated with N₂OR may play a role in stabilising the protein, thereby preventing Cu loss during purification [see Harrison *et al.* (1999) for a review on the roles of Cu chaperones]. This is certainly an issue that deserves further investigation.

The determination of the first crystal structure of a N₂OR (Brown *et al.*, 2000 and see section IV.2.2) was a long-awaited achievement and reveals interesting and quite unexpected features of this protein. It confirms that the Cuₐ site of N₂OR is, as correctly predicted by various spectroscopic and mutagenesis studies, a dinuclear Cu centre, very similar to that found in COX (see section IV.1.4.1 and references therein). However, the tetranuclear structure revealed for the catalytic site, Cu₉Cat, was quite unexpected and raises some intriguing questions. This is a novel type of Cu cluster, not previously seen in any protein, and the reasons why it is present in N₂OR or the exact way in which it catalyses the reduction of N₂O are not known. Riester *et al.* (1989) reported that four instead of two electrons per dimer were required to enter the catalytic centre to produce an EPR-active form. At the time, sulphur redox
chemistry at the site was postulated to resolve this discrepancy (Kroneck et al., 1990b; Dreusch et al., 1996). This can now also be explained by invoking the tetranuclear nature of the catalytic centre, although such an explanation would suggest that this centre is capable of undergoing more than one redox transition (Prudêncio et al., 2000). Clearly further studies are required in order to understand fully the mechanisms taking place in this novel structure.

The UV-Vis and EPR data collected for *Ps. nautica* N\textsubscript{2}OR are consistent with the presence of two independent, spectroscopically-active, centres in this enzyme. According to this model, the binuclear Cu\textsubscript{A} site, presumed to be the electron-transfer centre is capable of undergoing reversible one-electron redox transitions. It has a mid-point redox potential of approximately +275 mV, which is in good agreement with that reported by Coyle et al. (1985). In its oxidised, mixed-valence (Cu\textsuperscript{1.5+} Cu\textsuperscript{1.5+}) state, the Cu\textsubscript{A} centre is responsible for absorbance bands at ~480 nm, ~540 nm, and ~800 nm and for an EPR spectrum with a 7-line hyperfine splitting in the g\textsubscript{||} region. In the reduced (Cu\textsuperscript{1+} Cu\textsuperscript{1+}) state, the Cu\textsubscript{A} centre is both UV/Vis- and EPR-silent. It is the extent of the oxidation of this centre that determines the properties of the native forms A or B of purified, native N\textsubscript{2}OR. The other Cu site in the protein is the tetranuclear Cu\textsubscript{Cat} centre. This centre remains, in all conditions assayed, in a reduced (3Cu\textsuperscript{1+}, 1Cu\textsuperscript{2+}) state being responsible for an absorption band at ~650 nm and an EPR spectrum with an ill-defined 4-line hyperfine splitting in the g\textsubscript{\perp} region. This suggests a high redox potential for this site, compatible with the notion of an electron-rich centre and with the mechanistic implications outlined in section IV.2.2.4.

The model above provides the simplest explanation for the results obtained. However, other possibilities cannot be excluded and a very relevant aspect must be taken into account: the MCD studies of Farrar et al. (1991, 1998) and the RR studies of Dooley et al. (1987, 1991a) and Andrew et al. (1994) have clearly demonstrated thiolate ligation to the Cu\textsubscript{Z} centre, presumed to be the catalytic site of the enzyme. The X-ray structure now available shows that the Cu\textsubscript{Cat} site is coordinated by His residues and that no Cys residue ligates the Cu atoms in this centre. So, it is perhaps premature to state that the tetranuclear Cu\textsubscript{Cat} site is the same as what has been referred to in the literature as Cu\textsubscript{Z}. In other words, the species that generates the spectroscopic features observed in reduced *Ps. nautica* N\textsubscript{2}OR may not be Cu\textsubscript{Cat}, as assumed by the model outlined above and other possibilities need to be considered.
The model of Farrar *et al.* (1998) (section IV.1.4.3.2) suggested that the catalytic centre is spectroscopically silent and only variants of the Cu₄/Cu₂/Cu₂* electron transfer can be detected by the spectroscopic methods employed. In that case, the absorbance maximum at 650 nm and the 4-line EPR spectrum observed for form III of *Ps. nautica* N₂OR would not arise from the tetranuclear Cu centre but rather from the thiolate-bridged, binuclear, Cu₂/Cu₂* variant of the electron-transfer centre. In other words, CuCat and CuZ would be different entities. Of course this would imply that Cu₂/Cu₂* does not change its redox state within the experimental conditions assayed, contrary to what has been reported for the *Ps. stutzeri* enzyme. This would be compatible with the requirement that those features arise from a sulphur-ligated species, as suggested by the MCD and RR studies, and would agree with the proposal formulated by the model of Farrar *et al.*, (1998). However, this poses a major incompatibility with the spin quantifications of the *Ps. nautica* N₂OR in the redox states assayed (section IV.2.3), which indicated that the total spin of this enzyme oscillates between 1 and 2 spins per protein monomer, a notion that requires the contributions of two independent centres. Furthermore, it should be noted that the spin quantifications of Farrar *et al.* (1998) were made in relation to a protein estimated to have 3.2 to 3.9 Cu atoms per monomer, a number which is quite likely to be underestimated, possibly due to an overestimation of protein. Assuming that *Ps. stutzeri* N₂OR indeed contains 6 coppers per monomer, as implied by the conserved histidines in its sequence, the number of spins can be recalculated per 6 Cu atoms. For all three redox states of the samples, the sum of calculated spins for Cu₄, Cu₂ and Cu₂* gives 1.90 ± 0.01 spins / 6Cu, in good agreement with a model in which Cu₄ and Cu₂/Cu₂* are separate centres, with Cu₂/Cu₂* being the catalytic site.

It seems clear that the N₂OR enzyme from *Ps. nautica* differs from that of *Ps. stutzeri* at least with respect to the redox potential of the non-Cu₄ centre. However, it is also clear that this does not provide an explanation as to the exact chemistry of the Cu-centres in this enzyme and that discrepancies exist regarding the coordination of the Cu atoms in the catalytic site. One possibility that would at least help resolve this apparent contradiction is that at least one of the reported hydroxide ligands of CuCat could be a sulphide instead of a hydroxide ion. This would provide the sulphur-ligation to the tetranuclear Cu centre and thereby quite possibly explain the MCD and RR data. However, this possibility is not consistent with the crystallographic results:
the introduction of a sulphide ion instead of the central hydroxide ion resulted in a
negative electron density bulb, centred on the sulphur atom, indicating that the atom is
an oxygen and not a sulphur (Prof. Christian Cambillau, personal communication).

In the light of the present knowledge, the model presented above, with two
independent centres, CuA and CuCat, contributing towards the spectral properties of the
protein, is the one that provides the most likely explanation for the properties of the
metal centres in N2OR. Thus, it is very difficult to conciliate the model of Farrar et al.
(1998) with the results now at hand. In the absence of a chemical model that would
allow a more detailed analysis of the CuCat centre, the possibility that this site may
give rise to the MCD and RR features seen for the reduced form of other N2ORs
cannot be excluded. Thus, it is possible that the complex interactions between the Cu
atoms and their ligands are, indeed, responsible for those spectral features, thereby
explaining the spectroscopic data, in accord with the X-ray structure of the protein.

It seems clear that with the determination of the crystal structure of N2OR a
new chapter in the study of this enzyme is opened. The knowledge now at hand poses
interesting challenges and allows new questions to be formulated. What is the
physiological significance of the chaperonin-like peptide that is associated with Ps.
nautica N2OR? Why does the enzyme purified from other bacterial sources have a
consistently lower Cu content than that of Ps. nautica? What is the exact nature of the
ligands to the catalytic centre in the enzyme? How does reduction of N2O proceed at
this centre and what are the exact roles of the 4 Cu atoms in catalysis? Clearly, further
studies will be required in order to answer these questions and thereby allow a better
understanding of this interesting and ever-challenging enzyme.
CHAPTER V

REFERENCES


Chapter V. References

Chapter V. References


• Dooley, D.M. (1994) “Copper proteins with type 2 sites and copper-containing enzymes in denitrification”, in *Encyclopaedia of Inorganic Chemistry* (R.B. King, Ed.), Vol. 2, pp 905-924, John Willey and Sons Ltd., Chichester


Chapter V. References


- Ibrahim, S.K. (1992) “New electrochemical reactions of some metallo-sulphur compounds including the activation of NO and NHR ligands at Mo-S centres”, PhD Thesis, School of Chemical and Molecular Sciences, University of Sussex, UK


• Nurizzo, D., Silvestrini, M.-C., Mathieu, M., Cutruzzolà, F., Bourgeois, D., Fülpö, V., Hajdu, J., Brunori, M., Tegoni, M. Cambillau, C. (1997) “N-terminal arm exchange is observed in the 2.15 Å crystal structure of oxidized nitrite reductase from *Pseudomonas aeruginosa*,” *Structure*, **5**, 1157-1171

• Olesen, K., Veselov, A., Zhao, Y., Wang, Y., Danner, B., Scholes, C.P., Shapleigh, J.P. (1998) “Spectroscopic, kinetic and electrochemical characterisation of heterologously expressed wild-type and mutant forms of copper-containing nitrite reductase from *Rhodobacter sphaeroides* 2.4.3”, *Biochemistry*, **37**, 6086-6094


Chapter V. References


Chapter V. References

- Starkey, R.L. “A study of spore formation and other morphological characteristics of Vibrio desulfuricans”, Arch. Mikrobiol., 8, 268-304


• Veselov, A., Olesen, K., Sienkiewicz, A., Shapleigh, J.P., Scholes, C.P. (1998) “Electronic structural information from Q-band ENDOR on the type 1 and type 2 copper liganding environment in wild-type and mutant forms of copper-containing nitrite reductase”, Biochemistry, 37, 6095-6115


