Structural Plasticity and Function in Cytochrome $cd_1$ Nitrite Reductase

BY

TOVE SJÖGREN
ABSTRACT


Cytochrome cd$_1$ nitrite reductase is a bifunctional enzyme, which catalyses the one-electron reduction of nitrite to nitric oxide, and the four-electron reduction of oxygen to water. The latter is a cytochrome oxidase reaction. Both reactions occur on the d$_1$ haem iron of the enzyme.

Time resolved crystallographic studies presented here show that the mechanisms of nitrite and oxygen reduction share common elements. This is of interest from an evolutionary point of view since aerobic respiratory enzymes are thought to have evolved from denitrifying enzymes. Despite of similarities, the results also imply different requirements for the timing of electron transfer to the active site in these reactions.

Quantum chemical calculations suggest that nitric oxide, the product of nitrite reduction, is not spontaneously released from the haem iron while this is not the case with water. Reduction of the haem while nitric oxide is still bound to it would result in a tight dead-end complex. A mechanism must therefore exist for the selective control of electron transfer during the reaction.

Structural studies with a product analogue (carbon monoxide) combined with flash photolysis of the complex in solution revealed an unexpected proton uptake by the active site as the neutral CO molecule left the enzyme. This led to the suggestion that the increased positive potential of the active site triggers preferential electron transfer when the active site is empty.

Crystallisation and structure determination of the reduced enzyme revealed extremely large domain rearrangements. These results offer insights into the role of tethered electron shuttle proteins in complex redox systems, and suggests a mechanism for conformational gating in catalysis.

Tove Sjögren, Department of Biochemistry, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden.

©Tove Sjögren 2001

ISSN 1104-232X
ISBN 91-554-5089-X

Printed in Sweden by Uppsala University, Tryck och Medier, Uppsala 2001
Till mamma och pappa
Papers included in the thesis

This thesis is based on the following papers which will be referred to in the text by their Roman numerals.


*The articles are reprinted with permission from the copyright holders.*

Related publications


Contents

Structures discussed in this thesis .......................... 7

1 Introduction ............................................ 8
   1.1 The nitrogen cycle .................................. 8
   1.2 Denitrification ..................................... 10
   1.3 Cytochrome cdₙ nitrite reductase ..................... 12
      1.3.1 The structure of cytochrome cdₙ nitrite reductase . 12
      1.3.2 Haem ligand switching in cytochrome cdₙ ........... 13
      1.3.3 Comparison of cytochrome cdₙ nitrite reductases from different species 14
   1.4 Aim and outline of this thesis ......................... 14

2 Methods ................................................. 18
   2.1 Structure determination by X-ray crystallography ....... 18
   2.2 Time-Resolved Crystallography ....................... 20
      2.2.1 Reaction initiation and trapping of intermediates ... 20
      2.2.2 Microspectrophotometry .......................... 21
      2.2.3 Data collection on naked crystals at room temperature 21
   2.3 Studies of pre-steady state kinetics in solution by flash photolysis .......... 23

3 Results .................................................. 26
   3.1 Time-resolved structural studies of the oxidase reaction ... 26
      3.1.1 Preparation of reaction intermediates in the crystal .... 26
      3.1.2 X-ray mediated reduction of reaction intermediates during data collection .......... 27
      3.1.3 Structure of the bound dioxygen species in cytochrome cdₙ .... 28
   3.2 Studies of the complex between cytochrome cdₙ and carbon monoxide .... 29
      3.2.1 Structural studies of the cytochrome cdₙ:CO complex ...... 30
      3.2.2 Studies of the complex between cytochrome cdₙ and CO in solution 32
      3.2.3 Implications for the catalytic mechanism ............... 35
   3.3 Large redox-coupled conformational changes in cytochrome cdₙ .......... 36
      3.3.1 Anaerobic crystallisation of cytochrome cdₙ in its reduced form 36
      3.3.2 Molecular replacement and refinement of the structure ........ 38
      3.3.3 The overall structure ............................ 38
Contents

3.3.4 Conformational changes in the $d_1$ domain .................. 39
3.3.5 Catalysis in the tetragonal crystals ......................... 40

4 Discussion ............................................. 43
4.1 Multiple conformations of cytochrome $cd_1$ .................... 43
4.1.1 A comparison of the known conformers of cytochrome $cd_1$ 44
4.1.2 The role of the N-terminal arm .......................... 45
4.1.3 The tethered electron shuttle domain ...................... 46
4.2 Mechanisms of nitrite and oxygen reduction by cytochrome $cd_1$ 47
4.2.1 The reduction of nitrite ................................ 47
4.2.2 The reduction of oxygen ................................ 49

5 Future prospects ............................................ 50
5.1 Open questions and unfinished business ....................... 50
5.2 Epilogue ................................................. 52

Acknowledgements ............................................. 53

References .................................................... 55
## Structures discussed in this thesis

<table>
<thead>
<tr>
<th>PDB id.</th>
<th>Description</th>
<th>Resolution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1QKS</td>
<td>Oxidised, P2₁ crystals</td>
<td>1.28 Å</td>
<td>Fülöp et al. 1995, Baker et al. 1997</td>
</tr>
<tr>
<td>1AOF</td>
<td>Reduced, P2₁ crystals</td>
<td>2.0 Å</td>
<td>Williams et al. 1997</td>
</tr>
<tr>
<td>1AOM</td>
<td>Reduced and partially re-oxidised with nitrite, P2₁ crystals</td>
<td>1.8 Å</td>
<td>Williams et al. 1997</td>
</tr>
<tr>
<td>1AOQ</td>
<td>Reduced and partially re-oxidised with nitrite, P2₁ crystals</td>
<td>1.8 Å</td>
<td>Williams et al. 1997</td>
</tr>
<tr>
<td>1E2R</td>
<td>Reduced, complex with CN-, P2₁ crystals</td>
<td>1.59 Å</td>
<td>Jafferji et al. 2000</td>
</tr>
<tr>
<td>1HJ3</td>
<td>Reduced and partially re-oxidised with oxygen, P2₁ crystals</td>
<td>1.6 Å</td>
<td>Paper II</td>
</tr>
<tr>
<td>1HJ4</td>
<td>Reduced and partially re-oxidised with oxygen, X-ray re-reduced, P2₁ crystals</td>
<td>1.6 Å</td>
<td>Paper II</td>
</tr>
<tr>
<td>1HJ5</td>
<td>Reduced and re-oxidised with oxygen, P2₁ crystals</td>
<td>1.4 Å</td>
<td>Paper II</td>
</tr>
<tr>
<td>1DY7</td>
<td>Reduced, complex with CO, P2₁ crystals</td>
<td>1.57 Å</td>
<td>Paper III</td>
</tr>
<tr>
<td>1H9X</td>
<td>Reduced, P4₃2₁2 crystals</td>
<td>2.1 Å</td>
<td>Paper IV</td>
</tr>
<tr>
<td>1H9Y</td>
<td>Reduced, complex with CN-, P4₃2₁2 crystals</td>
<td>2.4 Å</td>
<td>Paper IV</td>
</tr>
<tr>
<td>1HCM</td>
<td>Oxidised, P4₃2₁2 crystals</td>
<td>2.5 Å</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Not deposited</td>
<td>Partially oxidised with nitrite, P4₃2₁2 crystals</td>
<td>2.8 Å</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>
1 Introduction

Proteins are one of the fundamental building blocks in life. The importance of this class of molecule was appreciated soon after their discovery which is reflected in the name, protein, coined by Jöns Jakob Berzelius in 1838. It is derived from the word *proteios*, which means “of the first rank”. Proteins consist of a chain of amino-acids linked together by peptide-bonds. The polypeptide is folded into a three-dimensional structure. The sequence of amino-acids and the three-dimensional structure makes each protein unique and allows for an incredible diversity.

Enzymes are a subset of proteins which act by catalysing a wide range of chemical reactions. Catalysis can be accomplished in many different ways. In enzymes, probably the most important factor is that the reactants can be kept in a favorable geometry in a chemically well-defined active site. The surrounding enzyme constitutes a scaffold which maintains the structure of the active site and permits transfer of substrates, products, electrons and protons to and from the active site. In order to understand how enzymes work it is important to know their three-dimensional structures.

In this thesis the three-dimensional structure of cytochrome *cd* nitrite reductase has been studied using X-ray crystallography, and structure-function relationships for this enzyme has been investigated.

Cytochrome *cd* nitrite reductase catalyses the committed step in denitrification, a process which plays a role in the global nitrogen cycle. In this chapter a brief introduction to the nitrogen cycle and especially to the process of denitrification is presented. This will be followed by a review of previous structural work on cytochrome *cd* and an outline of the major goals of this thesis.

1.1 The nitrogen cycle

Nitrogen occurs naturally with oxidation states ranging from -3 to +5 and can undergo a wide range of chemical reactions. Nitrogen compounds in the form of nitrogen oxides or ammonia are abundant in nature, and in the recent years there have been a lot of concerns regarding the biochemistry of these compounds. Nitrate is a pollutant of water and nitrous oxide is
1.1. The nitrogen cycle

Nitrogen is one of the major constituents of biomolecules. Higher organisms can not utilise simple nitrogen compounds for biosynthesis but have to obtain nitrogen by metabolic pathways where various foodstuffs are degraded. Bacteria, plants and fungi can use ammonium ions for biosynthesis. Ammonia is usually derived from nitrate which is reduced to nitrite and then reduced directly to ammonia in one step in the process of assimilation. Cyanobacteria and a small number of other bacterial strains including the symbiotic bacterium *Rhizobium* are able to incorporate gaseous nitrogen directly in the process of nitrogen fixation.

Nitrogen compounds can also be used in energy conservation or in detoxification. In these processes the end products are not used by the organism but are secreted. In dissimilatory nitrite reduction, nitrite is reduced directly from nitrite to ammonia in a process analogous to assimilation of nitrite. Dissimilatory nitrite reduction is found in bacteria such as *Escherichia coli* and *Wolinella succinogenes* (Cole, 1996).

Other bacteria such as *Paracoccus denitrificans*, *Pseudomonas aeruginosa* or *Alicigenes faecalis* have a more complex respiratory chain in which nitrate ($\text{NO}_3^-$) is reduced to nitrogen.
(N\textsubscript{2}) in four steps via nitrite(NO\textsubscript{2}\textsuperscript{-}), nitric oxide (NO) and nitrous oxide (N\textsubscript{2}O) in the process of denitrification.

In the reverse process, called nitrification, ammonia is oxidised to nitrite via hydroxylamine by ammonia monooxygenase and hydroxylamine oxidase (Arciero et al., 1998; Igarashi et al., 1997) in bacteria such as \textit{Nitrosomonas europaea}.

1.2 Denitrification

The enzymes in denitrification constitute a respiratory chain not unlike that found in aerobic organisms where protons are pumped and electrons transported to electron acceptors (Figure 1.2). In fact, an evolutionary relationship between the enzymes in denitrification and the aerobic respiratory chain has been suggested (van der Oost et al., 1994; Castresana et al., 1994; Saraste & Castresana, 1994).

Reduction of nitrate

The bacterial nitrate reductase implicated in denitrification is a membrane bound complex. Electrons are derived from ubiquinol and delivered via two $b$-type haems and Fe-S clusters to the active site on the cytosolic side of the membrane. Nitrate reductase is not a proton pump but the reaction itself generates a transmembrane proton motif force since the protons liberated upon oxidation of ubiquinol are released on the periplasmic side of the membrane and protons are consumed in the reduction of nitrate to nitrite on the cytosolic side. The reaction is catalysed by a bis-molybdopterin guanine dinucleotide (MGD) cofactor. MGD is also found in the dissimilatory and assimilatory nitrate reductases.

Reduction of nitrite

There are two known types of nitrite reductases involved in anaerobic respiration: one contains copper as a co-factor and one has haem co-factors. Both are soluble periplasmic enzymes. The copper containing enzyme is found in \textit{A. faecalis} and \textit{Rhodobacter sphaeroides}. The second type has been found in \textit{P. denitrificans}, \textit{Ps. aeruginosa}, and \textit{Ps. stutzeri}. In \textit{P. denitrificans} electrons are derived from ubiquinol which is reduced in the membrane by the $bc_1$ complex and are delivered to nitrite reductase by small electron shuttle proteins. Since the $bc_1$ complex is not ubiquitous in bacterial strains, other pathways may also exist. For example, members of the NapC/NirT family of membrane anchored tetra-haem proteins have
been suggested to be involved in $bc_1$ independent electron transfer to periplasmic oxidoreductases (Richardson & Watmough, 1999).

**Figure 1.2:** Schematic picture of the anaerobic respiratory chain in *P. denitrificans*. Dashed arrows indicate electron paths. "ET" denotes electron shuttle proteins such as pseudoazurin or $c_{550}$.

**Reduction of nitric oxide**

Reduction of NO in bacterial denitrification is carried out by a membrane bound complex. NO reductase is a member of the oxidase super-family of enzymes. It consists of a large membrane bound subunit which is homologous to subunit I of cytochrome $c$ oxidase. NO reductase contains two haems and one non-haem iron which forms a binuclear center together with one of the haems. In the two known isozymes, analogous to cytochrome $c$ oxidase and ubiquinol oxidase, electrons are delivered via small soluble electron shuttle proteins or ubiquinol respectively (Hendriks *et al.*, 2000).

**Reduction of nitrous oxide**

The reduction of nitrous oxide is carried out by a soluble dimeric protein. The recently solved three-dimensional structure of nitrous oxide reductase from *Pseudomonas nautica* (Brown *et al.*, 2000) confirmed suggestions, derived from spectroscopy, namely presence of one Cu$_A$ center and a second novel type of copper cluster denoted Cu$_Z$. The structure also revealed
an unusual dimeric structure suggesting that electrons are shuttled from the Cu\textsubscript{A} site of one monomer to the Cu\textsubscript{B} site of the other monomer.

1.3 Cytochrome cd\textsubscript{1} nitrite reductase

In this thesis the haem containing cytochrome cd\textsubscript{1} nitrite reductase from the soil bacterium *Paracoccus pantotrophus* \(^1\) has been studied. Cytochrome cd\textsubscript{1} was first isolated from this source by Moir *et al.* in 1993 but was known from *Ps. aeruginosa* as early as 1958 by Horio *et al.* Although there is a general agreement that the reduction of nitrite to nitric oxide is the physiological reaction performed by this enzyme, cytochrome cd\textsubscript{1} can also catalyse the reduction of dioxygen to water, and was in fact first identified as a soluble cytochrome oxidase.

1.3.1 The structure of cytochrome cd\textsubscript{1} nitrite reductase

The three-dimensional structure of the oxidised form of nitrite reductase from *P. pantotrophus* was determined to 1.5 Å from monoclinic P2\(_1\) crystals (Fülöp *et al.*, 1995). The quality of the data was so good that the sequence which was not known at the time could be determined from the electron density map. The resolution was later extended to beyond 1.3 Å (Baker *et al.*, 1997).

Each 60 kDa subunit of the homodimeric enzyme (Figure 1.3) contains one covalently attached c haem and one non-covalently bound d\textsubscript{1} haem.

The N-terminal part of the polypeptide chain (residues 1-135) forms a domain which in turn is composed by two elements: Residues 49-135 are folded into a cytochrome c like domain containing the c haem. The N-terminal arm (residues 1-48) has no counterpart in cytochrome c and the first 8 residues of this arm are disordered in the monoclinic crystals of the oxidised enzyme. The rest of the N-terminal arm is wrapped around the cytochrome c-like domain and provides ligands to both the d\textsubscript{1} haem (Tyr 25) and the c haem (His 17). This organisation results in an unusual His-His ligation of the c haem and has also been confirmed by EPR studies of the enzyme in solution (Cheesman *et al.*, 1997).

---

\(^1\)For clarity, a few words need to be said about the nomenclature of the bacterial strain. *Paracoccus pantotrophus* was first isolated in 1983 and was named *Thiosphaera pantotropha* (Robertson & Kuenen, 1983). In 1993 an analysis of the sequence of the 16S rRNA of *Thiosphaera pantotropha* led to a re-evaluation of the taxonomy and *Thiosphaera pantotropha* was assigned to *Paracoccus denitrificans* (Ludwig *et al.*, 1993). A second taxonomy re-evaluation was made which resulted in the transfer of *Thiosphaera pantotropha* as well as several strains of *Paracoccus denitrificans* to a new family named *Paracoccus pantotrophus* (Rainey *et al.*, 1999).
1.3. Cytochrome cd₃ nitrite reductase

Figure 1.3: Structure of cytochrome cd₃ nitrite reductase. The structure was determined to 1.5 Å from monoclinic crystals of oxidised enzyme (Fülöp et al., 1995). The enzyme is a homodimer with each polypeptide chain divided into two domains containing a covalently attached c haem and a non-covalently bound d₁ haem respectively.

The C-terminal (d₁) domain (residues 136-567) consists of an 8-bladed β-propeller structure and the d₁ haem is located in the central tunnel close to the domain-domain interface. Available evidence suggest that the role of the cytochrome c domain is to shuttle electrons to the d₁ haem which is the site of catalytic activity. The distal pocket of the d₁ haem is lined with catalytically important residues including two histidines, His 345 and His 388, which protonate the substrate during catalysis (Fülöp et al., 1995; Williams et al., 1997; Ranghino et al., 2000; Cutruzzola et al., 2001).

1.3.2 Haem ligand switching in cytochrome cd₃

The monoclinic crystals of cytochrome cd₃ used for structure determination were grown from oxidised protein in the presence of oxygen. The enzyme can be reduced in the crystal by small reducing agents such as dithionite. The structure of the reduced enzyme derived from such crystals revealed unexpected conformational changes which included religation of both haems (Williams et al., 1997). Partial disordering of the N-terminal arm and refolding of a loop in the cytochrome c domain switch the c haem ligation from His 69/His 17 in the oxidised to His 69/Met 106 in the reduced form. The His/Met ligation is a more common ligand combination in c-type cytochromes, and the isolated N-terminal domain (Gordon et al., 2001) of nitrite reductase adopts this haem ligation (Steensma et al., 2001). Reduction also releases Tyr 25 from the d₁ haem in the active site, giving access to the iron for substrate binding.
1. Introduction

Structural intermediates in the nitrite reduction were obtained by time resolved crystallographic methods where nitrite was soaked into dithionite reduced crystals of cytochrome cd₁ (Williams et al., 1997). The structures showed that nitrite binds as predicted (Fülöp et al., 1995), i.e. with its nitrogen atom ligated by the d₁ haem and with one of the nitrite oxygen atoms coordinated by the two active site histidines, His 345 and His 388. NO was found to bind in different geometries in two different structures. The structures of these intermediates provided starting points for quantum chemical calculations which led to the suggestion for a detailed reaction mechanism for nitrite reduction (Ranghino et al., 2000).

1.3.3 Comparison of cytochrome cd₁ nitrite reductases from different species

The structure of cytochrome cd₁ has also been determined from Ps. aeruginosa (Nurizzo et al., 1997; Nurizzo et al., 1998). The overall structure of this enzyme is similar to that of the reduced cd₁ from P. pantotrophus, with the exception that the N-terminal arm from one subunit extends into the active site of the other in the Ps. aeruginosa enzyme. An alignment of the known sequences of cytochromes cd₁ shows that there is considerable variation in sequence and length of the N-terminal arm, and some species lacks the N-terminal arm completely (Figure 1.4).

1.4 Aim and outline of this thesis

Cytochrome cd₁ nitrite reductase implements many processes relevant to a wide range of biological systems such as electron transfer and redox chemistry on a haem centre. The aim of the present study was to develop our understanding of the redox chemistry and structure-function relationships in this enzyme.

In chapter 2 a brief introduction to the methods used in this thesis will be given. It also includes the presentation of a device developed in the laboratory for data collection on naked crystals. This device which we call “the vapour stream” is presented in Paper I. The instrument was developed by Gisela Larsson, Gunilla Carlsson and myself, following an idea by Janos Hajdu.

The results of the present investigation are described in chapter 3, which is divided into three parts:

i) The initial objectives of this work was to study the oxidase reaction in cytochrome cd₁ using time-resolved structural studies based on cryogenic trapping methods. Although the reduction
1.4. Aim and outline of this thesis

of oxygen to water is not a physiological reaction in cytochrome cd\textsubscript{1} it has been awarded considerable amount of attention due to its significance for aerobic respiration. A structure of an early intermediate in oxygen reduction by cytochrome cd\textsubscript{1} is presented in Section 3.1.

ii) In section 3.2 a combined crystallographic and flash photolysis study of the complex between cytochrome cd\textsubscript{1} and carbon monoxide (CO) is presented. The flash photolysis experiments were done together with Dr. Margareta Svensson-Ek in collaboration with Professor Peter Brzezinski from Gothenburg University, later Stockholm University.

iii) In the structure of cytochrome cd\textsubscript{1} in its reduced form (Williams et al., 1997) it could not be excluded that lattice contacts hindered a full movement of the enzyme. Therefore the reduced enzyme was crystallised under anaerobic conditions. The structure derived from these crystals is presented in Section 3.3 along with the structure of its cyanide complex and the structure of an oxidised form.

A general discussion which includes all results is given in chapter 4 and prospects for future work in chapter 5.
1. Introduction

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>OEQVAPPKDPAALDEHKIDKNITPSLYTEVSQDVAAGPBGSSN</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>OEQVAPPKDPAALDEHKIDKNITPSLYTEVSQDVAAGPBGSSN</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>1</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>DQVPGESPRWKTDAPEIV</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>ATKAEQPAAEPKAAIPT</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>AAPE</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>AAPE</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>QSAI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>YFRCACGCHVRKAGTGGHGKDR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>QGTSNSDEYIQLLEALAVQSGSKILAPSDV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>YTLRTKHMLGLGILDSKLRTGH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>KTEWAVTLEQAPSKGLRQYSY</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pantotrophus</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>Ps. stutzeri Zobell</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>Ps. stutzeri JM300</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
<tr>
<td>M. magnetotactium</td>
<td>YQFEGAPEWFAASLPSEKAN</td>
</tr>
</tbody>
</table>
### 4.1. Aim and outline of this thesis

<table>
<thead>
<tr>
<th><strong>P. pantotrophus</strong></th>
<th>348</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. denitrificans</strong></td>
<td>348</td>
</tr>
<tr>
<td><strong>Ps. aeruginosa</strong></td>
<td>323</td>
</tr>
<tr>
<td><strong>Ps. fluorescens</strong></td>
<td>319</td>
</tr>
<tr>
<td><strong>R. eutropha</strong></td>
<td>315</td>
</tr>
<tr>
<td><strong>Ps. stutzeri Zobell</strong></td>
<td>314</td>
</tr>
<tr>
<td><strong>Ps. stutzeri JM300</strong></td>
<td>306</td>
</tr>
<tr>
<td><strong>M. magnetotacticum</strong></td>
<td>301</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>P. pantotrophus</strong></th>
<th>407</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. denitrificans</strong></td>
<td>407</td>
</tr>
<tr>
<td><strong>Ps. aeruginosa</strong></td>
<td>381</td>
</tr>
<tr>
<td><strong>Ps. fluorescens</strong></td>
<td>377</td>
</tr>
<tr>
<td><strong>R. eutropha</strong></td>
<td>373</td>
</tr>
<tr>
<td><strong>Ps. stutzeri Zobell</strong></td>
<td>372</td>
</tr>
<tr>
<td><strong>Ps. stutzeri JM300</strong></td>
<td>363</td>
</tr>
<tr>
<td><strong>M. magnetotacticum</strong></td>
<td>359</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>P. pantotrophus</strong></th>
<th>462</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. denitrificans</strong></td>
<td>462</td>
</tr>
<tr>
<td><strong>Ps. aeruginosa</strong></td>
<td>436</td>
</tr>
<tr>
<td><strong>Ps. fluorescens</strong></td>
<td>432</td>
</tr>
<tr>
<td><strong>R. eutropha</strong></td>
<td>428</td>
</tr>
<tr>
<td><strong>Ps. stutzeri Zobell</strong></td>
<td>432</td>
</tr>
<tr>
<td><strong>Ps. stutzeri JM300</strong></td>
<td>423</td>
</tr>
<tr>
<td><strong>M. magnetotacticum</strong></td>
<td>414</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>P. pantotrophus</strong></th>
<th>520</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. denitrificans</strong></td>
<td>520</td>
</tr>
<tr>
<td><strong>Ps. aeruginosa</strong></td>
<td>489</td>
</tr>
<tr>
<td><strong>Ps. fluorescens</strong></td>
<td>485</td>
</tr>
<tr>
<td><strong>R. eutropha</strong></td>
<td>482</td>
</tr>
<tr>
<td><strong>Ps. stutzeri Zobell</strong></td>
<td>487</td>
</tr>
<tr>
<td><strong>Ps. stutzeri JM300</strong></td>
<td>478</td>
</tr>
<tr>
<td><strong>M. magnetotacticum</strong></td>
<td>469</td>
</tr>
</tbody>
</table>

**Figure 1.4: Alignment of known sequences of cytochrome cd1 type nitrite reductase.** The sequences are from the top *Paracoccus pantotrophus* (Gordon et al., 2001), *Paracoccus denitrificans* (Saunders et al., 2000), *Pseudomonas aeruginosa* (Silvestrini et al., 1989), *Pseudomonas fluorescens* (Philippot et al., 2001), *Ralstonia eutropha* (Sann et al., 1994), *Pseudomonas stutzeri Zobell* (Jungst et al., 1991), *Pseudomonas stutzeri JM300* (Weeg-Aerssens et al., 1991) and *Magnetospirillum magnetotacticum* (extracted from the unfinished genome at http://www.jgi.doe.gov/). The sequences represent the mature enzyme (i.e. without the signal peptide). The start of the sequences were chosen based on experimental evidence or prediction by the program SignalP (Nielsen et al., 1997). The sequence alignment was made with the program BioEdit (Hall, 1999). 100% sequence identity is indicated with black. Grey shading indicates conservative sequence differences.
2 Methods

2.1 Structure determination by X-ray crystallography

X-rays which hit a molecule will yield a diffraction pattern which represents a spherical section of the Fourier transform of the molecule:

\[
\rho(X) = \int_S F(S) e^{2\pi i (S \cdot X)} + i\alpha(S) \, dS
\]

\(\rho(X)\) is the electron density at the point \((xyz)\) in real space, and \(F(S)\) is the amplitude and \(\alpha(S)\) the phase angle of the complex structure factor \(F(S)\) at the point \((S)\) in reciprocal space. The intensity of the diffraction pattern will be proportional to \(|F(S)|^2\) but the information about the phase angle \(\alpha(S)\) is lost in the image. With the X-ray sources available today it is impossible to obtain a strong enough image of a single molecule. A solution to this problem is to use a crystal containing millions of ordered molecules. By using a crystal the diffraction will be enhanced in certain directions according to Bragg’s law and results in a pattern of discrete points. The Fourier transform reduces to:

\[
\rho(xyz) = \frac{1}{V} \sum_h \sum_k \sum_l F(hkl) e^{2\pi i (hx+ky+lz)} + i\alpha
\]

where \(h, k\) and \(l\) are Miller indices and describe points in reciprocal space. The volume, \(V\) is included in the equation because the real vector \((xyz)\) is expressed in fractional coordinates.

There are a number of alternative methods for recovering the phase information. These include the multiple/single isomorphous replacement (MIR/SIR) and the multiple/single anomalous dispersion (MAD/SAD methods. In this thesis phasing was done by fourier methods or by molecular replacement. These methods will be described briefly here.

Phasing by Fourier methods

If a similar structure has already been solved initial phases can be obtained by calculating the structure factors for the known structures:
2.1. Structure determination by X-ray crystallography

\[ F_{\text{calc}}(hkl, \alpha) = V \int_{cde} \rho(xyz) e^{2\pi i (hx+ky+lz)} dxdydz \]

The calculated phases are not used directly since this would introduce considerable model bias. Instead the calculated and observed structure factor amplitudes are scaled together and the phases weighted according to the SigmaA procedure (Read, 1986) to produce a weighted 2mFo-DFcal map. This procedure was used in papers II and III.

Phasing by Molecular replacement

If no structure from an isomorphous crystal is available, phases from a similar structure can still be used in a procedure called molecular replacement, first described in 1962 (Rossman & Blow) (Rossman & Blow, 1962). The idea is to obtain as high agreement as possible between diffraction data of the unknown structure and calculated data from a known structure, the search molecule. This can be described by a six-dimensional problem where the search model is rotated and translated in order to find a maximum overlap:

\[ X' = CX + t \]

where \( C \) is the rotation matrix and \( t \) is the translation vector. \( X \) is the original set of coordinates for the search molecule and and \( X' \) denotes the transformed coordinate set. The maximum overlap is found using Patterson methods. Several programs for molecular replacement are available. AMoRe (Navaza, 1994) and CNS (Brünger et al., 1998) both adopt a method where the problem is divided into two parts, the rotation search and the translation search. EPMR (Kissinger et al., 1999), the program which was used to solve the structure of the alternative form of cytochrome \( cd_{1} \) (paper IV), uses stochastic search techniques in all six dimensions simultaneously.

The quality of the solutions is assessed by the linear correlation coefficient and the R-factor\(^1\). The linear correlation coefficient describes how well the observed intensities match the calculated ones of the solution. The correlation coefficient is a sensitive measure but the difference between the correct solution and a wrong solution is often small. The R-factor is a measure of how much the observed structure factors deviate from the calculated values. A high correlation coefficient and a low R-factor indicates possible solutions.

\(^1\)The R-factor is calculated as \[ \frac{\sum_{hkl} |F_{\text{calc}}|-|k|F_{\text{cal}}|}{\sum_{hkl} |F_{\text{obs}}|} \times 100\% \] where \( k \) is the scale factor for the intensities
2. Methods

Refinement

Once initial phases have been obtained the structure is refined by iterative rounds of manual fitting of atoms into electron density and refinement against a target. The refinement target of choice in this work has been the maximum likelihood function which maximises the likelihood of the model to match the observed amplitudes.

The quality of the model is assessed by the crystallographic R-factor and the free R-factor (Brünger, 1992). The free R-factor is calculated as the crystallographic R-factor but the data used for calculation is a fraction of the total data (typically around 5%) which is not included in the data used for refinement. The free R-factor thus becomes a less biased indicator of the quality of the model.

2.2 Time-Resolved Crystallography

As more and more structures have been solved there is an increasing interest for studying three dimensional structures of reaction intermediates and the area of time-resolved crystallography is expanding (for a review see Hajdu et al., 2000).

2.2.1 Reaction initiation and trapping of intermediates

In order to be able to capture a reaction intermediate, reaction initiation in the crystal must be uniform and fast with respect to the time of build-up and decay of the intermediate of interest.

For proteins which act upon light induction it is often possible to obtain fast and uniform reaction initiation with a short laser flash. Studies on bacteriorhodopsin (Edman et al., 1999; Royant et al., 2000) and the photo-active yellow protein (Genick et al., 1997; Perman et al., 1998) have contributed significantly to our understanding of photochemical energy transduction mechanisms.

Most enzymes do not use light in their function but act upon some other molecule. As a consequence the most common method of reaction initiation within such enzymes is diffusion of substrates into the crystal. The crystal is soaked in the substrate of interest and the reaction is stopped by plunging the crystal into liquid nitrogen. This handling is slow relative to the reaction rates usually seen in enzymes. However, the constraints of the crystal lattice often slow down the reaction speed significantly, often at specific step which results in the build-up of a particular intermediate. In this way the structure of cytochrome cd$_1$ nitrite reductase with a bound nitrite ion could be trapped (Williams et al., 1997) although this intermediate is expected to last only a few milliseconds in solution (George et al., 2000). The oxidase
reaction in cytochrome $\text{cd}_1$ was not slowed down to the same extent. In order to be able to capture an early reaction intermediate, the reaction had to be slowed down by performing the experiment at $-17^\circ\text{C}$ (paper II).

### 2.2.2 Microspectrophotometry

An invaluable tool in time resolved crystallographic studies on redox enzymes containing chromophores is the single crystal microspectrophotometer (Hadfield & Hajdu, 1993; Wilmot et al., 2001). Spectroscopic intermediates are often known from solution studies and by comparing single crystal spectra with solution spectra the redox state of the enzyme can be assessed and the state of the enzyme in the crystal can be placed along the reaction coordinate. A microspectrophotometer has the same components as any other spectrophotometer, a light source which is focused on the sample and a detector (Hadfield & Hajdu, 1993). The sample in this case is the crystal which is mounted on a goniometer head for positioning in the light beam. The crystal can be mounted either in a capillary or, if the microspectrophotometer is fitted with a cryo system or a vapour stream (see below), in a loop. In order to obtain good spectra of a single crystal care must be taken to align the crystal properly. Prism effects due to the shape of the crystal can distort the spectrum. In addition, the non-random organisation of the molecule in the crystal can make the spectrum look different from different directions. It is extremely helpful to have spectrum recorded of the enzyme in solution as a reference when aligning the crystal. However, it is important to keep an open mind about the spectrum, especially if one is looking for reaction intermediates for which absorption spectrum might not be available. In cytochrome $\text{cd}_1$ nitrite reductase there is also a discrepancy between the solution spectrum and the single crystal spectrum when the latter is recorded at cryogenic temperatures. In the solution spectrum recorded at room temperature the band of the $d_1$ haem there are two peaks at 645 and 710 nm respectively whereas the crystal spectrum recorded at 100 K the 710 nm peak is almost absent (Figure 2.1). This is due to a thermal equilibrium between two species and has also been reported for low temperature studies of the enzyme in a non-crystalline sample (Cheesman et al., 1997). If the crystal spectrum is recorded at room temperature on a crystal mounted in a capillary or in a vapour stream the spectrum is in agreement with the solution spectrum.

### 2.2.3 Data collection on naked crystals at room temperature

Another useful tool in time resolved crystallography is the vapour stream which has been developed in our laboratory. The idea is that the crystal is mounted in a loop of the same
2. Methods

![Absorbance vs Wavelength](image.png)

**Figure 2.1: Absorption spectra of oxidised cytochrome \(cd_1\).** The main panel shows the spectrum of the enzyme in solution at a concentration of 0.4 mg/ml. The sub-panel shows single crystal spectra of crystals of oxidised cytochrome \(cd_1\) recorded at 290 K (crystal mounted in capillary) and 100K (crystal mounted in cryo loop).

The type used for cryo-crystallography and is kept in a controlled atmosphere of humidified gas (0-30°C) which flows past the crystal in a laminar fashion.

Figure 2.2 shows the components of the vapour stream. An air stream is generated by a membrane pump and the flow is monitored and regulated by a flow meter. Gas is dispensed into the liquid used for humidification through a sintered metal diffuser. The liquid is kept in a glass bottle fitted with a gas tight lid with holes for inlet and outlet tubing. The humidified gas is led into a nozzle consisting of a plastic cylinder formed as to yield a laminar gas flow at the tip. For experiments requiring other than room temperature, the nozzle can be fitted with a Peltier device to allow temperature regulation in the range of 0-30°C at the crystal.

In order to get a laminar flow past the crystal the opening of the nozzle needs to be close to the crystal, typically 5-8 mm. The gas flow is calibrated as to prevent the crystal from drying or taking up excess humidity from its surrounding. Calibration is done by placing a droplet of the crystal mother liquor on the loop, and then gas flow is adjusted so that the drop neither shrinks nor grows. If gas is bubbled through room temperature water, typical gas flows required to keep the drop size stable is 0.5 l/min. The humidity of the gas can be manipulated by changing the temperature of the humidifying liquid or by changing its composition.

The vapour stream allows for a wide range of approaches of reaction triggering in a crystal, as the gas stream around the crystal can rapidly be changed to produce a new chemical atmosphere around and inside the crystal. The environment can be altered either by changing the reactant gas or by changing the humidifying liquid. A crystal of cytochrome \(cd_1\) reduced
2.3. Studies of pre-steady state kinetics in solution by flash photolysis

Figure 2.2: Components of the vapour stream experimental setup. 1) A membrane pump of the same type as used for a hobby aquarium. 2) A flow meter to monitor and regulate the flow. 3) Bottle fitted with gas-tight lid and a gas dispenser of sintered metal for dispersion of gas into the humidifying liquid. 4) Bottle with gas-tight lid for trapping of large droplets. 5) A simple air stream nozzle. 6) A more complicated nozzle fitted with a Peltier device for temperature control.

with sodium dithionite placed in a nitrogen stream humidified by oxygen free water stays reduced in the nitrogen stream for at least 2 hours, and no re-oxidation by molecular oxygen form the surrounding air can be detected. Figure 2.3 shows the spectral changes as crystals of cytochrome $cd_1$ following a switch of the gas flow from wet nitrogen to wet oxygen. The results showed that although the nitrite reduction by cytochrome $cd_1$ was slowed down by several orders of magnitude (Williams et al., 1997), the oxidase reaction in the crystal is fast (the enzyme is fully oxidised within 10 seconds after reaction initiation.

2.3 Studies of pre-steady state kinetics in solution by flash photolysis

Flash photolysis

The bond between carbon monoxide (CO) and the reduced $d_1$ haem iron of cytochrome $cd_1$ is photolabile and the CO molecule can be photo-dissociated from the $d_1$ haem with a short laser flash. The changes in the electronic environment of the haem which are visible as changes.
2. Methods

Figure 2.3: Spectral changes following oxygen diffusion into reduced cytochrome $cd_{1}$ crystal. Crystals grown under oxidising conditions were reduced with 20 mM dithionite and placed in a stream of wet nitrogen generated by the vapour stream. Oxygen was introduced by switching the gas from nitrogen to oxygen. Oxygen reaches the crystal by spectrum 8. A spectrum is recorded every 3 s.

in the visible absorption spectrum can be followed using an experimental setup where the laser flash, which induce the photodissociation of CO, triggers an oscilloscope connected to a photo multiplier which measures the intensity of a probe light (Figure 2.4).

In cytochrome $c$ oxidase it is possible to obtain a species where CO is bound to the binuclear center and the other cofactors are oxidised. Flash photolysis of the bond between CO and cytochrome $c$ oxidase in this mixed valence species allows for the study of internal electron transfer as electrons "trapped" by the CO are "released" upon photolysis.

Flow-flash spectroscopy

Flash photolysis can also be used to increase the time resolution in the study of pre-steady state catalytic events. Pre-steady state kinetics are usually studied using stopped flow techniques where enzyme and substrate are rapidly mixed prior to spectroscopic measurements. The limitation of this technique is that the mixing time (a few milliseconds) is often too long compared to the time-scale of the events of interests. However, by mixing the enzyme-CO complex with substrate and then initiating the reaction by photolysis of the enzyme-CO bond after completion of the mixing, events occurring in the µs time-range can readily be measured. This method was developed by Gibson & Greenwood (1963) and has been successfully used
2.3. Studies of pre-steady state kinetics in solution by flash photolysis

Figure 2.4: Schematic picture of a flash photolysis experiment.

for studies of members of the oxidase superfamily (Babcock & Wikström, 1992).

There are several requirements which have to be fulfilled for this method to work. First, recombination of the enzyme-CO complex must be slower than substrate (e.g. dioxygen) binding. Secondly, the dissociation of the enzyme-CO complex should be slow compared to the time between mixing with substrate and the laser flash so that the active site metal remains blocked until the laser flash releases the CO molecule.

**Studies of proton uptake and release**

Reaction steps where proton uptake or release takes place between the enzyme and the bulk solvent can be identified using pH dyes. An unbuffered sample is mixed with a pH dye such as phenol red or cresol red, depending on the pH range of interest. Proton uptake and release results in absorption changes due to a change in pH. If possible, a pH dye should be chosen such that its absorption maximum is found in a wavelength region where the absorbance of the sample is low and changes little during the reaction. However, a control experiment where the enzyme solution is buffered should always be performed. The difference in absorption due to pH changes is obtained by subtracting the absorbance changes in the buffered sample from those of the unbuffered sample.
3 Results

3.1 Time-resolved structural studies of the oxidase reaction

The reduction of dioxygen to water, also known as the oxidase reaction, is of greatest importance to aerobic life. Although the ability to catalyse this reaction is probably not of physiological significance for cytochrome cd$_1$, the enzyme serves as a valuable model system for oxygen chemistry at a haem centre. Unlike the oxidases, cytochrome cd$_1$ is soluble and good quality crystals can easily be obtained. Previous studies have shown that it is possible to reduce the enzyme in the crystalline state and to obtain structures of reaction intermediates by re-oxidising the crystal with nitrite (Williams et al., 1997). We set out to investigate the oxidase reaction in cytochrome cd$_1$ using a similar approach in time resolved X-ray crystallography. Paper II describes the structure of an early intermediate in the oxidase reaction and its implications for the mechanism of oxygen reduction.

3.1.1 Preparation of reaction intermediates in the crystal

For preparation of reaction intermediates in the crystal we used the same approach as for the studies of the nitrite reduction (Williams et al., 1997), i.e. crystals grown under oxidising conditions were reduced using dithionite and substrate was introduced by diffusion. However, several difficulties had to be overcome. First, oxygen is gaseous and the solubility in aqueous solution is only about 1 mM at room temperature. The enzyme concentration in the crystal is 5 mM. To increase the effective oxygen concentration we introduced the oxygen using a pressure cell. The reduction of crystals was carried out in a glove-box but to avoid oxygen leakage inside the box pressurising had to be done outside the box. It soon became apparent that although the nitrite reaction is slow in the crystal (Williams et al., 1997), oxygen reduction is not, and the enzyme became fully oxidised in 10-30 s at room temperature upon exposure to oxygen. The experimental procedure did not allow us to reduce the incubation time further. We therefore had to slow down the reaction. The cryoprotectant contained 18% glycerol, which lowered the freezing point to about -22°C. This allowed us to carry out
3.1. Time-resolved structural studies of the oxidase reaction

the experiment in a freezer room in which the temperature was maintained at -17°C. At this temperature it was possible to obtain intermediates with distinct spectral features. A by-effect of the low temperature was that the oxygen concentration increased, since the solubility of gas increases with decreasing temperature. Full oxidation of the enzyme at this temperature took more than 20 minutes (see Paper II for more details).

3.1.2 X-ray mediated reduction of reaction intermediates during data collection

The structure determined from a single crystal containing an intermediate prepared as described above showed density extending from the \( d_1 \) haem. However, the density could not readily be interpreted. It seemed to be a mixture between a diatomic ligand and a monatomic ligand (Figure 3.1). Single crystal spectrophotometry clearly showed that the haem centra were reduced after data collection. The electrons for this X-ray mediated reduction are

![Figure 3.1: Stereo view of the active site of subunit B in the X-ray reduced structure.](image)

Figure 3.1: Stereo view of the active site of subunit B in the X-ray reduced structure. The crystal used for structure determination was reduced with 20 mM dithionite and the exposed to oxygen under 15 atm. pressure at -17°C for 2 minutes. The 2mF\( _{obs} \)-DF\( _{calc} \) electron density map is contoured at 1.5 \( \sigma \) where \( \sigma \) is the root mean square electron density for the unit cell

generated when X-rays interact with atoms in the sample and electrons are emitted through the photo electric effect and associated processes (Ziaja et al., 2001). Electrons generated this way can travel over long distances in the crystal and an oxidised metal center can serve
as an electron sink. Conformational changes due to X-ray mediated reduction have been reported for ribonucleotide reductase (Logan et al., 1996). For data collection at cryogenic temperature the change in oxidation state is not expected to cause any dramatic changes to the three-dimensional structure since the enzyme is embedded in frozen solvent. However, small changes can occur even at cryogenic temperature such as movement of a ligand upon flash photolysis of the myoglobin:CO complex (Schlichting et al., 1994) or bond isomerisation and diffusion of solvent molecules upon light induction in bacteriorhodopsin crystals (Edman et al., 1999).

In order to be able to determine the structure of the intermediate present in the crystal a different data collection method had to be employed. Wedges of 10° of data were collected from 11 different crystals, each of which contained the same intermediate species as determined by microspectroscopy. The crystals were oriented for data collection to give maximum completeness. This was trivial since the anatomy of the monoclinic crystals resulted in an alignment in the loop so that the two-fold screw axis was perpendicular to the incident beam and the start orientation could be corrected after analysis of one short exposure. This data collection strategy has been successfully used to collect data from reduction sensitive species of NDO (Andreas Karlsson, personal communication) and horse radish peroxidase (Berglund et al., 2001).

### 3.1.3 Structure of the bound dioxygen species in cytochrome cd₁

The data collection strategy described above allowed us to visualise an early intermediate in the reduction of oxygen in cytochrome cd₁ nitrite reductase (Figure 3.2).

There are several possible electronic configurations for the structural intermediate in Figure 3.2. The diatomic molecule fitted into the density can be a neutral dioxygen molecule, a peroxide (one negative charge) or a superoxide with two negative charges. Depending on the nature of the ligand there are different possibilities for the electronic states of the haem irons. The difference in expected bond distance for the different possible ligands is too small to be useful as an indicator of the electronic configuration, instead other indicators must be used.

In this case, the single crystal spectra were not as useful as in other systems (e.g. horse radish peroxidase, Berglund et al. 2001) for assessing the electronic state since the two subunits react at different rates in the crystal, probably due to the difference in crystallographic environments. In all crystals used in this study, the reaction seemed to proceed faster in subunit A.

The puckering of the $d_1$ haem which is also seen in the structure of the reduced enzyme
3.2 Studies of the complex between cytochrome $cd_1$ and carbon monoxide

Figure 3.2: Stereoview of the active site of subunit B in the dioxygen species. The crystals used for structure determination were reduced with 20 mM dithionite and then exposed to oxygen under 15 atm pressure at -17°C for 2 minutes. The data set used for structure determination is composed by wedges of $10^5$ of data from 11 different crystals. The electron density shown is an “omit” map, calculated as $2mF_{\text{obs}}-DF_{\text{calc}}$ maps after omitting the dioxygen molecule from the model. The map is contoured at $1.5\sigma$ where $\sigma$ is the root mean square electron density for the unit cell (Williams et al., 1997) and in the complex between reduced $cd_1$ and carbon monoxide (Paper III) suggest that the $d_1$ haem iron was reduced. The $c$ haem of subunit B has His/Met ligation as in the fully reduced enzyme. These features suggest that both the $c$ and the $d_1$ haems of subunit B were reduced. In this case, the bound dioxygen species is most likely a neutral dioxygen molecule which has not yet been converted to other dioxygen species (e.g. superoxide or peroxide) by the enzyme.

3.2 Studies of the complex between cytochrome $cd_1$ and carbon monoxide

Diatom ligands such as NO, CN$^-$ and CO are widely used as dioxygen analogues in various types of experiments. Structures of cytochrome $cd_1$ complexed to NO (Williams et al., 1997) and CN$^-$ (Jafferji et al., 2000) are available. A structure of cytochrome $cd_1$ complexed to CO would add to our knowledge about binding of diatomic ligands. Moreover, detailed
knowledge about the enzyme:CO complex was required in order to assess the possibilities of studying pre-steady state kinetic events in solution using the flow-flash technique. The combined flash-photolysis and X-ray crystallographic study of cytochrome $cd_1$ in complex with CO presented in paper III gave insight not only about this complex but also had several mechanistic implications.

3.2.1 Structural studies of the cytochrome $cd_1$:CO complex

Preparation of enzyme:CO complex in the crystal

Crystals of oxidised cytochrome $cd_1$ were reduced in a glove box using dithionite according to the method of Williams et al. (1997). Soaking the reduced crystal in a solution saturated with CO (the solubility of CO in aqueous solution is 1 mM) did not result in complex formation. Instead the crystal was incubated with carbon monoxide under 15 atm. pressure in a pressure cell. The pressurising of the crystal was carried out outside the glove box. To prevent oxidation by atmospheric oxygen during the transfer of the crystal from the cryosolution to the pressure cell, the procedure was carried out in a freezing room at -17°C and the crystal was incubated for 20 minutes with CO at 15 atm.

The structure of reduced cytochrome $cd_1$ complexed to CO

Data were collected to 1.57 Å resolution. The crystals were isomorphous with the reduced monoclinic crystals and initial phases were obtained from the reduced structure (Williams et al. 1997, PDB id. 1AOF). The N-terminal domain of subunit A is disordered in the enzyme:CO complex but the overall structure of three ordered domains is similar to the structure used for phasing.

Electron density corresponding to a bound CO molecule was extending from the $d_1$ haem in both subunits. Unrestrained refinement of the enzyme:CO complex resulted in a bond length of 1.9 Å and a Fe-C-O bond angle of 158° (Figure 3.3).

Studies of CO bound to haem model complexes show a straight Fe-C-O bond. In cytochrome $cd_1$ such a binding mode is sterically hindered. However, calculations show that deviation of less then 25° result in very small energetic differences (Ghosh & Bocian, 1996). In the active site pocket of cytochrome $cd_1$ there are two histidine residues, His 388 and His 345, which are perfectly situated to coordinate the second atom of a diatomic ligand bound to the $d_1$ haem. This coordination is adopted by the dioxygen molecule in the structure of the intermediate of the oxidase reaction (Paper II). It is also the binding mode seen in the cyanide
3.2. Studies of the complex between cytochrome cd$_{1}$ and carbon monoxide

His 388

His 345

Haem d$_{1}$

His 200

CO

1.9 Å

158°

His 388

His 345

Haem d$_{1}$

His 200

CN

2.0 Å

164°

His 388

His 345

Haem d$_{1}$

His 200

O$_{2}$

1.8 Å

134°

His 388

His 345

Haem d$_{1}$

His 200

NO

1.8 Å

130°

Tyr 25

His 388

His 345

Haem d$_{1}$

His 200

NO

2.0 Å

127°

Tyr 25

His 388

His 345

Haem d$_{1}$

His 200

Figure 3.3: Structures of the active site pocket in complexes between cytochrome cd$_{1}$ and various diatomic ligands. (a) CO complex (Paper III) (b) dioxygen complex obtained by time resolved crystallographic methods (Paper II, see also section 3.1) (c) CN$^-$ complex obtained by soaking dithionite-reduced crystals with KCN (Jafferji et al., 2000) (d-e) Two different NO complexes obtained by soaking dithionite reduced crystals with nitrite (Williams et al., 1997).

complex (Jafferji et al., 2000) and in one of NO-complexes obtained by soaking nitrite into reduced crystals (Williams et al., 1997). In the structure of the oxidised enzyme this coordination site between the histidines is occupied by a solvent molecule (Fülöp et al., 1995). In the complex with CO, the oxygen atom is pointing away from the histidine coordination site. Instead a solvent molecule is coordinated by the two histidine residues. A similar arrangement is seen in another NO complex described by Williams et. al, (1997). In this structure NO is pointing away from the histidines and the phenolate oxygen of Tyr 25 is located in roughly the same position as the histidine-coordinated solvent molecules in the CO complex. This variation in binding geometries for diatomic ligands support the previous suggestion that dual conformations for NO is important for effective product release (Williams et al., 1997; Ranghino et al., 2000)
3. Results

3.2.2 Studies of the complex between cytochrome cd$_1$ and CO in solution

Despite the wealth of structural data available on cytochrome cd$_1$ nitrite reductase, the reaction kinetics of the enzyme from _P. pantotrophus_ is not well known. Pre-steady state studies of the nitrite reduction in _Ps. aeruginosa_ cytochrome cd$_1$ indicated that several fast kinetic phases were lost in the dead time of the stopped flow apparatus used (Silvestrini _et al._, 1990). We therefore set out to use the flow-flash technique (see Methods). This technique has not been used for cytochrome cd$_1$ before so first a careful study of the enzyme:CO complex was required.

Preparation of enzyme:CO complex in solution

Reduction of cytochrome cd$_1$ in the crystal by dithionite was shown to result in a structure where sulphur dioxide, a byproduct of reduction with dithionite was bound to the d$_1$ haem (Williams _et al._, 1997). To avoid any interference by dithionite we chose to use ascorbate which readily reduces cytochrome cd$_1$ from _Ps. aeruginosa_. However, reduction of the _P. pantotrophus_ enzyme was very slow, even in the presence of an electron mediator, phenazine methosulphate (PMS), reduction using stoichiometric amounts of ascorbate took over 15 h at 4°C. This was also documented by Koppenhöfer _et al._ (2000a) even if the authors used another electron mediator, hexaamineruthenium-(III)-chloride (HARC), in their experiments. The solution of reduced enzyme was flushed with gaseous carbon monoxide to prepare the complex. Upon introduction of CO the electronic absorption spectrum is altered (Figure 3.4). The major differences are in the wavelength regions corresponding to the electronic environment of the d$_1$ haem.

Kinetics of events following flash photolysis

Illumination of the sample with a laser flash of 10 ns duration with the wavelength of 532 nm releases the bound CO molecule from the d$_1$ haem. The recombination of the enzyme:CO complex was observed over a wide range of wavelengths and the second-order rate constant for the recombination was estimated to be $1.3 \times 10^4$ s$^{-1}$, which is comparable to values previously reported for the of the _Ps. aeruginosa_ enzyme (Parr _et al._, 1975; Wilson _et al._, 1999). The kinetic difference spectrum is in agreement with the static difference spectrum between

---

$^1$the difference in absorbance at $t\to\infty$ and $t=0$ for a kinetic phase
3.2. Studies of the complex between cytochrome cd$_{1}$ and carbon monoxide

![Figure 3.4: Solution spectra of cytochrome cd$_{1}$](image)

The reduced enzyme:CO complex was formed by flushing the reduced enzyme with gaseous CO for at least 5 minutes. The main absorption wavelengths attributable to the c and d$_{1}$ haems (Kobayashi et al., 1997) are indicated by solid bars.

Before the recombination, two rapid phases were observed with rate constants of $2.2 \times 10^4$ s$^{-1}$ and $4.0 \times 10^3$ s$^{-1}$ respectively. The kinetic difference spectrum for these phases are different from the kinetic difference spectrum of recombination, but display the most prominent features in the same wavelength region, i.e. the regions corresponding to the electronic environment of the d$_{1}$ haem, and are therefore interpreted as structural rearrangements in this environment. Similar rapid phases upon photolysis of the enzyme:CO complex were reported for the *Ps. aeruginosa* cytochrome cd$_{1}$ (Wilson et al., 1999).

Proton uptake and release following flash photolysis

Changes in the protonation state following flash photolysis were studied by adding a pH dye to unbuffered enzyme solution. The absorbance changes associated exclusively with alterations in the protonation state were extracted by subtracting absorbance changes measured under the same conditions but in the presence of 10 mM buffer (Figure 3.5). The correlation between the observed changes in absorbance and proton uptake/release was determined by measuring the absorbance change of the sample following additions of small volumes of an anaerobic HCl solution of known concentration.
3. Results

![Graph showing time-courses from flash-photolysis experiments in the presence of cresol red.](image)

**Figure 3.5**: Time-courses from flash-photolysis experiments in the presence of cresol red. Black traces were recorded with unbuffered enzyme solution, grey traces were recorded in the presence of 10 mM Tris-HCl pH 8.5. Experimental conditions: about 1 µM cytochrome cd₁, 2 mM ascorbate, 5 µM phenazine methosulfate, 36 µM cresol red, 0.1 M KCl, pH 8.5.

Our result show that CO recombination is associated with proton uptake and the slower of the two recombination phases (see above) is linked with proton release. Based on the structure of the complex between CO and reduced cytochrome cd₁ we suggest a sequential mechanism for proton uptake and release following flash photolysis (Figure 3.6). The model is based on the assumption that His 345, which is believed to have the higher pKa value of the active site histidines (Ranghino et al., 2000), is unprotonated in the complex. Following photodissociation of CO, there is one fast internal rearrangement possibly including internal proton transfer, and then a slower phase where a proton is taken up from solution so that in the fully reduced enzyme both histidine residues in the active site are protonated.

**Cytochrome cd₁ - a suitable candidate for flow-flash studies**

The rate constant for thermal dissociation of CO from the reduced enzyme was estimated to be $1.0 \pm 0.1$ s⁻¹. CO thus binds sufficiently well to the enzyme to allow for studies of pre-steady state kinetics using the flow-flash approach. Moreover, recombination following photolysis is slow compared to the events that should be detected. However, the unexpected biphasic behaviour is likely to complicate the picture.
3.2. Studies of the complex between cytochrome \( cd_1 \) and carbon monoxide

![Diagram of kinetic events following photodissociation of CO]

---

**Figure 3.6:** A model for the kinetic events following photodissociation of CO. Following photodissociation there are two rearrangements, the first one is fast and possibly associated with internal proton transfer, the second one include proton uptake from solution.

**Attempts to obtain a mixed valence species**

A mixed valence complex where the \( c \) haem is oxidised and CO is bound to a reduced \( d_1 \) haem (\( c^{3+} \) \( d_1^{2+} \)-CO) would be useful for studies of internal electron transfer in cytochrome \( cd_1 \) nitrite reductase. However, despite our efforts we were not able to obtain such a mixed valence species. Instead titrations with ferro/ferri-cyanide resulted in mixtures of fully reduced and fully oxidised enzyme. This is in contrast with results obtained with cytochrome \( cd_1 \) from *Ps. aeruginosa* where a mixed valence species could readily be obtained by gentle oxidation of the reduced enzyme:CO complex (Wilson *et al.*, 1999). This difference between cytochromes \( cd_1 \) from the two organisms is also reflected in results from redox titrations. For *Ps. aeruginosa* cytochrome \( cd_1 \), individual redox potentials for the haems can be obtained, around +280 mV for both haems (Silvestrini *et al.*, 1994). In the *P. pantotrophus* enzyme the haems are highly cooperative and, moreover, display a hysteretic behaviour (Koppenhöfer *et al.*, 2000b) where a reductive titration gives a midpoint potential of +60 mV and an oxidative titration results in a midpoint potential of +210 mV. The nature of this behaviour is unclear and needs to be clarified by further studies.

**3.2.3 Implications for the catalytic mechanism**

Proton release and uptake during the binding and the release of a neutral ligand (CO) changes the overall charge of the protein as a function of the ligation state of the \( d_1 \) haem. Such
3. Results

A process could modulate the redox potential of the active site and may offer a means for controlling electron transfer between the two different haem centra in the enzyme. In order to avoid product inhibition during catalysis through the formation of a stable haem $d_1$ Fe$^{2+}$ (NO) complex (Sharma et al., 1987), release of the NO product from the active site must precede electron transfer from haem $c$ to haem $d_1$. If NO release from the enzyme-NO complex is accompanied by a proton uptake in a similar manner as the release of CO, then such a mechanism may ensure preferential electron transfer to an empty $d_1$ haem and not to the enzyme-product complex.

3.3 Large redox-coupled conformational changes in cytochrome $cd_1$

Upon reduction of the enzyme in the crystal the N-terminal domain undergoes partial refolding resulting in religation of both haem groups (Williams et al., 1997). The movements triggered by reduction are among the largest observed within a crystal lattice, however, it can not be excluded that the crystal contacts prevent further movements. Therefore, crystallisation trials with reduced enzyme were set up under anaerobic conditions.

3.3.1 Anaerobic crystallisation of cytochrome $cd_1$ in its reduced form

Cytochrome $cd_1$ is readily reduced using dithionite, however, the structure of the reduced enzyme derived from crystals grown under oxidising conditions showed that a by-product of reduction, sulphur dioxide bound to the $d_1$ haem. We chose therefore to reduce the enzyme with ascorbate in the presence of the mediator phenazine methosulphate, the protocol developed for our earlier solution kinetics studies (Paper III). The enzyme was reduced over night after which the enzyme solution was dark green. Crystallisation was done using the hanging drop vapour diffusion technique in the glove box. 1 µl protein solution (20 mg/ml in 10 mM potassium phosphate buffer, 2 mM sodium ascorbate and 5 µM phenazine methosulphate) was mixed with an equal volume of reservoir solution containing 100 mM 2-(cyclohexylamino)-ethanesulfonic acid (CHES) and 2.0-2.1 M Ammonium sulphate. No additional reducing agent or oxygen scavenger was used to maintain the anaerobic crystallisation environment. However, care was taken that all solutions and plastic material used was de-aerated in the glove box for at least two days before use. Crystals appeared within a day (Figure 3.7). The
3.3. Large redox-coupled conformational changes in cytochrome cd

![Figure 3.7](image)

**Figure 3.7:** Different crystal forms of cytochrome cdₙ nitrite reductase. (a) Crystals of the oxidised enzyme, spacegroup P2₁. (c) Crystals of reduced enzyme, space group P₄₁2₁2. The crystal packing in the crystals in (a) and (c) are shown in (b) and (d) respectively. The d₁ haem domains are shown in black and the N-terminal domains in grey. The black frames indicate the unit cells.

The redox state of the crystals was assessed by microspectrophotometry and the spectra clearly showed that the enzyme was fully reduced. The morphology of the crystals was clearly different from the monoclinic crystals used in previous studies and the space group was determined to be P₄₁2₁2 with cell dimensions a=b=127.93 Å and c=263.2 Å. The asymmetric unit contained a dimer and the solvent content was 70 % . The crystals diffracted to 2.1 Å resolution at a synchrotron source.

Based on the crystal form used for structure determination, the different structures will from here on be referred to as monoclinic (crystallised from oxidised protein) or tetragonal (crystallised from reduced protein).
3. Results

3.3.2 Molecular replacement and refinement of the structure

Molecular replacement for initial phasing of the alternative, tetragonal, form of cytochrome \(cd_1\) was performed using a dimer of \(d_1\) domains as a search model. Reflections between 15 and 4 Å resolution were used. The search yielded a solution with a correlation coefficient of 48% and an R-factor of 46%. This solution was improved and the resolution extended to 3 Å with rigid body refinement in REFMAC (Murshudov et al., 1997). At this stage, parts of the N-terminal domains were visible in the electron density map. A free atom model of the N-terminal domain was generated by iterative rounds of ARP (Lamzin & Wilson, 1993) and REFMAC and the resulting electron density map was traced with O (Jones et al., 1991). The R-factor for the final model was 22% and the free R-factor 24%. The contacts between symmetry related molecules in the crystal include interactions both between the N-terminal domains and the \(d_1\) domains (Figure 3.7). This is in contrast with the monoclinic crystals grown under oxidising conditions where practically all crystal contacts are mediated by the \(d_1\) domains.

3.3.3 The overall structure

A comparison of the structure of cytochrome \(cd_1\) determined from the tetragonal crystals of the reduced enzyme and the structures of reduced and oxidised enzyme determined from monoclinic crystals is shown in Figure 3.8. A striking feature is the new position of the cy-

![Figure 3.8: Overall structure of different forms of cytochrome cd.](image)

Figure 3.8: Overall structure of different forms of cytochrome \(cd_1\). The structures of (a) reduced enzyme determined from tetragonal crystals grown from reduced protein (Paper IV), (b) reduced (Williams et al., 1997) and (c) oxidised (Fülöp et al., 1995) cytochrome \(cd_1\) determined from monoclinic crystals are shown in the same orientation.
3.3. Large redox-coupled conformational changes in cytochrome cd₁

tochrome c domain relative to the d₁ domain in the new structure. Within this structure there are no contacts between the ordered parts of the cytochrome c domains. The N-terminal arms are almost completely disordered, only 7 or 10 residues could be traced in subunit A and B respectively. Although the two monomers experience different crystallographic environments in the tetragonal lattice, the positions of the two cytochrome c domains are similar in both subunits within the asymmetric unit of the tetragonal crystals. The overall fold of the individual c and d₁ domains in the monoclinic and tetragonal forms of the reduced enzyme are similar. A structural alignment² of all the Cα atoms of the d₁ domain (residues 136-567) gives a root mean square deviation of 0.40 Å. The corresponding alignment for the cytochrome c domain (residues 49-130) yields a root mean square deviation of 0.32 Å. Notably, the c haem iron is coordinated by His 69 and Met 106 in both the monoclinic and tetragonal structures of the reduced enzyme.

The large differences in relative domain arrangement between the tetragonal reduced and the monoclinic oxidised structure seen in Figure 3.8 can be described by rigid body movements; A DYNDOM (Hayward & Berendsen, 1998) analysis of the structures identifies the c domain (residues 49-131) and the d₁ domain (residues 137-567) as rigid domains linked by a hinge region consisting of residues 132-136. The position of the cytochrome c domain in the two structures is related by a rotation of 59° about an axis which is nearly parallel to the central tunnel of the β-barrel of the d₁ domain (Figure 3.9). The different conformations give rise to different possible pathways for electron and proton transfer and for substrate/product uptake and release. In the monoclinic form of the reduced and oxidised enzyme there are unbroken networks of regular bonds and hydrogen bonds leading to the distal side (the active site pocket) of the d₁ haem. As a result of the alternative position of the c domain, a likely route of electron entry in the tetragonal form is via the proximal side (via the haem ligand His 200) of the d₁ haem.

3.3.4 Conformational changes in the d₁ domain

The d₁ domain is a rigid structure which changes little following reduction of the enzyme in the monoclinic crystalline form. However, in the structure of tetragonal reduced cytochrome cd₁ there are some important differences. The distal iron coordination site of the d₁ haem is empty and the iron is displaced out of the haem plane towards the proximal side by approximately 0.5 Å. The displacement of the haem iron is correlated with a displacement of the

²The least squares comparison of the two structures was done using the program O (Jones et al., 1991).
3. Results

![Diagram showing the relation between different conformations of cytochrome cd$_1$.](image)

Figure 3.9: Relation between the different conformations of cytochrome cd$_1$. Subunit B in the structures of (a) tetragonal reduced (Paper IV), (b) monoclinic oxidised (Fülöp et al., 1995) and (c) monoclinic reduced (Williams et al., 1997) are shown from the same orientation. The position of the cytochrome c domain in (a) is related to the position in (b) by a rigid body rotation of 59° about the indicated axis. Black large arrows indicate likely routes of electron transfer from the c haem to the d$_1$ haem.

proximal histidine ligand (His 200) and a rearrangement of the main chain structure of the loop holding this residue. Figure 3.10 shows a stereo view comparing the structures of this loop in the reduced tetragonal form to the structures of the monoclinic form. Tyr 197, which is located only three residues from the d$_1$ haem ligand makes hydrogen bonds to different parts of the N-terminal domain. In subunit B of the reduced and oxidised monoclinic forms, the phenolate oxygen of Tyr 197 makes a hydrogen bond to Asp 30 and Glu 34 respectively. In the reduced tetragonal form, the phenolate oxygen makes a hydrogen bond to the side chain of Glu 114. This residue is more than 20 Å away from Tyr 197 in the monoclinic structures.

Binding of cyanide to the enzyme in the tetragonal crystal form causes the haem iron to move into the plane of the haem (not shown, for details, see Paper IV). Changes on the proximal side of the haem are limited to a change in the sidechain of His 200 which adopts a more stretched conformation.

### 3.3.5 Catalysis in the tetragonal crystals

Exposure of the reduced tetragonal crystals to oxygen resulted in a rapid colour change from green to brown as well as an alteration in the absorption spectrum. The structure of the ox-
3.3. Large redox-coupled conformational changes in cytochrome cd

![Diagram of different conformations of $d_1$ domain loop near the proximal side of the $d_1$ haem.](image)

**Figure 3.10:** Stereoview of different conformations of $d_1$ domain loop near the proximal side of the $d_1$ haem. The tetragonal reduced structure is shown in dark grey and the monoclinic oxidised structure in a lighter shade.

Reduced enzyme derived from tetragonal crystals was determined to 2.5 Å. Unlike the large structural changes seen upon reduction in the monoclinic crystals, the backbones of the reduced and oxidised forms of the tetragonal crystals are virtually superimposable. Notably, the electron density clearly shows that the $c$ haem has His-Met ligation in both the oxidised and the reduced forms derived from the tetragonal crystals. A probable explanation for the lack of ligand switching in the crystal is that lattice contacts in the tetragonal crystal fix the structure.
3. Results

Figure 3.11: Stereo view of the active site of subunit A in the structure with a nitrogen containing compound bound. The 2.8 Å structure was determined from tetragonal crystals of reduced cytochrome $cd_{1}$ soaked in 20 mM nitrite for 10 minutes. The $2mF_{obs}-DF_{calc}$ electron density map is contoured at 1.5 $\sigma$ where $\sigma$ is the root mean square electron density for the unit cell.

Soaking of reduced tetragonal crystals in a nitrite solution did not result in a colour change detectable by the naked eye, however a shift in an absorption peak in the wavelength region diagnostic of the electronic environment of the $d_{1}$ haem was observed. The resulting spectrum is also similar to that of a stable spectral intermediate identified upon mixing the reduced enzyme with nitrite in solution (George et al., 2000), and was previously assigned to an equilibrium between two mixed-valence NO-bound complexes (George et al., 2000). A low resolution (2.8 Å) structure determined from a tetragonal crystal soaked in nitrite showed a ligand, possibly diatomic, bound to the $d_{1}$ iron (Figure 3.11). The observation that nitrite failed to oxidise the enzyme in the crystalline state will be discussed in the next chapter.
The results described in this thesis can be summarised in five points:

- There are several different binding modes for diatomic ligands to cytochrome $cd_1$ (Papers II-III).
- Binding and release of a neutral ligand, carbon monoxide, are accompanied by proton uptake and release (Paper III).
- In the monoclinic crystal form, the oxidase reaction proceed via a structural intermediate which is reminiscent of the enzyme:nitrite complex. (Paper II)
- While the oxidase reaction can readily proceed in both monoclinic and tetragonal crystals of cytochrome $cd_1$, the nitrite reduction is slow (Paper II and IV)
- Redox coupled conformational changes are larger than expected and include alternative domain arrangements (Paper IV).

The results have already been discussed to some extent in the previous chapter but here a more detailed discussion of a few topics will be given.

### 4.1 Multiple conformations of cytochrome $cd_1$

To me, the most surprising result described in this thesis was the large domain shift seen in the structure derived from crystals of the reduced enzyme. Previous speculations that the structure of the oxidised enzyme derived from monoclinic crystals would represent a resting state (Williams, 1996; Williams et al., 1997; Ranghino et al., 2000) have gained new actuality and the role of the N-terminal arm must be subjected to further investigations. The arrangement whereby the cytochrome $c$ domain is attached by a seemingly flexible hinge to the $d_1$ domain also raises some general questions with regard to this organisation.
4. Discussion

4.1.1 A comparison of the known conformers of cytochrome cd$_1$

The unusual His/His coordination of the c haem seen in the structure of the oxidised cytochrome cd$_1$ from P. pantotrophus (Fülöp et al., 1995) has been confirmed by Electron paramagnetic resonance (EPR) spectroscopy (Cheesman et al., 1997) and the conformation represents the structure of the “as isolated” oxidised enzyme in solution. Although the tetragonal reduced structure (Paper IV) is very different from the monoclinic oxidised one, the correlated conformational changes in the active site (see Figure 3.10) speak for the significance of this structure.

A comparison of domain-domain interactions in the known conformers of cytochrome cd$_1$ shows that the structure of the oxidised enzyme derived from monoclinic crystals has the largest domain-domain interface (Table 4.1). The interface is stabilised by 19 or 20 hydrogen bonds and salt bridges between the N-terminal part of the enzyme and the d$_1$ domain in subunit A and B respectively.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Subunit</th>
<th>Number of interactions</th>
<th>Buried surface area [Å$^2$]</th>
<th>PDB id.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised P$_2$</td>
<td>A</td>
<td>19 (10)</td>
<td>2740</td>
<td>1QKS</td>
<td>Fülöp et al. 1995</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>20 (11)</td>
<td>2790</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced P$_2$</td>
<td>A</td>
<td>3 (0)</td>
<td>650</td>
<td>1AOF</td>
<td>Williams et al. 1997</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>13 (7)</td>
<td>1430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced P$_4$</td>
<td>A</td>
<td>2 (0)</td>
<td>1340</td>
<td>1H9X</td>
<td>Paper IV</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3 (0)</td>
<td>1370</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Domain-Domain interactions within the subunit of cytochrome cd$_1$. The number of interactions includes direct hydrogen bonds and salt bridges (i.e. no hydrogen bonds via water molecules) between the N-terminal domain of the enzyme up to residue 135 (the N-terminal arm and the cytochrome c domain) and the d$_1$ domain. The buried surface area was calculated using using a probe radius of 1.4 Å. Only salt-bridges and direct hydrogen bonds are included. Numbers within barckets refer to the number of interactions between the N-terminal arm and the d$_1$ domain.

The domain interface in the structures of the reduced enzyme, both in the monoclinic (Williams et al., 1997) and in the tetragonal forms Paper IV, has less interactions and also a much smaller buried surface area and seem considerably more unstable. It should be noted that crystallisation could act as to “freeze out” specific conformational states and it is possible that the structure in solution is much more dynamic.
4.1. Multiple conformations of cytochrome cd₄

4.1.2 The role of the N-terminal arm

Of the 19 (20 in subunit B) interactions between the N terminal part and the d₁ domain 10 (11 in subunit B) are mediated by the N-terminal arm (residues 1-48). Moreover, the N terminal arm provides haem ligands to both the d₁ haem and the c haem and it thus constitutes an important structural element in the monoclinic oxidised structure (Fülöp et al., 1995). The role of the d₁ haem ligand, Tyr 25, in catalysis has been a matter of debate ever since the structure was solved. The initial suggestion (Fülöp et al., 1995) was that Tyr 25 played a role in product release. Some support for this is given by quantum chemical calculations (Ranghino et al., 2000). However, the conformational change seen upon reduction of the enzyme in the monoclinic crystal form also raised doubts; would conformational changes on this scale be in agreement with an enzyme continuously turning over substrate to product (Williams, 1996; Williams et al., 1997; Ranghino et al., 2000)? A sequence comparison of known cd₁ sequences show that Tyr 25 is not conserved, in some species the entire N-terminal arm is missing (Figure 1.4 and Cheesman et al. 1997). Moreover, the structure of Ps. aeruginosa cd₁ showed that although a tyrosine residue was present at position 10, it did not coordinate the iron directly, but via a water molecule (Nurizzo et al., 1997). Mutational studies also showed that this residue was not important for catalysis (Cutruzzola et al., 1997) in the Ps. aeruginosa enzyme.

![Figure 4.1: A comparison of the structure of the N-terminal part of cytochrome cd₁ and cytochrome c. a) Cytochrome c from tuna. The N-terminal portion of subunit A in the structures determined from b) tetragonal reduced (Paper IV), c) monoclinic reduced (Williams et al., 1997) and d) monoclinic oxidised (Fülöp et al., 1995) crystals of cytochrome cd₁. The N terminal arm (residues 1-48) is black and the cytochrome c domain (residues 49-135) is grey.](image)

In the structure of the reduced enzyme derived from tetragonal crystals (Paper IV) the N-terminal arm is almost entirely disordered, only 6 (9 in subunit B) residues are visible. The first residue to make contacts within the same subunit aligns with residue 1 of tuna
cytochrome $c$ (Figure 4.1). The space between the $d_l$ domain and the cytochrome $c$ domain occupied by the N-terminal arm in the monoclinic oxidised structure is completely eliminated in the structure of the reduced enzyme derived from tetragonal crystals and the area of the cytochrome $c$ domain which makes contacts with the N-terminal arm in the oxidised structure is involved in new contacts with the $d_l$ domain. In the light of the new structure it seems unlikely that the N-terminal arm including Tyr 25 and the $c$ haem ligand His 17 is directly involved in catalysis. It is more likely that the N-terminal arm plays a role in protecting the enzyme from oxygen damage in the presence of oxygen and in the absence of electrons where Fenton chemistry can occur. This also implies that the original structure of the oxidised enzyme represents a resting state. A recent study where one of the possible electron donors, pseudoazurin, was shown not to be able to reduce cytochrome $cd$ in the form as isolated but only after reduction with dithionite and subsequent re-oxidation by substrate is in favour of this view (Allen et al., 2000b). Based on spectroscopy this “active” oxidised form was suggested to have a His-Met ligation of the $c$ haem (Allen et al., 2000a).

4.1.3 The tethered electron shuttle domain

The arrangement with an electron shuttle protein (the cytochrome $c$ domain) attached to the enzyme (the $d_l$ domain) is rather curious, it seem to prolong the electron transfer chain by an extra member.

Electron transfer chains in biological systems are designed to satisfy metabolic energetic requirements. Some of the features employed by these chains have been identified. Electrostatic forces between surfaces of complementary charge on the acceptor/donor protein have been shown to be important (Rees, 1985). A survey of electron donor/acceptor pairs in the electron transfer chain of $P$. pantotrophus showed that although pseudoazurin and cytochrome $c_{550}$ have entirely different folds they both had similar surface charge distributions with one positive and one negative hydrophobic face (Williams et al., 1995). The “top” face of cytochrome $cd_l$ revealed a similar negative surface patch and a general rule was postulated where members of periplasmic redox chains which donate/accept electrons from electron shuttle proteins would have a negative hydrophobic surface patch.

An investigation of the domain interface shows that the (cytochrome $c$)/(d$_l$ domain) electron donor/acceptor pair of cytochrome cd$_l$ does not obey this rule. The opening of the beta barrel is surrounded by positively charged residues and a complementary surface with negatively charged residues is found on the domain interface area of the cytochrome $c$ domain. The positive patch on the $d_l$ domain is likely to serve to direct the negatively charged nitrite ion to the active site. A role for the tethered cytochrome $c$ domain could be to fulfill the require-
4.2. Mechanisms of nitrite and oxygen reduction by cytochrome cd₁

The structure of the bound dioxygen intermediate show that dioxygen binds in a manner similar to nitrite with an oxygen atom coordinated by the two active site histidines (Paper II). Initial elements in the reaction mechanisms of the two chemically very different reactions thus seem to be shared. However, the different reactivity in the crystalline state (Paper II and paper IV) tells us that the reaction after this point proceeds along different pathways.

4.2.1 The reduction of nitrite

The observation that the enzyme in the crystal can not readily turnover with nitrite may imply that the reduction of nitrite includes elements of conformational changes which can not be accommodated within the crystal lattice. However, recent pre-steady state studies of the oxidase reaction and the reduction of nitrite suggest a more complicated picture. Mixing of reduced Ps. aeruginosa cytochrome cd₁ and nitrite under pre-steady state conditions was reported to result in the formation of a dead end complex (Silvestrini et al., 1990). This observation was supported by recent work on P. pantotrophus cd₁ (George et al., 2000). The same authors also used Fourier transform infrared (FTIR) spectroscopy to pin down the possible structure of the complex and their results suggested that a mixture of the two mixed valence species $c^{2+}d_{i}^{3+}$-NO and $c^{3+}d_{i}^{2+}$-NO formed within 100 ms after reaction initiation. Formation of a dead end complex is clearly not compatible with a cycling enzyme and it is obvious that something is missing, both from the experiments in the crystals and in the pre-steady state.
experiments.

A common feature of pre-steady state studies and studies of enzyme in the crystal is that the reaction is initiated with fully reduced cytochrome \( cd_1 \). A suggestion put forward in Paper IV is that the enzyme under steady state condition is working by cycling between mixed valence species where electrons are fed into the system, possibly controlled by conformational changes linked to the redox state.

Cytochrome \( cd_1 \) has a tough task to deal with as the product of nitrite reduction, nitric oxide, is known to bind tightly to a reduced haem (Sharma et al., 1987) and nitric oxide would probably not be released spontaneously even from an oxidised haem (Ranghino et al., 2000). A mechanism for product release must thus exist.

At least four different possibilities for such a mechanisms have been suggested. The first mechanism was proposed by Fülöp et al. in 1995 when the structure was solved. They suggested that the side-chain of Tyr 25 in the form of a tyrosinate assisted in proton release. Quantum chemical studies refined this theory and it was shown that a hydroxyl ion or a nitrite ion could also facilitate product release (Ranghino et al., 2000).

Another possible mechanism, put forward in Paper III is based on the observation that a neutral ligand (CO) can affect the overall charge state of the enzyme. We suggest that if NO release from the enzyme-NO complex is accompanied by a proton uptake in a similar manner as the release of CO, then such a mechanism may ensure preferential electron transfer to an empty \( d_1 \) haem and not to the enzyme-product complex. Implications for such a mechanisms can be extracted from the work by Silvestrini et al. (1990) who observed a very slow internal electron transfer (1 s\(^{-1}\)) from \( c^{2+}d_1^{3+} \)-NO to form the dead end complex \( c^{3+}d_1^{2+} \)-NO. This slow rate of electron transfer is in contrast with the steady state turnover number of 8 s\(^{-1}\) reported for the \( Ps. \ aeruginosa \) enzyme (Cutruzzola et al., 2001).

A third possibility is that the reaction is controlled by conformational gating. This suggestion is based on the results presented in Paper IV and the observation that the different conformations give rise to different possible pathways for electron and proton transfer and for substrate/product uptake and release. Alterations between conformations could be a way to time and tune the reaction.

The fourth possibility is that the affinity for product and substrate can be modulated. The different conformations of the Tyr197-His 200 loop (Figure 3.10) and the corresponding difference in position of the \( d_1 \) haem iron relative to the haem plane is likely to affect the affinity of various ligands. An analogous mechanism is employed by haemoglobin where the affinity for oxygen is modulated by different positions of the E helix in the R and T states (Perutz et al., 1998).
4.2. Mechanisms of nitrite and oxygen reduction by cytochrome cd

These mechanisms are not mutually exclusive and it is possible that elements of all suggested mechanisms are implemented.

4.2.2 The reduction of oxygen

The reduction of oxygen does not pose the same problems as nitrite reduction in terms of product release. Interpretation of the studies of oxygen reaction has also proved to be more straightforward. Results from pre-steady state studies of the \textit{P. pantotrophus} (Koppenhöfer et al., 2000a) and \textit{Ps. aeruginosa} (Greenwood et al., 1978) enzymes show that both haems are reduced in a fast initial phase. This observation implies fast internal electron transfer (at least 100 s\(^{-1}\)) which is in contrast with the slow internal electron transfer reported for the reduction of nitrite (Silvestrini et al., 1990). Stopped flow-EPR studies of the oxidase reaction in the \textit{P. pantotrophus} enzyme showed the presence of a ferric \textit{c} haem on the same time-scale as the simultaneous oxidation of the haems (25 ms) but no signal which could be assigned to a ferric \textit{d} haem. Instead an organic radical was detected. These results suggest that the species formed following the initial fast phase is a Fe(IV)-oxo state.

A high oxidation state intermediate is highly reactive and could take up electrons either from the \textit{c} haem or from other sources. Promiscuity in the oxidase reaction is suggested by recent results from \textit{Ps. aeruginosa} cytochrome \textit{cd}, where it was shown that although mutations of either of the active site histidines abolished nitrite reductase activity, the oxidase activity remained unaltered (Cutruzzola et al., 2001).

The relative ease by which the oxidase reaction can take place under a wide range of conditions is in line with the view that the purpose of this reaction is to scavenge oxygen, which should have been a great advantage when the oxygen levels rose in the atmosphere. It might give a hint to how oxygen initially was reduced in ancestral oxidases before the development of the mechanism adopted by membrane bound oxidases today.
5 Future prospects

5.1 Open questions and unfinished business

There are a number of unresolved questions generated by the work presented here. Many of the suggestions regarding function put forward in the previous chapter are based on structural studies and further work remains to be carried out to obtain a deeper understanding of this enzyme.

Conformational changes during catalysis

Do the known structures, the oxidised and reduced forms represent ‘extremes’ (resting states) and can we in that case expect additional conformers (e.g. steady state conformers)? Further studies of samples undergoing steady state turnover using various spectroscopic techniques would be very valuable. One approach could be small angle X-ray scattering (SAXS) which allows the measurement of the radius of gyration of the molecule in solution before during and after turnover. Comparison of SAXS profiles for reduced, oxidised and enzyme undergoing steady state turnover with nitrite or oxygen could provide insight into the role of conformational changes in catalysis in cytochrome $cd_{1}$ in solution.

The role of specific residues in catalysis and conformational transitions

Based on the available structural evidence, a number of key residues have been identified. The haem ligands which are displaced upon reduction of the enzyme (Williams et al. (1997) and Paper IV), His 17 and Tyr 25 are likely to play a role in maintaining the structure of the N-terminal arm. Results presented in Paper IV suggest a role for the loop containing Tyr 197 and the proximal $d_{1}$ haem ligand His 200 in regulating affinity for ligands at the $d_{1}$ haem and/or in stabilising various conformations. The histidine residues in the distal pocket of the $d_{1}$ haem are suggested to play an important role in catalysis (Fülöp et al., 1995; Williams et al., 1997; Ranghino et al., 2000; Cutruzzola et al., 2001), and the present work also supports this.

A very important step towards understanding cytochrome $cd_{1}$ will include studies of site directed mutants. The main problem with mutant studies in cytochrome $cd_{1}$ is that despite ex-
tensive efforts it is still not possible to produce $d_1$ haem in *Escherichia coli* or other non-native strains. Fully functional mutants can be homologously produced in a strain with a deficient copy of the *nirS* gene, *i.e.* a strain which can not make cytochrome $cd_1$. Other mutants must be produced as semi-apo enzyme in *E. coli* and reconstituted with $d_1$ haem after purification. The production of foreign cytochromes *c* in *E. coli* is also not a trivial task as the yields are often very low (in the range of 1 mg/l). However, an improved expression system has been developed for this purpose (Gordon *et al.*, 2001) which was successfully used to produce high yields of the isolated *c* domain. A very similar system has been developed for the production of mutants in the semi-apo form. Reconstitution has been successfully accomplished with both wild-type and mutant forms of *Ps. aeruginosa* cytochrome $cd_1$ (Hill & Wharton, 1978; Cutruzzola *et al.*, 1997). However, it has proven difficult to reproduce the high efficiency of incorporation reported for the *Ps aeruginosa* enzyme (95 %) in the *P. Pantotrophus* mutants (Euan Gordon and Malin Löfqvist, unpublished results) and the protocols for reconstitution require refinement before structural studies and biochemical characterisation can be carried out.

**Factors affecting internal electron transfer**

An idea put forward in this thesis is that the mechanism of nitrite reduction could be regulated by tuning of electron transfer rates, either by alteration of the overall charge state of the enzyme (see also paper III) or by conformational gating (Paper IV). Further studies on intramolecular electron transfer would be of great value to discriminate between the suggested mechanisms.

A thought provoking work on intermolecular electron transfer in crystals was recently published by the group of Harry Gray (Tezcan *et al.*, 2001). They crystallised mixtures of cytochrome *c* in its native and Zn-reconstituted forms and studied kinetic events following photo-excitation in the crystal. Together with structural data this study provided a basis for discussion of factors affecting intermolecular electron transfer.

Similar studies could be envisaged with cytochrome $cd_1$ in its different crystal forms.

**Factors affecting intermolecular electron transfer**

Allen *et al.* (2000a&b) recently discovered that the enzyme “as isolated” could not form a productive ET complex with pseudoazurin. For the studies of the inter-molecular electron transfer, a structure of cytochrome $cd_1$ in complex with its electron donors, cytochrome $c_{550}$ and pseudoazurin would be of great value. Relatively few electron transfer donor/acceptor complexes are explored and such studies could give insights not only about the function of
cytochrome \( cd_1 \) but also about mechanisms of electron transfer in general. Co-crystallisation trials have previously been done with the oxidised enzyme and cytochrome \( c_{650} \) although no co-crystals were obtained in these experiments (Koppenhöfer, 1998). Results presented in this thesis (Paper IV) suggest a possible explanation for these observations as the alternative conformations of the N-terminal arm is likely to affect the interaction with electron donors. A rational approach would be to perform crystallisation trials under anaerobic conditions starting with various combinations of oxidation states for cytochrome \( cd_1 \) and its electron donors.

5.2 Epilogue

The treasure of structural data available on cytochrome \( cd_1 \) has made this enzyme an attractive platform for studies of structure-function relationship in complex redox enzymes which are of general interest to a broad range of scientists.

We are currently looking at an interesting development in “structural genomics” where high throughput studies are likely to reveal so far hidden knowledge. A combination of such studies with detailed pinpoint investigations as the one described in this thesis, where a key single entity is explored from all aspects, will play an important role in the pursuit of understanding of the mechanisms governing protein structure and function in the future.
Acknowledgements

I would like to thank my supervisor, Janos Hajdu for accepting me as a PhD student and for giving me freedom to do things my way through the years. I hope I have acquired some of your fantastic ability to see things from a different perspective and to expect the unexpected. However, I will never get accustomed to your way of dealing with deadlines...

A large part of the work presented in this thesis was a part of a EU-BIOTECH project (BIO4-CT96-0281) and I would like to thank everyone involved in this project for creating a stimulating scientific environment. I am especially thankful to Vilmos Fülöp and Pamela Williams who cleared the ground for me by solving the remarkable structures of cytochrome cd$_1$ which have provided the basis for my work and to Stuart Ferguson for enabling extensive exchange between Oxford and Uppsala.

Many thanks to Karin Valegård and Anke Terwisscha van Scheltinga who taught me crystallography and for your giggling sessions which cheered up our room. Margareta Ek and Peter Brzezinski - it was when we started to work together science became fun! Carrie Wilmot and Graziella Ranghino, I am happy I have got to know you, I hope our collaborations will not end here! For invaluable technical support I am indebted to Christer Andersson and Hans Pettersson, and without David and Remco and their magic touch with computers this thesis would not have been finished on time.

All the great students I have had over the years have made my teaching duties become a treat! Special thanks to my undergraduate project students Erik and Malin for being so great to work with and the “Methods in Biochemistry” bunch of 1999 (Malin, Katarina, Pia, Åsa, Malin, Sanna, Fredrik and Margareta) for their work which eventually resulted in the crystals of reduced cytochrome cd$_1$.

Without the atmosphere created by all the members of the Janos-Inger-So supergroup, now improved by Leif and his people, this period would have been dull. Susanna, I admire your patience with your membrane crystals, good luck with your fantastic structures! Linda, my room-mate for a long time, hope you get a new one who is better at keeping order than I am. Gisela, my very first summer student a long time ago, I wish you the best of luck with your PhD studies in Stockholm. Elles, I appreciated to have you around for discussions on cytochrome cd$_1$ and many thanks for your moral support during the Rome trip!!! Inger, So and Leif, thanks for always being around and always taking time to answer questions. Jeff,
the coffee breaks has become quiet since you left! I will miss discussions about gardening (Tom), whale fishing (Gunnar), NHL (Karl) and inverted sequences (Richard). Momi, Saeid, Diane, Beata, Nese, Jocke and Martin, I will miss you all!

Many thanks to past and present members of the structural biology unit in Uppsala who contributes to the great environment both with your skills and your personalities. Karin K., companion through both university and PhD studies and my best lunchkompis, I will miss our daily chats about everything under the sun. Thanks to all the people of the lunch bunch, Emma, Malin, Fredrik, Isabella, Anna, Elin, Seved, Torsten and many others for nice company in the lunch room! Andreas & Kenth, you are great company for synchrotron trips but lousy at mixing drinks. Jenny, Jimmy and Al, thanks for keeping up the beerclub tradition.

Big hugs to all my friends, Jenny O., Jenny B., Malin, Mia, Karin E., Per, Mattias, Anna, Ida, Mathias, Mattias B, Janne, Elle, Andreas, Nilo, Patrik, Tove, Ade, Jörgen, Fredrik, Anna-Karin and many others who makes life outside work fun!

Mamma and pappa, thanks for your unconditional support during all these years. Åse & Johan, Carl and Kajsa, thanks for helping me to keep the perspectives and not forget what is important in life.

Euan, for all your love and support, for proofreading this thesis, for putting up with me when I get stressed, for being such a wonderful person; Thank you!
References


References


References


