The 10th Nitrogen Cycle Meeting 2004

The emerging molecular structure of the nitrogen cycle: an introduction to the proceedings of the 10th annual N-cycle meeting

C.S. Butler*1 and D.J. Richardson†1

*Institute for Cell and Molecular Biosciences, University of Newcastle, Newcastle upon Tyne, NE2 4HH, U.K., and †School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K.

Abstract
Over the last 10 years, during the lifetime of the nitrogen cycle meetings, structural biology, coupled with spectroscopy, has had a major impact on our understanding enzymology of the nitrogen cycle. The three-dimensional structures for many of the key enzymes have now been resolved and have provided a wealth of information regarding the architecture of redox active metal sites, as well as revealing novel structural folds. Coupled with structure-based spectroscopic analysis, this has led to new insight into the reaction mechanisms of the diverse chemical transformations that together cycle nitrogen in the biosphere. An overview of some of the key developments in the field over the last decade is presented.

Introduction
The inorganic nitrogen cycle is driven by a diverse range of redox reactions, and provides a wealth of interesting systems with which to study bioenergetics and bioinorganic chemistry. These respiratory reactions involve the reduction of dinitrogen, nitrate, nitrite, nitric oxide and nitrous oxide, and the oxidation of ammonium and hydroxylamine. In each case, catalysis involves redox chemistry that takes place at metal centres. For example, the enzymes involved in denitrification (\(\text{Nitrate} \rightarrow \text{N}_2\)) have catalytic metal centres that include: mononuclear type 1 copper, \(\text{c haem}\) or \(\text{d}_1\) haem in nitrite reductases, the \(\text{Mo-}\text{bis-MGD}\) (\(\text{Mo-}\text{bis-guanine dinucleotide}\)) cofactor in nitrate reductases, a non-\(\text{haem Fe/haem Fe}\) dinuclear centre in nitric oxide reductase and a tetranuclear copper sulphide centre (\(\text{Cu}_4\)) in nitrous oxide reductase. Over the last 10 years, many structures of the redox centres of the nitrogen cycle enzymes have been solved (Figure 1).

And, when combined with spectroscopic, electrochemical and kinetic data for these enzymes, have significantly advanced our understanding of the mechanistic properties of the catalytic centres. Many of these developments have been reported by participants in the annual N-cycle meetings that have taken place during this exciting period, and in this review we consider each step of the nitrogen cycle in turn and highlight the recent advances in knowledge that have arisen from such structural studies, and also consider the unresolved issues that lie ahead for the next 10 years.

Periplasmic nitrate reductase (NAP)
Nitrate reductases catalyse the reduction of nitrate to nitrite (\(\text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}\)). There are two types of respiratory nitrate reductase in bacteria, one a periplasmic (NAP) enzyme, the other a membrane-bound (NAR) enzyme (see the next section). Periplasmic nitrate reductase enzymes can be subdivided into two groups: (i) heterodimeric periplasmic nitrate reductase, which purifies as a two-subunit enzyme complex containing a 16 kDa dihaem subunit (NapB) and a 90 kDa catalytic subunit (NapA) that binds an [4Fe-4S] cluster and \(\text{Mo-}\text{bis-MGD}\)...
Figure 1 | X-ray structures of the enzymes of the bacterial nitrogen cycle

Question marks represent unsolved structures. NAR, membrane-bound nitrate reductase; cd₁NIR, cytochrome cd₁ containing nitrite reductase; CuNIR, copper-containing nitrite reductase; NRF, cytochrome c nitrite reductase; NOS, Nitrous oxide reductase; N₂ase, Nitrogenase; HAO, Hydroxylamine oxidoreductase.

cofactor [1–3]; and (ii) monomeric periplasmic nitrate reductase, which comprises only the [4Fe-4S]/Mo-bisMGD cofactor containing NapA. The X-ray crystal structures of the monomeric NapA from Desulfovibrio desulfuricans [4] and Escherichia coli (B. Jepson, S. Mohan, J. Cole, C. Butler, E. Flores, A. Herrero, J. Butt and D. Richardson, unpublished work) emerged in 1999 and 2004 and that of the heterodimeric NapAB complex from Rhodobacter sphaeroides in 2003 [5]. In all cases, NapA folds into four domains, but the surface electrostatic properties of the monomeric and heterodimeric NapAs are quite different, which may influence the strength on interaction with the NapB (or equivalent) redox partner. One of the domains is formed entirely from an N-terminal segment of the polypeptide, and binds the [4Fe-4S] cluster co-ordinated by four cysteine ligands. The remaining three domains have structure and fold around the Mo-bisMGD cofactor, providing numerous H-bonds to both of the MGD moieties. The MGD cofactor lies at the bottom of a substrate funnel between domains II and III, and residues lining this cleft and at its base will play a
role in defining substrate specificity and orientating the substrate onto the Mo atom. Of particular significance is a conserved arginine at the base of the pocket that may interact with nitrate oxygens of the substrate. The structures also show that the molybdenum amino acid ligand in periplasmic nitrate reductases is a cysteine residue. The Mo ion is additionally co-ordinated by four sulphur ligands provided by the two MGD moieties (predicted previously by EXAFS; [6]) and a water/hydroxo ligand. The structures are most likely those of a Mo(VI) redox state, and consideration of the Mo(VI)/V) and Mo(V/IV) redox couples, in conjunction with protein film voltammetry and Mo(V) EPR, has led to the suggestion that nitrate binds to the Mo(V) redox state in the catalytic cycle [38]. A similar proposal has also been made for the cytoplasmic cyanobacterial assimilatory nitrate reductase, NarB, which is very closely related to NapA [39].

The structure of NapB has been solved both in the NapAB complex (from *R. sphaeroides* in 2003) [5] and as a truncated form of NapB alone (from *Haemophilus influenzae* in 2002) [7]. NapB is a soluble 16 kDa c-type cytochrome subunit, which transfers the electrons to NapA. NapB contains two c-type haems that are both *bis*-his ligated [8], are parallel and stacked with an iron-to-iron distance of 9.9 Å (1 Å = 0.1 nm). The structure of the NapA–NapB complex shows that NapB extensively interacts with NapA, with one of the NapB haems (haem II) in the vicinity of the NapA [4Fe-4S] cluster and the other (haem I) solvent exposed. This architecture suggests that haem I interacts with the electron donor (NapC), and that direct electron transfer to the MGD, via haem II, can occur without requiring the electrons to go through the [4Fe-4S] cluster in NapA [5]. It is noted, however, that there is close proximity between one of the cysteine residues that covalently attaches haem II and one of the cysteine residues that co-ordinates the [4Fe-4S] cluster in NapA. Whether this is an important structural feature for electron transfer remains to be demonstrated.

**The membrane-bound nitrate reductase (NAR)**

Recently, two crystal structures for the membrane-bound nitrate reductase from *E. coli* have been reported. The structure reported in 2003 by Bertero et al. [9] is of the complete NarGHI trimer, whereas that reported in 2004 by Jormakka et al. [10] is of the soluble NarGH components only. However, it is the analysis of both structures in combination that provides novel insight as to the function of the membrane-bound nitrate reductase.

Membrane-bound nitrate reductase is a heterotrimer with subunits NarG (≈140 kDa), NarH (58 kDa) and NarI (20 kDa) forming a ‘flower-shaped’ structure with dimensions of 90 × 128 × 70 Å [9]. NarI anchors the other subunits to the cytoplasmic membrane mainly through hydrophobic interactions. NarI contains two *bis*-his co-ordinated *b*-type cytochromes, and has the Q-binding site that receives electrons from the quinol pool. NarI has five transmembrane helices, two short horizontal helices and a C-terminal tail that interacts with both NarH and NarG. The architecture dictates that one of the *b*-type haems is 8.9 Å from the [3Fe-4S] clusters in NarH, allowing for rapid electron transfer [9]. NarH contains three [4Fe-4S] clusters and one [3Fe-4S] cluster that provides a molecular wire by which electrons are transferred from NarI to NarG [9,10].

NarG contains an unusual [4Fe-4S] cluster, previously undetected by EPR spectroscopy, co-ordinated by one histidine and three cysteine ligands [9,10]. NarG also contains the Mo-*bis*-MGD co-factor. Interestingly, the two NarG structures show a different Mo co-ordination: in the structure reported by Bertero et al. [9], the Mo is co-ordinated by six ligands, four *cis*-dithiolene sulphur atoms from *bis*-MGD and a bidentate ligand from both side chain oxygen atoms from a carboxylate group of an Asp²²² residue [9]. In contrast, the structure reported by Jormakka et al. [10] shows the Mo co-ordinated by four *cis*-thiolate groups and a single carboxylate oxygen from an Asp²²² residue. An o xo group has also been assigned as a sixth Mo ligand. It has been suggested that this structural difference may explain the observed pH dependent activity. In the structure presented by Jormakka et al. [10], Asp²²² is also hydrogen-bonded to a conserved His⁵⁴⁶ residue. It is speculated that transition from the active (low-pH form) to the inactive (high-pH form; pKa ≈8) that His⁵⁴⁶ becomes protonated and dissociates from Asp²²², which in turn may rotate to form a bidentate Mo ligand, as seen in the structure of Bertero et al. [9], blocking the binding of nitrate.

**The nitrite reductase (NRF)**

The six-electron reduction of nitrite to ammonium is catalysed by the cytochrome *c* nitrite reductase, NrfA (*NO₂⁻ + 8H⁺ + 6e⁻ → NH₄⁺ + 2H₂O). The crystal structures of cytochrome *c* nitrite reductase from *Sulfurospirillum deleyianum*, *Wolinella succinogenes* and *E. coli* were determined in 1999, 2000 and 2002 respectively [11–13]. In all three systems, NrfA crystallized as a homodimer, with four of the five haems within each monomer closely packed to form arrangements of near-parallel and near-perpendicular haem pairs that have also been observed in hydroxylamine oxidoreductase [14]. In all the structures, the active site haem displays a unique co-ordination, with a distal lysine ligand and proximal oxygen ligand that is likely to arise from water or hydroxide. This catalytic haem lies buried in the protein at the bottom of a putative substrate inlet channel with positively charged electrostatic potential, and a putative product efflux channel that exhibits a more negative electrostatic potential. In the case of the *W. succinogenes* enzyme, NrfA structures with sulphate, nitrite, hydroxylamine and azide bound in the active site have additionally been reported [13], which suggest the nitrite nitrogen is co-ordinated to the haem iron with the two oxygen atoms being hydrogen-bonded by His⁶⁴⁶ and Arg¹¹⁴ (*E. coli* numbering). In the hydroxylamine co-ordinated derivative, the single oxygen is hydrogen-bonded by Arg¹¹¹ [12]. An active site calcium ion is conserved across all three structures, and this plays an important structural role in positioning key histidine and tyrosine residues that may be involved in substrate binding and proton delivery.
The nitrite reductase (cd1Nir)

The reduction of nitrite to nitric oxide is catalysed by both the cytochrome cd1 and the Cu nitrite reductase (NO2− + H+ + e− → NO + H2O). The crystal structures of the oxidized dimeric cytochrome cd1 from Paracoccus pantotrophus and Pseudomonas aeruginosa were first reported in 1995 and 1997/8 [15–17]. In both cases, the d1 haem was enclosed in an eight-bladed β-propeller structure. However, the co-ordination of the haem d1 Fe(III) differed. In P. pantotrophus the Fe(III) had a proximal histidine and distal tyrosine ligands, but in Ps. aeruginosa the distal ligand was a hydroxide that was hydrogen-bonded to Tyr10 from the opposite polypeptide chain of the dimer. Differences were also observed in the cytochrome c domain. Although the fold of this was similar in both enzymes, in the P. pantotrophus enzyme the c-type haem was co-ordinated by two His ligands [15], but in the Ps. aeruginosa enzyme it exhibited His/Met co-ordination.

Some convergence of the P. pantotrophus and Ps. aeruginosa cytochrome cd1 structures, however, do emerge in different redox states. Reduction of the crystals of P. pantotrophus cytochrome cd1 resulted in loss of the tyrosine ligand (residue 25) from the d1 haem, thus allowing substrate binding. His57, a c-type haem ligand on the same polypeptide loop as Tyr25, was also shown to dissociate and be replaced by the sulphur of Met150, leading to a large conformational change [18,24]. Although it was initially proposed that these ligand switches may be important during the catalytic mechanism, experiments under turnover conditions have shown that the c-type haem maintains histidine plus methionine, rather than bis-histidinyl, co-ordination [19–22]. Furthermore, substitution of Tyr25 with serine yields a protein that is active, but the Tyr25Ser protein retains the d1 haem less well than the wild-type protein. Thus it has been suggested that Tyr25 may play an important role in stabilizing the binding of the d1 haem cofactor [23]. A reduced state structure of the Ps. aeruginosa enzyme has only been obtained with NO bound to the d1 haem iron [17,25]. The haem c domain was unaltered by reduction, but Tyr10 moved away from the haem d1 iron.

The nitrite reductase (CuNir)

The copper-containing nitrite reductase, CuNir, is widely distributed amongst Bacteria. However, most biochemical studies have focused on the enzymes from Achromobacter cycloclastes and Alcaligenes xylosoxidans, which were the earliest structures to emerge for denitrification enzymes when they were published in 1991 and 1994. The copper-containing nitrite reductases are homotrimers which bind three type I copper centres. The type I copper is co-ordinated by two cysteine, one methionine and one histidine residue. The type II copper is co-ordinated by two cysteine, one methionine and one histidine residue. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme. The type II copper site is the active site of the enzyme.

Nitrous oxide reductase (NOS)

Nitrous oxide reductase catalyses the last step in denitrification, the reduction of nitrous oxide to dinitrogen gas (N2O + 4H+ + 2e− → N2 + 2H2O). The X-ray structure of nitrous oxide reductase from Pseudomonas nautica [29] and Paracoccus denitrificans [30] were both published in 2000. Nitrous oxide reductase is a homodimer of a 65 kDa copper-containing subunit that binds a di-nuclear CuA, electron entry site and a tetra-nuclear Cu2 catalytic centre. Each monomer is made up of two domains: the ‘CuA domain’ that has a cupredoxin fold and the ‘CuB domain’, which is a seven-bladed propeller of β-sheets. The CuA site in nitrous oxide reductase is very similar to the CuA site in cytochrome c oxidase. The binuclear copper atoms (Cu1 and Cu2) are bridged by two cysteines (Cy604 and Cy612; P. denitrificans numbering) in a distorted square-planar structure. Histidine residues His599 and His630 also ligate Cu1 and Cu2 respectively. In addition, Cu1 has a methionine ligand (Met641) and Cu2 has a tryptophan ligand (Trp632). The Cu2 centre belongs to a new type of metal cluster, in which four copper ions are liganded by seven histidine residues, two hydroxide molecules and a bridging inorganic sulphide. It has been suggested that, to allow for fast electron transfer avoiding the formation of dead-end products, N2O binds to the Cu2 centre via a single copper ion, with the remaining copper ions acting as an electron reservoir [29,31]. Electron input into Nos occurs at the CuA site and is usually via monohaem c-type cytochromes or cupredoxins. The two subunits of the nitrous oxide reductase dimer are arranged head-to-toe, such that during catalysis, following reduction of CuA, inter-dimer electron transfer must take place between the CuA centre of one monomer and the Cu2 centre of the second monomer [29,31].

Nitrogenase

Nitrogenase catalyses the conversion of dinitrogen gas to ammonia, and the first structure of this enzyme emerged in 1992. The nitrogenase complex consists of two distinct protein components referred to as the Fe protein and the MoFe protein [32], both of which have been structurally resolved [32,33]. The Fe protein contains a [4Fe-4S] cluster that transfers electrons to the FeMo protein. The FeMo protein contains two types of metal cluster: the P-cluster and the FeMo cofactor. The P-cluster is an [8Fe-7S] cluster that, in its reduced state, can be considered as two typical [4Fe-4S] clusters covalently attached via a corner S atom. Two cysteine residues, which co-ordinate Fe atoms from each cluster, also

©2005 Biochemical Society
bridge the two cubane clusters. The function of the P-cluster is to mediate the electron transfer from the \( [4\text{Fe}-4\text{S}] \) cluster in the Fe-protein to the active site in the FeMo protein. The crystal structures solved in a number of defined oxidation states have shown that the P-cluster undergoes a redox-dependent rearrangement, whereby two Fe ligands are exchanged from the shared S atom (reduced) to a serine oxygen to one Fe and an amide nitrogen from the peptide back-bone to the other. The significance of this rearrangement is currently unknown, but it has been suggested that it may provide a mechanism to couple electron transfer to proton uptake. The FeMo cofactor is the active site where nitrogen binding and reduction to ammonia occurs. The FeMo cluster comprises a \([\text{Mo-3Fe-3S}]\) cluster bridged to a \([4\text{Fe-3S}]\) cluster via three sulphur atoms and a putative nitrogen atom that is hexa-coordinately bound to three Fe atoms of each cluster [32].

The Mo moiety of the cluster is also attached to homocitrate, which is bound to the Mo via two oxygen ligands. The FeMo cluster is covalently attached to the protein via a cysteine ligand to the terminal Fe atom and a histidine ligand to the Mo. Despite the high-resolution structure of nitrogenase (\( \approx 1 \text{Å} \)), the mechanism by which nitrogen binds and is reduced to ammonia still remains to be established.

The hydroxylamine oxidoreductase (HAO)

Hydroxylamine oxidoreductase from *Nitrosomonas europaea* is a water-soluble periplasmic protein that catalyses the conversion of hydroxylamine into nitrite (NH\( _{2} \text{OH} + \text{H}_{2}\text{O} \rightarrow \text{NO}_{2}^{-} + 5\text{H}^{+} + 4\text{e}^{-} \)). The structure of hydroxylamine oxidoreductase was resolved in 1997 [14]. Each monomer in the trimeric enzyme has eight covalently attached c-type cytochromes. One of these cytochromes, the P460 centre, is penta-coordinate and sits at the bottom of a pocket, and is the active site for hydroxylamine binding. The oxidation of hydroxylamine is considered to occur via two successive abstractions of electron pairs, with the concomitant release of five protons. The structure shows that haem P460 is located close to an aspartate residue, a histidine residue and covalently attached to a tyrosine residue. It has been suggested that these are all potential candidates as proton acceptors. The structure reveals that adjacent to haem P460 are two other haems. These haems may be required to rapidly accept a pair of electrons removed from the substrate: this might prevent slower single electron transfer, resulting in toxic nitric or nitrous oxide intermediates being released. The additional c-type cytochromes within hydroxylamine oxidoreductase provide a number of possible routes for onward electron transfer, ultimately resulting in the reduction of the cytochrome c554 molecule, for which the structure was solved in 1998 and a model for a 36-haem electron-transfer complex was proposed [40].

Unresolved structures

The structure of a number of key nitrogen cycle enzymes remain unsolved, including nitric oxide reductase, ammonia mono-oxygenase, and nitrite oxidase. Bacterial nitric oxide reductase is composed of two subunits \([\text{NorC (17 kDa) and NorB (53 kDa)}]\). NorC contains one c-type cytochrome, and NorB contains two b-type cytochromes and non-haem iron. The active site is a dinuclear centre comprising one haem b and non-haem iron [34]. Although the X-ray structure of nitric oxide reductase has yet to be solved, a structural model for NorB has been constructed based upon the sequence homology between nitric oxide reductase and cytochrome c oxidase [35] and a considerable body of spectroscopic data is emerging on this enzyme [41–43]. Obtaining the structure of ammonia mono-oxygenase presents a real challenge, since bacteria that catalyse nitrification grow only to a low cell density, and obtaining pure ammonia mono-oxygenase is difficult due to lability during the purification process. However, current opinion points towards a polynuclear copper centre at the catalytic site. Nitrite oxidoreductase oxidizes nitrite to nitrate in *Nitrobacter* sp. The enzyme has yet to be studied in detail at a biochemical or structural level, but sequence analysis indicates that it is a membrane-bound enzyme analogous to the membrane-bound nitrate reductase, in that it comprises a Mo catalytic \( \alpha \) subunit and a FeS containing a \( \beta \)-subunit [36]. Therefore the recent structures of membrane-bound nitrate reductase may also provide some mechanistic information as to how nitrite can be oxidized to nitrate. The process of ANAMOX (anaerobic ammonium oxidation; NH\( _{3} + \text{NO}_{2}^{-} \rightarrow \text{N}_{2} + \text{H}_{2}\text{O} \)) is also of considerable interest. The recent genome and proteome analysis of *Kueneia stuttgartiensis* will undoubtedly identify a number of novel ANAMOX enzymes and solving the three-dimensional structures of these enzymes will be essential for the understanding of the complex reaction mechanisms that underpin this intriguing process.

Conclusions

Structural biology has significantly increased our understanding of the enzymology of the N-cycle over the past 10 years. Not only are structures of many key enzymes now available, but multiple high-resolution structures of the same enzyme from different sources and/or in different states are now being resolved. It is this comparative structural analysis that will undoubtedly provide the greatest insight into the complex enzymic mechanisms that underlie the interconversion of nitrogen species within the global nitrogen cycle.

We thank the BBSRC for supporting work from the authors’ laboratories, and all those who have actively participated in the N-cycle meeting during the past 10 years.

References


©2005 Biochemical Society