Review

Nitric oxide in bacteria: synthesis and consumption

Nicholas J. Watmough a,*, Gareth Butland a, Myles R. Cheesman b, James W.B. Moir c, David J. Richardson a, Stephen Spiro a

1. Introduction

Bacteria in their natural environments may be exposed to nitric oxide (NO) from a variety of sources.

In the case of denitrifying bacteria, it is now established that NO is generated endogenously because it is an obligate intermediate [18] in the sequential reduction of nitrate to dinitrogen (reviewed in [10,133]) (Fig. 1). However, until quite recently there was considerable debate as to whether NO is a freely diffusible intermediate in denitrification (reviewed in [46]), an important consideration since NO is not only cy-
totoxic but also able to inhibit a number of metalloproteins found in bacterial respiratory chains. It is now clear that both the cytochrome $cd_1$ and copper nitrite reductases catalyse formation of NO rather than N$_2$O as the principal reaction product. Several lines of evidence proved a role for NO as a free intermediate in denitrification. These include: (i) trapping of extracellular NO, formed from nitrate ($NO_3^-$) and nitrite ($NO_2^-$), in suspensions of bacterial cells by either binding to haemoglobin or reduction through the action of the NADH:NO oxidoreductase activity of inside-out cytoplasmic membrane vesicles prepared from Paracoccus denitrificans [20,54]; (ii) displacement of the low steady-state [NO] from a suspension of cells with a stream of argon [55]; (iii) use of very sensitive approaches for measuring steady-state [NO] in suspensions of denitrifying cells with the outcome that NO is estimated as varying between $10^{-9}$ M and $10^{-7}$ M [55].

Some aspects of bacterial NO metabolism are quite well understood. For instance, mechanisms by which NO is formed by the nitrite reductases have been illuminated by high-resolution X-ray structures of both the copper nitrite reductase [41,85] and the cytochrome $cd_1$ nitrite reductase [89,124] in which substrate (NO$_3^-$) or product (NO) is bound at the active site. These structures provide insight into the mechanisms of these enzymes, in particular they suggest how the protein architecture around the active site might attenuate the usually very strong metal–NO interactions that could otherwise impair product release. The subsequent reduction of NO to nitrous oxide (N$_2$O) is catalysed by nitric oxide reductase (NOR). It is now clearly established, both as the result of primary sequence alignments [116] and detailed spectroscopic analysis [24] that this enzyme is closely related to respiratory haem–copper oxidases typified by the mitochondrial cytochrome $c$ oxidase (CrO).

It is also likely that non-denitrifying bacteria in
complex soil communities are exposed to NO generated by their denitrifying neighbours. In addition, pathogenic bacteria infecting mammalian hosts may also be exposed to significant amounts of host-generated NO, particularly around phagocytic cells. Thus it is likely that many species of bacteria have not only evolved the ability to recognise NO in their environments, but are also able to respond to its presence by eliciting a series of defensive mechanisms designed either to remove NO or protect them against its toxic effects. Some of these adaptive responses to the presence of NO have been elucidated in recent years and will be reviewed briefly.

The purpose of this article is to consider some of the recent progress in our understanding of bacterial NO metabolism and response mechanisms and draw lessons that apply to other areas of contemporary biochemistry and cell biology.

2. Biosynthesis of nitric oxide by denitrifying bacteria

Three different types of respiratory nitrite reductase have been found in bacteria. The cytochrome c nitrite reductase (which also may have a role in NO consumption (see Section 3.2)) reduces nitrite to ammonia while the other two classes of nitrite reductase (cytochrome cd₁ nitrite reductase and a trimeric copper containing nitrite reductase) catalyse the one electron reduction of nitrite (NO$^{2-}$) to NO. The copper and cytochrome cd₁ nitrite reductases have similar roles in denitrifying bacteria and no single organism has yet been identified which can express both types of the enzyme. In addition a copper nitrite reductase has been identified as part of the mitochondrial respiratory chain of some fungal species [73].

2.1. Cytochrome cd₁ nitrite reductase

Cytochrome cd₁ has been isolated from a number of denitrifying organisms and the corresponding structural gene (nirS) cloned and sequenced from Paracoccus denitrificans, Pseudomonas aeruginosa, Pseudomonas stutzeri andRalstonia eutropha (reviewed in [10,133]). The enzyme has a subunit molecular mass of around 60 kDa and functions as a homodimer [7,49,90]. Each subunit contains one covalently bound c-type haem and one non-covalently bound molecule of the unique d₁-type ‘haem’ (isobacteriochlorin), which can be extracted with organic solvents resulting in an inactive semi-apoprotein [62].

The three-dimensional structures of the oxidised (as isolated) cytochromes cd₁ from P. denitrificans GB17 (formerly Thiosphaera pantotropha) and P. aeruginosa have been solved to high resolution [49,90]. Both crystallise as dimers, with each monomer comprising two domains. The structure of the P. denitrificans enzyme reveals the smaller N-terminal domain (residues 1-134) which contains the c-haem has the form of a distorted class I c-type cytochrome. The larger domain (residues 135–567) forms an eight-bladed β-propeller structure. This fold is also found in another periplasmic enzyme methanol dehydrogenase [125], whilst related structures have been reported in a number of other enzymes (see [6] and references therein). The d₁ haem is centrally located in the large domain.

Surprisingly the haem c in the P. denitrificans enzyme has bis-histidine axial ligation [49]. Bis-histidine co-ordinated haems usually exhibit very low mid-point redox potentials, typically +50 mV. This low value would not be compatible with the presumed role of haem c in mediating electron transfer from potential electron donors such as pseudoazurin or cytochrome c₅₅₀ to the catalytic centre. Moreover, the d₁ haem was found to be six-co-ordinate, with not only a proximal histidine ligand (His-200), but a distal oxygen ligand being provided by a tyrosine residue at position-25, only eight residues away from His-17 which is one of the axial ligands to haem c [49]. The combination of an atypical c-type cytochrome domain and the putative substrate binding site being blocked suggested that the enzyme might undergo some structural rearrangement upon reduction to accommodate substrate binding.

This notion of conformational change during the catalytic cycle is consistent with earlier studies of the P. aeruginosa enzyme using CD spectroscopy which showed the ‘β-sheet’ content of the protein to decrease from 48% to 35% on reduction [112]. As evidenced by variable temperature MCD and EPR spectroscopies, reduction also causes the low-spin d₁ haem in the oxidised enzyme to become high-spin [68,119]. Such high-spin states of haemns are often associated with one rather than two protein ligands.
to the iron atom, suggesting that the $d_1$ haem loses an axial ligand upon reduction.

In an imaginative study, which combined single crystal microspectrophotometry with structural analysis Williams and co-workers [124] showed that not only did the $d_1$ haem shed its tyrosine axial ligand upon reduction, but reduction was associated with a switch of axial ligands to the haem $c$ such that His-17 was replaced by the conserved methionine residue at position 106. The mechanistic significance of this ligand switch at haem $c$ is not yet clear, although it is of interest that the oxidised enzyme from P. aeruginosa contains an N-terminal domain which looks like a typical class I cytochrome $c$ with His-Met axial ligation of the haem. It is important to note that unusual co-ordination of the haems seen in the oxidised P. denitrificans enzyme is not an artefact of crystallisation. Detailed spectroscopic analysis of the enzymes from P. denitrificans, P. aeruginosa, and P. stutzeri clearly shows that only the P. denitrificans enzyme has His-histidine axial co-ordination in solution, whilst both Pseudomonad enzymes have His-Met co-ordination (see [22] and references therein).

A possible role in catalysis for the distal tyrosine was suggested when Williams et al. went on to expose the crystals of reduced cytochrome $cd_1$ to nitrite. These authors observed in one polypeptide chain of the dimer, nitrite bound to the $d_1$ haem iron through its nitrogen, so leaving one of the oxygen atoms in hydrogen bonding contact with two histidine residues (His-345 and His-388) that lie in the haem $d_1$ distal pocket. On longer timescales a structure containing NO bound to the $d_1$ haem was observed in which the sidechain of Tyr-25 had moved back into the haem distal pocket [124]. This has been taken to imply a mechanistic role for Tyr-25 (Fig. 2A), in displacing NO from Fe(III) $d_1$ haem prior to transfer of a second electron into this site from the $c$-haem, thus preventing formation of a dead end product Fe(II)-NO which has been observed in single turnover experiments with the fully reduced enzyme from P. aeruginosa [108].

Unfortunately, this attractive proposal appears difficult to reconcile with the fact that the enzyme from P. aeruginosa in common with all other cytochromes $cd_1$ of known sequence lacks a homologue of Tyr-25 [22]. Moreover, the crystal structure of the oxidised P. aeruginosa enzyme shows the sixth ligand to the $d_1$ haem to be an hydroxide ion hydrogen bonded to Tyr-10 from the opposite polypeptide chain [90]. The issue is further clouded by the fact that site-directed mutagenesis suggests that Tyr-10 is not essential for catalytic activity [31]. In fact the ability to dissociate the product and avoid formation of the dead-end ferrous-nitrosyl species, may not be exclusively endowed by the protein architecture of the distal pocket. Haem $d_1$ is a prosthetic group unique to the active site of nitrite reductases. The reasons why such an unusual structure is preferred for this particular reaction are far from clear and will remain so until the consequences of each individual modification relative to the parent protoporphyrin IX structure are understood. It is already known that for the low-spin ferric state the 4-electron reduced haem $d_1$ macrocycle stabilises $(d_{xz},d_{yz})^4(d_{xy})^1$ relative to the more usual $(d_{xz})^2(d_{yz},d_{xy})^3$ electronic configuration [22,23]. This ‘inverted’ ground state represents a relocation of unpaired spin density from orbitals extending above and below the haem plane to an orbital lying in that plane and this will have important consequences for the binding of π-un saturated ligands such as nitric oxide, in this case the product molecule. Ground state inversion also occurs for haem $d$ [97] but in relation to cytochromes $cd_1$, it should be noted that nitric oxide synthase (NOS) also shares this property. NOS contains a cysteine-ligated haem group spectroscopically indistinguishable from that of the cytochromes P450, EPR and MCD studies of which show that cysteine ligation also stabilises an inverted low-spin ferric ground state (M.R.C., H.E. Seward, A.J. Thomson, unpublished results). Also, a detailed analysis of the MCD properties of high-spin ferrous P450 and myoglobin reveals the same $d_{xz},d_{yz}$ stabilisation [91] and it is to be expected that haem $d_1$ will behave similarly in the high-spin ferrous state. This particular electronic property may therefore have important consequences for more than one spin-oxidation-state of these enzymes.

2.2. Copper nitrite reductase

Copper nitrite reductases have been identified in a number of bacterial genera and purified from several Gram-negative bacteria (reviewed by Berks et al.
and one Gram-positive organism, *Bacillus halodenitrificans* [39]. The structural gene (*nirK*) encoding the copper type nitrite reductase has been sequenced from a number of species, including *Alcaligenes faecalis* [86], *Pseudomonas* sp. strain G179 [128], *Pseudomonas aureofaciens* [52] and *Rhodobacter sphaeroides* 2.4.3 [113]. The primary structure of the enzyme from *Achromobacter cycloclastes* has also been determined by protein sequencing [45]. Based on detailed analysis of the primary sequence, Berks et al., proposed that the *aniA* gene product of *Neisseria gonorrhoeae* is also likely to be a copper nitrite reductase [10]. This proposition was recently shown by mutational analysis to be correct [81], suggesting that successful gonococcal infection relies at least in part upon the anaerobic reduction of nitrite by this outer membrane lipoprotein, although it should be noted that the *aniA* mutant retains its virulence.

The crystal structures of the copper nitrite reductases, including those purified from *A. cycloclastes* [53] and *A. faecalis* S-6 [75] reveal them to be homo-trimers which bind three type I and three type II copper centres. Each monomer forms two β barrel domains (domain I, residues 8 to 160 and domain II, residues 161 to 340). Domain I provides four ligands for the type I copper centre (His145, Met150, His95 and Cys136). The type II copper is liganded by His100 and His135 of domain I, a solvent molecule (presumably either water or hydroxyl) and His306...
from domain II of a different subunit. The type II copper ligands are, unusually, arranged as a regular tetrahedron. The two copper molecules are spaced 12 Å apart and are co-ordinated by adjacent amino acids: the type II copper ligand His135 and the type I copper ligand Cys136. All the type I and type II copper ligands are conserved in the sequenced copper nitrite reductases.

The type I copper centre serves to transfer electrons from donor proteins (cupredoxins and cytochromes) to the type II centre. The type II copper centres of copper nitrite reductases had been proposed to be the site of substrate binding based upon density difference maps of oxidised nitrite reductase crystallised in the absence and presence of nitrite [53], perturbation of ENDOR and EPR signals assigned to the type II copper centres in the oxidised enzyme by the addition of nitrite [63], and inhibitor binding studies. This role has now been confirmed by the observation of nitrite bound to the type II copper centres of the enzymes from *A. faecilis* [85] and *Alcaligenes xylosoxidans* [41]. In contrast to the cytochrome cd$_1$ nitrite reductase which binds substrate to the reduced d$_1$ haem via the nitrogen atom, type II copper centres co-ordinate nitrite in a bidentate fashion via the oxygens. Moreover, one of the oxygens is hydrogen bonded to a nearby conserved aspartic acid (Asp-98).

Based on the structures of the oxidised and reduced forms of the *A. faecilis* enzyme in the presence and absence of nitrite, Murphy et al. [85] have proposed a mechanism in which nitrite binds to an oxidised type II copper centre displacing a hydroxide ion. This centre is in turn reduced by transfer of an electron from the type I centre. Nitrite decomposes after protonation at the reduced centre to give a transient intermediate which has NO and hydroxide ion bound. The catalytic cycle is completed when the bound NO is released. In this mechanism, unlike that proposed for cytochrome cd$_1$ which involves the participation of a metal–nitrosyl intermediate (Fig. 2A, [120]), neither substrate, nor product are bound through the nitrogen during the catalytic cycle (Fig. 2B). However, based both on the structure of the nitrite bound form of the *A. xylosoxidans* enzyme [41,42] and an earlier EXAFS study [109], Dodd et al. [41,42] have proposed an alternative mechanism which does involve participation of a bound nitrosyl (Cu(I)–NO$^+$) intermediate (Fig. 2B). One feature of this alternative mechanism is that it more readily accounts for the formation of N$_2$O as a minor product during normal turnover of copper nitrite reductases by the reaction of the product (NO) with the bound nitrosyl intermediate [66]. This rebinding reaction, which is sensitive to the addition or removal of NO from the assay system [66], appears not to be catalysed by cytochrome cd$_1$ nitrite reductase where N$_2$O accounts for only 0.5% of the reaction product [66].

3. Reduction of nitric oxide by denitrifying bacteria

3.1. Nitric oxide reductase

For a long time it was thought that nitrite reductase catalysed the sequential reduction of nitrite into nitrous oxide via NO. First proof that NO reduction was carried out by the distinct enzyme nitric oxide reductase (NOR) came from the isolation of a mutant of *P. stutzeri* that lacked cd$_1$ nitrite reductase, but which was still competent in the reduction of NO [135]. In addition it was demonstrated that inside-out vesicles from *P. denitrificans* in which nitrite reductase was absent, possessed NADH–nitric oxide oxidoreductase activity [20]. Later it was discovered that deletion of the copper–nitrite reductase from *Pseudomonas* sp. strain G-179 also did not abolish nitric oxide reductase activity [127], thus establishing that a distinct NOR is present irrespective of the type of nitrite reductase. Confirmation that this NOR participates in denitrification came from the work of Braun and Zumft [13], who deleted the gene for this enzyme from *P. stutzeri* and showed that the resulting mutant was unable to denitri f'y owing to the accumulation of toxic NO.

The two-electron reduction of NO to N$_2$O is an unusual reaction in biology in that it involves formation of an N–N bond. Apart from the bacterial NOR, other examples of metalloproteins able to catalyse this reaction include copper nitrite reductase [66], bacterioferritin [78], ribonucleotide reductase [57], haemocyanin [118], CeO [15] and a P450 type enzyme from *Fusarium oxysporum* [96]. Of these, only the fungal P450$_{NOR}$ is part of a denitrification pathway, which can reduce nitrate as far as N$_2$O.
The NO reductase activity of the other proteins, all of which contain dinuclear metal centres, is probably adventitious. However, in the case of CcO the NO reductase activity is perhaps unsurprising given the close relationship of this enzyme to NOR. Indeed, reaction of NO with CcO has been proposed both as a possible route for the catabolism of NO [12] and as a mechanism for regulating respiration [14].

The fact that NOR is a close relative of a superfamily of haem–copper oxidases that evolved from a common ancestor is clearly demonstrated by the analysis of the amino acid sequences of NOR from a number of different organisms [103,116]. Respiratory haem–copper oxidases are widely distributed in Archaea and Eubacteria, but the best understood remains the CcO of eukaryotic mitochondria. Recently the structure of the 13-subunit bovine mitochondrial CcO has been solved to 2.3 Å resolution [115,129], and the structure of a simpler four subunit bacterial CcO from *P. denitrificans* has been solved to 2.7 Å resolution [65,92]. Members of this superfamily of haem–copper oxidases possess a low spin *bis*-histidine co-ordinated haem and a dinuclear active site formed by a haem group and a copper ion, CuB, which will bind and reduce dioxygen. Analysis of the amino acid sequences of subunits I (which contains both the dinuclear centre and the low-spin haem) of all members of this superfamily shows that they share the protein architecture of 12 transmembrane helices as revealed by the crystallography. Transmembrane helices II, VI, VII and X contain the six invariant histidines that are responsible for ligating both haems and CuB and are absolutely conserved in all members of the HCO superfamily and in NOR [116].

NOR which is usually isolated as a heterodimer, NorBC, has been purified from *P. stutzeri* [59], *P. denitrificans* [19,40,51], *P. denitrificans* [48], *Paracoccus halodenitrificans* [101] and *A. cycloclastes* [69]. The *P. stutzeri* enzyme exhibited a *Km*(NO) of 40×10⁻⁶ M when PMS was the electron donor [59]. In contrast the *Km*(NO) of the *P. denitrificans* enzyme assayed with cytochrome *c*₅₅₀ was sub-micromolar [19], which in view of the success of NOR in whole cells in keeping [NO] below 10⁻⁷ M [55] seems a more reasonable value.

The catalytic subunit, NorB is a 53 kDa (apparent molecular mass 37 kDa by SDS–PAGE) polypeptide that binds haem b. NorC is predicted to have a single transmembrane helix at the N-terminus that anchors the periplasmic C-terminal domain to the cytoplasmic membrane. A single Cys–Xaa–Xaa–Cys–His *c*-type haem binding motif has been identified in this domain which is consistent with biochemical analysis which shows NorC to be a membrane-anchored 17 kDa mono-haem *c*-type cytochrome. Exceptions to this have recently been discovered in *R. eutropha* H16 [28] and in *Synechocystis* sp. strain PCC6803 [71], both of which appear to have no NorC homologue and an N-terminal extension of approximately 250 amino acids to NorB, which is proposed to encode a further two transmembrane helices connected by a hydrophilic domain of 203 amino acids. This is proposed to provide a topological and functional substitute for NorC [28], although as yet no redox centre has been identified in this domain.

Recently it has been shown using a combination of MCD and EPR spectroscopies that the cytochromes *b* of NOR from *P. stutzeri* have the same protein derived ligation as their equivalent centres in haem–copper oxidases [24]. One of the key differences between the haem–copper oxidases and NOR is the elemental composition of the dinuclear centre. In the haem–copper oxidase superfamily, high-spin ferric haem is magnetically coupled to a copper ion known as CuB, but in NOR copper is absent. Analysis shows that iron is the only metal present at a level of approximately three ions per subunit I [60]. It has therefore been suggested that CuB is replaced by one (or possibly two) non-haem iron (Fe₃B). The insertion of a metal in a specific site is in part dictated by the co-ordination sphere provided by the protein architecture. Therefore since Fe₃B requires extra ligands from the protein, the insertion of iron rather than copper would be favoured only if the protein provides an octahedral co-ordination sphere, rather than the tetrahedral site favoured by copper ions. It is possible that the ancestral oxidase from which NOR and CcO both evolved utilised iron rather than copper at this site because, under the highly reducing conditions of the primordial biosphere, ferrous ions would be more readily available than insoluble cuprous ions. This could be important in understanding the evolution of aerobic life on earth since the substitution of iron by copper in the ancestral oxidase allowed it to reduce oxygen efficiently.
Castresana and Saraste [21] have argued that denitrification preceded aerobic respiration in the early biosphere so that the primary function of the ancestral oxidase was NO reduction. However, it is likely that the primordial oxidase must have been able to catalyse the reduction of trace amounts of both NO and O₂. In this context it is of interest that the NOR purified from \textit{P. denitrificans} has oxidase activity [48], although the reported \( K_{\text{m}} \) of 0.9 mM would make it rather ineffective in the early biosphere. Perhaps a more pertinent question concerns the rate at which cytochrome \textit{cbb₃}-type oxidases (FixNOQP) [99] catalyse NO reduction. Although these oxidases which function at very low oxygen concentrations contain Cu₉, the organisation around the dinuclear centre is very different to CrO (Fig. 3).

As first pointed out by Berks and colleagues [10] NorB contains a highly conserved glutamate residue (Glu-267) which secondary structure predictions place in the middle of transmembrane helix VIII (Fig. 3). The high energetic cost of introducing a charged residue into such a hydrophobic region of a protein led these authors to propose that this res-

![Fig. 3. Amino acid sequence identity within transmembrane helices VI, VII and VIII of NOR and two classes of haem-copper oxidase. Amino acid residues that are conserved in all three classes of haem-copper oxidase are shown in bold. The consensus sequences represent those amino acids that are conserved within each class of haem-copper oxidase. Cu₉ ligands are represented by *. Proposed Fe₉ ligands are represented by #. Conserved components of the so-called D and K proton input channels of CrO that lead from the cytoplasmic face of the enzyme to the dinuclear centre are indicated by ▲ and ▼, respectively. Components of a possible proton input pathway from the periplasmic face of NOR to the dinuclear centre are indicated by ●. EMBL accession numbers for the DNA sequences and references are as follows: 1J01394 [2], 2X14910 [105], 3X05829, 4X62654 [106], 5J05492 [26], 6U34353 [34], 7L07487 [99], 8AE000536 [111], 9U28078 [33], 10AB010889 [102], 11AF000233 [8], 12Z28384 [134], 13D38133, 14AF002217 [28], 15D90917 [71].]
idue could contribute to the ligation sphere of FeB [10]. In fact this is just one of five glutamate residues that are absolutely conserved in all eight NorB sequences currently available (Fig. 3). Clearly, these carboxylate residues cannot all be involved in FeB ligation and it is therefore reasonable to consider other functions for these carboxylate side chains, including the movement of protons involved in catalysis to the active site.

The structures of CcO reveal two pathways, leading from the N-face of the membrane to the dinuclear centre, that could function as proton input channels. It was originally thought that the purpose of these channels was to separate the substrate and the pumped protons. However, site-specific mutagenesis combined with detailed biophysical analysis has shown that one channel (the K-channel) delivers the two protons required for (re)duction of the dinuclear centre during turnover, whilst the remaining six protons enter via the D-channel [72]. Analysis of the primary and secondary structures of NOR shows that all the key elements that make up the D- and K-channels are missing. This is perhaps unsurprising as NOR is not electrogenic and is unlikely to pump protons, presumably taking substrate protons required by the stoichiometry of the overall reaction from the periplasm [9,107].

It is clear that any model of the NOR dinuclear centre must take account of the need for a proton channel from the periplasm to the active site and a co-ordination sphere that favours iron rather than copper. We are currently using site directed mutagenesis to explore such a model which is based on both the primary amino acid sequence of six NORs (Fig. 3) and the three-dimensional structures of CcO. This proposes that all three invariant histidines are retained as ligands to FeB and a fourth ligand is provided by Glu-267 [10]; two solvent molecules complete the co-ordination sphere. A putative proton input channel is formed by four conserved glutamic acids: two in the periplasmic facing loop between helices III and IV, and two in transmembrane helix VI which might be considered as an ‘upside down’ version of the D-channel. It is of interest to note that a highly conserved region of transmembrane helix VI (HLWVEG-E) aligns with different consensus sequences that are totally conserved within the CcO and cytochrome cbb3 groups of haem-copper oxides (Fig. 3). This suggests that together with the elemental composition of the dinuclear centre, this region of the protein may act to control the specificity and affinity of the active sites for their substrates.

As already mentioned, a number of proteins containing dinuclear metal centres will catalyse the reduction of NO to N2O. Therefore it is necessary to ask what catalytic advantage is conferred by a haem–non-haem iron dinuclear centre of bacterial NOR. It has been argued that NO reduction takes place at dinuclear metal centres because such centres can orientate two bound nitroxy lons in such a way as to favour N–N bond formation. Such a model has been put forward to account for NO reduction by NOR [51] and recently Hendriks and colleagues have identified EPR signals present during turnover of the enzyme that they assign to a Fe(II)–NO::ON–Fe(II) ligated dinuclear centre [60]. It is argued that this species would yield N2O and an oxidised dinuclear centre containing a µ-oxo (or hydroxo) bridge which may mediate magnetic coupling between the Fe(III) haem and FeB(III) [51]. A variable temperature MCD study of oxidised NOR from P. stutzeri provides evidence for a His–Fe(III)–X–Fe(II) ligated high-spin species in the dinuclear centre at room temperature [24]. This observation is difficult to reconcile with room temperature resonance Raman data on the oxidised P. denitrificans enzyme which suggests that the dinuclear centre haem is a pure penta-co-ordinate species [84]. Interestingly at liquid helium temperatures (the conditions under which EPR spectra of NOR are normally recorded) the MCD spectrum reveals all three haems, including the dinuclear centre haem to be low-spin hexa-co-ordinate, with a suggestion that the distal ligand to the dinuclear centre haem bridges to FeB [24].

It has been shown using inorganic models that most mononuclear iron centres can catalyse NO reduction, and that the efficiency of this reaction is greatly enhanced if the centre has two exchangeable co-ordination sites. If our proposed model of co-ordination for FeB proves to be correct, this suggests that NO reduction in NOR could be catalysed at that site (see [4] and references therein). However, such a mechanism takes no account of the fact that the active site of NOR contains a high-spin haem and that the overall architecture of the enzyme permits rapid haem–haem electron transfer [16]. An
dication as to how both centres could participate in catalysis can be found by considering another member of the superfamily of haem–copper oxidases, *E. coli* cytochrome *bo*$_3$. It has recently been shown by Thomson and colleagues that not only can oxidised cytochrome *bo*$_3$ can bind two equivalents of NO to Cu$_B$ [17], but also in common with other haem–copper oxidases it can also stabilise an oxyferryl haem o species [122]. These two observations led to a proposal for a plausible mechanism for the reduction of NO by haem–copper oxidases [121], a modified version of which may be applicable to NOR.

### 3.2. Cytochrome *c* nitrite reductase

The cytochrome *c* nitrite reductase is a 50 kDa periplasmic enzyme which can bind five *c*-type haems. It can reduce both nitrite and hydroxylamine to ammonia. The $V_{\text{max}}$ values for nitrite and hydroxylamine are similar but the $K_m$ for hydroxylamine is much higher than for nitrite [70]. The enzyme can also reduce NO to nitrous oxide at a rate comparable to that of nitric oxide reductase [27]. Thus it is likely that NO and hydroxylamine are enzyme-bound intermediates in the reduction of nitrite to ammonia. The full reaction mechanism may proceed via nitrosyl (NO$^+$), one electron-reduced NO, two electrons-reduced nitroxy (NOH) and four electrons-reduced hydroxylamine (NH$_2$OH) intermediates. Following reduction and oxidation by nitrite, some EPR studies have detected signals assigned as nitric oxide bound to the high spin iron [27,79], leading to suggestions that a pair of haems known to be close enough to show weak magnetic coupling is the site of nitrite binding and that reduction proceeds via a nitrosyl intermediate. In this case the site of nitrite binding and formation of the nitrosyl species would be a penta-co-ordinate high-spin haem with the bound nitrogen intermediates providing the sixth haem ligand. Recently it has been shown that the *E. coli* enzyme contains an unusual *c*-type haem with histidine/lysine axial co-ordination [43] which may contribute to the active site. This would be consistent with the suggestion of Blackmore et al. [11] that the proposed high-spin/low-spin haem pair at the active site is bridged by a single histidine residue which only acts as a sixth ligand to the high-spin haem during some parts of the catalytic cycle.

### 4. Nitric oxide buffering by haem proteins?

Evidence is emerging that, in addition to the catalytic reduction of NO by NOR, other haem proteins may counteract NO toxicity by binding NO to reduce its intracellular concentration. Investigations of denitrifying bacteria using EPR spectroscopy of whole cells showed that after exposure to nitrate under anaerobic conditions, those denitrifying bacteria that contained cytochrome *c*' exhibited the characteristic three-line EPR signal of five-co-ordinate haem–nitrosyl species [130,131]. Such spectra were not observed in denitrifying bacteria that do not synthesise a cytochrome *c*'. The implication is that cytochrome *c*' binds NO produced in vivo during denitrification. The possibility that another haemoprotein is responsible for this signal cannot be completely excluded; but the signal does not derive from the cytochrome *cd*$_1$ nitrite reductase since the NO derivative of this enzyme that forms during its reaction cycle is a six-co-ordinate haem–nitrosyl [68,108].

Cytochrome *c*' is a periplasmic cytochrome with unusual spectroscopic and structural features [83]. Widely distributed amongst the proteo-bacteria, complete amino acid sequences have been determined for cytochrome *c*' from both denitrifying and photosynthetic bacteria of the *Rhodospirillaceae* and *Chromatiaceae* families [1]. The cytochrome is also synthesised by sulphur-oxidising [104] and methano trophic bacteria [132]. Despite detailed biophysical and biochemical characterisation, the physiological role is yet to be defined. X-Ray crystal structures have been solved for the cytochrome from four photosynthetic bacteria (*Rhodospirillum molischianum* [123], *Rhodospirillum rubrum* [126], *R. capsulatus* [110] and *Chromatium vinosum* [100]), and two denitrifying species (*Achromobacter xylosoxidans* and *R. eutropha*) [5]. The structures consist of a four helix bundle, to which the *c*-haem is covalently attached near to the C-terminus. The iron of the haem is penta-co-ordinate in the absence of added ligands. Access to the vacant sixth co-ordination site is hindered by bulky hydrophobic residues, which limit ligand binding to small predominantly uncharged molecules, particularly NO and CO supporting the possibility of a physiological role for the cytochrome in NO metabolism.
The demonstration that in *P. denitrificans* binding of NO by cytochrome c' is reversible under physiological conditions (J.W.B.M., unpublished results) offers a mechanistic explanation of the control of NO toxicity by cytochrome c'. The cytochrome may reversibly bind (buffer) NO in order to keep its free concentration low to prevent it damaging cellular processes. Even non-denitrifying bacteria may use cytochrome c' to prevent the intracellular accumulation of toxic concentrations of NO which may arise as a result of NO production processes occurring in their natural habitat. However, this poses the question as to why some species of bacteria do not synthesise cytochrome c'. Presumably, organisms lacking cytochrome c' either do not encounter environments containing NO at potentially toxic concentrations or they possess alternative mechanisms to control the toxicity of NO. Evidence for the latter can be found in the Gram-positive denitrifying bacterium *B. halodenitrificans*. This organism possesses a soluble haemoprotein estimated to contain 6 b-haems per protein molecule [38]. This green protein reversibly binds NO and has been suggested to function as an NO buffer in vivo.

5. Nitric oxide signalling in bacteria

5.1. Denitrifying bacteria

In the case of organisms which generate NO as an intermediate of denitrification, that NO is most easily dealt with through the NO-inducible expression of a nitric oxide reductase. The first indication of this mode of regulation came from the observation that in a nitrite reductase mutant of *Paracoccus denitrificans* (which does not generate NO) expression of nitric oxide reductase is reduced [32]. In the case of *Pseudomonas stutzeri*, expression of NO reductase is at 5–20% of wild-type levels in a nitrite reductase deficient strain, although in vivo rates of NO reduction remained similar to wild-type [134]. This was taken to indicate that much of the enzyme is inactive in wild-type strains [134], though an alternative explanation might be that flux through denitrification is relatively insensitive to the activity of NO reductase (it has a low flux control coefficient). Körner [74] has shown that exogenous NO at concentrations up to 25 μM produce some induction of NO reductase in *P. stutzeri*. Higher concentrations of NO inhibited growth in these experiments [74]. Expression of reporter fusions to both the nir and nor genes is severely impaired in a nitrite reductase deficient strain of *Rhodobacter sphaeroides*, and this has been taken to indicate that NO is required for the activation of both nir and nor [76,113]. Virtually wild-type levels of expression could be restored in the nitrite reductase mutant by addition of exogenous sodium nitroprusside, which is an NO-generating reagent, providing further evidence that NO is an activator of nir and nor expression [76]. The rationale for the apparent co-ordinate regulation of nitrite and NOR by NO is generally thought to be that it is a strategy to help maintain low steady-state levels of NO. In a denitrifying strain of *Rhodobacter sphaeroides*, the NO-activation of nir and nor expression requires the product of a regulatory gene designated *nrr* which encodes a member of the Fnr family of transcriptional activators [76,114]. This suggests that NnrR is a NO-dependent activator of gene expression, though it remains to be established that the effect of NO on NnrR is direct. A similar regulatory protein has been found in *Paracoccus denitrificans* where it is also required for maximal expression of nitrite reductase and nitric oxide reductase [117]. Experiments with gene fusions and NO-generating agents have demonstrated that, as in *R. sphaeroides*, Nnr is either directly or indirectly required for NO-mediated activation of the nir and nor genes (R.J.M. Van Spanning, personal communication). Assuming NnrR and Nnr are direct sensors of NO, then there are few clues from their sequences to suggest possible sensing mechanisms. The prototype member of this family of proteins, Fnr, activates gene expression in response to anoxia, and has an oxygen-sensitive iron–sulfur centre [77]. The cysteine residues which provide ligands to the Fe–S centre are not conserved in Nnr(R) which must therefore have a different mechanism. Another Fnr homologue, CooA from *R. rubrum*, is a haem-containing carbon monoxide sensor [3,58], indicating that members of this family have diverse signal recognition mechanisms. Thus, it is tempting to speculate that Nnr and NnrR have redox active centres which are responsible for inter-
acting with NO, although there are very few conserved residues which could potentially provide ligands for such a centre.

Some rhizobial species denitrify and may therefore also require mechanisms for defence against endogenously generated NO. The membrane-associated FixL protein is a haem-containing oxygen sensor found in a number of Rhizobia which interacts with a soluble transcriptional regulator, FixJ, to regulate expression of nitrogen fixation [47]. It has recently been demonstrated that two engineered soluble domains of FixL from *Rhizobium meliloti* form nitrosyl complexes, although the regulatory and physiological consequences of NO binding (if any) remain unknown [80].

### 5.2. Pathogenic bacteria

Responses to NO in pathogenic bacteria have not been well characterised, though there are some observations that have been made in enteric bacteria which may have more general significance. Several bacterial regulatory proteins have been characterised which act as sensors of oxygen. The Fnr protein, found in *E. coli* and several other species, has an oxygen-sensitive Fe-S cluster. It seems possible from a chemical point of view that the Fe-S centre of Fnr might be sensitive to NO and this could form the basis of an NO signalling mechanism. However, the reactivity of Fnr towards NO has yet to be tested.

*Escherichia coli* has a soluble flavohaemoglobin (Hmp) with an N-terminal domain which has sequence similarity to eukaryotic globins [98]. This protein forms a nitrosyl complex [64] and may therefore have some role in either protecting the cell against the toxic effects of NO or in an NO-dependent signal transduction pathway. Recently, a flavohaemoglobin deficient mutant of *Salmonella typhimurium* has been constructed and shown to have an increased sensitivity to NO generating agents, under both aerobic and anaerobic conditions [29]. The *hmp* gene encoding Hmp is activated during aerobic growth by NO (10–20 μM) in *E. coli*; the mechanism of NO activation is not known, but it does not involve the SoxRS system [98]. Flavohaemoglobin-deficient mutants of *E. coli* are also sensitive to NO, and it has been demonstrated that the *E. coli* Hmp has an NO dioxygenase activity with nitrate as the reaction product [50]. The pattern of expression of *hmp* and the phenotype of the *hmp* mutant together with the catalytic activity of Hmp suggest that it has a specific role in the defence against NO. Proteins similar to Hmp appear to be very widely spread in bacterial species [82] and may therefore have a general role in the response to NO.

The most important NO-responsive regulators yet to be characterised are the SoxR and SoxS proteins of *E. coli* (reviewed by Demple [37]). The SoxR protein is sensitive to superoxide-generating compounds and to NO, and in response to these signals it activates transcription of the *soxS* gene, the product of which (SoxS) is another transcriptional activator. SoxS in turn activates expression of genes belonging to the Sox regulon; these include genes encoding enzymes which remove superoxide radicals (Mn superoxide dismutase) and which initiate repair of oxidatively damaged DNA (endonuclease IV). Two TCA cycle enzymes (aconitase A and fumarase C) are also activated by the Sox system in response to superoxide radicals or NO.

Purified SoxR is a homodimer containing one [2Fe–2S] centre per monomer, the centres have a redox potential of approximately −285 mV [61]. SoxR binds to DNA irrespective of whether the Fe-S centres are reduced or oxidised, but activates transcription only when they are oxidised. It is suggested that SoxR undergoes one-electron redox chemistry, with the reduced form being inactive with respect to transcription activation. It is possible that SoxR is maintained in its reduced state in vivo in non-stressed cells by a reductase activity [61]. The biochemical mechanism of activation of SoxR in vivo by NO is not clear, but might involve direct oxidation of the Fe–S centre by NO and/or inhibition of the reductase activity (either directly or indirectly) by NO [61].

The SoxRS regulon is activated by free NO and is also activated following incubation of *E. coli* with mouse macrophages [87]. Macrophage activation is almost certainly mediated by NO since it is prevented by *N*-G-monoethyl-L-arginine, an inhibitor of NO synthase [87]. Furthermore, a *soxRS* mutant is less viable than its wild-type parent when cultured with macrophages, which is consistent with the idea that the SoxRS regulon mounts a response against the
toxic effects of NO [88]. However, this may not be a ubiquitous defence mechanism since: (a) there are no soxRS homologues in the genomes of some other pathogens for which genome sequences are available (e.g., Helicobacter pylori), and (b) in Salmonella typhimurium soxS is not required for resistance to NO or to macrophage killing [44]. One member of the SoxRS regulon is a Mn-superoxide dismutase, which might have a specific role in detoxifying NO. In the case of Salmonella typhimurium, a periplasmic Cu,Zn-superoxide dismutase has been shown to protect against phagocyte derived NO [35].

Homocysteine has a role in counteracting the toxicity of NO and nitrosothiols in S. typhimurium [36]. Mutation of metL which encodes an enzyme on the homocysteine biosynthetic pathway causes hypersensitivity to NO releasing compounds such as S-nitrosothiophene. This hypersensitivity can be complemented by exogenously added homocysteine. Also, the mutation in metL attenuates the virulence of S. typhimurium in a mouse model, which suggests that homocysteine reacts with NO in order to counteract its cytotoxic effects in vivo. In S. typhimurium and Mycobacterium tuberculosis there appears to be an alkyl hydroperoxide reductase which has a role in the protection against reactive nitrogen species including NO and its derivatives [25].

5.3. Parallels with eukaryotic systems

It is unlikely that the bacterial NO signalling mechanisms will have direct counterparts in eukaryotic organisms. Nevertheless, there are some intriguing comparisons that can be drawn. One in particular concerns the regulation of the tricarboxylic acid (TCA) cycle by NO and other oxidative stresses in bacteria and eukaryotes. As mentioned above, some E. coli genes encoding TCA cycle enzymes are members of the SoxRS regulon and so are induced by oxidative stress (and presumably by NO). The acnA gene encoding one of two aconitases in E. coli is activated by oxidative stress in a SoxR-dependent fashion [30]. Further, the fumC gene which encodes an oxygen-stable fumarase (one of three isoenzymes) is also activated by the SoxRS system [95]. The fumarases and aconitases are [4Fe–4S]-containing enzymes which are, in general, sensitive to oxidation by a variety of oxidising agents. The rationale for SoxRS regulation of acnA and fumC (which encode normally minor isoenzymes of aconitase and fumarase, respectively) might be that these isoenzymes are particularly resistant to oxidation and so are induced by exposure to oxidants in order to maintain flux through the TCA cycle. In higher eukaryotic organisms, some TCA cycle enzymes are regulated by the iron response proteins (IRP-1 and IRP-2) which are themselves homologous to mitochondrial aconitase [56]. IRP-1 contains a labile Fe-S centre; in iron-replete cells this centre is assembled and the protein has aconitase activity and is inactive with respect to regulation of gene expression. In iron-limited cells, the Fe–S centre is disassembled, the protein loses aconitase activity and becomes active as an mRNA binding protein. When bound to mRNA, IRP-1 may down-regulate gene expression (by inhibiting translation when occupying an IRE in the 5′ untranslated region) or up-regulate gene expression (by stabilising mRNA by binding to a site near the 3′ end). Exposure to nitric oxide mimics the effect of iron limitation, shifting IRP-1 into its active conformation [67,93]. Mammalian mitochondrial aconitase mRNA has an iron response element (IRE) in its 5′ untranslated region to which IRP-1 binds [56]. The location of the cis-acting IRE is consistent with translation of the aconitase mRNA being repressed by the active form of IRP-1. Since activation of IRP-1 can be brought about either by iron depletion or by treatment with NO, it can be inferred that aconitase expression is down-regulated by oxidative stress [56,67] It has been suggested that NO activates IRP-1 not by reacting with its Fe–S centre, but rather by affecting cellular iron availability [94]. A similar suggestion had been made to explain the mode of activation of SoxR by NO [37], but direct interaction of NO with the SoxR Fe–S centre is the currently favoured model [61]. Thus there is evidence to indicate that the TCA cycle is regulated by NO in both prokaryotes and eukaryotes. In both cases regulation involves proteins containing Fe–S centres (SoxR and IRP), though the mechanisms of activation may be different, and the response is different; E. coli aconitase A is activated by SoxR (and therefore probably by NO) whereas mammalian mitochondrial aconitase seems likely to be down-regulated by IRP, which can itself be activated by exposure to NO. Whether there are mechanistic par-
alleles which can be drawn between other NO-responsive transcription factors remains to be established.

6. Concluding remarks

The high interest in the nitrogen cycle and in particular reduction of N-oxides by bacteria has led to rapid advances in our understanding of the metalloproteins that participate in denitrification. An ability to purify large amounts of these enzymes, in particular the nitrite reductases, has allowed their study to benefit from detailed structural and spectroscopic analysis. This, as we discussed earlier, has led to a greater understanding as to how the enzymes which catalyse the formation or reduction of NO avoid the greater understanding as to how the enzymes which mediate also acting as an inhibitor. In this context the demonstration that the $d_1$ haem has an altered electronic configuration which is also found in $P450$ enzymes may ultimately have a significant impact on our understanding of the eukaryotic NO synthases. So although there is no direct counterpart of the denitrification pathway in eukaryotes, a greater understanding of the principles governing the active site chemistry of not only the nitrite and NO reductases, but also the molybdoproteins that catalyse nitrite reduction, will lead to advances in the biochemistry of enzymes containing related cofactors in other organisms. This principle clearly applies to our as yet incomplete understanding of NO signalling in bacteria where redox active metal centres appear to be involved in sensing NO. Elucidation of the mechanisms of NO interaction with these sensing elements may allow the identification of previously unrecognised components of NO signalling pathways in higher organisms.

Acknowledgements

Much of our ongoing work on bacterial NO metabolism is underpinned by continued EPSRC/BBSRC support for the Centre for Metalloprotein Spectroscopy and Biology at UEA. N.J.W. is in receipt of a Wellcome Trust Career Development Award, and G.B. thanks the EPSRC for a Special Studentship. We would particularly like to thank our colleagues Prof. Stuart Ferguson (University of Oxford) and Prof. Andrew Thomson (UEA) for a number of helpful discussions concerning the mechanisms of cytochrome $cd_1$ and NOR, respectively.

References


23. M.R. Cheesman, F.A. Walker, Low temperature MCD studies of low spin ferric complexes of tetramesitylporphyrinate: evidence for the novel \((d_{2}z_{2}d_{y}d_{x}y)^{1}\) ground state which models the spectroscopic properties of heme d, J. Am. Chem. Soc. 118 (1996) 7373–7380.


26. V. Chepuri, L. Lemieux, D.C. Au, R.B. Gennis, The sequence of the cyo operon indicates substantial structural similarities between the cytochrome o ubiquinol oxidase of Escherichia coli and the aa3-type family of cytochrome c oxidases, J. Biol. Chem. 265 (1990) 11185–11192.


[56] K.E. Hill, D.C. Wharton, Reconstitution of the apoenzyme of cytochrome oxidase from *Pseudomonas aeruginosa* with heme *d* and other heme groups, J. Biol. Chem. 253 (1978) 489–495.


N.J. Wattmough et al. / Biochimica et Biophysica Acta 1411 (1999) 456–474


[95] S.J. Park, R.P. Gunsalus, Oxygen, iron, carbon, and superoxide control of the fumarase *fumA* and *fumC* genes of *Es-


