ELSEVIER

Biochimica et Biophysica Acta 1232 (1995) 97–173

BBA Biochimica et Biophysica Acta

Review

Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions

Ben C. Berks [a,b], Stuart J. Ferguson [b,*], James W.B. Moir [a,b], David J. Richardson [a]

[a] Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK
[b] Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK

Received 25 April 1995; accepted 12 May 1995

Keywords: Enzyme; Respiration; Oxyanion reduction; Nitrogen oxide reduction; Electron transport; Denitrification

Contents

1. Introduction 98

2. The organisation and bioenergetics of electron transport pathways to reductases for nitrate, nitrite, nitric oxide and nitrous oxide 98
   2.1. *Paracoccus denitrificans* 98
   2.2. *Escherichia coli* 102
   2.3. *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* 103
   2.4. Other organisms 104

3. Regulation of N-oxide respiration 104
   3.1. Regulation of gene expression 104
   3.2. Regulation of electron transport and substrate transport 106

4. Roles of dissimilatory nitrogen oxide reduction 106
   4.1. Photo-respiration of nitrogen oxides 106
   4.2. Aerobic nitrogen oxide respiration 108
   4.3. Resistance to toxic nitrogen oxides 111

5. Respiratory nitrate reductases 113
   5.1. Periplasmic respiratory nitrate reductase 113
   5.2. Eubacterial assimilatory nitrate reductase 118
   5.3. Membrane-bound respiratory nitrate reductase 121
   5.4. Structure/function analysis of the membrane-bound, periplasmic and eubacterial assimilatory nitrate reductases 122
   5.5. Nitrite oxidase 130

6. Transport of nitrate and nitrite across the cytoplasmic membrane 130
   6.1. NarK and nitrite efflux 131
   6.2. Nitrate transport 131

Abbreviations: CD, circular dichroism; DDC, diethyldithiocarbamate; DCCD, *N,N'*-dicyclohexylcarbodiimide; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; ESE, electron spin echo; ESEEM, electron spin echo envelope modulation; EXAFS, extended X-ray absorption fine structure; HQNO, 2-n-heptyl-4-hydroxyquinoline *N*-oxide; MCD, magnetic circular dichroism; MGD, molybdopterin guanine dinucleotide; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium; Δp, protonmotive force (proton electrochemical gradient in units of mV); PHB, poly(3-hydroxybutyrate); PMS, phenazine methosulfate (*N*-methyldibenzopyrazine methyl sulfate); PES, phenazine ethosulfate (*N*-methyldibenzopyrazine ethyl sulfate) (U)Q, (ubi)quinone; (U)QH_2, (ubi)quinol. MQ/MQH_2, menoquinone/menoquinol.

* Corresponding author: Fax: +44 865 275259.

0005-2728/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0005-2728(95)00092-5

7. Nitrite reductases 132
7.1. Energy-conserving bacterial nitrite reductase systems 132
7.2. Cytochrome cd_1 nitrite reductase 134
7.3. The copper nitrite reductase 140
7.4. The cytochrome c nitrite reductase 143

8. Nitric oxide reductase 144
8.1. Nitric oxide as a freely diffusible intermediate in denitrification 144
8.2. The molecular properties of the nitric oxide reductase 144
8.3. The catalytic mechanism 146
8.4. Is the nitric oxide reductase a proton pump? 146

9. Nitrous oxide reductase 147
9.1. Molecular properties 147
9.2. Catalytic properties 148
9.3. The electron transfer (Cu_A) centre 148
9.4. The catalytic site (Cu_Z centre) 150
9.5. Reduction of nitrous oxide 152
9.6. The nature of electron transfer between the Cu_A and Cu_Z centres 153
9.7. Biosynthesis of nitrous oxide reductase 153

10. Concluding remarks 154

11. Note added in proof 154

Acknowledgement 155

References 155

1. Introduction

Nitrogen is present in the environment in a number of oxidation states (Fig. 1). Interconversion between these oxidation states is predominantly biological (Fig. 1). In this review we consider those parts of the nitrogen cycle that involve the reduction of nitrogen oxyanions and nitrogen oxides (henceforth collectively termed N-oxides) by respiratory electron transport chains. Respiratory reduction of nitrate to dinitrogen via nitrite, nitric oxide and nitrous oxide is termed denitrification (Fig. 1). This metabolism is phylogenetically widespread in the Eubacteria and has also been identified in both kingdoms of the Archaea [572a,615]. Respiratory reduction of nitrite to ammonium (Fig. 1) is common in bacteria with a fermentative metabolism. In Section 2 we examine the organisation and bioenergetics of the electron transport systems involved in N-oxide respiration. In Sections 3 and 4 we consider the role of such systems in bacterial physiology and how N-oxide respiration interacts with other modes of cellular metabolism. The N-oxide reductases bind a wide range of redox-active metallocofactors: molybdopterin, mononuclear and dinuclear copper centres, iron porphyrins, iron-sulfur clusters and probably mononuclear non-haem iron. In Sections 5, 7, 8 and 9 we examine these reductases emphasising in particular the insights to be gained by comparing each enzyme with structurally related proteins binding the same metallocofactors. In Section 6 we consider how extracellular nitrate reaches its intracellular site of reduction and how the toxic product, nitrite, is transported back to the external environment. A number of recent reviews have dealt with individual aspects of N-oxide respiration [91,120,121,168] [170,233,302,307,524,530,532,544,615] [616], but here we give an integrated, critical, account focusing on advances that have taken place in the last 5 years. Literature coverage is to February 1995.

2. The organisation and bioenergetics of electron transport pathways to reductases for nitrate, nitrite, nitric oxide and nitrous oxide

Details of the electron transport pathways involved in N-oxide respiration are available for only a very limited number of organisms. Here we examine electron transport to N-oxide reductases in *Paracoccus denitrificans* (α Proteobacterium) a free-living denitrifying organism, *Escherichia coli* (γ Proteobacterium) an enteric bacterium that reduces nitrate to ammonium, and the closely related photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* (α Proteobacteria) which utilise N-oxides as auxiliary electron acceptors. We then consider how electron transport pathways in other organisms may differ from these examples.

2.1. *Paracoccus denitrificans*

P. denitrificans is a facultative aerobe with a versatile metabolism that includes the ability to grow anaerobically

using the reactions of denitrification [531]. Another extensively studied bacterium, *Thiosphaera pantotropha*, has recently been suggested to be a strain of *P. denitrificans* (*P. denitrificans* GB17 [336a,463]). For the purposes of this review, although we assume that these bacteria are identical in all aspects except where proven otherwise, we retain the *T. pantotropha* designation for the GB17 strain to avoid confusion when citing the literature on the organism. During anaerobic denitrifying growth *P. denitrificans* employs a standard tricarboxylic acid cycle. Thus, the electron donors to the electron transport chain are primarily NADH and succinate. The cell membrane contains electron transport components which are in many respects similar to the classical complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase) and III (ubiquinol-cytochrome *c* oxidoreductase [cytochrome bc_1 complex]) of the mitochondrial respiratory chain (Fig. 2). The reductases for nitrite and nitrous oxide are soluble periplasmic proteins, while the nitric oxide reductase and membrane-bound nitrate reductase are integral membrane proteins (Fig. 2).

The membrane-bound nitrate reductase receives electrons directly from the ubiquinol pool in an electron transfer process that is coupled to the generation of a transmembrane proton electrochemical gradient [410,412]. All available data are consistent with a model [63,273a] of energy coupling in which ubiquinol (UQH_2) is oxidised at the periplasmic face of the cytoplasmic membrane. The two protons produced are released into the periplasmic compartment, while the two electrons are transported through the protein across the membrane to the site of nitrate reduction in the cytoplasm, resulting in a separation of positive and negative charge and a $H^+\uparrow:2e^-$ of two (Fig. 2). The molecular details of this mechanism are considered further in Section 5.4. It should be noted that the overall energy conservation in the nitrate reductase reaction may differ from $2H^+\uparrow:2e^-$ because of the need to take into account the energetics of nitrate movement into, and nitrite egress from, the cell. This topic is explored further in Section 6.

In contrast to the membrane-bound nitrate reductase, electrons from ubiquinol are delivered to the nitrite, nitric oxide and nitrous oxide reductase via the cytochrome bc_1 complex and periplasmic electron transport proteins (Fig. 2). The involvement of the cytochrome bc_1 complex in the transfer of electrons from UQH_2 to nitrite, nitric oxide and nitrous oxide contributes to the generation of a protonmotive force associated with these reactions. Delivery of a pair of electrons by the cytochrome bc_1 complex from UQH_2 to the periplasmic electron transfer proteins results in the net translocation of two protons from the cytoplasm to the periplasm; this is the same stoichiometry as for reduction of nitrate (Fig. 2; further details of the mechanism of the cytochrome bc_1 complex see [390]). For all

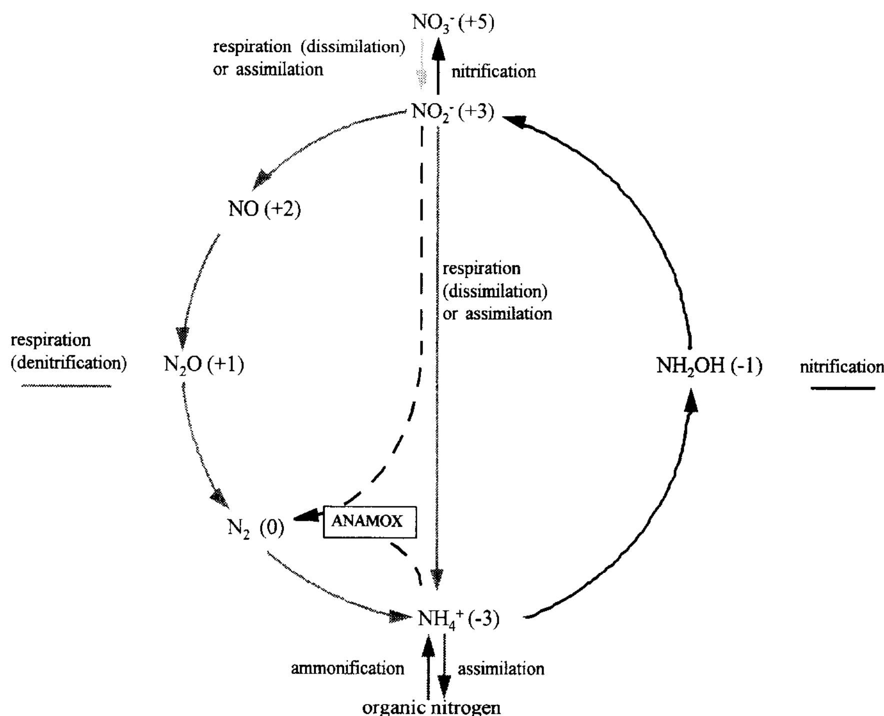


Fig. 1. The nitrogen cycle. The oxidation state of nitrogen is given in brackets for each intermediate. The terms respiration and dissimilation are often used interchangeably. Both processes involve energy conserving electron transport pathways, but dissimilation has historically been used to define nitrate and nitrite reduction that leads to production of ammonia rather than nitrogen oxides. ANAMOX, ANaerobic AMmonia OXidation is an as yet poorly characterised process in which nitrite and ammonia combine to produce dinitrogen and water [447a]. The oxidation of ammonia to nitrite, via hydroxylamine and the oxidation of nitrite to nitrate are both considered reactions of nitrification but have never been found together in a single bacterial species.

four N – oxides the stoichiometry of net proton translocation ($2H^+ \uparrow:2e^-$) in *P. denitrificans* is less than occurs in this organism when ubiquinol is oxidised by oxygen via the cytochrome bc_1 complex and cytochrome aa_3 oxidase ($6H^+ \uparrow:2e^-$). This is because the cytochrome aa_3 oxidase is not only a proton pump, but also effectively brings the electrons back to the cytoplasmic surface of the membrane where they combine with oxygen and protons [390]. (Note that this description of the energetics of nitric oxide reduction assumes that nitric oxide is reduced at the periplasmic side of the membrane and that the nitric oxide reductase is not a proton pump. The evidence for these assumptions is given in Section 8.)

The cytochrome bc_1 complex is obligatory for nitrite, nitric oxide and nitrous oxide reduction [14,98,380]. In contrast, no single periplasmic electron carrier has been shown to be essential for these processes. *P. denitrificans* possesses a periplasmic cytochrome *c*-550 that is structurally related to mitochondrial cytochrome *c*. As the expression of cytochrome *c*-550 is markedly increased during growth under denitrifying conditions, it had seemed likely that cytochrome *c*-550 would be involved in electron transfer between the bc_1 complex and the terminal reductases. However, disruption of the gene coding for cytochrome *c*-550 is not accompanied by any dramatic change in the rate of electron transport to the cytochrome bc_1-dependent reductases involved in denitrification [380,381,564]. This demonstrated that cytochrome *c*-550 is

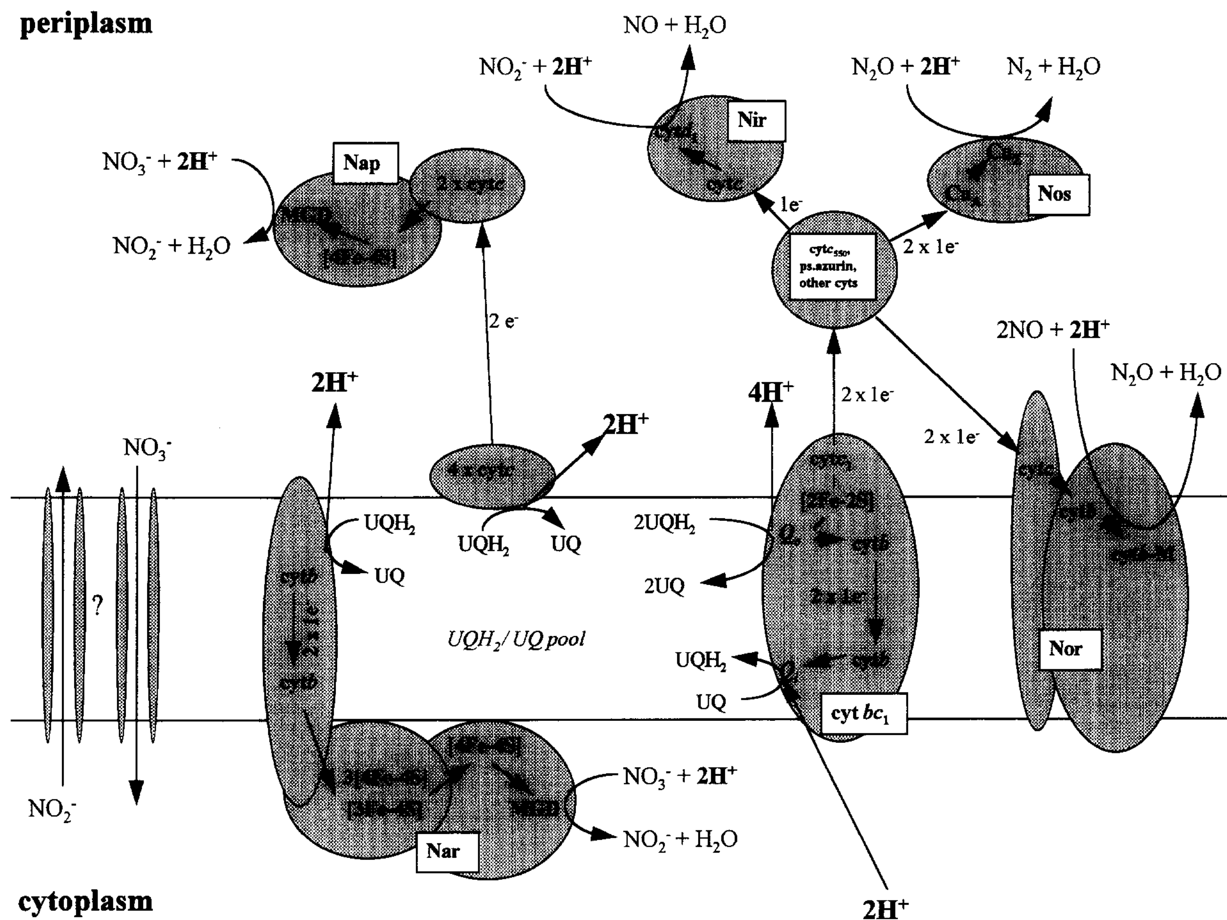


Fig. 2. Organisation of the electron transport system from ubiquinol to the reductases for nitrate, nitrite, nitric oxide and nitrous oxide in *Paracoccus denitrificans*. Ubiquinone is reduced by NADH and succinate dehydrogenases (see Fig. 4). The flow of two electrons from ubiquinol to nitrate via the membrane-bound nitrate reductase involves the net movement of two positive charges out of the cell, the same stoichiometry as associated with electron movement to nitrite, nitric oxide and nitrous oxide reductases. (Note, however, that the organisation and absence of proton pumping ability of nitric oxide reductase is not yet fully confirmed, see Section 8.4). The mechanism of electron transfer and net proton translocation catalysed by the cytochrome bc_1 complex is best described by a Q cycle mechanism (see, for example, [390] for more detail). This involves the oxidation of two molecules of ubiquinol at the Q_o site, with the accompanying release of four protons into the periplasm and the reduction of one molecule of ubiquinone at the Q_i site with the uptake of two protons from the cytoplasm. The pathway of electrons from ubiquinol to the periplasmic nitrate reductase is not fully elucidated (see Sections 2.1, 2.3 and 6.1). The nature of the nitrate and nitrite porter systems has not yet been conclusively established (see Section 6). The M in the nitric oxide reductase denotes a metal, probably iron (Section 8.2). It should be noted that two electrons per nitrogen are consumed in the reduction of nitrate to nitrite but only one electron per nitrogen in each of the reductions of nitrite, nitric oxide and nitrous oxide. Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; cyt bc_1, cytochrome bc_1 complex; ps. azurin, pseudoazurin.

not an obligatory component of these electron transport chains. Electron transport to the reductases in the c-550$^-$ mutant, but not the wild-type, is essentially abolished by the copper chelator diethyldithiocarbamate (DDC) [381]. This observation strongly suggest that electrons are carried between the bc_1 complex and the reductases by both cytochrome c-550 and one or more copper proteins. The copper protein is likely to be pseudoazurin, which in *T. pantotropha* is only expressed at high levels during anaerobic denitrifying growth [380]. Purified *T. pantotropha* pseudoazurin has been shown to act as electron donor to nitrite and nitrous oxide reductases in vitro, and to readily lose its copper atom to DDC [61,382]. Substitution of a c-type cytochrome by a copper protein has precedent both in methylotrophic bacteria [28] and algae [414,595].

Consideration of the dentrification systems reveals that the topological organisation of the electron transport system has to be elucidated in order to understand energy conservation. The standard reduction potentials ($E^{o\prime}$) for the four N-oxide couples are: nitrate/nitrite +420 mV, nitrite/nitric oxide +375 mV, nitric oxide/nitrous oxide +1175 mV and nitrous oxide/nitrogen +1355 mV. The $E^{o\prime}$ for oxygen/water is +800 mV. Consideration of these values in isolation indicates that the free energy change associated with nitric oxide and nitrous oxide reduction by NADH ($E^{o\prime}$ NAD^+/NADH = −320 mV) is greater than with oxygen, yet the topological organisation of the electron transport chain results in a greater conservation of this energy by proton translocation when oxygen is reduced.

P. denitrificans has recently been shown to synthesise a

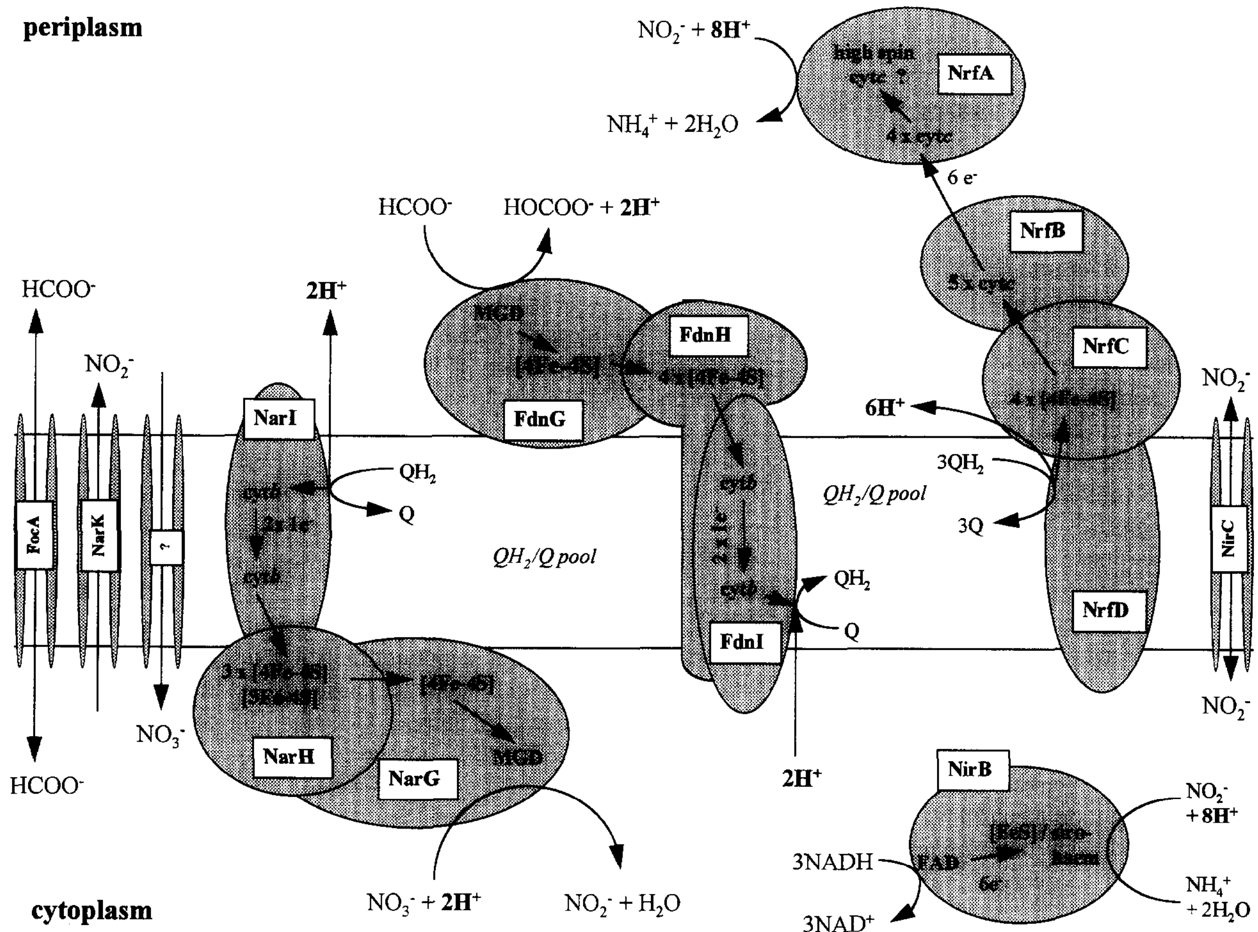


Fig. 3. Suggested organisation of the electron transport system from formate to nitrate or nitrite in *Escherichia coli*. During anaerobic growth of *E. coli* with nitrate as electron acceptor, menaquinone is the major membrane quinone and formate, via the membrane-bound nitrate-inducible formate dehydrogenase-N (FdnGHI), the major electron donor to the quinone pool. Formate, produced by cytoplasmic pyruvate-formate lyase, is probably transported to its site of oxidation in the periplasm by the *focA* gene product (Section 6.2). The major membrane-bound nitrate reductase (NarGHI; Section 5.3) is essentially the same enzyme found in *P. denitrificans* (Section 2.1; Fig. 2). Oxidation of quinol by this nitrate reductase results in the generation of Δp by a redox loop mechanism. Reduction of quinone by FdnGHI likewise generates Δp and, as shown in the figure, a redox loop mechanism has also been proposed for the coupling mechanism of this enzyme [63]. The electron transport pathway by which nitrite is reduced by menaquinol using the cytochrome c nitrite reductase (NrfA) is speculative. Details of the interpretation shown can be found in Section 7.4.2. NarK is a nitrite exporter (Section 6.1) and NirC may also be a nitrite transporter (Section 6.2). NirB is the cytoplasmic sirohaem nitrite reductase. There is uncertainty about the nature of the iron-sulfur centre(s) bound by this enzyme; both [4Fe-4S] and [2Fe-2S] may be present. Not shown in the figure are the minor membrane-bound nitrate reductase (NarZYV), the periplasmic nitrate reductase (Nap) system (Section 5.1) or minor alternative electron donors to the quinone pool.

second type of respiratory nitrate reductase, located in the periplasmic compartment and which is expressed during aerobic growth (Section 5.1) [[51,62,481] (note that most of the work on this enzyme has been with the system from *T. pantotropha*)]. This enzyme is thought to receive electrons from the quinol pool by a cytochrome bc_1 complex-independent route and our current view is that ubiquinol oxidation may not be associated with the generation of a protonmotive force (Δp). In our model (Fig. 2) UQH_2 is oxidised at the periplasmic face of the cytoplasmic membrane with the two protons released in the periplasm. Electrons also pass into the periplasm and, together with two protons, are used to reduce nitrate. Thus there is no charge separation associated with this route of electron transfer. This model requires that any putative component acting as a quinol oxidase before the periplasmic nitrate reductase does not function as a proton pump. These suggestions mean that electron transport to the periplasmic nitrate reductase is not energy conserving if electrons originate from succinate, but if electrons originate from NADH the primary dehydrogenase will provide a coupled electron transfer process.

2.2. *Escherichia coli*

The enteric bacterium *E. coli* is able to use nitrate and nitrite as electron acceptors during anaerobic growth. In the presence of nitrate, oxidation of the carbon source proceeds only as far as the conversion of pyruvate to acetyl-CoA and carbon dioxide. The principal electron donor to the electron transport chain is believed to be the formate produced when pyruvate is converted to acetyl-CoA by pyruvate-formate lyase [524]. The electron transport chain comprises a nitrate-inducible membrane-bound formate-menaquinone oxidoreductase (formate dehydrogenase-N; FdnGHI) and a menaquinol-nitrate oxidoreductase (membrane-bound nitrate reductase-A; NarGHI) (Fig. 3). The membrane-bound nitrate reductase is essentially the same enzyme found in *P. denitrificans* and so generates a protonmotive force of $2H^+ \uparrow:2e^-$. The formate dehydrogenase is also energy conserving ($2H^+ \uparrow:2e^-$) probably, like the membrane-bound nitrate reductase, by means of a redox loop mechanism (63; Fig. 3). An alternative mode of anaerobic metabolism of pyruvate, involving direct conversion to acetyl-CoA and carbon dioxide by pyruvate dehydrogenase, can also be important when cells are respiring nitrate [280]. In this reaction reductant is conserved as NADH which is presumably used to reduce the quinone pool via the two NADH-quinone oxidoreductases [585].

Electron donors other than formate and NADH (e.g., glycerol 3-phosphate, D-lactate and molecular hydrogen) could, in principle, act as alternative electron donors to the menaquinone pool and thus the membrane-bound nitrate reductase. However, at least in the case of molecular hydrogen as sole electron donor, this has been shown not to occur [365]. It is also notable that the typical aerobic metabolites L-lactate and succinate have been reported to support respiration with nitrate but not with other anaerobic electron acceptors [258]. This could be a reflection of the ability of the membrane-bound nitrate reductase to use ubiquinol as well as menaquinol as electron donor [418,579], thus allowing the use of ubiquinone-linked dehydrogenases in the electron transport pathway.

E. coli expresses isozymes of both NarGHI (nitrate reductase-Z; NarZYV, and FdnGHI (formate dehydrogenase-O; FdoGHI), constitutively at low levels [74,254,417,418]. These isozymes may assist in the transition from aerobic to anaerobic growth. Recent DNA sequencing shows that *E. coli* possesses a periplasmic nitrate reductase system (Section 5.1). The physiological role of this system is currently unclear.

The nitrite produced in the cytoplasm by the action of the membrane- bound nitrate reductase may be electrogenically exported from the cell by the NarK protein (see Section 6). Alternatively, under most anaerobic growth conditions a substantial amount of this nitrite is reduced to ammonia by a cytoplasmic sirohaem-containing NADH-nitrite oxidoreductase (Section 7.1.1). This reaction is not linked to energy conservation and does not function primarily in nitrite assimilation. Extracellular nitrite may also be reduced to ammonia by a periplasmic respiratory cytochrome-*c* nitrite reductase (Section 7.4). This enzyme system, which is never expressed to very high levels, would appear to be synthesised only as nitrate levels fall [405,553,553a]. The cytochrome-*c* nitrite reductase pathway probably receives its electrons from the menaquinol pool (Fig. 3). The proteins involved in this electron transport pathway are discussed in Section 7.4.2. It is notable that formate is able to donate electrons to the cytochrome-*c* nitrite reductase not just via the membrane-bound formate dehydrogenases but also via FdhF [139], the formate dehydrogenase component of the formate hydrogenlyase pathway (FdhF is not normally a donor to the membrane-bound nitrate reductase because its synthesis is indirectly repressed by nitrate; [453a]). The formate hydrogenlyase pathway does not involve the quinol pool [579]. As the membrane-bound formate dehydrogenases which are quinone reductases act as electron donors to the cytochrome-*c* nitrite reductase this suggests that either: (i) FdhF can donate electrons to an alternative electron acceptor complex that is a menaquinone reductase (one candidate would be the complex described in [17]) or (ii) the hydrogen evolved by the formate hydrogenlyase system is used to reduce the quinone pool via one of the quinone-linked uptake hydrogenases (hydrogenase-1 or -2).

E. coli and other enteric bacteria can emit nitric oxide, nitrous oxide and dinitrogen during anaerobic metabolism in nitrate- or nitrite-containing medium [77a,91a,263a]. This can be ascribed to one or a combination of: (i) the non-specific action of the membrane-bound nitrate reductase which may be able to act as a nitrite reductase

releasing gaseous N-oxide as products [263a,470a,500a]; (ii) chemodenitrification, whereby nitrite is reduced to nitric and nitrous oxide by Fe^{2+} [91a] which in some cases is a product of dissimilatory Fe^{3+} reduction [334a] and (iii) the activity of a non-specific and as yet poorly characterised nitrous oxide reductase (K_m $N_2O > 3$ mM) [283a].

2.3. Rhodobacter capsulatus and Rhodobacter sphaeroides

The photosynthetic bacteria *R. capsulatus* and *R. sphaeroides* are phylogenetically very closely related to *P. denitrificans*. During anaerobic photosynthetic growth a protonmotive force is generated by cyclic electron transfer in which the light-activated photosynthetic reaction centre reduces ubiquinol ($2H^+ \uparrow : 2e^-$). Ubiquinol is reoxidised by a cytochrome bc_1 complex ($2H^+ \uparrow : 2e^-$) which returns the electrons to the reaction centre via various electron transport proteins (Fig. 4) [390]. The ubiquinol/ubiquinone (UQH_2/UQ) pool links cyclic electron transport to other electron transport chains including those terminating in N-oxide reductases (Fig. 4) [170a,361,459,556]. The role of these reductases during photosynthesis is discussed in Section 4.1.

There is significant variation in the reductases present

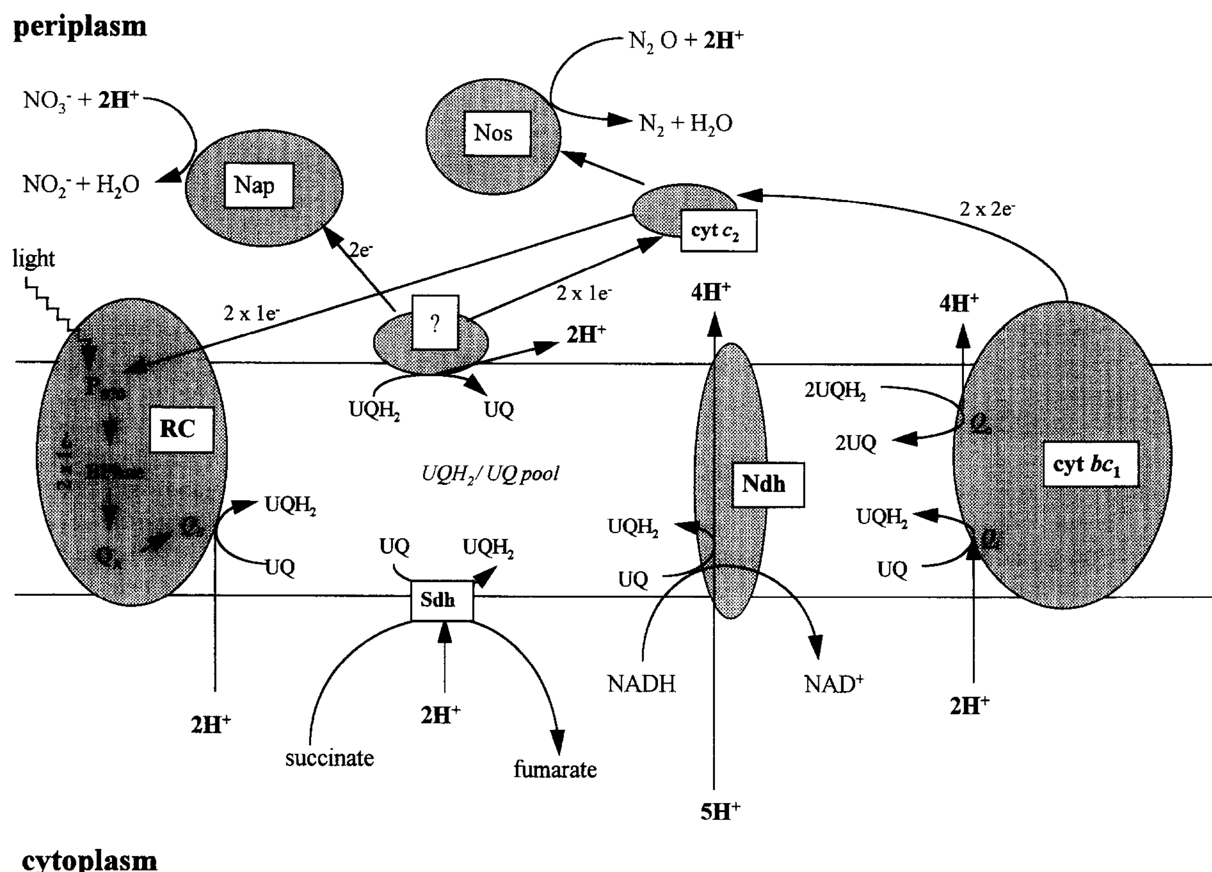


Fig. 4. The interaction between the cyclic photosynthetic electron transport pathway and the pathways of electron transport to nitrate and nitrous oxide in *Rhodobacter capsulatus*. In this scheme the photosynthetic electron transport pathway comprises the reaction centre and the cytochrome bc_1 complex, both intrinsic membrane proteins, a pool of water soluble periplasmic cytochrome c_2 and a pool of lypophilic ubiquinone/ubiquinol that can diffuse freely in the membrane bilayer. Light-driven oxidation of P_{870} (a pair of bacteriochlorophyl molecules) leads within the reaction centre to the sequential reduction of ubiquinone at two distinct binding sites (the Q_A and Q_B sites). P_{870} is subsequently re-reduced by ferro cytochrome c_2. The net result is a charge separation across the cytoplasmic membrane that contributes to the generation of Δp. Ubiquinol is released from the Q_B site and diffuses to the Q_o site of the cytochrome bc_1 complex. The oxidation of two molecules of ubiquinol at this site, the reduction of one molecule of ubiquinone at the Q_i site and the reduction of two molecules of cytochrome c_2 comprise the complete protonmotive turnover of the cytochrome bc_1 complex (see Fig. 2 for more details). The poise of the cyclic photosynthetic electron transport pathway can be disturbed by electron input into the ubiquinone pool by primary dehydrogenases, such as the NADH and succinate dehydrogenases, or the fatty acyl-CoA dehydrogenase-linked electron-transferring flavoprotein (not shown). Ubiquinol can be oxidised to repoise the cyclic photosynthetic electron transport pathway by the passage of electrons to the nitrate and nitrous oxide reductases as described in Section 4.1. Electrons pass to the periplasmic nitrate reductase independently of the cytochrome bc_1 complex and cytochrome c_2. Electrons can pass to the nitrous oxide reductase via cytochrome bc_1 complex-dependent or independent pathways. In either case electron transfer is dependent on the presence of cytochrome c_2. A membrane-bound *b*-type cytochrome (shown as ? in the figure) may serve as a common quinol oxidase for the cytochrome bc_1 complex independent pathways to nitrate and nitrous oxide. A membrane-bound cytochrome variously termed cyt c_y or c_x [262,272](not shown in the figure) can replace cytochrome c_2 in cyclic photosynthetic electron transfer and perhaps in nitric oxide reduction, but not in nitrous oxide reduction. For more details of the redox centres in Nap, Nos and cyt bc_1 see Fig. 2. The nitric oxide reductase and nitrite reductase have been omitted. RC, Reaction Centre; Sdh, succinate dehydrogenase; Ndh, NADH dehydrogenase.

between different strains. All strains of *R. capsulatus* tested possess nitric oxide reductase activity [52] and most have nitrous oxide reductase [358]. When present, the nitrate reductase is normally the periplasmic enzyme, but the membrane-bound enzyme can be present and one strain of *R. sphaeroides* can express both types [35,94]. Only one strain of *R. capsulatus* and three strains of *R. sphaeroides* are reported to be able to completely denitrify nitrate to dinitrogen. All four denitrifying strains possess a membrane-bound nitrate reductase and are the only strains known to have a nitrite reductase [373,435a,470,471].

In the case of nitrous oxide and nitric oxide reduction, the cytochrome bc_1 complex is implicated in the delivery of electrons, since the cytochrome bc_1-specific inhibitor myxothiazol inhibits electron flow [52,434]. In contrast to observations with *P. denitrificans* [412] such inhibition is never complete and a mutant lacking a functional cytochrome bc_1 complex can still reduce both nitrogen oxides, albeit at lower rates than the parent strain [52,434]. These observations indicate that there must be an alternative quinol oxidase that provides a by-pass around the cytochrome bc_1 complex (Fig. 4). It is also found that cytochrome c_2 is dispensable for nitric oxide reduction in *R. capsulatus*, suggesting that, as with the equivalent cytochrome *c*-550 in *P. denitrificans*, a missing periplasmic electron transport protein can be replaced by another protein [52]. This redox protein may plausibly be the membrane-anchored cytochrome c_Y/c_X that substitutes for cytochrome c_2 in cyclic electron flow [252,272]. While cytochrome c_2 is not obligatory for nitric oxide reduction, lack of cytochrome c_2 in *R. capsulatus* prevents physiological electron transport to the nitrous oxide reductase [436].

2.4. Other organisms

There are no detailed biochemical studies of the organisation of electron transport systems to N-oxides in organisms other than *P. denitrificans, E. coli, R. capsulatus* and *R. sphaeroides*. The organisation of some systems can be inferred from identification of genes encoding redox proteins that cluster with structural genes for the N-oxide reductase (see for instance the *nir* and *nap* operons discussed in Sections 7.2 and 5.1). There are indications (mostly derived from the use of specific inhibitors) that the cytochrome bc_1 complex is widespread in denitrifiers [311] and thus is probably widely involved in transferring electrons from quinols to both the periplasmic nitrite and nitrous oxide reductases and the membrane-bound nitric oxide reductase, in each case via a connection provided by *c*-type cytochromes or cupredoxins. However, the identification of a number of novel quinol oxidising systems is also anticipated (see Sections 5.1.3 and 5.1.4). In Gram-positive organisms which, by definition, lack a periplasm it is probable that the 'periplasmic enzymes' will be anchored to the cytoplasmic membrane (either by a polypeptide chain or covalently attached lipid) but that their redox centres will be outside the confines of the bilayer [167,169]. This appears to be the case for the copper nitrite reductase isolated from *Bacillus halodenitrificans* [146]. However, Urata and Satoh [559] have argued that in *Bacillus firmus* the dissimilatory nitrite reductase is membrane-bound with its active site facing the cytoplasm. This conclusion was reached from measurements of proton uptake, an approach that can be ambiguous. Nitrate reductases have been purified from Gram-positive organisms, e.g., *Bacillus licheniformis*, and as expected have a similar organisation to those of the membrane-bound enzymes of Gram-negative bacteria [566]. Membrane-bound nitric oxide reductases are also expected to occur in Gram-positive organisms although their presence has not been directly proved; it is notable that there are currently rather few examples of Gram-positive bacteria that can denitrify nitrate to dinitrogen [167,168].

Until recently it has been the general view that respiratory (or dissimilatory) N-oxide reduction is restricted to prokaryotes. One eukaryotic organism, the protozoan *Loxodes*, was reported in 1983 [171] to be capable of dissimilatory nitrate reduction with the activity possibly associated with the mitochondria. No further information about this system has been forthcoming. More recently it has been shown that the fungus *Fusarium oxysporum* can reduce nitrate to nitrous oxide [491–493]. It is notable that the system is repressed by aeration but not by ammonium, suggesting a role in anaerobic metabolism [491,493]. The fungal system involves a nitrate- and nitrite-inducible cytochrome *P*-450 nitric oxide reductase termed P-450nor [386] that shows highest amino acid sequence similarity to bacterial rather than eukaryotic *P*-450 molecules. A blue copper protein that may also be involved in this fungal denitrification has been tentatively identified as a nitrite reductase [386]. It is not yet clear how any of the denitrification reactions are associated with energy conservation. One other eukaryote, the fungus *Neurospora crassa*, may also, according to an old report [389], catalyse the dissimilatory reduction of nitrate to nitrite, but there is no indication that it can further reduce the nitrite to gaseous nitrogen oxides or nitrogen.

3. Regulation of N-oxide respiration

3.1. Regulation of gene expression

The presence of both oxygen and N-oxides during growth of bacteria can regulate expression and in vivo activity of the N-oxide reductases [14,130,135,296]. In general, the enzymes are not expressed under aerobic conditions [295,296] (but see also Section 4.2 on aerobic denitrification) ensuring that respiration of oxygen, which is highly coupled, occurs in preference to N-oxide respiration.

Much has been learned concerning the expression of N-oxide reductase genes in *E. coli* (reviewed in [216,218,257,323,342,516–518]), but studies in other organisms are much less advanced. In *E. coli* the transcriptional modulator Fnr has a central role in regulating the expression of genes, including the nitrate and nitrite reductases, involved in anaerobic metabolism [517,554]. Four cysteine residues, three at the extreme N-terminus of the protein, are conserved between *E. coli* Fnr and probable Fnr homologues in other bacteria [38,516]. Site-directed mutations of these residues in the *E. coli* protein has demonstrated that these cysteines are essential for Fnr function [487]. Iron is also required for Fnr activity ([208,209] and references therein) and is thought to be ligated by the conserved cysteine residues [547]. However, the in vivo activity of Fnr does not appear to be regulated by intracellular iron levels [391]. Instead, the bound iron atom is suggested to be involved in sensing such that when it is in the ferrous state the protein activates the transcription of appropriate genes. Fnr appears not to sense oxygen directly but may instead respond to some indicator of the cell redox state [554,554a]. This could explain the expression of some putative Fnr-regulated genes in denitrifying bacteria under aerobic conditions (Section 4.2). As judged by sequence comparisons, the presumed DNA binding domain of Fnr is likely to be structurally very similar to that of the *E. coli* catabolite activator protein (Cap). Further, both proteins have similar target DNA binding sequences. The *E. coli* Fnr DNA binding motif (TTTGATXXXXATCAAA; 'Fnr box') is found 35 to 50 bases upstream of the transcriptional start point of Fnr-regulated genes. Upon activation, Fnr appears to dimerise [318], possibly allowing cooperative binding of each subunit to one of the two (inverted) repeat sequences in the Fnr box. The DNA-Fnr complex probably activates RNA polymerase by direct physical contacts.

Sequences with similarity to that of the *E. coli* Fnr box have been detected upstream of some genes involved in anaerobic nitrogen oxide respiration in denitrifying bacteria [21–23,135,240,276,392,501,516,606,624], while Fnr-like proteins have been identified in a number of non-*E. coli* organisms including denitrifying bacteria (listed in [516]). In *Pseudomonas aeruginosa* and the denitrifying symbiotic nitrogen-fixing bacterium *Bradyrhizobium japonicum* there is reasonable genetic evidence that these Fnr-like proteins regulate nitrate reductase expression [19,181]. Cuypers and Zumft [136] have recently suggested that the denitrifying bacterium *Pseudomonas stutzeri* possesses two Fnr-like systems, only one of which specifically regulates anaerobic expression of genes involved in denitrification. Two Fnr-like proteins may also be present in the denitrifying bacterium *Shewanella putrefaciens*, one of which is involved in regulating nitrite but not nitrate reductase expression [89,461]. *B. japonicum* is a third denitrifying organism with two Fnr-like proteins [19]. Control of denitrification by anaerobiosis is further complicated in this organism by oxygen regulated expression of the Fnr proteins mediated by the FixLJ system. FixL and FixJ are the sensor and response regulator components of a two component regulatory system. In such systems (for reviews see [408,528]), interaction of a signal molecule with a sensor domain in the sensor protein, activates a kinase domain in the same protein. In the FixLJ system the signal is loss of an oxygen (or possibly nitric oxide) molecule bound to a haem group in FixL [196]. The activated sensor kinase domain phosphorylates a receiver domain in the response regulator. This results in activation of an output domain in the response regulator. For FixJ the response presumably involves interactions with elements in the *fnr* promoter region. FixJL-like systems have not been implicated in the regulation of N-oxide reductase expression in other organisms.

Nitrate and nitrite regulation of gene expression in *E. coli* is by means of two, interacting, two-component regulatory systems, NarXL and NarQP (reviewed in Refs. [218,342,525]). Activation of the membrane-spanning sensor proteins NarX and NarQ is thought to be triggered by the binding of nitrate or nitrite to the periplasmic domains of these proteins. A consensus TACY-KT (Y = C or T, K = A or C) DNA binding site ('NarL-heptamer') has been proposed for the response regulators NarL and NarP the relative affinities of the two response regulators for this site being context dependent [553,553a]. The NarL-heptamer appears variously as a monomer, as a direct or inverted repeat, in either orientation, and between $+20$ and -220 with respect to the regulated gene transcriptional start site. The interactions between nitrate and nitrite, the two sensors and two regulators is complex [423]. An approximate summary of the current hypotheses [423,525] is that nitrate stimulates both sensors to phosphorylate both regulators, that nitrite stimulates NarQ to phosphorylate both regulators and that in the absence of nitrate NarX acts as a NarL-phosphate phosphatase.

In denitrifying organisms nitrate, nitrite, nitric oxide and nitrous oxide have all been reported to act as inducers of at least one of the reductases (e.g., [295,296]). The molecular basis of this regulation is as yet poorly understood. In the case of nitrate and nitrite regulation the operation of a NarLX/NarQP type system is a possibility but such a system has not yet been identified in a denitrifying organism. Studies with both *P. stutzeri* and *P. denitrificans* have suggested that there is some degree of co-regulation of the nitrite and nitric oxide reductases. In *P. denitrificans* mutants that fail to assemble a functional nitrite reductase system are also deficient in nitric oxide reductase activity [142]. This suggests that the nitric oxide reductase may be substrate induced, with the phenotype arising from the failure to synthesise nitric oxide from nitrite [142]. A similar phenotype is also apparent in transposon Tn*5* insertion mutants deficient in nitrite respiration in *P. stutzeri* [622]. Inactivation of the nitric oxide reductase in *P. stutzeri* results in decreased levels of nitrite

reductase and of electron flux to the enzyme in vivo [84]. The nitrite reductase gene clusters of *P. denitrificans, P. stutzeri* and *P. aeruginosa* contain a potential regulatory gene *nirQ* [22,142,277]. NirQ shows sequence similarity to NtrC, the σ^{54} RNA polymerase-specific response regulator of a two-component regulator system. The phenotype of a *P. stutzeri nirQ* mutant is complex but results in lesions of in vivo nitrite and nitric oxide reductase activities [277].

The amount of nitrous oxide reductase expressed in *P. stutzeri* is regulated by nitrate, nitrous oxide and anaerobiosis [296]. Direct sensing of nitrous oxide is a difficult chemical proposition (see Section 9). Because of its high reduction potential, metabolism of nitrous oxide is likely to lead to a relatively oxidised electron transport chain. Nitrous oxide might thus be sensed indirectly by measuring the redox poise of an electron transport chain component. Such considerations might explain why repression of nitrous oxide reductase by oxygen is not as severe or complete as that experienced by the other enzymes of denitrification [61,295,296,380]. On the basis of transposon insertion mutants, *nosR*, a gene divergently transcribed from the nitrous oxide reductase structural gene, is thought to encode a trans-acting activator of nitrous oxide reductase expression [137]. NosR is predicted to be an integral membrane iron-sulfur protein (discussed in more detail in Section 5.1.4).

3.2. Regulation of electron transport and substrate transport

The activities of most of the enzymes of denitrification are reversibly inhibited under aerobic conditions via a variety of mechanisms. It was shown many years ago that the inhibitory effect of oxygen on reduction of nitrate by anaerobically grown *P. denitrificans* [265] does not operate in inside out vesicles, or in cells when the cytoplasmic membrane has been permeabilised to a limited extent with a detergent [12]. As the site of nitrate reduction by the membrane-bound nitrate reductase is in the cytoplasm these observations suggest that the control of nitrate respiration by oxygen is exerted at the level of transport of nitrate across the cytoplasmic membrane (see also Section 6) [12]. This conclusion has since been confirmed with *E. coli* and other organisms [226,393]. A further facet to this control is that it was shown that oxygen itself was not the controlling factor. Both nitrous oxide and hexacyanoferrate mimicked the effect of oxygen, and inhibition of nitrate reduction by either of these two electron acceptors could be attenuated by restricting the flow of electrons to them by blocking the electron transport chain with antimycin [13,14,309]. This suggests that the redox state of an electron transport component, plausibly the quinol/quinone pool, is primarily responsible for regulating the putative nitrate transporter.

Oxygen can also reversibly inhibit respiratory nitrite reduction in *P. denitrificans*. This is a consequence of competition for electrons between the oxidase and nitrite reductase, which itself is active in the presence of oxygen. Such competition arises because there are redox components common to both electron transport pathways (e.g., ubiquinol and cytochrome bc_1) [14]. Similar competition for electrons has also been observed for other N-oxide reductases [14].

4. Roles of dissimilatory nitrogen oxide reduction

The most widely recognised role for dissimilatory N-oxide reduction is anaerobic respiration. Here reduction of the N-oxide provides an alternative means to oxygen respiration for the generation of a protonmotive force of sufficient magnitude, either to facilitate growth and maintenance of the organism on non-fermentable carbon sources or to supplement energy generation during growth on fermentable carbon sources. This role has been well documented elsewhere [168], and some general energetic considerations were discussed in Section 2. It is not, however, the only function of these reactions and in this section some alternative roles for dissimilatory N-oxide reduction will be explored.

4.1. Photo-respiration of nitrogen oxides

A number of members of the Rhodospirallaceae family of photosynthetic bacteria have the capacity to respire N-oxides. These include *R. capsulatus* (Section 2.3), *R. sphaeroides* (Section 2.3) and *Roseobacter denitrificans* [150,538]. In *R. capsulatus*, the reduction of nitrate does not support anaerobic dark growth on non-fermentable carbon substrates. For many years this led to confusion between the processes of respiratory (dissimilatory) and assimilatory nitrate reduction in this organism. However, following confirmation that some strains could couple the reduction of nitrate to the generation of Δp [355,356], it became clear that dissimilatory nitrate reduction is important during photoheterotrophic growth of *R. capsulatus* on reduced carbon substrates [433]. This is also true of nitrous oxide reduction [433], although this process can support growth of the organism under anaerobic dark conditions on non-fermentable carbon sources [358].

4.1.1. Redox balancing during photoheterotrophic growth

Cellular redox balancing is the process by which bacteria utilise electron sinks to dissipate excess reductant generated during photometabolism of a carbon substrate that is more reduced than the average oxidation state of the cell biomass [170a,361,433]. A carbon source that falls into this category is butyrate. Many species of Rhodospirallaceae family are only able to photometabolise butyrate anaerobically in the presence of carbon dioxide. Excess reductant generated through the oxidative photometab-

olism of butyrate is consumed by the reductive fixation of CO_2, resulting in extensive deposition of poly 3-hydroxybutyrate (PHB). However, CO_2 is not likely to be present at high concentrations in all environments in which phototrophs encounter reduced carbon substrates and alternative redox balancing mechanisms must therefore be available. One such mechanism, which has been characterised in detail in *R. capsulatus*, is the reduction of N-oxides. In the absence of CO_2, photoheterotrophic growth of *R. capsulatus* on butyrate can be facilitated through the reduction of nitrate, nitrite, nitric or nitrous oxide [433,435]. This involves the transfer of reductant from the cytoplasm, via the membrane associated electron transfer chain, to periplasmic oxido-reductases responsible for nitrate and nitrous oxide reduction (Fig. 4). Under energy-rich conditions, in the presence of both CO_2 and an N-oxide, reductive CO_2 fixation is the selected mechanism by which NAD^+ is regenerated [431], presumably because it conserves carbon. However, CO_2 fixation also consumes ATP and the choice of mechanism may be different under energy-limited (i.e., light-limited) conditions.

In order for nitrate or nitrous oxide reduction to effectively consume excess reductant, it may be advantageous if the transfer of electrons from ubiquinol to the N-oxide reductases can continue in the presence of a substantial light-dependent Δp. Accordingly, oxidation of ubiquinol by nitrate, via the periplasmic nitrate reductase, is not thought to be coupled to the generation of Δp (Fig. 4, Section 2.1) and will not therefore be subject to thermodynamic back-pressure mediated by light-dependent Δp. This may also be true of the cytochrome bc_1 complex-independent pathway of electron transfer to nitrous oxide discussed in Section 2.3. It is notable that when *R. capsulatus* is grown photoheterotrophically on butyrate, 75% of the total capacity of electron flow to nitrous oxide can proceed independently of the cytochrome bc_1 complex (Richardson, D.J., unpublished observations) compared to only 20% in cells grown on malate, a more oxidised substrate [434].

4.1.2. *Maintenance of the redox poise of the cyclic photosynthetic electron transport pathway*

N-oxide reduction in phototrophs can also regulate the redox poise of the cyclic photosynthetic electron transport pathway. A number of redox components are common to both the cyclic photosynthetic and N-oxide-reducing electron transfer pathways (Fig. 4). Under illuminated conditions, the photosynthetic reaction centre will turn over only if there is a supply of oxidised quinone electron acceptor (Q_A, Q_B and UQ in the UQH_2/UQ pool), whilst the cytochrome bc_1 complex requires the provision of both UQ and UQH_2 (the protonmotive Q-cycle consumes 2 molecules of UQH_2 and 1 molecule of Q; Fig. 2 see [390]). The UQ/UQH_2 ($E^{o'} = +80$ mV) pool can be coupled to low potential electron donors such as NADH ($E^{o'}$ NAD^+/NADH $= -330$ mV). This could lead to extensive reduction of the UQH_2/UQ pool, restricting the rate of cyclic electron transport. During steady-state cyclic electron transfer, a major factor which prevents this is the membrane potential (approximately $+200$ mV) across the coupling site of the NADH dehydrogenase. This would maintain the potentials of the NAD^+/NADH and UQ/UQH_2 couples $+400$ mV apart assuming that four protons are translocated per two electrons transferred from NADH to UQ [390]. Thus, at high light intensities, nitrate respiration by bacteria utilising malate (and therefore predominantly NADH) as electron donor is sensitive to inhibition by the light-dependent Δp [357,433]. However, nitrate is reduced extensively during photoheterotrophic growth on butyrate [433], suggesting that the NADH/NAD^+ couple is sufficiently reduced to transfer electrons to the UQ pool and/or that there is extensive reduction of the UQ-pool via the activity of the quinone-dependent flavoprotein dehydrogenases involved in fatty acid metabolism. In the absence of nitrate, electrons would accumulate in the UQH_2/UQ pool, leading to over-reduction and perturbation of the redox poise of the cyclic photosynthetic electron transport pathway.

The capacity of N-oxides to specifically repoise the cyclic electron transport pathway has been demonstrated in experiments using the redox mediators PMS and PES [359]. Addition of these redox dyes to intact cells of *R. capsulatus* results in the attenuation of cyclic electron transport. This can be restored through addition of either nitrate or nitrous oxide. The rationale for this is that PMS and PES mediate the equilibration of the NADH/NAD^+ and UQ_A redox couples, perturbing the redox poise of the electron transfer system. Photochemistry is restored by addition of nitrogen oxides which, through the activity of the N-oxide reductases, remove electrons from the UQH_2/UQ pool and so repoise the system.

Intermittent periods of darkness or prolonged periods of low light intensity could result in the redox poise of the photosynthetic electron transport system becoming disturbed, even during photometabolism of relatively oxidised carbon sources. Under these conditions the light-dependent protonmotive force could collapse to as low as $+50$ mV, holding the potentials of the NADH/NAD^+ and UQH_2/UQ couples as little as 100 mV apart. The effect of prolonged periods of anaerobic dark incubation, in malate medium, on the redox poise of the electron transfer system of intact cells of *R. capsulatus* has been examined [273]. Trains of flash excitation were utilised to drive single turnovers of the reaction centre in the presence of myxothiazol, which inhibits turnover of the quinol oxidase site of the cytochrome bc_1 complex. Under these conditions quinol generated at the quinone reductase site of the reaction centre cannot be reoxidised by the cytochrome bc_1 complex. However, since there are > 10 molecules of UQ/UQH_2 per reaction centre, if the UQH_2/UQ-pool were fully oxidised sufficient quinone should be available for turnover of the reaction centre throughout a train of at

least 10 flashes. In fact, photooxidation of P_{870} only occurred following the first and second flashes, indicating that the UQ/UQH_2 pool was extensively reduced. If nitrate or nitrous oxide were included during the period of anaerobic incubation, the reaction centre could turnover throughout the train of 10 flashes. This indicated that the UQ/UQH_2 pool was now substantially more oxidised and that by drawing electrons from this pool (Fig. 4) the N-oxide reductases were serving to maintain the optimal redox poise of the cyclic electron transfer pathway during periods of darkness. It is notable that in these experiments reduction of nitrous oxide must have been proceeding via the cytochrome bc_1 complex-independent pathway.

The light intensity on sediment surfaces is probably rarely as high as routinely used for growth of photosynthetic organisms in laboratory cultures. At low light intensities (and therefore low light-dependent protonmotive force), the reduction of nitrate, during growth of *R. capsulatus* on oxidised carbon substrates such as malate and succinate, is more extensive than at high light intensities [431,433]. This may be related to the electron transfer system being more susceptible to overreduction under these conditions.

Perhaps the most striking example of the use of N-oxide reduction as a means of regulating redox poise of the cyclic photosynthetic electron transport chain of purple non-sulfur photosynthetic bacteria can be found in *R. denitrificans* [150,399,538]. Despite having all the components required for a photosynthetically competent protonmotive cyclic electron transfer pathway, this organism cannot grow photosynthetically under anaerobic conditions unless oxidants, such as nitrate and nitrite, are present [399]. The reaction centre complex of *R. denitrificans* is very similar to that of the photosynthetically competent *Rhodobacter* species, with the exception that the redox potential of the primary acceptor quinone (Q_A) is higher ($E^{\alpha'} = +35$ mV compared to -20 mV in *R. sphaeroides*) [399]. Thus the UQH_2/UQ-pool has to be maintained in a very oxidised state (i.e., at a high redox potential) in order for the reaction centre to turnover. The failure to grow phototrophically under anaerobic conditions suggests that the organism is incapable of holding the UQH_2/UQ-pool at a sufficiently high potential in the absence of a respiratory oxidant. This has been confirmed using single flash turnover studies on the photosynthetic electron transport system [538], which have also demonstrated that the system can be repoised by the removal of electrons through reduction of nitrate and nitrite [538].

4.2. Aerobic nitrogen oxide respiration

Until relatively recently it was thought that N-oxide respiration was confined to anaerobic conditions. However, the work carried out by Robertson, Kuenen and colleagues, predominantly with *T. pantotropha* [443,444], has led to the proposal that co-respiration of oxygen and N-oxides can take place [312,445,447]. One point to note at the outset of this discussion is that in *T. pantotropha*, with the exception of nitrate reductase, many, if not all, of the enzymes and electron transfer proteins associated with aerobic denitrification are those that are involved in anaerobic denitrification [60,61,380,382]. We see no reason to believe that this will not be so in other bacterial species. Concerning the nitrate reductase, there is clear evidence that the membrane-bound enzyme is most intimately involved with anaerobic nitrate reduction whilst the periplasmic enzyme is expressed and active in aerobically grown cells [51,53,437]. As discussed in Section 2.1, there is likely to be a greater degree of energy conservation associated with nitrate reduction by the membrane-bound enzyme. It is notable that mutants deficient in this enzyme, but which can express the periplasmic enzyme under anaerobic conditions, have lower growth rates and yields when grown anaerobically with nitrate as electron acceptor [53] (Richardson, D.J., unpublished observations). It makes bioenergetic sense to express the membrane-bound nitrate reductase during anaerobic growth when nitrate respiration is required to be a major generator of Δp. The rationale for expressing a periplasmic nitrate reductase under aerobic conditions is a simple one. As discussed in Section 3.2, oxygen exerts a regulatory effect on nitrate respiration via the membrane-bound nitrate reductase through inhibiting (probably indirectly) nitrate transport across the cytoplasmic membrane to the active site of the enzyme. The location of a nitrate reductase in the periplasm effectively by-passes this site of oxygen inhibition.

4.2.1. Redox balancing and redox poising in chemoheterotrophs

The capacity of a number of different species to catalyse aerobic nitrate respiration has become increasingly widely reported and may indeed be a potential property of all N-oxide respiring bacteria (see for instance a discussion of some 15 species in [447]). Despite this, the physiological role of aerobic nitrogen oxide respiration during chemoheterotrophic metabolism remains somewhat cryptic. Studies with *T. pantotropha* have led Robertson and Kuenen [312,447,448] to suggest that it serves as a mechanism by which reductant can be dissipated to maintain the cellular redox balance of the cell or the redox poise of the electron transport chain. In many ways this role would be similar to that of photo-respiration (Section 4.1) and consistent with ubiquinol oxidation by the aerobically active periplasmic nitrate reductase being energy dissipating rather than energy conserving. A major factor that is likely to influence the destination of electrons entering the respiratory chain from low potential electron donors is the redox state of the QH_2/Q-pool, since this is the only redox component common to both oxygen and all the N-oxide respiratory pathways (Fig. 2).

During chemoheterotrophic growth on reduced carbon sources the carbon substrate must be oxidised to the level

at which it can be assimilated. If this oxidation results in the release of more reductant than needed for the generation of the ATP needed for the metabolism of the carbon, some means must be found of disposing of reductant or the growth rate will be slowed to that allowed by the reoxidation of NADH by cell maintenance reactions. One means of disposing of the reductant is by the membrane-associated electron transport pathways involved in oxygen reduction. However, the combination of a high $NADH/NAD^+$ ratio and the inability of efficiently-coupled pathways (e.g., cytochrome aa_3 oxidase dependent pathways) to operate in the presence of a high steady-state Δp means that maximum rate of utilisation of growth substrate is only possible if electrons are disposed of by relatively uncoupled pathways. Thus the reduction of N-oxides, particularly by the periplasmic nitrate reductase, are more favourable methods of disposing of reductant via respiratory pathways than oxygen reduction. Failure to dispose of electrons via a poorly coupled pathway will result in the QH_2/Q ratio increasing, which will in turn limit turnover of Q-dependent redox proteins such as the NADH dehydrogenase and the cytochrome bc_1 complex. In the case of the NADH

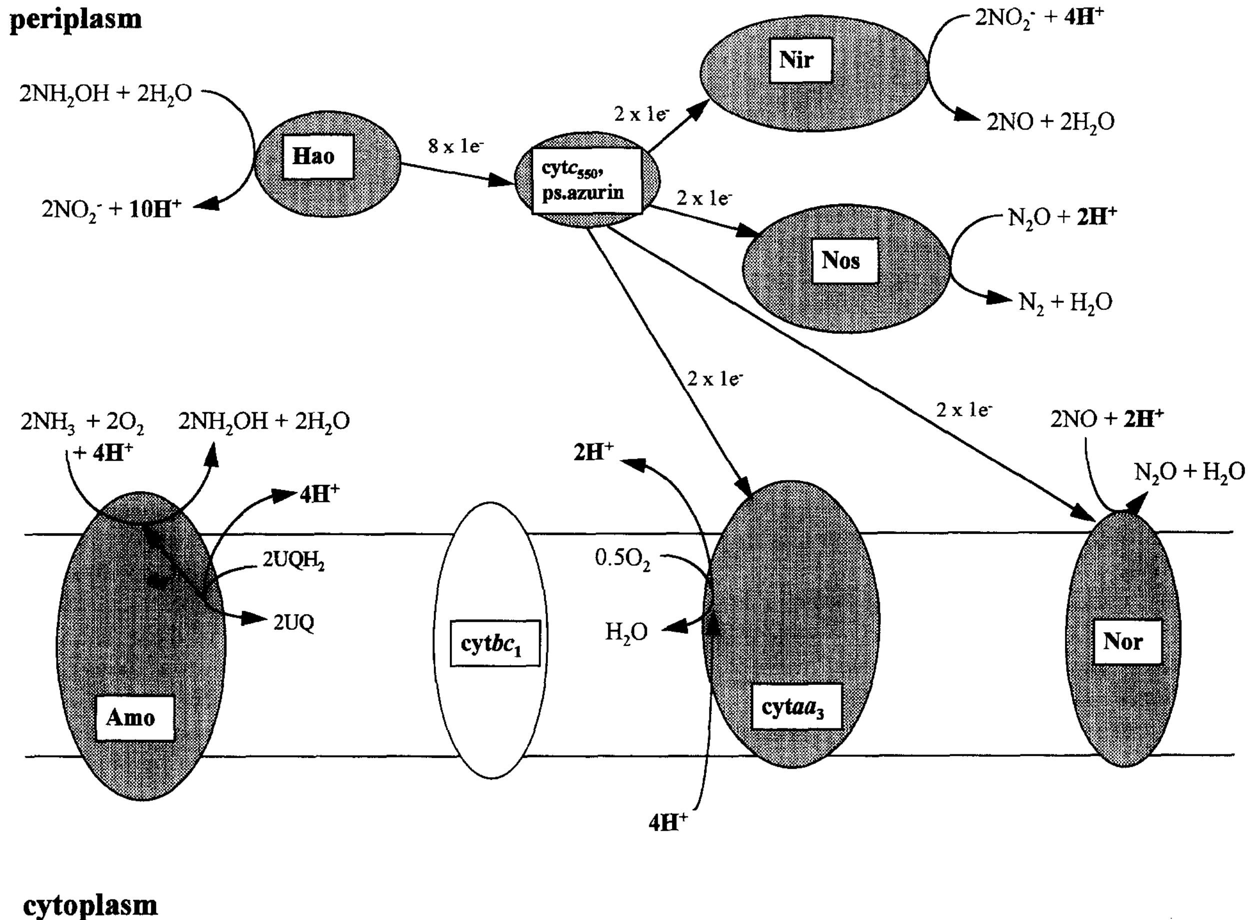


Fig. 5. A model for heterotrophic nitrification coupled to aerobic denitrification in *T. pantotropha*. This figure is adapted from Wehrfritz et al. [584]. In this scheme the oxidation of two molecules of ubiquinol by two turnovers of the ammonia monooxygenase (Amo) is coupled to the generation of two molecules of hydroxylamine from ammonia. The two molecules of hydroxylamine are oxidised to two molecules of nitrite by the hydroxylamine oxidase (Hao) resulting in the generation of eight molecules of reduced cytochrome *c*-550 (or pseudoazurin) and ten protons. Cytochrome *c*-550 and pseudoazurin are also electron donors to the denitrification enzymes and cytochrome aa_3 oxidase. Thus, the turnover of Hao provides the substrates required for complete conversion of two molecules of nitrite to dinitrogen: i.e. nitrite, six molecules of ferro cytochrome *c*-550 (or cuprous pseudoazurin) and ten protons. The remaining two molecules of ferro cytochrome *c*-550 (cuprous pseudoazurin) can be used to reduce an atom of oxygen to water. The net result of this model is the removal of two molecules of ubiquinol from the ubiquinol/ubiquinone pool accompanied by the translocation of only two protons (by cytochrome aa_3 oxidase) across the cytoplasmic membrane. The consumption of two protons from the cytoplasm by oxygen reduction and production of two protons from hydroxylamine oxidation will also contribute to the generation of a proton electrochemical gradient. In the absence of nitrification coupled to denitrification, the complete oxidation of two molecules of ubiquinol via the cytochrome bc_1 complex and the cytochrome aa_3 oxidase would be predicted to be accompanied by the net translocation of twelve protons across the cytoplasmic membrane. Thus the coupling of nitrification to denitrification in this model can provide an effective mechanism for oxidising ubiquinol via a poorly coupled electron transport pathway that proceeds independently of the cytochrome bc_1 complex. Although the site of ubiquinol oxidation by the ammonia monoxoygenase is not known with certainty, we predict that it is towards the periplasmic face of the cytoplasmic membrane with the protons being released into the periplasm and consumed in the conversion of ammonia to hydroxylamine. Hao, hydroxylamine oxidase; Amo, ammonia monooxygenase. For more details of the redox centres of Nir, Nos and Nor see Fig. 2.

dehydrogenase this will lead to the build up of pyridine nucleotides in the cytoplasm, thus upsetting cell redox balance. The requirement of the cytochrome bc_1 complex for Q suggests that at high QH_2/Q cytochrome bc_1 complex-independent pathways will be favoured for removing electrons. Such pathways are generally poorly coupled.

The above model predicts that the need to dissipate reductant is most acute during the metabolism of a reduced carbon substrate under conditions that are both oxygen and energy sufficient. This suggestion is supported by the observation that aerobic denitrification in carbon (acetate) limited (and therefore energy-limited) chemostat cultures of *T. pantotropha* increases with specific growth rate, which would correspond to decreasing energy (carbon) limitation [447,448]. Turning to conditions of carbon and energy excess, in batch culture (with acetate as carbon source) *T. pantotropha* exhibits highest growth yields with oxygen as electron acceptor, intermediate yields with oxygen plus nitrate and lowest yields with nitrate only [444]. This is in agreement with the relative potentials for energy conservation associated with reduction of oxygen and nitrogen oxides and suggests that a significant proportion of electron flux into the electron transport chain is being diverted to N-oxide respiration when both types of electron acceptor are available. In general bacteria seek to maximise growth rate rather than yield and nitrogen oxide metabolism during aerobic growth appears to achieve this [444,445]; the aerobic growth rate is faster when nitrate as well as oxygen is present while anaerobic growth with nitrate as electron acceptor is slower than either type of aerobic growth.

Laboratory cultures of denitrifying bacteria are routinely grown on relatively oxidised carbon substrates such as succinate or acetate. However, higher levels of intracellular reductant may be generated through the oxidation of more reduced substrates, such as fatty acids, to carbon intermediates suitable for biosynthesis and assimilation. Problems in maintaining the redox poise of the electron transport chain may be accentuated with such substrates and it is notable that the expression of the periplasmic nitrate reductase is 10-fold higher following growth of *T. pantotropha* aerobically with butyrate or caproate compared to malate or succinate and 5-fold higher compared to acetate [437]. The problems in maintaining the redox poise of the electron transport chain during fatty acid metabolism could be accentuated by the obligatory involvement of flavoprotein dehydrogenases in fatty acid oxidation, which transfer electrons directly into the QH_2/Q-pool. This may also explain the recent failure to observe aerobic nitrate reduction in cultures of *T. pantotropha* growing with acetate as carbon source [541].

The degree to which N-oxide respiration is utilised by bacteria to dispose of reductant will be dependent on the alternative mechanisms available. These may include the formation of reduced storage polymers, such as PHB, or heterotrophic nitrification [446–448]. In the absence of nitrate, under conditions when PHB production is not favoured (i.e., carbon limitation), it has been suggested that heterotrophic nitrification (the oxidation of ammonia to nitrite) may play an important role in the maintenance of redox balance [447,448]. The mechanism by which an oxidative process can play a role in the disposal of reductant appears to be intimately linked to aerobic denitrification. Studies on the biochemistry of heterotrophic nitrification in *T. pantotropha* have led us to suggest a model, shown in Fig. 5 [584], which demonstrates how nitrification can be coupled to aerobic denitrification through the common redox component cytochrome *c*-550. Nitrite and reduced cytochrome *c*-550 are produced as a result of ammonia oxidation via hydroxylamine (Fig. 5). The nitrite feeds into the series of denitrification reactions that lead to the production of nitrogen and which utilise electrons provided by cytochrome *c*-550. The process serves to remove reductant from the QH_2/Q pool, dissipating energy and explaining the low growth yields observed in actively nitrifying chemostat cultures of *T. pantotropha* [448]. A major factor which limits the aerobic production of gaseous nitrogen oxides and nitrogen from either nitrate or ammonia is the expression of nitrite reductase, which can be very low under some oxic growth conditions [380].

4.2.2. Microaerobic growth

Studies on the magnetotactic bacterium, *Magnetospirillum magnetotacticum*, have demonstrated that it can denitrify nitrate to dinitrogen gas. The organism cannot grow under either anaerobic or highly aerated conditions. Optimum growth is under microaerophillic conditions. Under these conditions the presence of nitrate increased the cell yield [45]. A simple interpretation of these data is that the oxidases are not saturated at these low oxygen tensions. This could ultimately raise the extent of reduction of the Q-pool, driving the electron transfer to N-oxide reductases such that the energy conserved as Δp by reduction of the nitrogen oxides supplements that conserved by oxygen respiration. These arguments apply equally to a number of other organisms in which microaerobic denitrification has been demonstrated (see [447] for a list). Little is known about the properties of the N-oxide reductases of this organism, but our analysis of the data of Blakemore and co-workers [45,490] leads us to suggest that soluble nitrate reductase reported may be of the periplasmic type.

4.2.3. Autotrophic bacteria

Although classically associated with heterotrophs, denitrification reactions are increasingly being identified in autotrophic nitrifying bacteria [174,419,420]. The enzymes are largely restricted to copper nitrite reductases, of which that from *Nitrosomonas europeae* has been purified [149,376]. However, at least one species of *Nitrosomonas* may also have a nitrous oxide reductase [419]. Anaerobic autotrophic growth using nitrite as electron acceptor has not been demonstrated and the role of N-oxide reduction

remains cryptic. However, nitrite, generated from ammonia through nitrification, is reduced during autotrophic growth in the presence of oxygen. A scheme for ammonia oxidation in *N. europeaea* is shown in Fig. 6. In this model cyt *c*-552 serves as a mediator for electron transfer between the cyt bc_1 complex and the cyt aa_3 oxidase. In principle, cyt *c*-552 could mediate electron transfer to the denitrification enzymes. This could be important under microoxic conditions, where nitrite reduction may serve as an alternative means to oxygen for generation of Δp, conserving the available oxygen for the ammonia monoxygenase. A copper nitrite reductase, that reduces nitrite to nitric oxide, has also been identified in *Nitrobacter vulgaris*, and is expressed and active in cells nitrifying under aerobic conditions [8]. The role of nitrite reduction under these growth conditions is not clear.

4.3. Resistance to toxic nitrogen oxides

Many bacteria are not furnished with the entire complement of N-oxide reductases required to convert nitrate to dinitrogen. For instance, some strains of *R. capsulatus* express only nitric oxide and nitrous oxide reductases and at least one strain only expresses a nitric oxide reductase (Section 2.3; [52]). The production of nitric oxide in the environment may be widespread, arising as a product of

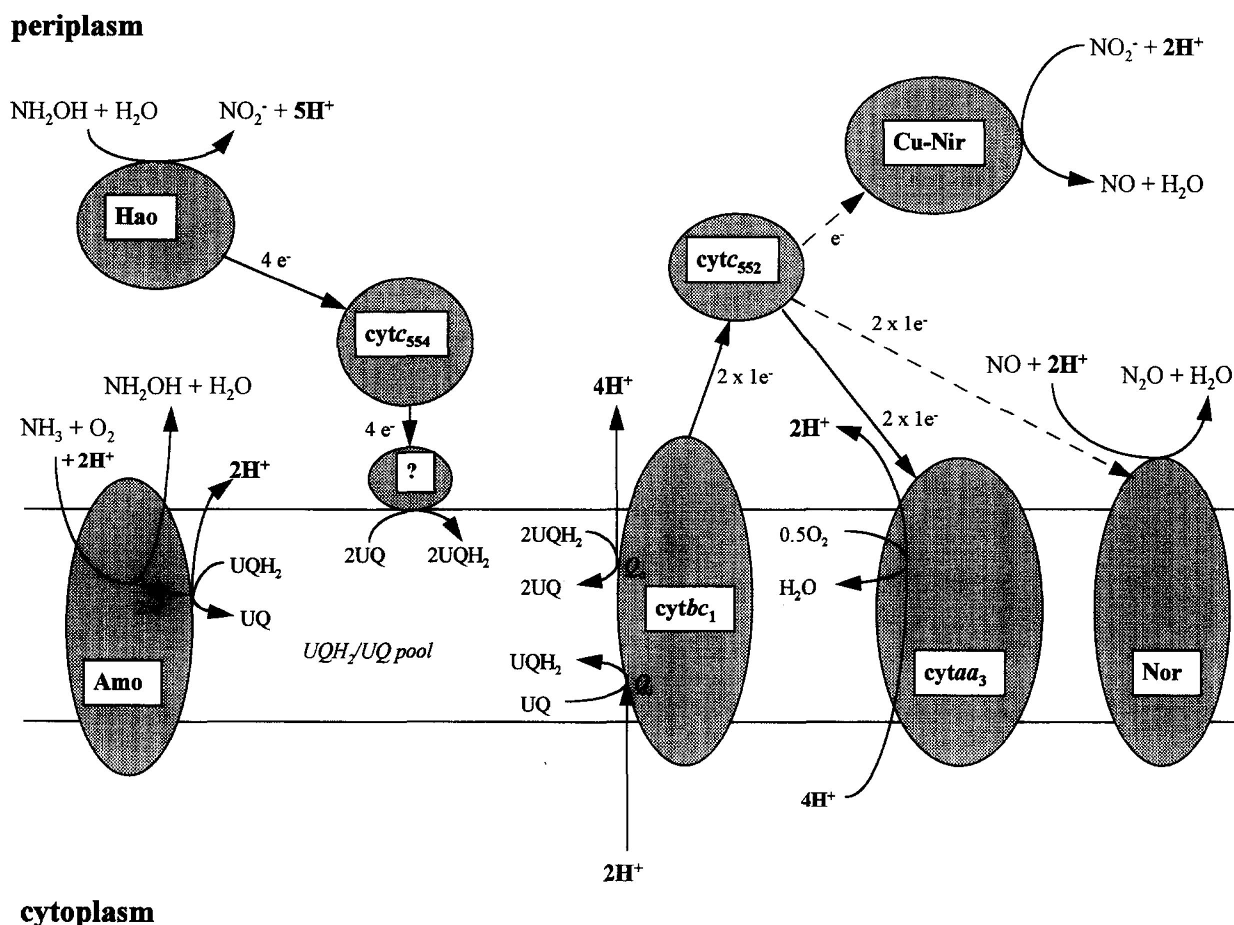


Fig. 6. The interaction between the electron transfer pathways involved in nitrification and nitrite reduction in *Nitrosomonas europeae*. The nitrification pathway is essentially based on that of Wood [596]. The scheme depicts the oxidation of one molecule of ubiquinol, by the ammonia monooxygenase (Amo), which is consumed in the oxidation of ammonia to hydroxylamine. The hydroxylamine is oxidised to nitrite by the hydroxylamine oxidase (Hao), generating four electrons and five protons. The four electrons generated pass to tetrahaem cytochrome *c*-554 and then, with four protons, are used to reduce two molecules of ubiquinone to two molecules of ubiquinol. One net molecule of ubiquinol is oxidised by the cytochrome bc_1 complex, with the two electrons proceeding via two molecules of monhaem cytochrome *c*-552 to the cytochrome aa_3 oxidase to reduce one atom of oxygen to water. The second ubiquinol molecule is used to refurbish the Amo with reductant. A small proportion of electrons (approximately two for every twenty ammonia molecules oxidised) are used to reduce NAD^+. Despite often being depicted as a quinone reductase it is unlikely that the soluble cytochrome *c*-554 can play this role directly. Thus, in this figure the quinone reductase is depicted as ?. It is possible that this could be a membrane-bound tetra-*c*-type haem protein (Orf2) [59a] of the NirT family (Section 5.1 [64]), the gene for which is linked to that coding for one copy of cytochrome *c*-554. As in Fig. 5 we place the site of quinol oxidation by the ammonia monoxygenase at the periplasmic face, although this is not proven. The presence of nitric oxide reductase has not been directly demonstrated but is inferred from the reports of dinitrogen generation from nitrite [77b]. Note that in this figure the nitrification reactions are given for the oxidation of one molecule of ammonia, whereas a scheme for the oxidation of two molecules of ammonia was given in Fig. 5 in order to balance the subsequent denitrification reactions.

microbial nitrification and denitrification, chemodenitrification and combustion of fossil fuels. Nitric oxide can serve as a ligand to a number of metal ions and as such is toxic to many microbial processes that are dependent on metalloenzymes. It has been proposed that cytochrome *c*′ may have a role in scavenging nitric oxide [609], but the widespread occurrence of the gas suggests that a range of protective mechanisms may eventually become apparent in different species of bacteria. The simplest mechanism would be the reduction of NO using nitric oxide reductase. We have argued that nitric oxide reductases may be widespread amongst bacteria [52]. In addition, the cytochrome *c* nitrite reductase may also have the capacity to reduce NO (Section 7.4.1) and the copper nitrite reductases can rebind NO generated from nitrite reduction to form nitrous oxide (Section 7.1.2), but the importance of both these processes in vivo is unclear.

Nitrite is also highly toxic to bacteria and Cole [120] has commented that the reduction of nitrite to ammonia by the sirohaem or *c*-type cytochrome nitrite reductases, serves

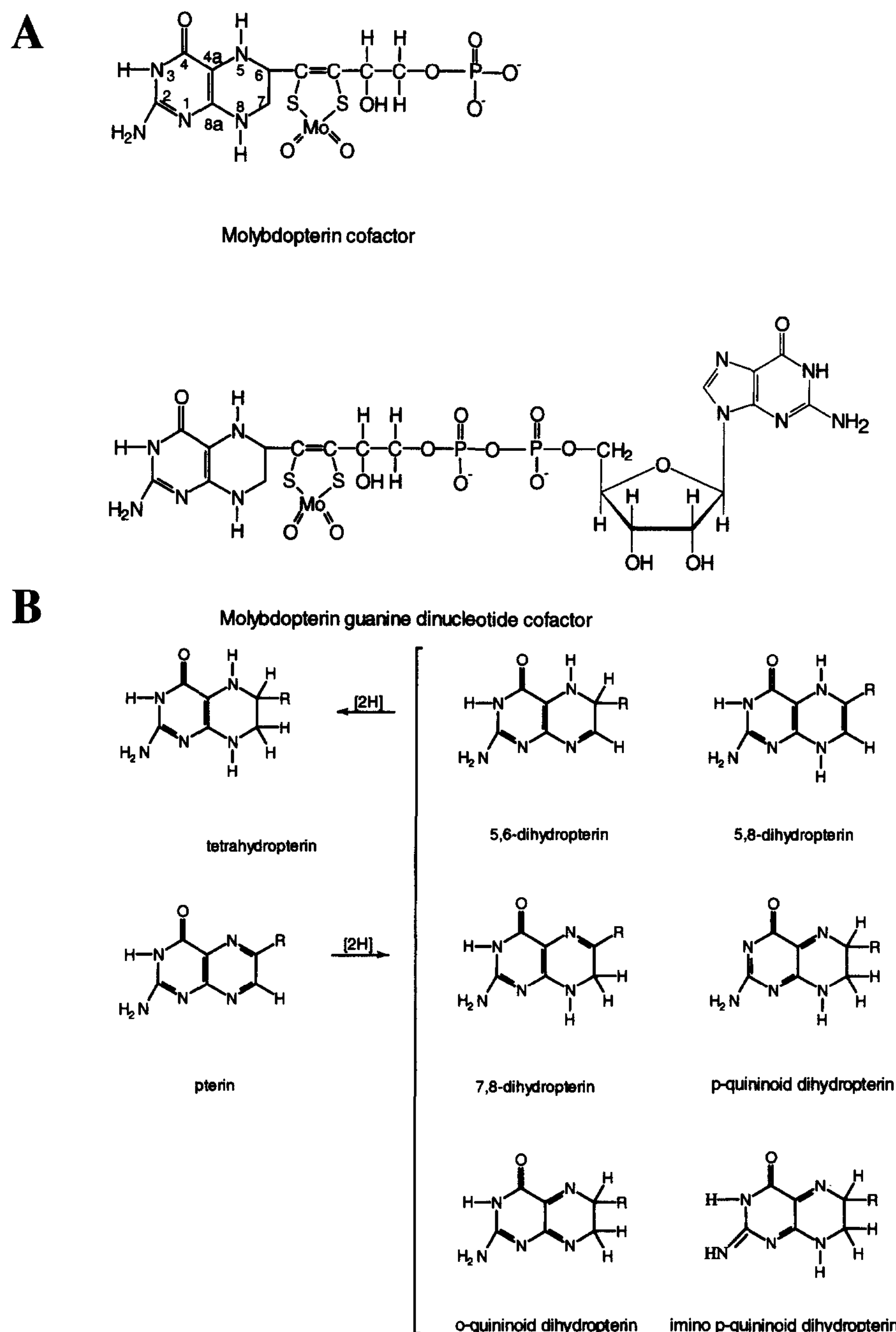


Fig. 7. The molybdopterin cofactor. (A) Proposed structure of the molybdopterin [299] and molybdopterin guanine dinucleotide (MGD) [266] cofactors. The pterin is shown in the fully reduced state. The molybdenum coordination sphere depicted is just one of a number of possibilities. (B) Possible oxidation states of the pterin portion of the molybdopterin cofactors.

to remove this toxic chemical from intracellular and extracellular environments, respectively. Indeed, it has been proposed that this may be the most important function of this enzyme [405]. The detoxification of nitrite also appears to be the specific role of the copper nitrite reductase of *Rhizobium heydysari* strain HCNT1 [101,102]. The reduction of nitrite cannot support growth of this organism but serves to remove the N-oxide, which has a toxic effect on the cytochrome-*o* oxidase. Since this reaction occurs in the presence of oxygen, it can be considered as an additional function of an aerobic denitrification reaction. The rebinding of nitrite and subsequent formation of nitrous oxide by the cytochrome-cd_1 nitrite reductase (Section 7.1) may also be a protective mechanism under some growth conditions.

5. Respiratory nitrate reductases

Four classes of nitrate reductases have been identified; the bacterial membrane-bound and periplasmic respiratory enzymes introduced earlier (Section 2.1) and the distinct cytoplasmic assimilatory enzymes of bacteria and eukaryotes. All four types of nitrate reductase have a molybdopterin cofactor at their active sites. This cofactor, the proposed general structure of which is shown in Fig. 7a, functions in oxygen atom transfer (or hydroxylation reactions) between substrate and water. The bacterial nitrate reductases contain a variant of the basic molybdopterin cofactor in which the cofactor is conjugated to guanosine monophosphate (Fig. 7a). This has been termed molybdopterin guanine dinucleotide (MGD) by Rajagopalan and Johnson [427]. On the basis of amino acid sequence all MGD-binding proteins are thought to be structurally related to one another, but structurally distinct from proteins binding other forms of the molybdopterin cofactor (Section 5.4.1). The membrane-bound nitrate reductase is a member of a subgroup of MGD-dependent enzymes that are heterotrimeric membrane-bound complexes. In order of diminishing size and of transcription of the corresponding structural genes the subunits are (i) a MGD-binding extrinsic membrane protein (α subunit), (ii) an iron-sulfur cluster-binding extrinsic membrane protein (β subunit) which is thought to mediate electron transfer between the other two subunits and (iii) an intrinsic membrane protein (γ subunit) the structure of which show considerable variation between enzymes of this subgroup. The γ subunit invariably acts as the membrane anchor for the complex. It carries a site of interaction with the cytoplasmic membrane quinol/quinone pool and may bind up to two *b*-haems.

The eukaryotic assimilatory nitrate reductase is a cytoplasmic, NAD(P)H-linked enzyme possessing molybdopterin (mononucleotide), haem (cytochrome *b*) and FAD as noncovalently bound cofactors, the binding sites for which are arranged on a single polypeptide in that order (reviewed in [511]). The structure of molybdenum active site and the mechanism of nitrate reduction by the enzyme appear to resemble those of the membrane-bound nitrate reductase. Beyond these similarities we do not consider this enzyme further.

5.1. Periplasmic respiratory nitrate reductase

Periplasmic nitrate reductases (Nap) have been purified from the closely related α Proteobacteria *R. capsulatus* [11,360,435], *R. sphaeroides* f.sp. *denitrificans* [469] and *T. pantotropha* [62] as well as the β Proteobacterium *Alcaligenes eutrophus* H16 [495]. Sequences have been obtained for the *A. eutrophus* structural genes and for the complete periplasmic nitrate reductase (*nap*) loci of *T. pantotropha* and *E. coli* [64,438,495]. The *E. coli nap* locus was originally designated *aeg-46.5* (anaerobically expressed gene at 46.5 minutes on the *E. coli* chromosomes). In discussing the *E. coli* system, we will employ the revised gene nomenclature recently advanced by Valley Stewart (personal communication). Amino acid sequences from peptide fragments of the *R. sphaeroides* f.sp. *dentrificans* nitrate reductase [460] clearly indicate that this is the same enzyme as found in those species for which gene sequences have been obtained. While the *nap* operon in *E. coli* has a chromosomal location, the *nap* genes of *A. eutrophus* H16 are found on a megaplasmid (pHG1) together with the nitrous oxide reductase structural gene and others involved in denitrification [450,451,475, 495,581,624]. The genes coding for the periplasmic nitrate reductase of *R.capsulatus* AD2, *T. pantotropha* and *P. denitrificans* may also be located on megaplasmid DNA [480,593]. Although it has been purified from only a relatively small number of sources, the periplasmic nitrate reductase is probably widespread amongst the Proteobacteria. For example, 40 species of α, β and γ Proteobacteria expressing the enzyme have been isolated from soil and freshwater environments near Norwich, UK [100,100a].

A role for the periplasmic nitrate reductase in aerobic denitrification and redox balancing was discussed in Sections 4.1 and 4.2. That aerobic nitrate reduction may not be the only physiological rôle of the periplasmic nitrate reductase in chemoheterotrophs is suggested by the observation that *P. denitrificans* PD1222, despite expressing the periplasmic nitrate reductase during aerobic growth, is incapable of aerobic nitrate reduction [481]. Furthermore, in *A. eutrophus* the periplasmic nitrate reductase is expressed in stationary phase only after aerobic growth. This pattern of expression would generally be thought to be unlikely for an enzyme system that functions to dissipate Δp (e.g., [611]). The physiological rôle of periplasmic nitrate reductases may thus vary between different organisms.

The periplasmic nitrate reductase is not primarily involved in nitrate assimilation in the organisms currently studied. Expression of the periplasmic nitrate reductase in

A. eutrophus, *T. pantotropha*, *P. denitrificans* PD1222, *R. capsulatus* and *R. sphaeroides* f.sp. *denitrificans* is not repressed by ammonium [355,437,469,481,581] and *A. eutrophus*, at least, possesses a separate soluble assimilatory nitrate reductase [581]. Further, an *A. eutrophus* mutant specifically deficient in the periplasmic nitrate reductase (*nap*) assimilates nitrate normally [495]. When this mutant strain was grown aerobically to stationary phase, then incubated anaerobically in the presence of nitrate, there was a reduction in growth rate [495]. This was interpreted as implying a rôle for the periplasmic nitrate reductase in adaptation to anaerobic growth. A similar rôle has been proposed for the minor membrane-bound nitrate reductase (Nar-Z) of *E. coli* (Section 2.2) [254]. A relationship in *A. eutrophus* H16 between periplasmic nitrate reductase expression and formate metabolism has been suggested [495].

The periplasmic nitrate reductase does not appear to be primarily involved in anaerobic denitrification. Expression of the periplasmic nitrate reductase is repressed during anaerobic growth of *T. pantotropha*, *P. denitrificans* PD1222 and *A. eutrophus* [51,481,581], while of the *Rhodobacter* species that are known to possess the enzyme, only *R. sphaeroides* f.sp. *denitrificans* is a denitrifier and this organism also expresses a membrane-bound nitrate reductase [94]. In *A. eutrophus* H16 a mutant lacking membrane-bound nitrate reductase activity (the nature of this mutation was not determined) was unable to grow anaerobically [581], while a mutant with a specific insertion in *napA* was unaffected in anaerobic growth on nitrate [495]. These results reinforce the suggestion that the periplasmic nitrate reductase in this organism plays no part in anaerobic denitrification. In contrast, a *T. pantotropha* mutant lacking the membrane-bound nitrate reductase *will* denitrify under anaerobic conditions, apparently reducing nitrate by means of the periplasmic enzyme [53]. This suggests that the periplasmic nitrate reductase might function in denitrification in some organisms. It is tempting to speculate that the enzymes of the ancestral denitrification pathway were periplasmically located and included a periplasmic nitrate reductase. Organisms that later acquired the membrane-bound nitrate reductase would probably have a selective advantage during anaerobic growth on nitrate because quinol oxidation via the membrane-bound, but not periplasmic nitrate reductase, is thought to be energy conserving (Sections 2.1 and 4). A periplasmic nitrate reductase active during the dissimilatory reduction of nitrate to ammonia has recently been reported in the sulfur-reducingbacterium *Sulfurospirillum deleyianum* ('*Spirillum* 5175') [478].

The regulation of expression of the *E. coli aeg-46.5* locus (putative periplasmic nitrate reductase system) has been reasonably well characterised [116,117,423]. Like the enzyme from *R. capsulatus* [435], but in contrast to the enzymes of *T. pantotropha* [437] and *A. eutrophus* [581], the *aeg-46.5* locus is induced by nitrate. The locus is also

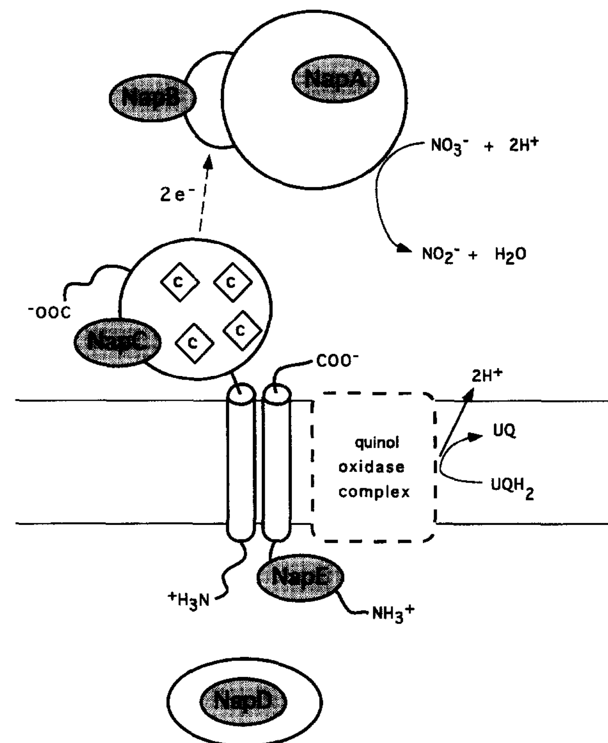


Fig. 8. Models for the structure and function of proteins coded by the *nap* locus of *T. pantotropha*. The suggested subcellular locations of the NapEDABC proteins are shown. The question of whether NapC is a quinol oxidase, and whether a NapGH complex (Fig. 10) or cytochrome *b* function in this role is discussed in the text (Section 5.1).

induced by nitrite. Induction by both nitrate and nitrite is mediated by NarP while NarL strongly represses *aeg-46.5* expression (see Section 3.1 for a description of the NarXLPQ system). Two potential NarL-binding sites arranged as an inverted repeat are present in a promoter region. The locus is induced approximately 10-fold by anaerobiosis if nitrate is also present. Consistent with this, the promoter region contains four possible Fnr binding sites and induction by anaerobiosis is abolished in an *fnr* mutant.

5.1.1. Structural and catalytic properties

The periplasmic nitrate reductase is a heterodimer (Fig. 8). NapA (90 kDa) binds MGD [64,427,438,495] while NapB (16 kDa), is a dihaem cytochrome *c*-552 [62,64,438,495] (note that there is currently no evidence that *Rhodobacter* NapB subunits bind more than one haem [435,607]). It has recently been demonstrated that the periplasmic nitrate reductase of *T. pantotropha* contains a $[4Fe\text{-}4S]^{2+,1+}$ cluster ($E_{m,7.4} = -160$ mV) [88]. This cluster is most plausibly ligated by a conserved four cysteine motif found at the N-terminus of NapA and some other MGD-dependent proteins [88]. The reactions and functions of the periplasmic nitrate reductase MGD and [4Fe-4S] cofactors are discussed in Sections 5.4.1–3.

From amino acid sequence conservation [64] and EPR

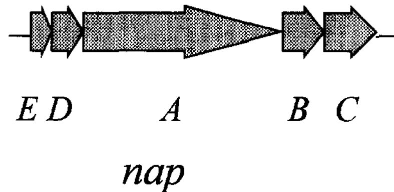
measurements [88], it can inferred that both NapB haems are *bis*-histidine ligated. In the *T. pantotropha* enzyme the haems have reduction potentials (pH 7.0) of − 15 mV and + 80 mV [62]. These potentials are relatively high for *bis*-histidine ligated *c*-type cytochromes. The NapB proteins of *T. pantotropha* and *R. capsulatus* N22DNAR$^+$ characteristically fail to bind the dye Coomassie brilliant blue [62,435]. The *T. pantotropha* protein is also unusual in that it cannot be detected by electrospray mass spectrometry [62]. Analysis of the amino-acid sequence of *T. pantotropha* NapB has not, however, revealed the reason for this unusual behaviour [64].

NapA and NapB most probably form a one-to-one complex [62]. At least in the case of the enzyme from *R. capsulatus* N22DNAR$^+$ the interaction between the subunits is predominantly hydrophobic in nature and some dissociation of the subunits during purification can occur [435]. Loss of the NapB subunit from the *R. capsulatus* enzymes leads to a marked loss of enzyme activity when reduced viologens are the electron donors [360,435].

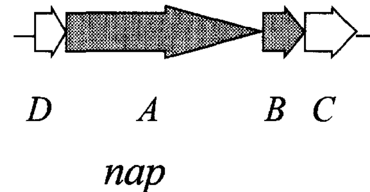
The periplasmic nitrate reductase is catalytically distinct from the membrane-bound nitrate reductase [384]. The membrane bound, but not periplasmic nitrate reductase, is sensitive to competitive inhibition by azide anion at micromolar concentrations [133,571a]. Further, the membrane-bound nitrate reductase can reduce chlorate (and bromate) while the periplasmic nitrate reductase shows complete specificity for nitrate as substrate [51,62,355,384]. Chlorate is tetrahedral, while nitrate is planar so that it is perhaps the ability of the membrane-bound enzyme to reduce chlorate rather than the inability of the periplasmic enzyme to do so that is surprising. In vivo, the specificity of the membrane-bound enzyme will also be a function of the discrimination of the cytosolic membrane transport systems against alternative substrates.

In intact cells the periplasmic nitrate reductase can be readily distinguished from the membrane-bound nitrate reductase using viologen-linked assays. The active site of the membrane-bound enzyme is located at the cytoplasmic face of the plasma membrane and so accessible to the membrane-permeant benzyl viologen radical but not to the membrane-impermeant methyl viologen radical. In contrast, the active site of the periplasmic enzyme is readily accessible to both redox dyes [51].

Thiosphaera pantotropha



Alcaligenes eutrophus



2 kbp

Escherichia coli aeg-46.5

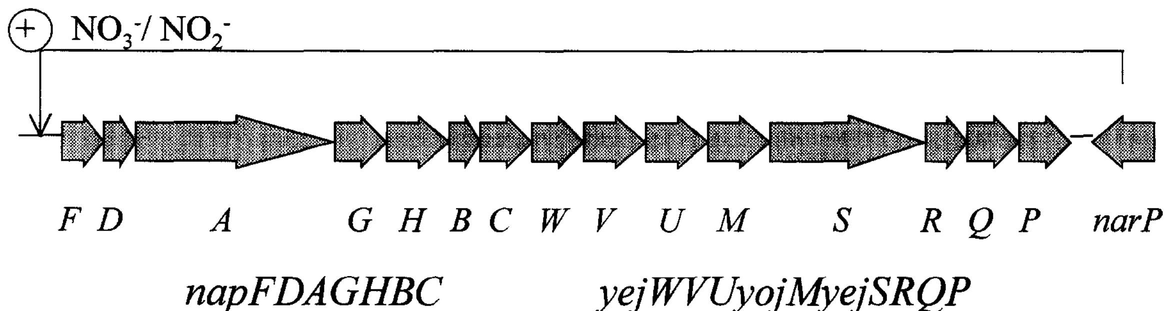


Fig. 9. Organisation of the *nap* loci of *T. pantotropha*, *A. eutrophus* H16 and the *aeg-46.5* locus of *E. coli*. Sequences are from [64,438,495]. Nomenclature for the *E. coli nap* genes follows that suggested by V. Stewart (personal communication). *narP* codes for a nitrate/nitrite response regulator. Sequence similarities of the non-*nap* gene products coded by the *aeg-46.5* locus to proteins implicated in *c*-type cytochrome biogenesis in other bacteria can be found in Table 1. Note that the sequence of the *A. eutrophus* locus is incomplete, with the unfilled arrows representing genes that are only partially sequenced.

5.1.2. The nap loci

The organisation of the sequenced *nap* loci are compared in Fig. 9. All three loci contain the gene products *napDABC* in that order. The *E. coli* locus contains three additional genes *napFGH* coding for probable iron-sulfur proteins. Like the *E. coli* operon encoding the cytochrome-*c* nitrite reductase (Section 7.4.2), the *E. coli* nap locus contains genes that are probably involved in periplasmic addition of haem to apocytochromes *c* (Table 1; Fig. 9). The *E. coli nap* locus is linked to the gene coding for nitrate/nitrite response regulator NarP (Section 3.1). The *T. pantotropha* gene *napE* is not present at the *E. coli* locus, but a possible homologue is found divergently transcribed from the copper nitrite reductase structural gene of *Pseudomonas* sp. strain G-179 [64].

NapD is predicted to be a soluble cytoplasmic protein of around 12 kDa (Fig. 8). The function of NapD is unknown but from its predicted cellular location, the lack of conserved residues likely to ligand redox cofactors, and the overlap of *napD* and *napA* genes in all three operons implying translational coupling and stoichiometric synthesis of NapD with napA, it has been argued that the most probable role of NapD is in some aspects of the maturation of the NapA protein [64]. As discussed in the next section, the remaining accessory *nap* genes are probably involved in electron transfer to the periplasmic nitrate reductase.

5.1.3. Electron transport to the periplasmic nitrate reductases

The proposed minimal electron transport pathway to the periplasmic nitrate reductase in *R. capsulatus* and *T. pantotropha* and its bioenergetic consequences were discussed in Sections 2.1 and 4 (Figs. 2 and 8). Recall that there is predicted to be no energy conservation in the span ubiquinol to nitrate, consistent with the proposed rôle of the periplasmic nitrate reductase pathway in redox balancing. The electron transport chain between ubiquinol and the periplasmic nitrate reductase in *R. capsulatus* [435], *R. sphaeroides* f. sp. *denitrificans* [607] and *T. pantotropa* (Richardson, D.J. and Berks, B.C., unpublished observations), involves membrane-bound haemoprotein(s) which, from their α-band maxima (around 560 nm in redox difference spectra), were suggested to be *b*-type cytochromes. These cytochromes '*b*' are distinct from the cytochrome bc_1 complex. For *R. capsulatus* it has also been determined that the electron transfer from ubiquinol to the periplasmic nitrate reductase is inhibited by the menaquinone analogue 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO [432,435]). It has been suggested that this HOQNO-sensitive ubiquinol oxidase is also involved in the cytochrome bc_1 complex-independent nitrous oxide reduction and the cytochrome bc_1-independent cyclic photosynthetic electron transfer found in this organism ([434]; Section 2.3).

NapC (around 23–27 kDa) is a member of the NirT family of tetrahaem *c*-type cytochromes (Section 7.2; see [64] for extensive sequence comparisons). From sequence analysis, the bulk of NapC containing the four haem groups is predicted to be located in the periplasm and anchored to the cytoplasmic membrane by an N-terminal transmembrane helix [64] (Fig. 8). NapC is likely to be the direct electron donor to the periplasmic nitrate reductase. As no other haemoproteins have been identified in the *nap* operons, we suggest that NapC corresponds to the membrane-bound cytochrome '*b*' identified spectroscopically in the *Rhodobacter* species and ascribe the unusual *c*-type cytochrome α-band maximum to haem-haem interactions. While NapC could also be the quinol oxidase (see [64] for a discussion of this point) we think that a separate quinol oxidase is a more likely possibility. In *E. coli* we would suggest that some or all of the predicted iron-sulfur cluster-binding proteins NapF, NapG and NapH constitute the quinol oxidase and that equivalent proteins fulfilling this function in *T. pantotropha* are coded at a locus distinct from the *nap* operon. We propose that in *T. pantotropha* the predicted monotopic integral membrane protein NapE mediates the interaction of this oxidase with NapC (Fig. 8). The involvement of iron-sulfur proteins in electron transport between quinol and NapC is supported by the high sensitivity of the activity of the periplasmic nitrate reductase pathway, [49,557,435] relative to that of the periplasmic nitrate reductase itself ([469]; Berks, unpub-

Table 1
Proteins involved in bacterial *c*-type cytochrome biogenesis in *B. japonicum* and *R. capsulatus* that show sequence similarity to putative gene products coded by the *E. coli aeg-46.5* locus and *nrf* (cytochrome *c* nitrite reductase) operon

Escherichia coli aeg-46.5 locus[438]	*Escherichia coli nrf* operon[77,250]	*Bradyrhizobium japonicum* 110Rif15	*Rhodobacter capsulatus* SB1103
YejP[405a]	NrfF + NrfG[405a]	CycL[543a] + CycH[441]	Ccl2[48] + ?
YejQ	–	TlpB[543a]	Helx[47]
YejR	NrfE	CycK[543a]	Ccl1[48]
YejS	–	CycJ[543a]	–
YojM	–	CycX[543a]	HelD[47]
YejTU	–	Orf263[428]	HelC[48]
YejV	–	CycW[428]	HelB[48]
YejW	–	CycV[428]	HelA[48]

lished data), to inhibition by the iron chelator bathophenanthroline or by cyanide. Notably, in *R. capsulatus* cyanide was shown to act between ubiquinol and the cytochromes '*b*' and *c* [435].

5.1.4. The NapFGH proteins

E. coli NapF (formerly YojG) is predicted to be a soluble protein of around 20 kDa. NapF does not appear to possess a signal peptide so a cytoplasmic location is indicated (the DNA sequence corresponding to the N-terminus is somewhat uncertain and so this interpretation should be treated with caution). The cysteine motif Cys-X-X-Cys-X-X-Cys-X-X-X-Cys-(Pro)(2.2.3.(P) spacing) repeated once, is typical of proteins binding two [4Fe-4S]$^{2+,1+}$ clusters (see Section 5.4.3 for details). NapF contains four cysteine motifs, spacings 2.2.3, 2.2.3.P, 7.2.3 and 2.2.3.P, and so is likely to bind four [4Fe-4S] clusters. NapF does not, however, show strong sequence similarity to other ferredoxins.

NapG (around 20 kDa mature protein, the sequence is not yet finalised; formerly YojBA) and NapH (32 kDa; formerly YejZ) show strong sequence similarity to two proteins, MauM and MauN respectively, present in the methylamine dehydrogenase gene clusters of *Methylobacterium extorquens* AMI, *Methylophilus methylotrophus* WSA1-NS and *P. denitrificans* [114,115,563]. The association of the genes for the two proteins in each instance (the genes overlap in most cases) suggests that NapG and NapH may form a complex. The function of MauMN is unclear as insertional mutagenesis of the gene coding for either protein had no detectable effect on growth on methylamine or synthesis of the methylamine dehydrogenase [114,563]. NapG/MauM contain four cysteine motifs with spacings of 2.2.3.P, 2.4.3.P, 7.2.3.P and 2.2.3. These pro-

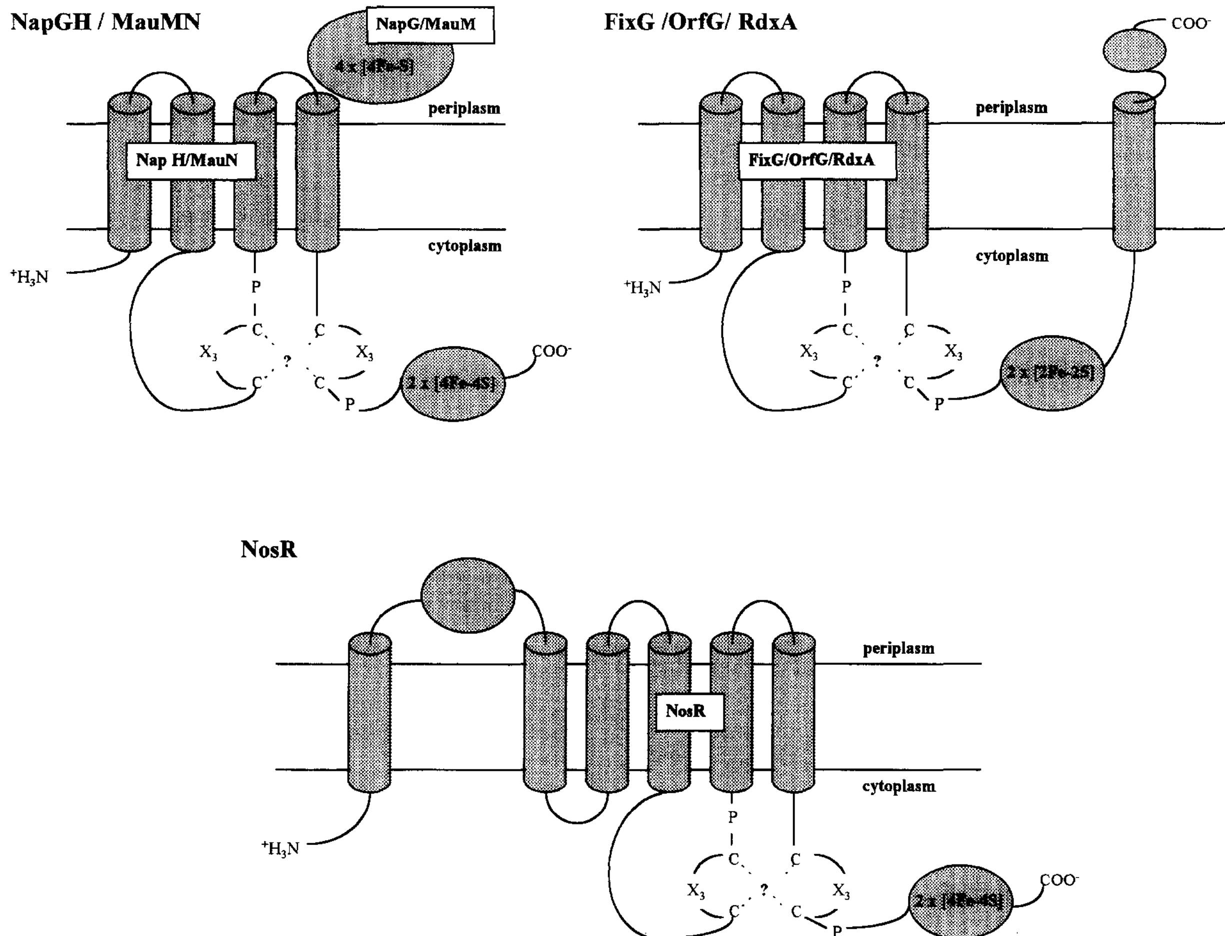


Fig. 10. Comparison of the predicted topological organisation of NapGH/MauMN with those of other structurally related proteins. NapGH are coded at the *aeg-46.5* (putative periplasmic nitrate reductase) locus of *E. coli* (Fig. 9) [438]. MauMN are coded by the methylamine dehydrogenase gene clusters of several bacteria [114,115,563]. Genes coding for FixG/OrfG are found adjacent to the operons coding for the 'cytochrome cbb_3' oxidases [278,279,344,543]. *R. sphaeroides rdxA* forms an independent transcriptional unit [387]. The gene for NosR is linked to that for nitrous oxide reductase (Sections 3.1, 9.1, Fig. 22) [137]. NosR has been proposed to be a trans-acting regulator of nitrous oxide reductase expression. The function of the other proteins is not known. The proposed topological structures of the integral membrane proteins contain a core of four transmembrane helices with two similarly positioned Cys-(Xaa)$_3$-Cys-Pro motifs and two consensus [4Fe-4S]-binding motifs. The ? indicates that the Cys-(Xaa)$_3$-Cys-Pro may be linked by disulfide bonds or a metallocofactor, possibly an iron-sulfur cluster. The region of NosR proposed to form a helix-turn-helix DNA binding domain in NosR [137] corresponds in our model to the periplasmic domain between helices one and two.

teins are therefore likely to bind four iron-sulfur clusters, probably of the [4Fe-4S] type. NapG/MauM appear to possess signal peptides indicating a periplasmic location for the mature proteins (Fig. 10). NapH/MauN are predicted to be integral membrane proteins with four transmembrane helices arranged so that both the N- and C-termini of the polypeptide are cytoplasmic (Fig. 10). The proposed cytoplasmic C-terminal domain contains two four-cysteine motifs, spacing 3.2.3.(P) and 2.2.3.(P). This region of the polypeptide is thus likely to bind two [4Fe-4S] clusters (Fig. 10). NapH/MauM also contains two cytoplasmic Cys-$(Xaa)_3$-Cys-Pro motifs, one each in the C-terminal domain, and in the cytoplasmic loop between helices 2 and 3 (Fig. 10). We propose that these motifs are involved either in binding a metal centre, perhaps another iron-sulfur cluster, or are involved in thiol interchange reactions.

The large number of probable redox centres in NapGH/MauMN immediately suggests an electron transfer function for these proteins. The proposed iron-sulfur clusters in NapH/MauN are, however, predicted to be located at the cytoplasmic side of the membrane. If these clusters are involved in electron transfer from donor molecule to NapG/MauM then additional electron carriers, almost certainly *b*-type haems, are required to transfer the electrons across the membrane bilayer. However, there are no conserved histidines that could ligate *b*-haem groups either in the predicted transmembrane helices or periplasmic loop regions.

The basic NapH/MauN structure of four transmembrane helices with the four cysteine motifs at appropriate positions, can be recognised in several other proteins (NosR, RdxA, FixG/OrfG) that do not otherwise show significant sequence similarity to NapH/MauN (Fig. 10). The similarity of the independently determined topology models of these proteins to those for NapH/MauM as well as limited topological mapping by alkaline phosphatase fusions of the RdxA protein [387], supports our proposed NapH/MauN topology (Fig. 10). Can we detect a functional similarity amongst these structurally similar proteins that could indicate the role of NapGH?

In *R. meliloti* and *Azorhizobium caulinodans fixG* is the first gene in an operon *fixGHIS* required for symbiotic nitrogen fixation but not free-living diazotrophic growth [278,279,344]. In these and other organisms the *fixG / orfG* gene clusters with an operon coding for the microaerophilic cbb_3 oxidase operon [543]. From sequence comparisons, FixI is identified as a P-type integral membrane ATPase that is probably involved in export of Cu^{2+} or a similar heavy metal cation [496,507] perhaps the copper atom of the oxidase dinuclear centre.

The *rdxA* gene of *R. sphaeroides* apparently forms an independent transcriptional unit [387]. The only phenotype detected in a mutant in which *rdxA* was insertionally inactivated was impairment of reduction of tellurite to tellurium metal [387].

It has been proposed from the phenotype of insertion mutants that NosR in *P. stutzeri* is a trans-acting positive regulator of nitrous oxide reductase expression (Section 3.1) [137]. In support of this idea a possible helix-turn-helix DNA binding region has been advanced [137]. Note, however, that in our topology model this region is unambiguously periplasmic (Fig. 10). An electron transport role for NosR could also be consistent with the insertion mutant phenotypes. Insertions in the region of *nosR* coding for the NapH-like portion of the polypeptide still allow low-level synthesis of active nitrous oxide reductase [135,569,620]. However, there is no nitrous oxide reductase activity with physiological electron donors and the mutants cannot grow with nitrous oxide as terminal electron acceptor. If nitrous oxide reductase expression were to be regulated by the redox state of some component of the electron transport chain (Section 3.1), then, if NosR is an obligate electron carrier to the reductase, the low level of reductase expression in *nosR* mutants may reflect the inability of nitrous oxide to poise the electron transport chain.

While it is difficult to discern a definite functional theme amongst the NapH-like proteins one possibility is regulation by the redox state of the cell. Perhaps the NapH-like proteins change conformation with E_h, allowing them to act as redox sensors coupled to diverse physiological processes. NapH might then function to control electron flow to the periplasmic nitrate reductase pathway in response to redox poise by regulating either the activity of a quinol oxidation site in NapH, or transfer of electrons from this site to NapG and then NapC.

5.2. Eubacterial assimilatory nitrate reductase

Eubacterial assimilatory nitrate reductases (Nas) are single subunit cytoplasmic enzymes, structurally distinct from the eukaryotic assimilatory enzyme. From the amino acid sequences of the enzymes from *Klebsiella pneumoniae* (NasA, 93 kDa; [324]), *Bacillus subtilis* (NarB, [397a]) and *Synechococcus* PCC 7942 (NarB, 80 kDa; [18]) it can

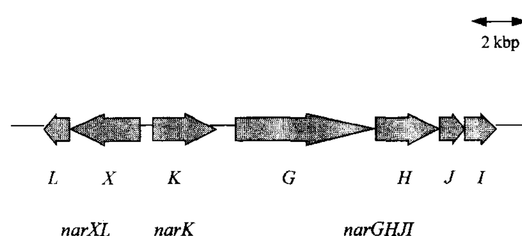


Fig. 11. Operon structure of the major membrane-bound nitrate reductase of *E. coli* (Nar-A). *narGHI* are the structural genes for the α, β and γ subunits. *narJ* codes for a protein that is probably involved in assembly of the mature nitrate reductase (Section 5.4) [74,504]. *narK* [394] codes for a nitrite exporter (Section 6.1); *narXL* [392a] codes for a two-component regulatory system that senses nitrate and probably also nitrite (Section 3.1).

be deduced that the enzymes bind MGD and an N-terminal [4Fe-4S] cluster. The sequence similarity to the periplasmic nitrate reductase NapA subunit is very high. *K. pneumoniae* NasA contains a C-terminal extension relative to NapA and the other assimilatory nitrate reductases. From the high sequence similarity this extension shows to a portion of the NifU protein, it is likely that this sequence binds a [2Fe-2S] cluster ([402,178]; possibly one of the ligands is histidine rather than cysteine). Consistent with these inferences, the biochemically characterised bacterial assimilatory nitrate reductases are single subunit enzymes binding a molybdopterin cofactor and iron-sulfur cluster(s) including at least one [2Fe-2S] cluster as judged by CD [182,375,482]. In contrast to the eukaryotic assimilatory nitrate reductases, the bacterial enzymes do not use NAD(P)H as an electron donor. The *nas* operon of *K. pneumoniae* contains a gene, *nasC*, essential for nitrate but not nitrite assimilation, that codes for a protein with sequence similarity to a number of NADH-dependent reductases [325]. NasC is likely to be the electron donor to the

```
Segment I

E. coli   NarG (43-107)  DKIVRST HGVNCTGSCS WKIYVKNGLV TWETQQTDYP RTRPDLPNHE PRGCPRGASY SWYLYSAN
E. coli   NarZ (43-107)  DKIVRST HGVNCTGSCS WKIYVKNGLV TWEIQQTDYP RTRPDLPNHE PRGCPRGASY SWYLYSAN
T. pant.  NapA (41-97)   ITWSKAP C.RFCGTGCG VMVGVKE... ....GRVVAT HGDLLAEVNR GLNCVKGYFL SKIMYGAD
A. eutr.  NapA (41-97)   LKWSKAP C.RFCGTGCG VTVAVKD... ....NKVVAT QGDPQAEVNK GLNCVKGYFL SKIMYGQD
E. coli   NapA           ?KWDKAP C.RFCGTGCG VLVGTQQ... ....GRVVAC QGDPDAPVNR GLNCIKGYFL PKIMYGKD
K. pneu.  NasA (1-57)    MTETRTT C.PYCGVGCG VIA....... SRAPHGQVSV RGDEQHPANF GRLCVKGAAL GETVGLEG
B. subt.  NarB (-15-43)  EKTYDTQ C.PFCSMQCK MQLV......EQTIVTRKKYT AIGIDNPTTQ GRLCIKGMNA HQHALNSS
Synoc.    NarB (16-83)   IDTAKTL C.PYCGVGCG LEAV....(17).....IWQI RGDRQHPSSQ GMVCVKGATT VAESVSKS
M. form.  FdhA (3-59)    IKYVPTI C.PYCGVGCG MNLVVKD... ....EKVVGV EPWKRHPVNE GKLCPKGNFC YEIIHRED
E. coli   FdhF (1-56)    MKKVVTV C.PYCASGCK INLVVDN... ....GKIVRA EA.AQGKTNQ GTLCLKGYYG WDFINDTQ
W. succ.  FdhA (54-110) SKKVKTI C.TYCSVGCG IIAEVVD... ....GVWVRQ EVAQDHPISQ GGHCCKGADM IDKARSET
E. coli   FdoG (43-106) TRETRNT C.TYCSVGCG LLMYSLGDGA KNAKASIFHI EGDPDHPVNR GALCPKGAGL VDFIHSES
E. coli   FdnG (43-106) AKEIRNT C.TYCSVGCG LLMYSLGDGA KNAREAIYHI EGDPDHPVSR GALCPKGPGL LDYVNSEN
E. coli   DmsA (27-89)  EKVIWSA CTVNCGSRCP LRMHVVDG.. EIKYVETDNT GDDNYDGLHQ VRACLRGRSM RRRVYNPD
B. subt.  OrfX (2-59)   SKVHQSA CPLNCWDSCG FLVTVDDG.. .....KVTKV DGDPNHPITE GKICGRGRML ETKTNSPD
W. succ.  PsrA (44-100) AKFVPSI C.EMCTSSCT IEARVEG... ....DKGVFI RGNPKDKSRG GKVCARGGSG FNQLYDPQ

E. coli   TorA (45-98)  DAVISKE GILTGSHWGA IRATVKDG.. ..RFVAAKPF ELDKYPSKMI A.......GL PDHVHNAA
                        ↑   ↑   ↑                                                ↑ +

Segment II

E. coli   NarG (108-184) RLKYPMMR.(38).GR GGFVRSSWQE VNELIAASNV YTIKNYGPD
E. coli   NarZ (108-184) RLKYPLIR.(38).GR GGFIRSNWQE LNQLIAAANV WTIKTYGPD
T. pant.  NapA (98-143)  RLTQPLLR.(8)...D GEFTPVSWEE AFDTMAAQAK RVLRDKGPT
A. eutr.  NapA (98-143)  RLTRPLMR.(8)...N GDFAPVTWDQ AFDEMERQFK RVLKEKGPT
E. coli   NapA           RLRQPLLR.(8)...E GEFTPITWDQ AFDVMSEKFK TALKEKGPE
K. pneu.  NasA (58-84)   RMLFPEVD........ ..GERATWPQ AGGGRFAPAG D........
B. subt.  NarB (44-82)   RITRPLLK......KN GEFMPVSWEE ALNHIKDQVT MIQTEHGHD
Synoc.    NarB (84-124)  RLKYPMFR....ASLD DPFTEISWDE ALDRLCDRIQ QTQADYGKD
M. form.  FdhA (60-94)   RLTTPLIK......EN GEFREATWDE AYDLIASKLG A....YDPN
E. coli   FdhF (61-101)  RLKTPMIR....RQRG GKLEPVSWDE ALNYVAERLS AIKEKYGPD
W. succ.  FdhA (111-149) RLRYPIEK......VG GKWRKTSWDS AMDKIAKQLQ DLTQKYGPD
E. coli   FdoG (107-139) RLKFPEYR:...APGS DKWQQISWEE AFDRIAKLMK E........
E. coli   FdnG (107-139) RLRYPEYR....APGS DKWQRISWEE AFSRIAKLMK A........
E. coli   TorA (99-152)  RIRYPMVR.(14).GD NRFVRVSWDE ALDMFYEELE RVQKTHGPS
E. coli   BisC (18-70)   RVRFPMVR.(14).GQ DEFVRVSWDE ALDLIHQQHK RIREAYGPA
E. coli   DmsA (90-132)  RLKYPMKR.(4)..GE GKFERISWEE AYDIIATNMQ RLIKEYGNE
B. subt.  OrfX (60-97)   RLRYPMKK......QN GEFVRISWEQ ALDEIADKL. REIKETSET
W. succ.  PsrA (101-135) RLVKPIMR..VGERGE GKWKEVSWDE AYTFIAKKL. DEIKQKHGA
                         +     (+)

Segment III

Membrane-bound nitrate reductases

E. coli   NarG (185-242) RVAGFSPIPA MSMVSYASGA RYLSLIGGTC LSFYDWYCDL PPASPQTWGE QTDVPESA..
E. coli   NarZ (185-242) RVAGFSPIPA MSMVSYAAGT RYLSLLGGTC LSFYDWYCDL PPASPMTWGE QTDVPESA..

Soluble nitrate reductases/molybdenum-dependent formate dehydrogenases

T. pant.  NapA (145-203) .LGMFGSGQW TIFEGYAATK LMRAGFRSNN LDPNARHCMA SAAYAFMRTF GMDEPMGCYD
A. eutr.  NapA (145-203) .VACSAPAQW TVWEGYAAAK LYKAGFRSNN IDPNARHCMA SAAAGFMRTF GMDEPMGCYD
E. coli   NapA           .IGMFGSGQW TIWEGYAASK LFKAGFRSNN IDPNARHCMA SAVVGFMRTF GMDEPMGCYD

K. pneu.  NasA (92-149)  .AVRFTSGQL LTEDYYAANK LMKGFIGAAN IDTNSRLCMS SAVTGYKRAR WGT.VVPCSY
B. subt.  NarB (84-142)  .MAVYGSASI TNEEAYLLGK FARVGLQTKY IDYNGRLCMS AAATAANQTF GADRGLTNPL
Synoc.    NarB (126-184) .ICFYGSGQF QTEDYYIAQK LVKGCLGTNN FDTNSRLCMS SAVSAYSLCL GSD.GPPACY

M. form.  FdhA (96-154)  IGFFCCARS. PNENIYVNQK FARIVVGTHN IDHCARLCHG PTVAGLAASF GSGAMTNSYA
E. coli   FdhF (103-162) IQTTGSSRGT GNETNYVMQK FARAVIGTNN VDCCARVUHG PTVAGLAASF GNGAMSNAIN
W. succ.  FdhA (151-208) VMFIGGSKC. SIEQSYYFRK FAA.FFGTNN LDTIARICHA PSVAGLHQSV GYGGMTNHLA
E. coli   Fdog (160-218) STGNLCASAS SNETGYLTQK FSR.ALGMLA VDNQARVUHG PTVASLAPTF GRGAMTNHWV
E. coli   FdnG (160-218) STGMLCASGA SNETGMLTQK FAR.SLGMLA VDNQARVUHG PTVAGVSNTL GRGAMTNHWV
                                   •   *    +  (+)       - ▫•+  !   ▫         *

S- and N-oxide reductases

E. coli   TorA (158-216) S.GWQSTGMF HNASGMRAKR IALHGNSVGT GGDYSTGAAQ VILPRVVGSM EVYEQQTSWP
E. coli   BisC (76-135)  SYGWRSNGVL HKASTLLQRY MALAGGYTGH LGDYSTGAAQ AIMPYVVGGS EVYQQQTSWP
E. coli   DmsA (142-197) TLGGTMTRSW PPGNTLVARL MNCCGGYLNH YGDYSSAQIA EGLNYTYGGW A....DGNSP
B. subt.  OrfX (100-154) VLHSHDYANN GLLKALDQRF FNGYGGVTEI VGSICWGSGI EAQSWDFGRS YGHG.....P
                                         * (+)       *      ***

Polysulphide reductase

W. succ.  PsrA (136-194) .IKQKHGAHT VAFTARSGWN KTWFHHLAQA YGSPNIFGHE STCPLAYNMA GRDVFGGSMN
```

assimilatory nitrate reductase in this organism. Ferredoxins or flavodoxins have been suggested as electron donors in other cases [182,375,482].

All three eubacterial assimilatory nitrate reductase operons also code for a sirohaem-dependent nitrite reductase (Section 7.1.1) and a periplasmic binding protein-dependent nitrate uptake system (Section 6.2) [18,325,397a,400]. The *K. pneumoniae nasFEDCBA* operon is linked to a gene, *nasR*, encoding a nitrate- and nitrite-responsive positive regulator of the operon [203] as well as to the two

Segment IV

```
E. coli   NarG (243-325) DWYNSSYI IAWGSNVPQT RTPDAH.... ....FFTEVR YKGTKTVAVT PDYAEIAK.. ..LCDLWLAPK QGTDAAMALA MGHVMLREFH LDNPSQ
E. coli   NarZ (243-325) DWYNSSYI IAWGSNVPQT RTPDAH.... ....FFTEVR YKGTKTIAIT PDYSEVAK.. ..LCDQWLAPK QGTDSALAMA MGHVILKEFH LDNPSD
T. pant.  NapA (204-288) DFEAADAF VLWGSNMAEM HPILWT........RVADRRL GHPHVKVAVL STFTHRSS.. .DLADIPIVFK PGTDLAILNY IANHIIQTGR VNRDFV
A. eutr.  NapA (204-288) DFEAADAF VLWGSNMAEM HPILWT.... ...RVTDRRL SHPKTRVVVL STFTHRCF.. .DLADIGIIFK PQTDLAMLNY IANYIIRNNK VNKDFV
E. coli   NapA           DIEQADAF VLWGANMAEM HPILWS.... ...RITNRRL SNQNVTVAVL STYQHRSF.. .ELADNGIIFT PQSDLVILNY IANYIIQNNA INQDFF
K. pneu.  NasA (150-240) DVENSDLV VLVGSNAAWA HPVLYQ.... ..RLAQAKRD NPQMRVVVID PRRTATCD.. ..IADRHLALA PGSDGGLFVG LLNAIAASRR ISDQRA
B. subt.  NarB (144-226) DIPHTRVI ILAGTNIAEC QPTIMP.... ....YFEKAK ENGAYFIAID PRETATTK.. ..IADLHLKIK PGTDAALANG LVKIIIDEQL INEDFI
Synoc.    NarB (185-268) DLDLADCL LIVGSNTAEC HPILFN.... ...RYRKRHK QGGTNLIVVD PRCTPTAE.. ..VADLHLALK PGSDVALLNG LGWLLYQMGY VKKDFI
M. form.  FdhA (155-237) SFEDADLI FSIGANSLEA HPLVGR.... ...KLMRAK. MNGAYFIVAD PRYTPTAK.. ..QADQYIPFK TGTDVALMNA MMNVIISEGL EDKEFI
E. coli   FdhF (163-245) EIDNTDLV FVFGYNPADS HPIVAN.... ...HVINAK. RNGAKIIVCD PRKIETAR.. ..IADMHIALK NGSNIALLNA MGHVIIEENL YDKAFV
W. succ.  FdhA (209-291) DMMHSKAI FIIGGNPAVN HPVGMV.... ...HILRAK. EAGAKIIVVD PHFSRTAT.. ..KADHYVRLR NGTDVAFMYG MIRHIVKNGL EDKEFI
E. coli   FdoG (219-302) DIKNANLV VVMGGNAAEA HPVGFR.... ...WAMEAKI HNRREADCDR SCFTRTAA.. ..VADYYAPIR SGTDIAFLSG VLLYLLNNEK FNREYT
E. coli   FdnG (219-302) DIKNANVV MVMGGNAAEA HPVGFR.... ...WAMEAKN NNDATLIVVD PRFTRTAS.. ..VADIYAPIR SGTDITFLSG VLRYLIENNK INAEYV
E. coli   TorA (218-316) VLQNSKTI VLWGSDLLKN QQANWW..(11)..QLKRKSA AGEIEVISID PVVTSTHEYL GGEHVKHIAVN PQTDVPLQLA LAHTLYSENL YDKNFL
E. coli   BisC (136-230) VLEHSDVV VLWSANPLNT LKIAWNASDE QGLSYFSALR DSGKKLICID PMRSETVDFF .GDKMEWVAPH MGTDVALMLG IAHTLVENGW HDEAFL
E. coli   DmsA (199-286) DIENSKLV VLFGNNPGET RMSGGGVT.. ..YYLEQARQ KSNARMIIID PRYTDTGA.. .GREDEWIPIR PGTDAALVNG LAYVMITENL VDQAFL
B. subt   OrfX (156-238) DIYNSKHV VVWGRNVSRT NMHLYH.... ....HLQQVK KKGATITVID PIFNPTAK.....LADRYISVK PGMDGWLAAA VLKVLIEMGR TDETFI
W. succ.  PsrA (196-279) DFAKAKYI INMGHNVFEG IVISYV.... ...RQYMEAI ENGAKVVTLE PRLSVMAQ.. ..KASEWHAIK PGHDLPFVLG FMHTLIFENL YDKKFV
                                  * *                                                           (-)
```

Segment V

```
E. coli   NarG (500-587) VKAYTPAWA EQITGVSRSQ IIRIAREFADN ADKTHGASM IIVGAGLNHW YHLDMNYRGL INMLIFCGCV GQSGG.(6).DQEKLRPQTG
E. coli   NarZ (499-586) IKPYTPAWG EQITGVPRQY IETIAREFADT AHKTHGRSM IILGAGVNHW YHMDMNYRGM INMLIFCGCV GQSGG.(6).GQEKLRPQTG
B. subt   NarG                                                                                            GQEKLRPAEG
T. pant.  NapA (336-415) VSEYTLDKV SELSGVEPAF LEQLAELYADP D....RKVM SLWTMGFNQH VRGVWANQMV YNLHLLTGKI SEPGN.....SPFSLTGQAS
A. eutr.  NapA (336-415) VSKYDADYV SKLSAVPKAK LDQLAELYADP N....IKVM SLWTMGFNQH TRGTWANNMV YNLHLLTGKI ATPGN.....SPFSLTGQPS
E. coli   NapA           VAEYTLEKT AEMTGVPKDQ LEQLAQLYADP N....KKVI SYWTMGFNQH TRGVWANNLV YNLHLLTGKI SQPGC.....GPFSLTGQPS
K. pneu.  NasA (243-322) AQDWDLDKV AQFCGLPRQQ IADFYREFIAA .....PRAI TLYTMGINQS ASGSDKCNAI INVHLACGKY GRPGC.....GPFSLTGQPN
B. subt.  NarB (240-318) TDSLDLNDI AEQTSVSLVD IRKAAVKFAKE .....TSGM LFTARGIEQQ TDGTAAVKGF LNMVLITGKI GKPYS.....GYGAITGQGN
Synoc.    NarB (282-360) IEDYPPQRT AELTGLAVAE LVRAADLIASA .....QRWL SLWSMGVNQS IQGTAKATSL INLHLLTRQI GLPGC.....GPFSLTGQPN
M. form.  FdhA (251-329) VSKYTPEMA EEITQVPADV IRDIAIKYAKA .....DKAA IVYSLGITEH SHGVDNVMQT ANLAMLTGNI GRLGT.....GVNPLRGQNN
E. coli   FdhF (259-337) VEGYTPESV EDITGVSASE IRQAARMYAQA .....KSAA ILWGMGVTQF YQGVETVRSL TSLAMLTGNL GKPHA.....GVNPVRGQNN
W. succ.  FdhA (305-383) CEQYTPEVV EEVTGVPAQQ LIEITEIFAKA .....KPAS LIWGMGLTQH TTGTSNTRLA PILQMILGNI GKRGG.....GTNVLRGHDN
E. coli   FdoG (368-448) VSRYTPDVV ENICGTPKDA FLKVCEYIAET SAHD.KTAS FLYALGWTQH SVGAQNIRTM AMIQLLLGNM GMAGG.....GVNALRGHSN
E. coli   FdnG (368-448) VSRYTPDVV ENICGTPKAD FLKVCEVLAST SAPD.RTTT FLYALGWTQH TVGAQNIRTM AMIQLLLGNM GMAGG.....GVNALRGHSN
E. coli   TorA (336-412) GQPKDAAWA EKLSGIDAET IRGLARQMAA. .....NRTQ IIAGWCVQRM QHGEQWAWMI VVLAAMLGQI GLPGG......GFGFGWHYN
E. coli   BisC (251-326) GIAKTAEWA AEICGVGAAK IRELAAIFHQ. .....NTTM LMAGWGMQRQ QFGEQKHWMI VTLAAMLGQI GTPGG......GFGLSYHFA
E. coli   DmsA (320-396) GVAKTPEWA SQITGVPADK IIKLAREIGST .....KPAF ISQGWGPQRH ANGEIATRAI SMLAILTGNV GINGG......NSGAREGSY
B. subt.  OrfX (260-329) FIVKTETSM EELEYLAGLY AD......... .....GPVS TFMGLGMQRY KNGGGTIRWI DALVAASGNV GIKGG.....GANFGNVQIG
W. succ.  PsrA (282-373) IEPCTPEKM ALECDIPADT IKRLAREFAKA A....PKAI FDFGHRVTFT PQELELRRAM MMVNALVGNI ERDGGMY..FGKNASFYNQF
```

Segment VI

```
B. taur.  75kD (580-623)                           AD.....VILPGA AYTEKSATYV NTEGRAQQTK.....VAVTPP GLAREDWKII
N. cras.  78kD (584-627)                           AD.....IVLPGA AYTEKAGTYV NTEGRVQMTR.....AATGLP GAARTDWKIL
P. deni.  66kD (536-579)                           RD.....IILPGA CYTEESGLFV NTEGRPQLAM.....RANFAP GEGKENWAIL
E. coli   NouG (603-646)                           AH.....LVLSAA SFAESDGTVI NNEGRAQRFF.....QVYDPA YYDSKTVMLE

E. coli   NarG (765-836) KLD LVVTLDFRLS STCLY.SD.....IILPTA TWYEKDDMNT SDMHPFIHPL S...AAVDPA WEAKSDWEIY KAIAKKFSE
E. coli   NarZ (764-835) KLD LLVTLDFRMS STCLF.SD.....IVLPTA TWYEKDDMNT SDMHPFIHPL S...AAVDPA WESRSDWEIY KGIAKAFSQ
B. subt.  NarG           KLD LLINLDFRMA GTALY.SD.....IV.PAA TWYEKHDLSS TDMHPFIHPF A...PAISAP WESKSDWDIF KALSKAVSD
T. pant.  NapA (504-574) PDN FIVVSDAYPT VTALA.AD.....LILPAA MWVEKEGAYG NAERRTHVWH ....QLVEAP GEARSDLWQM MEFSTRFTT
A. eutr.  NapA (504-574) PAN FIVVSDAYPT VTALA.AD.....LVLPSA MWVEKEGAYG NAERRTQFWH ....QLVDAP GEARSDLWQL VEFAKRFKV
E. coli   NapA           PRN FIIVSDPYPT VSALA.AD.....LILPTA MWVEKEGAYG NAERRTQFWR ....QQVQAP GEAKSDLWQL VQFSRRFKT
K. pneu.  NasA (405-475) PVS LVIISDVADT DTGRF.AH.....IRFPAL AWGEKSGTVT NSERRISLQR ....AFMPPP GEARAAGGLS PAGRGLAGL
B. subt.  NarB (403-473) RLT FFVAIDLFIS ETAKY.AD.....LVLPAS SYLEDEGTMT NVEGRVTLRE ....ASRPCP GEAKHDWQII CDLASALGK
Synoc.    NarB (446-517) RSP FTVLQDAYHP TETTTYAH.....LLLPAA QWSEKTGTMT NSERRVTLCQ ....AFRQPP GEARADWQIF AEVGRRLGF
M. form.  FdhA (412-483) NLD FFVVQDIFMT DTAEF.AD.....VVLPAA CWAEQEGTFT NGERRVQLIR ....KAVDAP GESKYDWEIF CDLAKKMGA
E. coli   FdhF (421-491) DLE LVIVQDIFMT KTASA.AD.....VILPST SWGEHEGVFT AADRGFQRFF ....KAVEPK WDLKTDWQII SEIATRMGY
W. succ.  FdhA (485-560) KMD LVVFFDPYFN DAAALTNRKDNL.YILPAA TQMETSGRVA ATNRSYQWRS ....MVMKPL FECRPDEEIL FDLAKRLGF
E. coli   FdoG (575-662) KLK FLVTIDPLNT ETSNFWQ.(17).FRLPST CFAEENGSIV NSGRWLQWHW....KGADAP GIALTDGEIL SGIFLRLRK
E. coli   FdnG (575-662) KLK YMVVIDPLVT ETSTFWQ.(17).FRLPST CFAEEDGSIA NSGRWLQWHW ....KGQDAP GEARNDGEIL AGIYHHLRE
E. coli   TorA (503-576) NVE TVIAIDNQWT STCRF.AD.....IVLPAT TQFERNDLDQ YGNHSNRGII AMK.QVVPPQ FEARNDFDIF RELCRRFNR
E. coli   BisC (409-482) KPE LVVISECFWT AAAKH.AD.....IVLPAT TSFERNDLTM TGDYSNQHLV PMK.QVVPPR YEARNDFDVF AELSERWEK
E. coli   DmsA (476-550) KCE LIVVIDCHMT SSAKYAD......ILLPDC TASEQMDFAL DASCGNMSYV IFNDQVIKPR FECKTIYEMT SELAKRLGV
B. subt.  OrfX (392-464) KVP MTVAIDSIMT DTAELCD......YVLPTA TVFEEEDIYY SSMYHHYVQY GK..KLVEPQ GEAKSDSWIW SELAKRLGF
W. succ.  PsrA (466-540) SMD LVVCVDIQVS DTAWFAD......VVLPDT TYLERDEEFT AGGGKNPSFG IGRQKVVEPL GDAKPGWKIA KELSEKMGL
                                        (-)                 -                                (- +)
```

Segment VII

```
E. coli   NarG (1118-1158) VWLS EADAKDLGIA DNDWIEVFNS NG.ALTARAV VSQRVPAG
E. coli   NarZ (1117-1157) VWIS ETRCRELTIV DNDWVEVFNA NG.ALTARAV VSQRVPPG
B. subt.  NarG             VWMN KDDAEDTDIK DNDWIECFNR NG.VVVARAV LSHRIPKG
T. pant.  NapA (749-790)   CFMN PEDARDMGFN QGAEVRIVSR RGEIRSRVET RGRNRMPR
A. eutr.  NapA (749-790)   VFMH PEDAKALGLR RGVEVEVVSR RGRMRSRIET RGRDAPPR
E. coli   NapA             LFIH PLDAKARDLR RGDKVKVVSR RGEVISIVET RGRNRPPQ
K. pneu.  NasA (597-635)   VEVP PAEPQRYHLL EGELARVRSP KG...VMVAK VTIGFANG
B. subt.  NarB (595-635)   MEIH PQTAATYNIE DRVLVKIESP RG.SITVRSK LSEQIRKD
Synoc.    NarB (638-678)   VEIH PRDADRYNIS EGQAVVIRSR RG.EGCFPAK VTTAISPG
M. form.  FdhA (599-639)   VEIN TEDAAELGIA NKEKVKVKSR RG.EIEIAAR VTDDIVKG
E. coli   FdhF (608-648)   AQIN TEDAKRLGIE DEALVWVHSR KG.KIITRAQ VSDRPNKG
W. succ.  FdhA (836-878)   VEIH PETATDLGVK HGDMVWVHGT NGAKILVKAR HSYKVNKT
E. coli   FdoG (921-958)   VEIG ESLANKLGIA QGDTVKVSSN RG.YIKAKAV VTKRIRTL
E. coli   FdnG (921-958)   VEIS ETLAAAKGIN NGDRVTVASK RG.FIRAVAV VTRRLKPL
E. coli   TorA (723-762)   VFIN PQDASARGIR NGDVVRVFNA RGQVMAGAVV SDRYAPGV
E. coli   BisC (629-669)   VTIH PDDAQERGIQ DGDTVRLWNA RG.QILAGAV ISEGIKPG
E. coli   DmsA (695-735)   MWIN PLDAQKRGIH NGDKVRIFND RG.EVHIEAK VTPRMMPG
B. subt.  OrfX (584-624)   VDIS PDIAAGQDLQ DGDEVVIFND RG.SMKGKVK VMKQAHAK
B. pul.   Orf              PHMN EQDAKERQIE DGDMVRVLNH RG.ECELIVS VGQNVLSG
W. succ.  PsrA (672-712)   IWIH PKTASKLGIK NGDAIELYNK FS.SQKSKAL ITEGVRED
                                                     *
```

Fig. 12 (continued).

component nitrate/nitrite response regulatory system *narXL* (Section 3.1) The latter does not, however, regulate the *nasFEDCBA* operon [526].

5.3. Membrane-bound respiratory nitrate reductase

The membrane-bound nitrate reductase (Nar) has a wide distribution, being found not only in the denitrifying bacteria but also in many enteric bacteria. Substantive biochemical characterisation is limited to the enzymes of *E. coli* and *P. denitrificans / T. pantotropha* and so we limit our discussion to the reductases from these sources. The molecular masses of the three subunits are around 140 kDa (α), 60 kDa (β), and 25 kDa (γ). The subunits and their bound cofactors are discussed in detail in Section 5.4.

Two membrane-bound nitrate reductases are found in *E. coli* (and probably also in *Salmonella typhimurium*; [44]) (Section 2.2). The α, β, δ and γ proteins of the major membrane-bound nitrate reductase Nar-A are coded by the *narGHJI* operon situated at 27 min on the *E. coli* chromosome (Fig. 11). This operon is linked to those coding for the nitrite exporter NarK (Fig. 11; Section 6.1) and to the two-component nitrate/nitrite sensing regulatory system NarXL (Fig. 11; Section 3.1). The α, β, δ and γ proteins of the minor membrane-bound nitrate reductase Nar-Z are coded by the *narZYWW* operon situated at 32.5 min on the *E. coli* chromosome [74]. The *narZYWV* operon appears to be linked to a gene coding for a probable NarK homologue [74]. Nar-Z and Nar-A have similar specific activities [254]. Their sequences are, therefore, assumed to be of equal weight in structural comparisons. Further, Blasco and co-workers have shown by expressing combinations of complementary subunits from the two operons that, in the combinations tested and allowing that the heterologous proteins are relatively unstable, the subunits are functionally interchangable [75,76]. *T. pantotropha*, in contrast to *E. coli*, possesses a single copy of the membrane-bound nitrate reductase operon [53]. A partial sequence of an operon coding for a membrane-bound nitrate reductase has been reported for *B. subtilis* [238a]. Regulation of expression of *E. coli* Nar-A has been extensively studied. References to this work and a brief summary are found in Section 3.1. In contrast, little is known about mechanisms that control expression of the membrane-bound nitrate reductase in denitrifying organisms.

5.3.1. Subunit interactions

The γ subunit of the membrane-bound nitrate reductase anchors the α and β subunits to the cytoplasmic side of the cytoplasmic membrane [34,338,339,505,527] (Figs. 2 and 3). An $\alpha\beta$ complex can be released (irreversibly) from inside-out membrane vesicles of *P. denitrificans* by washing in the absence of magnesium or presence of EDTA [34]. This suggests a rôle for magnesium in the interaction of the soluble subunits with the γ subunit and/or membrane phospholipids. The γ subunit is also easily lost during purification of the *P. denitrificans* and *E. coli* Nar-A enzymes [133,162]. Loss of the γ subunit is accompanied by an apparent reduction in size of the β subunit as determined by SDS-PAGE from 61 kDa to 58 kDa (so-called β'). Such a β'-like band is also generated by limited proteolysis of the enzymes, suggesting that the change in electrophoretic mobility is most likely the result of proteolytic cleavage ([145]; note that a post-translational modification event has also been postulated [107,108]). β' has an unmodified N-terminus [145], suggesting that the proteolytic modification is at the C-terminus. The inference is that contact between $\alpha\beta$ complex and γ subunit is mediated solely by the β subunit and in a region near the carboxy end of the polypeptide chain. The extreme C-

Fig. 12. Segments of amino acid sequence similarity in MGD-dependent enzymes. Amino acid residues that are identical in all sequences are shown in bold. Also shown in bold are: the possible membrane-bound nitrate reductase Segment I histidine iron-sulfur cluster ligand; the proposed cysteine, selenocysteine or serine molybdenum ligand in Segment III of the soluble nitrate reductases, formate dehydrogenases and S-/N-oxide reductases; the methionine in BisC Segment V which is replaced by isoleucine in a mutant strain deficient in BisC activity [414a]. * indicates residues that are conserved in all but one sequence. Strict conservation of amino acids with charged side chains is indicated by +(Arg/Lys) and −(Asp/Glu). ↑ indicates amino acids proposed to bind an iron-sulfur cluster. In the Segment III comparisons ! indicates a residue that is conserved in the formate dehydrogenases but is a different but conserved amino acid in the soluble nitrate reductases; ● an amino acid conserved in the formate dehydrogenases and assimilatory nitrate reductases but not the periplasmic nitrate reductases; □ an amino acid conserved amongst the soluble nitrate reductases but not found in the formate dehydrogenases; ◆ an amino acid conserved between the formate dehydrogenases and periplasmic nitrate reductases. Sequences are those of the *E. coli* major membrane-bound nitrate reductase catalytic subunit (NarG) [73,597]; *E. coli* minor membrane-bound nitrate reductase catalytic subunit (NarZ) [74]; *B. subtilis* major membrane-bound nitrate reductase catalytic subunit partial sequence (NarG) [238a]; the catalytic subunits of the periplasmic nitrate reductases of *T. pantotropha* (NapA) [64], *A. eutrophus* (NapA) [495] and *E. coli* (NapA) [438]; *K. pneumoniae* assimilatory nitrate reductase (NasA) [324]; *B. subtilis* assimilatory nitrate reductase (NarB; note that we include an N-terminal extension relative to that originally reported) [397a]; *Synechococcus* PCC7942 assimilatory nitrate reductase (NarB) [18]; *Methanobacterium formicicum* F_{420}-dependent formate dehydrogenase catalytic subunit (FdhA) [494]; *E. coli* formate dehydrogenase component of the formate hydrogenlyase system (FdhF) [614]; *Wolinella succinogenes* major membrane-bound formate dehydrogenase catalytic subunit (FdhA) [79]; *E. coli* membrane-bound formate dehydrogenase-O catalytic subunit (FdoG; [417]); *E. coli* nitrate-inducible membrane-bound formate dehydrogenase catalytic subunit (FdnG)[59]; *E. coli* periplasmic TMAO reductase (TorA) [364]; *E. coli* biotin sulfoxide reductase (BisC) [506]; *E. coli* dimethyl sulfoxide reductase catalytic subunit (DmsA) [67]; a protein encoded by a *Bacillus subtilis* partial open reading frame which may be a cytoplasmic S- or N-oxide reductase (OrfX) [397]; *Bacillus pumilus* partial xylanase-linked *orf* [178a] ; *W. succinogenes* polysulfide reductase MGD-binding subunit (PsrA) [298]. For Segment VI the MGD-binding protein alignment is compared with a portion of the largest subunit of the proton-pumping NADH-quinone oxidoreductase (complex I/NDH-1); bovine 75 kDa subunit [457b], *Neurospora crassa* 78 kDa subunit [420a], *P. denitrificans* 66 kDa subunit (Nqo3) [601a] and *E. coli* 91 kDa subunit (NuoG) [585].

terminus of the β subunit of the structurally related membrane-bound MGD-dependent *E. coli* enzyme dimethylsulfoxide reductase has also been implicated in integral membrane subunit binding [462].

The relative stoichiometry of the membrane-bound nitrate reductase subunits is uncertain. It is clear that the soluble nitrate reductase $\alpha\beta$ fragment has a stoichiometry of $\alpha_1\beta_1$ (e.g., [34]). In the *E. coli* nitrate reductase operons, successive open reading frames overlap the stop codon of the previous gene. This suggests that translational coupling [401] is operative, resulting in an equal stoichiometry of synthesis of the subunits. There appears to be no objection to such a $(\alpha_1\beta_1\gamma_1)_n$ stoichiometry on mechanistic grounds. It should be noted that, if this stoichiometry is correct, the integral membrane portion of the enzyme (γ subunit) comprises only about 10% of the total protein mass.

5.3.2. Enzymology

The presence of the γ subunit is necessary for the membrane-bound nitrate reductase to oxidise quinols [34,162,384,527]. It is, however, more convenient to assay nitrate reductase activity using reduced viologens ($E^{o\prime} < -350$ mV) as electron donors. The viologens sustain a higher V_{max} but have a higher K_m (electron donor) than the quinols. Although the exact site(s) at which electrons from the viologens enter the complex has not been elucidated, it can be via the α and/or β subunits, as the γ subunit is not required for viologen-linked nitrate reductase activity.

Morpeth and Boxer [384] have proposed that *E. coli* Nar-A with reduced viologen as electron donor operates a compulsory order mechanism in which nitrate (two electron acceptor) binds before the reduced viologen (one electron donor). Nitrite release was proposed to be rate-limiting. With UQ_1 as electron donor, either reduction of the enzyme by quinol or quinone release was rate-limiting. With *P. denitrificans* nitrate reductase the *b*-cytochromes are reducible by duroquinol in the absence of nitrate (but note that the kinetic competence of this reaction has not been established [34]). Thus, with the physiological electron donor, an 'enzyme substitution' or two-site ping-pong mechanism, in which binding of substrates is of random order, may apply.

The membrane-bound nitrate reductase is activated by prolonged exposure to very reducing conditions [54]. The mechanism involved is unclear but has been suggested to be a consequence of the reduction of the lowest potential [Fe-S] clusters (Section 5.4.3).

5.4. Structure / function analysis of the membrane-bound, periplasmic and eubacterial assimilatory nitrate reductases

5.4.1. The MGD-binding subunit

Alignment of the sequences of the MGD-binding subunits of the nitrate reductases with other bacterial MGD-dependent proteins reveals that certain sections of the polypeptide chains are highly conserved (Fig. 12; for earlier analyses see the sequence references in the Fig. 12 legend and in particular [79,597]). Although some regions of sequence similarity conceivably correspond to distinct domains, we shall be cautious and refer to the regions as Segments. The minimum size of the conserved regions (Segments II–VIII) is around 350 amino acids (about 40 kDa), while the smallest MGD-binding proteins are around 80 kDa. The sections of polypeptide between the segments are of variable length. They are most extensive in the case of the membrane-bound nitrate reductases. It is notable that the membrane-bound nitrate reductase β subunit also contains similar extensive inserts (see later). The function of these inserts is unclear, but they are possibly involved in forming a transient binding site for the δ subunit during assembly of the enzyme. In both subunits the inserts are predicted to have substantial regions of α-helix.

Segment I is not found in all of the MGD binding polypeptides (Fig. 12). It appears to bind a [4Fe-4S] cluster and is discussed further in Section 5.4.3. Segments II, and IV–VIII are conserved in all the MGD-dependent enzymes. These segments are likely to be involved in binding the MGD cofactor. Known nucleotide binding motifs are not, however, apparent. Several regions of weak sequence similarity with other proteins have been identified. Rivers and co-workers [442] have suggested sequence similarity between *E. coli* MoaA and MoaD, two proteins thought to be involved in synthesis of the pterin portion of the MGD cofactor, and Segments IV and V of the MGD-enzymes, the implication being that these Segments may bind the pterin ring (note that the MoaA/Segment V alignment is not well conserved in possible *Methanobacterium formicicum* and *Bacillus subtilis* MoaA homologues [200,590]). Luque and co-workers suggested sequence similarity between the *modA* gene product of *Azotobacter vinelandii* [337], which might code for a molybdopterin binding protein, and *E. coli* FdhF. Chow and co-workers [118] suggest sequence similarity between residues 290 to 512 of *M. formicicum* FdhA and residues 455 to 660 to the 75 kDa subunit of mammalian complex I (proton pumping NADH-ubiquinone oxidoreductase). Of these suggestions, only the similarity with the 75 kDa complex I subunit is statistically significant when applied over all MGD proteins and this only over a portion of Segment VI (Fig. 12). The functional significance of this similarity is not clear, as current models do not ascribe a nucleotide binding function to the 75 kDa subunit of complex I [164,578]. Note that the 75 kDa protein would be the fifth subunit of complex I to show structural similarity to a component (FdhF) of the formate hydrogenlyase system [78,578].

By analogy with other enzymes binding nucleotide cofactors, each of the phosphate groups of the MGD cofactor is likely to be liganded by at least one ionic interaction with a basic amino acid side-chain in addition to multiple hydrogen-bonding interactions. The most likely

candidate for a basic phosphate ligand is the absolutely conserved arginine in Segment II (Fig. 12). A second, almost conserved, basic residue in the same Segment is another strong candidate (Fig. 12) with the conserved basic residue in Segment VII (Fig. 12) also a possibility.

The Segment III region shows sequence similarity between enzymes with similar types of substrate but not between enzymes of different substrate type (Fig. 12). This segment has thus been suggested to be involved in substrate binding and catalysis [59,64,79,495]. One distinct Segment III grouping ('formate dehydrogenase site') comprises the molybdenum-dependent formate dehydrogenases and the soluble nitrate reductases (periplasmic nitrate reductase and eubacterial assimilatory nitrate reductases). The similarity of the active sites of the soluble nitrate reductases inferred from sequence comparisons is also apparent from the very similar Mo(V) EPR spectra of the two types of enzyme [56,182]. The possible structural relationship of the substrate-binding sites of formate dehydrogenases and nitrate reductases is also supported by the observations that nitrate is both a stabiliser and competitive inhibitor of formate dehydrogenase [31,175,284, 301,474]. A second distinct Segment III grouping can be recognised that contains enzymes that reduce S- or N-oxides (the 'S-oxide site'). It is notable that Segment III of the membrane-bound nitrate reductase is distinct from either the 'formate' or 'S-oxide sites'. The different structures of the soluble and membrane-bound nitrate reductase catalytic sites implied by this sequence comparison are consistent with the contrasting catalytic properties of the periplasmic and membrane-bound enzymes and the distinct Mo(V) EPR signals of the soluble and membrane-bound enzymes [56].

Formate and nitrate are both approximately trigonal planar monovalent anions. The two conserved basic residues in the 'formate site' might thus form salt bridges to the anionic substrate. Compared to formate and nitrate, S- and N-oxides are uncharged and relatively bulky, possibly explaining the absence of absolutely conserved charged residues in the 'S-oxide site' and the presence of three conserved glycines which might permit entry of large substrate molecules into the active site (Fig. 12).

Selenocysteine replaces cysteine at the 'conserved Cys' position in the three *E. coli* formate dehydrogenases (Fig. 12). This amino acid is incorporated during translation [224]. Recent EPR investigations of *E. coli* formate hydrogenlyase-linked formate dehydrogenase (FdhF) have clearly demonstrated that the selenium directly coordinates the molybdenum atom of the cofactor [198]. The equivalent selenocysteine/cysteine in the other formate dehydrogenases and the soluble nitrate reductases (Fig. 12) is likewise expected to be a molybdenum ligand. Variable temperature MCD spectroscopy produced no evidence for molybdenum cysteine coordination in periplasmic dimethylsulfoxide reductases [57,172] consistent with the absence of a conserved cysteine in the 'S-oxide site' (Fig. 12). The Mo(V) EPR signals of *T. pantotropha* periplasmic nitrate reductase have been interpreted as indicating an additional -SR molybdenum ligand relative to periplasmic dimethylsulfoxide reductase: an analysis that is likewise consistent with the sequence comparisons [56,64] (Fig. 12). Cysteine coordination of the cofactor molybdenum atom is also probable in the membrane-bound nitrate reductase. EXAFS of *E. coli* Nar-A shows a (sulfur/chlorine) scatter additional to those attributed to the dithiolene ligation and any bound chloride [193] while comparison of the nitrate reductase Mo^{V} g_{av} values with those of model compounds also suggests that more than two sulfur ligands could be present [597]. This additional sulfur ligand is presumably one of the two conserved cysteines present in the membrane-bound nitrate reductase Segment III (Fig. 12).

The reason why nitrate reductases and formate dehydrogenases, but not S- and N-oxide reductases, have cysteine/selenocysteine ligation is obscure. Mutagenic replacement of the selenocysteine in *E. coli* formate dehydrogenase-H (FdhF) by cysteine leads to a drastic reduction in k_{cat} due to a decrease in the rate of the catalytic step in which bound formate is oxidised, i.e., the reactivity of the MGD active site is affected [32]. The mutant enzyme also binds formate significantly more tightly than the selenocysteine-containing enzyme [32]. Both the wild-type and SelCys → Cys mutant are inhibited by the alkylating agent iodoacetamide but only in the presence of formate [32]. From the pH dependencies of this reaction in the two enzymes it was inferred that the inhibition was due to alkylation of the active site cysteine/selenocysteine [32] with the implication that binding of formate and/or reduction of the molybdenum atom releases the selenocysteine/cysteine as the free selenolate/thiolate [32,224]. This idea is, however, difficult to reconcile with the EPR evidence for direct coordination of selenium to the molybdenum atom in formate-reduced FdhF [198]. A terminal sulfur (= S) molybdenum ligand seen in the Mo^{VI} state of other molybdopterin-dependent hydroxylases, and which is present as a thiol agent-reactive Mo^{IV}-S-H function in the reduced enzyme [191,191a,191b,218] has been demonstrated for the formate dehydrogenase of *M. formicicum* [41]. We suggest that in the experiments described above iodoacetamide reacts with such a terminal thiolate molybdenum ligand and not the active site cysteine/selenocysteine.

5.4.2. The rôle of the MGD cofactor in nitrate reduction

Various aspects of the chemistry of the molybdopterin cofactor (Fig. 7) have been reviewed in detail in [86,125,132,160,173,231,232,425–427,520,597] (note that the nomenclature of *E. coli* genes involved in the metabolism of molybdenum and synthesis of the molydopterin cofactor has recently been rationalised [484]). All available evidence indicates that the molybdenum atom of the cofactor cycles between the Mo(IV) and Mo(VI) oxidation states using both one and two electron steps. There is

considerable evidence that the molybdenum atom is bound by the organic portion of the cofactor via the dithiolene function (Fig. 8; [57,172,189,213,415,416]; other possible modes of molybdenum coordination by the cofactor are discussed in [160]). In addition to the dithiolene coordination, EXAFS studies on several enzymes suggest that the molybdenum atom in all three oxidation states is ligated by at least one oxo-group (e.g., [194]; but see Section 5.4.2.1). The remainder of the molybdenum coordination sphere appears both to differ between enzymes and to undergo change during the catalytic cycle. In the nitrate reductases there is almost certainly a protein cysteine thiolate ligand (Section 5.4.1).

Complete oxo-transfer cycles between water and substrate have now been demonstrated for synthetic mononuclear oxo-molybdenum complexes ([160,173,241]; important recent papers include [195,601]; the system described by Craig and Holm [131] is also of interest, as it catalyses the reduction of nitrate to nitrite). These model compound studies strongly support the idea that the oxygen atom is transferred via the molybdenum coordination sphere. They also suggest that the organic part of the cofactor may not play a direct role in the oxo-transfer reaction. What, then, is this organic portion doing? Fraústo da Silva and Williams [173] have argued that the dithiolene moiety, in contrast to protein ligands, provides a binding site for molybdenum that discriminates against other metal ions or oxoanions. The basis of the specific binding is proposed to be the extremely 'soft' (deformability of valence electron orbitals leading to a propensity for covalent bonding) nature of both molybdenum and the dithiolene ligand. These workers suggest that the pterin ring acts as a molecular adaptor allowing proteins to recognise and bind the molybdate-dithiolene complex.

The pterin might function in electron transfer between the molybdenum atom and other redox centres in the enzyme. As we argue below for iron-sulfur clusters (Section 5.4.3), the pterin ring need not redox cycle to perform this function as the delocalised electron orbitals could provide an electron transfer pathway of lower energy than that provided by the protein matrix. We note also that spin coupling measurements show no evidence of coupling between Mo^{V} and any of the other (paramagnetic) redox centres of the periplasmic [56], and probably also the membrane-bound [217], nitrate reductases, indicating a separation of molybdenum and electron donor of greater than 2 nm [66]. Electron transfer over distances of 2.5 nm or greater is too slow to be physiologically viable [385]. The large donor-acceptor distance suggested by the absence of spin coupling may therefore indicate the presence of an additional redox centre, most likely the pterin ring, in the electron transfer pathway.

The pterin ring is in principle able to undergo redox changes between oxidised, dihydro (of which there are six possible tautomers) and fully reduced tetrahydro forms (Fig. 7b). Microcoulometry (necessarily in the absence of substrate) of several molybdenum enzymes has given no evidence for electron uptake by the pterin ring [513–515], while what has been interpreted as reduction of the pterin ring from a dihydro- to a tetrahydro-state in recent EPR studies appears to occur only at physiologically unreasonable potentials [55,56]. What evidence there is suggests, then, that the pterin is unlikely to change its redox state during catalysis. If, however, the pterin is in the dihydro state, it is an attractive possibility that a change of tautomer might be a mechanism to couple reactions at the molybdenum atom to those of the pterin without requiring a change in pterin oxidation state.

The reduction potentials of the molybdenum atoms of molybdopterin-dependent enzymes range over at least 0.6 V for either the Mo^{V}/Mo^{IV} or Mo^{IV}/Mo^{V} couples. For *E. coli* Nar-A, values of $E_{m,8.3}(Mo^{V}/Mo^{IV}) = +80$ mV, $E_{m,8.3}(Mo^{VI}/Mo^{V}) = +190$ mV, $E_{m,7.1}(Mo^{V}/Mo^{IV}) = +180$ mV and $E_{m,7.1}(Mo^{VI}/Mo^{V}) = +220$ mV have been determined [217,571]. In the 7,8-dihydro or 5,8-dihydro states the dithiolene π bonds are conjugated to those of the pterin ring system (Fig. 7b). Relative to the tetrahydro state, where such electron delocalisation from the molybdenum-dithiolene unit does not occur, we would expect that the stabilisation of the more reduced molybdenum states in these dihydro tautomers would lead to higher molybdenum reduction potentials. As discussed by Bennett and co-workers [55], evidence presented by Gardlick and Rajagopalan [186,187] indicates that the pterin in mammalian xanthine oxidase, which has molybdenum couples around -350 mV, is in the tetrahydro state, while, in agreement with the theoretical argument, it is in a dihydro state in mammalian sulfite oxidase, where the molybdenum couples are around 0 mV. Exposure of the periplasmic nitrate reductase or a periplasmic dimethylsulfoxide reductase to highly reducing conditions gives rise to a change in EPR properties, from those typical of enzymes with high molybdenum reduction potentials to those typical of enzymes with low reduction potentials [55,56]. These changes were postulated to be caused by reduction of the pterin portion of the cofactor [55,56]. Control of molybdenum redox potential by pterin oxidation state might be a way to reconcile the similarity of the active sites of the soluble nitrate reductase and the molybdenum-dependent formate dehydrogenase with the observation of 'high-potential' EPR signals from the former [56,182] and 'low potential' signals from the latter (e.g., [40]) as well as the difference of around 0.7 V in the probable average midpoint potentials of the two types of enzyme.

5.4.2.1. The molybdenum environment in the membrane-bound nitrate reductase. Two types of Mo^{V} EPR signal of apparent physiological relevance have been described for *E. coli* Nar-A [571]. Because these signals were originally detected at different pH values, they were named Low pH and High pH. It is now clear that these signals in fact correspond to anion-bound and anion-free forms. Subtly

different forms of the Low pH signal can be generated using a range of different anions – chloride, fluoride, bromate, chlorate, dihydrogenphosphate, formate, sulfonic acid-containing buffer anions – with two forms for each of nitrate and nitrite [192,193,571]. Strong hyperfine coupling of Mo^V to fluorine ($I = 1/2$) in the Low pH Fluoride signal indicates that at least in this case the anion is directly bound to the molybdenum [192]. Anion-dependent Mo^V states have also been described for eukaryotic assimilatory nitrite reductases and sulfite oxidases [87,191, 194,287,315].

Low pH species contains a strongly coupled, and High pH a weakly coupled, exchangeable proton [192]. It is likely that during the catalytic cycle the oxygen atom transferred to the molybdenum from nitrate should be protonated and released as hydroxide or water. Also, model compound studies suggest that protonation of an oxo-group is likely to accompany the Mo(VI) → Mo(V) transition. For these reasons, the possibility that the High pH to Low pH transition corresponds to concerted anion binding and protonation of an oxo-group was initially attractive. However, the failure to detect the ^{17}O coupling expected from enzyme in $H_2^{17}O$ if an exchangeable hydroxyl ligand was present [193], and the possible increase rather than expected decrease in oxo-coordination associated with anion binding measured by EXAFS [193], argue against such an interpretation. Note also that the EXAFS data suggest that, in contrast to other molybdopterin-dependent enzymes, a substantial proportion of Nar-A molecules lack oxo-groups in the Mo^{IV} state [193].

5.4.2.2. The molybdenum environment in the bacterial soluble nitrate reductases. In contrast to the membrane-bound nitrate reductase, only a single Mo^V EPR signal of possible physiological relevance has been observed in the soluble nitrate reductases [56,182]. This signal, termed High-*g* Split, exhibits splitting by an $I = 1/2$ nucleus, presumably a proton [1]. However, in contrast to the proton seen in the membrane-bound nitrate reductase signals, the High-*g* Split proton is non-exchangeable. This observation, together with the weakness of the coupling, indicates that the proton is not on a hydroxyl or thiol ligand but probably on a relatively distant portion of a protein ligand or organic portion of the cofactor. Anions and pH changes do not affect the High-*g* Split signal, suggesting, in contrast to the membrane-bound enzyme, that the molybdenum in this state has no readily accessible coordination position. The high catalytic specificity of the periplasmic nitrate reductase relative to the membrane-bound nitrate reductase may reflect this property. Nitrate in the absence of reductant does not perturb High-*g* Split, indicating that the substrate binds only to Mo^{IV}. Turnover of the enzyme leads to very subtle changes in the High-*g* Split parameters. The physical nature of these changes is unclear but may be related to bound product [56].

Speculation on the mechanism of the soluble nitrate reductases is difficult. As with the membrane-bound nitrate reductase, the absence of a potential Mo^V-OH state is troublesome. If the similarity of the active site between the soluble nitrate reductases and molybdenum-dependent formate dehydrogenases (which presumably catalyse the reaction $H\text{-}COOH + H_2O \rightarrow H\text{-}O\text{-}COOH + 2e^- + 2H^+$, although what evidence there is suggests that the final product leaving the enzyme is CO_2) indicates a similarity of mechanism, then future studies on formate dehydrogenases may give insight into the soluble nitrate reductases.

Additional Mo^V states can be generated in the periplasmic nitrate reductase under non-physiological conditions [56]. As discussed in Section 5.4.2, addition of excess reductant leads to signals (Pseudo Rapid) that resemble those from molybdenum hydroxylases catalysing low potential reactions. In contrast to the High-*g* Split signal, an exchangeable hydroxyl proton can be observed in one variant of Pseudo Rapid. Bennett and co-workers ascribe the production of Pseudo Rapid to a change in the redox state of the pterin ring of the cofactor [56]. Addition of cyanide to Pseudo Rapid generates a novel axial signal termed Very High-*g* Split. Like High-*g* Split, this has a weakly coupled non-exchangeable proton. Very High-*g* Split has also been observed with the assimilatory nitrate reductase [182].

5.4.3. The iron-sulfur clusters

A $[3Fe\text{-}4S]^{1+,0}$ cluster ($E_{m,8.3} = +60$ mV; centre 2) and three $[4Fe\text{-}4S]^{2+,1+}$ clusters ($E_{m,8.3}$ of $+80$ mV (centre 1), -200 mV (centre 3) and -400 mV (centre 4)) have been identified in *E. coli* Nar-A $\alpha\beta$ complex [217,269]. Centre 1 is present in two different forms and shows an anticooperative redox interaction of 45 mV with centre 2. In the reduced state the [4Fe-4S] clusters are magnetically coupled, suggesting that they are relatively close in space. In *E. coli* Nar-Z $\alpha\beta$ complex, the corresponding centres are a $[3Fe\text{-}4S]^{1+,0}$ cluster ($E_{m,8.3} = -40$ mV) and three $[4Fe\text{-}4S]^{2+,1+}$ clusters ($E_{m,8.3}$ of -20 mV, -200 mV and -400 mV; [217]). Thus, in Nar-Z the two highest-potential centres have lower reduction potentials than in Nar-A. Recall that the periplasmic nitrate reductase of *T. pantotropha* contains a single $[4Fe\text{-}4S]^{2+,1+}$ centre with an $E_{m,7.4}$ of -160 mV [88].

The reduction potentials of the periplasmic nitrate reductase [4Fe-4S] centre and of the two lower potential [4Fe-4S] centres of the *E. coli* membrane-bound nitrate reductases are considerably lower than those of the potential electron donor molecules; the ubiquinol pool ($E_{m,7.0} = +80$ mV) and NapB *c*-type cytochromes ($E_{m,7.0} = -20$ mV and $+80$ mV) in the case of the periplasmic nitrate reductase, the menaquinol pool ($E_{m,7.0} = -80$ mV) and

[1] A second non-exchangeable $I = 1/2$ nucleus, with splitting too weak for the nucleus to be in the Mo co-ordination sphere is present (Table 1 in [56]).

b-type cytochromes ($E_{m,7.0}$ = + 10 mV and + 125 mV) of the γ subunit, for the *E. coli* membrane-bound nitrate reductases. Thus these centres are not thermodynamically reducible by the physiological electron donor. Such physiologically irreducible redox centres appear to be a common feature of complex electron transfer proteins, a particularly well-studied example being the tetrahaem cytochrome *c* of the *Rhodopseudomonas viridis* reaction centre [155,183]. Because these centres are not physiologically reducible, they cannot function to store electrons. They are, however, still capable of functioning in electron transfer. If such a centre lies on a path between an electron donor and acceptor, localisation of the moving electron onto the centre, even if as determined by the reduction potential of the centre the time averaged probability is very low, results in a large enhancement of electron transfer rate as a consequence of the exponential dependence of electron transfer rate on distance [96,385,429]. Alternatively, one could consider that the electron transfer rate is enhanced because the accessible energy levels of the low-potential [Fe-S] centre are closer in energy to those of donor and acceptor than are those of the protein matrix.

The identity of amino acids that ligate the iron-sulfur clusters of respiratory nitrate reductases can be inferred by

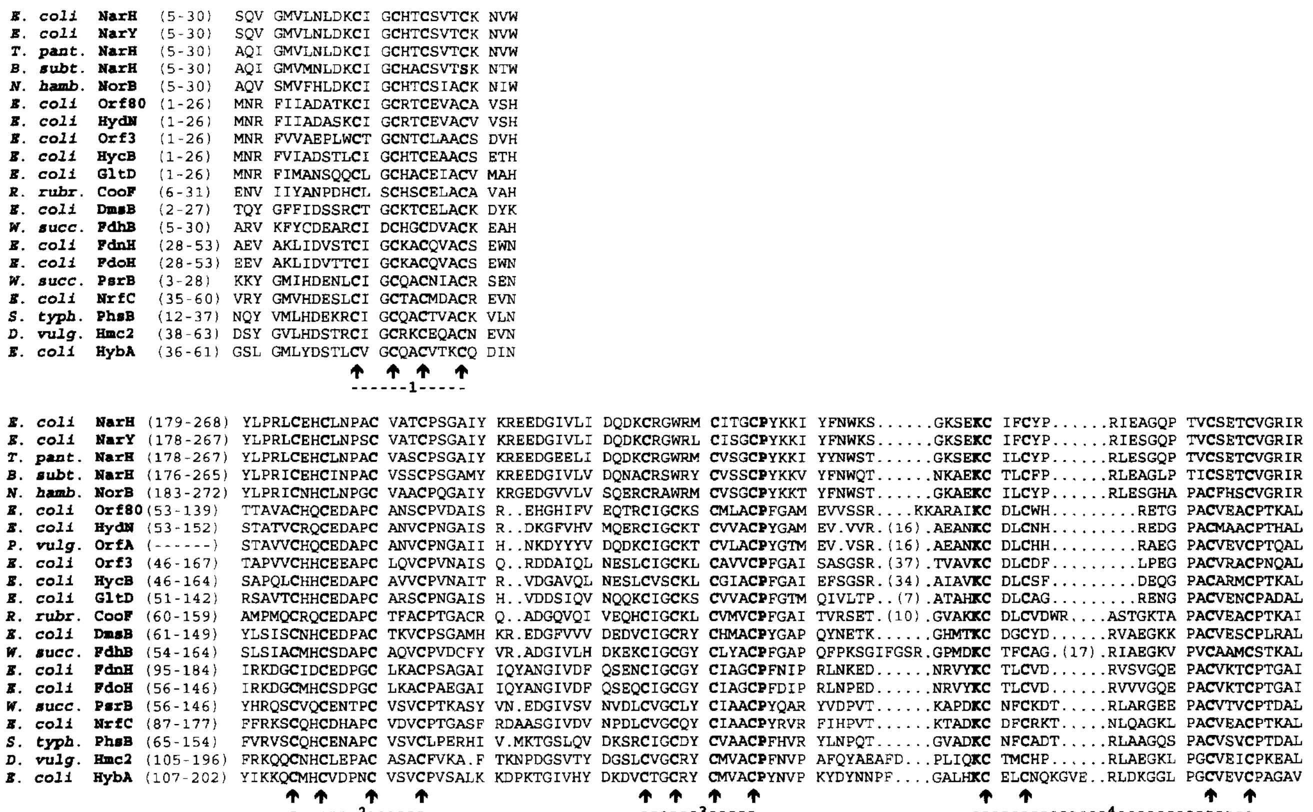


Fig. 13. Regions of amino acid sequence similarity between the Iron Sulfur Protein (β) subunits of the membrane-bound MGD-dependent enzymes and other structurally related Iron Sulfur Proteins. Amino acid residues that are identical in all sequences are shown in bold. ↑ indicates the position of possible iron-sulfur centre ligands. The numbering of the cysteine motifs is that used in Fig. 14. Sequences are those of the *E. coli* major membrane-bound nitrate reductase β subunit (NarH) [73,597]; *E. coli* minor membrane-bound nitrate reductase β subunit (NarY) [74]; *T. pantotropha* membrane-bound nitrate reductase β subunit (NarH) [63]; *B. subtilis* membrane-bound nitrate reductase β subunit (NarH) [238a]; *N. hamburgensis* nitrite oxidase β subunit (NorB) [290]; *orf* at approximately 80 min on the *E. coli* chromosome (Orf80; note that this *orf* is not identified in the reference; it is a translation from base 155,834 to 155,360 with a frameshift at approximately 155,630) [506]; *E. coli* HydN (the operon also codes for HypF [formerly HydA] a gene that in other organisms has been implicated in nickel metabolism related to hydrogenase biosynthesis) [252]; *Proteus vulgaris* partial protein sequence coded by a fumarate reductase-linked operon (OrfA; this gene is almost certainly functionally equivalent to *E. coli* HydN as it is followed by a HypF [HydA] homologue) [122]; predicted gene product of an unidentified open reading frame at approximately 53 min on the *E. coli* chromosome (Orf3) [17]; *E. coli* formate hydrogenlyase hydrogenase operon HycB (this possibly acts as a β subunit to FdhF, the gene for which is unlinked to the hydrogenase operon) [78]; *E. coli* nitrate- and anaerobically-regulated gene product divergently transcribed from the nitrate/nitrite sensor protein NarQ and which is either fused to, or part of the same operon as, the gene coding for a glutamate-synthase (glutamine amide 2-oxoglutarate amidotransferase; GOGAT) small subunit (GltD) homologue (GltD) [111]; *Rhodospirillum rubrum* carbon monoxide dehydrogenase CooF subunit [289]; *E. coli* dimethyl sulfoxide reductase β subunit (DmsB) [67]; *W. succinogenes* major formate dehydrogenase β subunit (FdhB) [79]; *E. coli* nitrate-inducible formate dehydrogenase β subunit (FdnH) [59]; *E. coli* formate dehydrogenase-O β subunit (FdoH) [417]; *W. succinogenes* polysulfide reductase β subunit (PsrB) [298]; *E. coli* cytochrome *c* nitrite reductase operon protein NrfC (Section 7.4) [77,250]; *S. typhimurium* protein involved in production of hydrogen sulfide from thiosulfate, a partial *orf* in the same operon has high sequence similarity to the MGD-binding protein of *W. succinogenes* polysulfide reductase (PhsB) [10]; *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough *Hmc orf2* gene product (Hmc2; this protein is proposed to be part of a complex that transfers electrons from periplasmic hydrogenases to cytoplasmic sulfate reductases) [453]; *E. coli* hydrogenase-2 small subunit (HybA) [365].

comparison with simple ferredoxins of known three-dimensional structure [25,92,95,242,351]. In these proteins $[4Fe\text{-}4S]^{2+,1+}$ clusters are bound by three closely spaced cysteines in a Cys-$(Xaa)_2$-Cys-$(Xaa)_2$-Cys motif, while a fourth cysteine ligand is distant in the primary sequence. In $[3Fe\text{-}4S]^{1+,0}$ clusters the second cysteine is absent or replaced by a weak ligand. '8Fe' ferredoxins bind two $[4Fe\text{-}4S]^{2+,1+}$ centres by means of two Cys-$(Xaa)_2$-Cys$(Xaa)_2$-Cys$(Xaa)_3$-Cys motifs, the final cysteine in each motif being the distal cysteine for the other cluster. A proline is also found after the fourth cysteine in at least one of the two motifs. As shown in Fig. 13, cysteines motifs resembling two copies of the '8Fe' ferredoxin motif are conserved between a homologous group of iron-sulfur proteins, including the β subunits of membrane-bound MGD-dependent enzymes. These '16Fe' ferredoxins appear in many cases to mediate electron transport between the integral membrane and peripheral membrane redox subunits of membrane-bound electron transport complexes. From sequence comparisons it is likely that these proteins bind four [4Fe-4S] clusters in two '8Fe' ferredoxin-like domains [586]. The membrane-bound nitrate reductase/nitrite oxidase (Section 5.5) β subunits are almost twice the size of the other '16Fe ferredoxins', the extra length in the nitrate reductase/nitrite oxidase polypeptides being due to an insert between iron-sulfur cluster motifs A and B and an extension of the C-terminus.

In the membrane-bound nitrate reductase/nitrite oxidase β subunit, the second cysteine in the third cysteine motif is replaced by a tryptophan residue (Fig. 13), indicating that this motif may bind the [3Fe-4S] centre 2 (Fig. 14). Some support for this suggestion has been adduced from experiments in which the corresponding cysteine in the β subunit of another MGD-dependent membrane bound enzyme, *E. coli* dimethylsulfoxide reductase (Fig. 13), was replaced by tryptophan, serine, tyrosine or phenylalanine, resulting in the conversion of a [4Fe-4S] cluster into a [3Fe-4S] cluster [456]. The mutant dimethylsulfoxide reductases were deficient in electron transport from menaquinol to dimethylsulfoxide, most likely as a consequence of an increase of at least 200 mV in the reduction potential of the cluster. This result suggests that the corresponding centre in the membrane-bound nitrate reductases is a compulsory electron carrier between quinol and MGD. When the tryptophan in the third cysteine motif of *E. coli* NarH, Trp220, was replaced with a cysteine the [3Fe-4S] cluster (centre 2) was still present. However, changes at this position did alter the reduction potential of both the high-potential centres and partially inhibited benzyl viologen-linked nitrate reductase activity [26]. These mutagenesis experiments suggest that the residue at position 220 is folded away from the centre and cannot act as a ligand (supported by modelling studies according to [26]), although the possibility that cysteine motif 3 does not bind the [3Fe-4S] cluster cannot be excluded.

Mutation of the first or second cysteines in *E. coli NarH* motif 1 (Fig. 13) to alanine or serine leads to specific loss of centre 1, suggesting that this high-potential [4Fe-4S] cluster is bound by motif 1 [27] (Fig. 14). The retention of some menaquinol-dependent activity in these

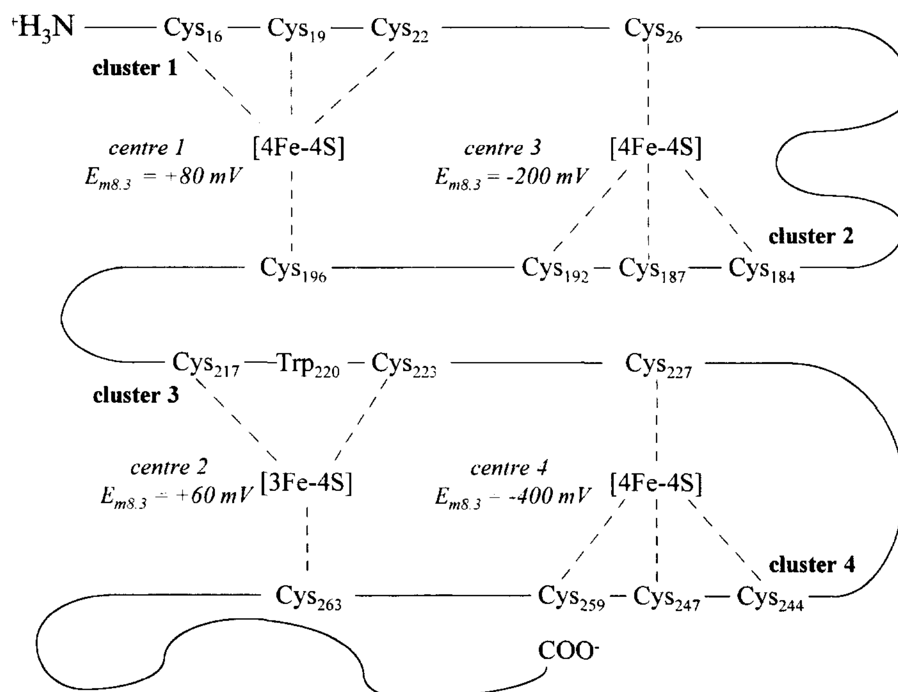


Fig. 14. Proposed assignment of membrane-bound nitrate reductase β subunit cysteine cluster motifs to spectroscopically defined centres. This figure is based on the work of Augier et al. [26,27]. The sequence numbering is that of *E. coli* NarH and cluster numbering is that shown in Fig. 13. Reduction potentials are versus the standard hydrogen electrode. The assignment of the two low-potential clusters is tentative [26,27].

mutants indicates that centre 1 is not obligatory for electron transfer between quinol and nitrate but may be required for an optimal electron transfer rate. As loss of cluster 1 does not affect the other redox centres, this cluster does not have an important structural role. In contrast, substitution of the first cysteine in each of the other three motifs resulted in most cases in enzymes completely lacking both iron-sulfur clusters and MGD [27]. The most probable assignment of clusters to β subunit cysteine motifs is summarised in Fig. 14. In *B. subtilis* NarH the fourth cysteine of the first motif is substituted by serine (Fig. 13). This suggests that one of the iron-sulfur clusters in this protein has a serine ligand. While natural iron-sulfur clusters with serine ligation have not been reported, it is frequently found that serine can be substituted experimentally for cysteine iron-sulfur cluster ligands without disturbing the cluster structure (e.g., [26,27,109a,372,549]; spectroscopic evidence that the serine is a cluster ligand has been presented [109a,372]).

The [4Fe-4S] cluster of the periplasmic nitrate reductase is almost certainly bound by a four cysteine motif (2.3.27 spacing) at the N-terminus of the MGD-binding subunit NapA (Fig. 12) [64,88]. These cysteines are conserved in Segment I of the most other MGD binding subunits (Fig. 12), suggesting that these proteins, which include the assimilatory and membrane-bound nitrate reductases, also bind a [4Fe-4S] cluster. This cluster would presumably be the direct electron donor to the molybdenum cofactor at the active site. Some biochemical evidence for this cluster in enzymes other than the periplasmic nitrate reductase is available (reviewed in [88]; and see also Section 5.2). Recently, Trieber and co-workers have carried our site-directed mutagenesis in the MGD-binding subunit of *E. coli* dimethylsulfoxide reductase of the last three cysteines in this motif [549]. In the majority of cases the mutants were affected in electron transfer from quinol to dimethylsulfoxide but not from reduced benzyl viologen, suggesting a role for these cysteines in physiological electron transport within the complex. Although not suggested by Trieber et al., ligation of an iron-sulfur cluster by these residues would account for this phenotype. In the *E. coli* nitrate reductases the first cysteine in the cluster motif appears to be substituted by histidine (Fig. 12) which we suggest replaces the cysteine as a ligand to the [4Fe-4S] cluster [88]. This suggestion is not unreasonable. A histidine has recently been shown to be a ligand to one of the [4Fe-4S] clusters of *Desulfovibrio gigas* [NiFe] hydrogenase [572]. Histidine is also a ligand of the [2Fe-2S] Rieske cluster [140,190] and possibly other [2Fe-2S] clusters [178,372]. Some support for the presence of a histidine iron-sulfur cluster ligand in the membrane-bound nitrate reductase can perhaps be adduced from experiments with the *E. coli* dimethylsulfoxide reductase, where replacement of the first cysteine in the Segment I motif (Fig. 12) with histidine did not affect physiological activities [549]. Because both spectroscopically detectable high-potential iron-sulfur clusters of the membrane-bound nitrate reductase have been convincingly ascribed to the β subunit by the mutagenesis studies described above (Fig. 14), if the α subunit does bind an iron-sulfur cluster then this cluster is likely to be part of the pool of clusters with reduction potentials of -200 mV and below. Such a value would be reasonably consistent with that determined for the equivalent iron-sulfur cluster in the periplasmic nitrate reductase.

Why might the membrane-bound nitrate reductase possess two unusual iron-sulfur clusters that are not seen in the other MGD-dependent enzymes, a [3Fe-4S] cluster and a [4Fe-4S] cluster with a histidine ligand? In cases where [3Fe-4S] and [4Fe-4S] clusters have been interconverted by mutagenesis the [3Fe-4S] form of the cluster has the higher reduction potential (e.g., [346] or the dimethylsulfoxide reductase mutant described above [456]). Substitution of histidine ligand for a cysteine ligand would be expected to raise the cluster potential. The nitrate/nitrite couple is the most electropositive utilised by MGD-dependent enzymes. The membrane-bound nitrate reductase probably evolved from an enzyme working at a lower substrate redox couple. If the potentials of the [Fe-S] clusters in nitrate reductase needed to be raised relative to those of the ancestral enzyme then, given the established protein framework, the easiest evolutionary options may have been the cluster and ligand conversions seen in the contemporary enzyme.

5.4.4. The transmembrane (γ) subunit of the membrane-bound nitrate reductase

The membrane-bound nitrate reductase is believed to generate a proton electrochemical gradient by the redox loop mechanism discussed in Section 2.1. in which quinol is oxidised at the periplasmic side of the membrane with the electrons, but not protons produced, being transferred across the membrane to the cytoplasmic site of nitrate reduction [63,273a]. Quinol oxidation and transmembrane electron transport are ascribed to the integral membrane γ subunit. It is now recognised that transfer of electrons across the width of the membrane bilayer at physiological rates requires at least two, appropriately spaced, redox centres. The presence in the γ subunit of two *b*-type haems (*b*-557) with midpoint potentials (pH 7.0) of +95 mV and +210 mV has been inferred from potentiometric titrations of the purified *P. denitrificans* enzyme [34]. These haems are reducible by duroquinol and reoxidisable by nitrate. *E. coli NarI* probably also binds two *b*-haem groups (*b*-555 [505]) the reduction potentials of which in a partially purified enzyme preparation were estimated by Hackett and Bragg to be +10 mV and +125 mV [219]. These potentials are around 100 mV less than those of the *P. denitrificans* enzyme and may reflect the use of a lower potential electron donor ($E_{m,7}(MQ/MQH_2) = -80$ mV cf. $E_{m,7}(UQ/UQH_2) = +80$ mV) by the *E. coli* enzyme.

The membrane-bound nitrate reductases of *E. coli* have been shown by the use of ubiquinol and menaquinol-defi-

cient mutants to oxidise both ubiquinol-8 and menaquinol-8/demethylmenaquinol-8 [418,579], though it is likely that during anaerobic growth menaquinol is the most important donor molecule. Brito and co-workers have reported the copurification of menaquinone with Nar-A [90], suggesting that the enzyme binds quinone very tightly and possibly non-exchangeably. The physiological electron donor to the membrane-bound nitrate reductases of *P. denitrificans / T. pantotropha* is presumably ubiquinone-10. Oxidation by the *P. denitrificans* enzyme of the ubiquinol analogue duroquinol is sensitive to inhibition by the menaquinone analogue 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) [133].

A structural model for the γ subunit has recently been proposed from sequence comparisons of *E. coli* NarI and NarV and *T. pantotropha* NarI [63]. The subunit is predicted to possess five transmembrane helices, N-terminus periplasmic, C-terminus cytoplasmic. Four conserved histidines have been identified as potential *b*-haem ligands. Two of these are found on helix **b**, with a spacing of 9 amino acids, and two on helix **e**, with a spacing of 17 amino acids. This distribution of haem ligands allows placement of the haems in different halves of the membrane bilayer as required by the redox loop model. Two conserved polar residues have been identified in the proposed transmembrane helices, a glutamine in helix **d** and an arginine in helix **e**. These residues were proposed to be of functional importance. Note that the glutamine is not present in the recent partial *B. subtilis* NarI sequence [238a]. A number of glycine residues are conserved in the transmembrane helices that may be important for packing the bulky haem molecules. Amino acid residues important for binding quinol have not yet been identified, but by analogy to the cytochrome bc_1 complex [134,190,291] they are expected to lie in loop regions at the periplasmic face.

5.4.5. The 'δ subunit' and biosynthesis of the membrane-bound nitrate reductase

The membrane-bound nitrate reductase operons encode not only the three structural subunits of the enzyme but also a fourth, expressed [504] open reading frame *narJ / W* (Fig. 11). The gene product (25 kDa) has been referred as the 'δ subunit' [76] although the protein does not copurify with the enzyme (e.g., [505]). The δ subunit is predicted to be a soluble cytoplasmic protein. It has no obvious sequence similarity to other proteins. Secondary structure prediction strongly suggests an all-helical structure [63]. The δ subunit is presumably produced at a stoichiometry equal to that of the other nitrate reductase subunits (Section 5.3.1).

E. coli mutants with deletions in *narJ* or *narW* express normal levels of the three nitrate reductase structural subunits [75], but are devoid of [76] or have very low [158] physiological and viologen-linked nitrate reductase activities. The three subunits can still assemble as a membrane-bound complex, although there is an increase in soluble $\alpha\beta$ pairs [76,158]. The iron-sulfur centre composition of the enzyme is normal [76] and the enzyme contains a pterin derivative, though whether this is authentic MGD has not been determined [76]. In vivo the β subunit, irrespective of cellular location, is sensitive to degradation of the β' state in mutants lacking NarJ, while the α subunit is similarly subject to a small decrease in polypeptide molecular size but only if the subunit fails to assemble on the membrane [158]. A similar pattern of $\alpha\beta$ degradation is seen if soluble $\alpha\beta$ complexes are purified from a *narJI* mutant (our interpretation of the results presented in [76]). This purified inactive $\alpha\beta$ complex is completely digested by trypsin [76] in contrast to the active $\alpha\beta\gamma$ enzyme which is usually subject to, at most, a specific cut in the β subunit (Section 5.3.1). The apparent instability of the enzyme in *narJ* mutants suggests a number of possibilities. Perhaps the inactive enzymes are either not fully folded or incorrectly and unstably folded as suggested by Blasco and coworkers [76]. In this case the molybdopterin cofactor is either incorrectly inserted into, or requires a δ subunit-mediated modification after binding to, the α subunit. This model would suggest that molybdenum cofactor is inserted at a site that is occluded by the membrane or γ subunit in the intact $\alpha\beta\gamma$ complex. Alternatively, as speculated by Dubourdieu and DeMoss [158], the δ subunit might bind to and protect the soluble $\alpha\beta$ complex prior to its attachment to the membrane.

Some comments can now be made on the assembly of *E. coli* Nar-A. Expression of subunits of *E. coli* Nar-A and Nar-Z from different transcription units, homologous or heterologous combinations, resulted in a relatively high efficiency of assembly of the subunits. This indicated that translation and assembly of the subunits are not tightly coupled precesses [75]. Mutants lacking a functional γ subunit, because of either deletion of the γ subunit structural gene [505,527] or because of haem deficiency [339], accumulate $\alpha\beta$ complexes in the cytoplasm. This suggests that formation of the $\alpha\beta$ complex in the cytoplasm precedes binding of the complex to the γ subunit. Assembly of the $\alpha\beta\gamma$ complex is not dependent on the functional assembly of either iron sulfur centres or the MGD active site as mutant enzyme lacking both these cofactors [26] forms a membrane-bound three polypeptide complex. However, molybdenum cofactor synthesis-deficient mutants *mob* (formerly *chlB*), *moe* (formerly *chlE*) and *mog* (formerly *chlG*), or cells grown with the molybdate antagonist tungstate, accumulate inactive cytoplasmic $\alpha\beta$ complexes [197,465,527]. This suggests that, while assembly of $\alpha\beta$ complexes is possible in the absence of correct cofactor insertion, membrane assembly does not occur, a result in conflict with the experiments with the iron-sulfur centre-deficient mutants. The most interesting of the molybdenum cofactor mutants is *mob* which has a defect in the synthesis of MGD from molybdopterin [267]. The inactive $\alpha\beta$ complex from such a strain contains pterin

and stoichiometric amounts of molybdenum [83,465]. This could be interpreted to mean that the GMP moiety is added to molybdopterin after molybdopterin is bound by the α subunit. Alternatively, in the absence of MGD, the unconjugated molybdopterin is inserted, but this is unable to produce an active enzyme. The presence of an inactive pterin in the *mob* mutant is reminiscent of the phenotype of the *narJ / narW* mutants. Functional insertion of MGD into the nitrate reductase starting from molybdopterin requires Factor FA, which is the product of the first open reading frame at the *mob* locus [406,417], GTP and a soluble protein Factor X, of size 35–60 kDa by gel permeation chromatography [267,465]. While Factor X is most likely the product of the second open reading frame at the mob locus (predicted mass 18.9 kDa [417]) it might instead be the δ subunit. Mutants in which the β subunit [Fe-S] clusters fail to assemble contain no molybdenum cofactor [26,27], suggesting that insertion of iron sulfur clusters precedes MGD insertion but might alternatively mean that binding of MGD to the α subunit is stabilised by correctly assembled β subunit.

5.5. Nitrite oxidase

Members of the genus *Nitrobacter* are capable of chemoautotrophic growth using nitrite as electron donor and oxygen as electron acceptor. Oxidation of nitrite to nitrate ($E_{m,7.0}$ = +420 mV) is catalysed by a membrane-bound enzyme which is also capable of the reverse (nitrate reduction) reaction. It has been apparent for some time that a soluble subcomplex of this nitrite oxidase can be prepared that resembles the $\alpha\beta$ complex of the membrane-bound nitrate reductases [33]. The nitrite oxidase α and β subunits show similar molecular masses to the corresponding membrane-bound nitrate reductase subunits [535]. Further, the nitrite oxidase $\alpha\beta$ complex contains molybdopterin [253,308,363] and iron-sulfur clusters, including a possible $[3Fe\text{-}4S]^{1+,0}$ centre [363]. The close structural relationship of the two enzymes is confirmed by the recently determined amino-acid sequence of the β subunit (NorB) of *N. hamburgensis* nitrite oxidase [290]. This shows a very high similarity, including the conservation of the proposed iron-sulfur centre ligands, to the β subunits of the membrane-bound nitrate reductases (Fig. 13). The *norB* gene appears to be part of an operon coding for a membrane-bound nitrate reductase α subunit homologue (NorA) and a 24 kDa, predicted soluble cytoplasmic protein NorX [290]. We have detected sequence similarity between NorX and *E. coli* SlyD [452], *E. coli orf149* gene product [82] and *P. fluorescens orf149* gene product [255]. The function of these proteins is unclear, but they do contain a region with significant sequence similarity to the FK506-binding proteins (FKBPs) which have peptidyl-prolyl *cis-trans* isomerase (rotamase) activity and probably other functions in protein folding [548]. NorX could, therefore, be the functional equivalent of the membrane-bound nitrate reductase δ subunit, although the predicted secondary structure elements are very different. Note that a peptidyl-prolyl isomerase of a different structural class (NifM) has been implicated in the insertion of the iron-sulfur cluster into nitrogenase reductase [457a]. A cytoplasmic location has been indicated for the active site of nitrite oxidases (e.g., [119,166]). Thus NorA and NorB, like the NarG/Z and NarH/V homologues, are probably located at the cytoplasmic face of the plasma membrane.

How is the nitrite oxidation reaction connected to the electron transport chain? It is certain on thermodynamic grounds that the enzyme cannot reduce ubiquinol ($E_{m,7.0}$ = +80 mV) but must employ some more oxidising electron acceptor, probably cytochrome *c* ($E_{m,7.0}$ around +250 mV) [119,166,390]. Even so, the reaction is highly unfavourable ($E_m(NO_3^-/NO_2^-)$ = +420 mV) and is probably dependent both on a substantial excess of nitrite over nitrate and, consistent with the inhibitory effect of uncouplers on nitrite oxidation [119], on the membrane potential being used to assist movement of electrons ($\downarrow 2H^+{:}2e^-$) from the cytoplasmic active site to periplasmic cytochrome *c*. Tamaka and co-workers reported the presence of cytochrome a_1 in nitrite oxidase [537]. This redox centre is likely to be involved in transfer of electrons across the lipid bilayer. It is probably necessary for the short electron transport chain from *c*-type cytochrome to oxygen to employ a proton-pumping cytochrome oxidase (i.e., $\uparrow 4H^+{:}2e^-$) in order for there to be net energy conservation by the nitrite oxidation pathway [390]. Note also that nitrite uptake and nitrate excretion need to be taken into account when considering the overall energetics of nitrite oxidation.

Nitrite oxidase $\alpha\beta$ complexes can catalyse both nitrite oxidation and nitrate reduction. In contrast, both the three-subunit membrane-bound nitrate reductase and the $\alpha\beta$ subcomplex are unable to oxidise nitrite ([34]; Berks, B.C., unpublished observations), despite the probable structural similarity to nitrite oxidase. Parallels exist: for example, *E. coli* succinate dehydrogenase and fumarate reductase are structurally similar enzymes whose physiological roles are in the catalysis of opposite reactions; only the fumarate reductase can catalyse the reverse of the physiological reaction [533,534].

6. Transport of nitrate and nitrite across the cytoplasmic membrane

A consequence of the cytoplasmic location of the active site of the membrane-bound nitrate reductase is that nitrate has to be delivered across the lipid bilayer against the membrane potential, and nitrite subsequently delivered back across the membrane to the periplasmic nitrite reductases. These translocation processes are poorly understood, but it is clear from early work on *P. denitrificans* that discrimination by the nitrate reductase of intact cells against

chlorate and the control by oxygen on nitrate reduction is lost in parallel upon permeabilisation of the cell membrane [12,265]. Thus the nitrate porter(s) is (are) highly specific for nitrate, discriminating against chlorate [12,265], and reversibly inhibited, either directly or indirectly, by oxygen (see Section 3.2).

6.1. NarK and nitrite efflux

In *E. coli* the genes coding for the major membrane-bound nitrate reductase are clustered with a gene, *narK* (Fig. 11), coding for a highly hydrophobic protein that is predicted to contain twelve membrane-spanning helices (Fig. 3) [394]. Possible *narK* homologues are found adjacent to the *narZYWV* operon [74] and in *S. typhimurium* [239]. Deletion of the *narK* gene does not prevent anaerobic growth with nitrate as electron acceptor, but there is a decrease in extracellular nitrite accumulation during growth [144]. Nitrate electrode studies revealed a decrease in the steady-state rate of nitrate reduction by intact cells, which could be restored by permeabilising the cell membrane with detergent [144,394]. Studies following the uptake of $^{13}NO_3^-$ by intact cells have revealed no significant difference in the initial rate of uptake between wild-type and *narK* mutant cells [457]. However, intracellular ^{13}N accumulation was much more extensive in the *narK* mutant. Virtually no nitrate uptake was observed in a nitrate reductase (*narG*::Tn*10*) mutant, indicating that nitrate uptake is closely coupled to reduction. This could be as a result of the Δp generated by nitrate reduction and implicates an active process for nitrate uptake (see Section 6.2). Whatever the reason, the close coupling of nitrate uptake to reduction suggested that the accumulated ^{13}N in the *narK* mutant was almost certainly nitrite [457]. Nitrate uptake was also sensitive to uncoupling agents and oxygen.

In order to dissect nitrate uptake from nitrate reduction Rowe et al. [457] performed a further series of experiments with proteoliposomes in which nitrite and the fluorophore *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) were trapped. The fluorescence of MQAE is quenched by nitrite but not nitrate, enabling nitrite efflux from the proteoliposome to be followed by monitoring increases in fluorescence. The rate of nitrite efflux from proteoliposomes prepared from *narK*$^-$ cells was 10- to 15-fold lower than in proteoliposomes prepared from either wild-type or nitrate reductase mutants. Consideration of the activation energies for the efflux process in the three systems suggested that efflux in the wild-type and nitrate reductase mutant was enzyme-catalysed (i.e., a transporter) but efflux from the *narK* mutant was by passive permeation. Efflux from wild-type proteoliposomes was enhanced when a negative-inside membrane potential was present and was decreased when a positive inside-potential was applied. Taken together, all the data available on NarK mutants are consistent with NarK being a nitrite efflux porter. The increased intracellular nitrite concentration that occurs as a result of NarK deficiency could lead to inhibition of nitrate reduction (by competitive inhibition or by mass action) and in turn to the apparent decrease observed in steady-state nitrate uptake and reduction [394]. Permeation by detergents can presumably overcome the absence of NarK by increasing nitrite efflux.

In addition to *narK*, genes for possible nitrite transporters have been identified in *E. coli* (*nirC*; [413]), *S. typhimurium* (*nirC*; [598]) and *B. subtilis* (*ipa-48r*; [199]). In *E. coli*, *nirC* is transcribed as part of the assimilatory NADPH-linked nitrite reductase operon [222]. The NirC proteins show high sequence similarity to the FocA protein coded at the pyruvate-formate lyase locus of *E. coli* [535a]. FocA is almost certainly a bidirectional (although physiologically probably functioning as an exporter) formate-specific transporter [535a]. This sequence similarity suggests that NirC is probably a nitrite transporter, although whether an importer or exporter is unclear. NirC/FocA have high sequence similarity to FdhC, the product of a gene co-transcribed with the structural genes of the formate dehydrogenase of *Methanobacterium formicicum* and which is presumably involved in formate uptake [589,591], a potential gene product (frameshifted reading frame discernible in [501a]) linked to the glutamate dehydrogenase structural gene of *Peptostreptococcus asaccharolyticus* (a glutamate transporter?) and the product of *Saccharomyces cerevisiae* chromosome VII open reading frame YHL008c [269a]. Alkaline phosphatase and beta-galactosidase fusion experiments support a topological model for FocA consisting of six transmembrane helices with both termini in the cytoplasm [535a]. Sequence analysis indicates that this model is applicable to the other members of this FocA protein family ([535a], our analysis). A large number of polar residues that are likely to be involved in transport of the oxyanion substrate are conserved in the proposed transmembrane helices of the FocA-family.

6.2. Nitrate transport

A number of mechanisms for nitrate uptake have been considered, including: a passive nitrate uniport; an ATP-dependent uniport; Δp-dependent NO_3^-/nH^+ ($n \geq 1$) symport and a nitrate/nitrite antiport. The principal argument against a major role for a nitrate uniport is that in respiring bacteria the presence of a Δp of approx. 200 mV (periplasm positive with respect to the cytoplasm) would severely limit the flux of nitrate into the cytoplasm. Furthermore, the intracellular concentration of nitrate would be restricted to approximately one-thousandth of the external concentration. Since it is known that cells of *P. denitrificans* continue to respire nitrate at maximum rates even as the external nitrate falls below 5 μM [410], and the lowest observed K_m values for isolated nitrate reduc-

tase are of the order of 10 μM [133], reliance on a uniporter to supply nitrate to its reductase appears improbable.

Some researchers advocate an NO_3^-/nH^+ symport operating during respiratory nitrate reduction [81,457]. However, experimental data are contradictory; in *E. coli* $^{13}NO_3^-$ uptake is sensitive to protonophores [457] but nitrate respiration by *P. denitrificans* is not and osmotic swelling studies have not provided any evidence in support of a proton symport [13,411]. Uptake by a NO_3^-/nH^+ symport would have energetic consequences, since it would consume Δp, reducing growth yield. For example, an $NO_3^-/2H^+$ symport would consume 17%, 50% and 25% of the Δp generated during the reduction of nitrate by NADH, succinate and formate, respectively. The energetic consequences of a uniport coupled to ATP hydrolysis would be even greater. Assuming one ATP is hydrolysed per nitrate ion translocated and that the inward translocation of $3H^+$ is needed for each ATP synthesised [390], three protons would effectively be consumed per nitrate anion transported. This is not an energetically feasible proposition.

For many years, an NO_3^-/NO_2^- antiporter was considered to be the appropriate mechanism for nitrate and nitrite movements because it would involve no net charge movement across the cytoplasmic membrane. Boogerd et al. [81] observed that collapse of the membrane potential in intact cells resulted in a lag before nitrate reduction commenced. The lag was abolished if nitrite was added. The interpretation of the data was that nitrate respiration is started by a nitrate-proton symport, but following initial reduction of nitrate intracellular accumulation of nitrite drives an antiport. In the absence of Δp the symport could not operate, but HNO_2 (pK_a $HNO_2 = 3.4$) could enter the cytoplasm by passive diffusion, providing the cytoplasmic NO_2^- needed for the antiporter. A number of other researchers, measuring osmotic swelling in intact cells in response to nitrate [188,411] and nitrite uptake in proteoliposomes [457], have failed to find evidence supporting the presence of a nitrate/nitrite antiporter in either *P. denitrificans* or *E. coli*.

In contrast to respiratory nitrate uptake, there is clear evidence for energy-consuming nitrate transport systems associated with assimilatory nitrate reduction. The *CHL1* locus of the plant *Arabadopsis* encodes a nitrate-inducible protein with twelve predicted membrane-spanning helices that is responsible for chlorate uptake [550] and when expressed in *Xenopus* oocytes Chl1 functions as a low-affinity proton-linked nitrate transporter. Another possible proton symporter is the *crnA* gene product of the fungus *Aspergillus nidulans*, which is linked to genes involved in nitrate and nitrite assimilation and believed to encode an alkali metal ion-independent nitrate uptake protein [555]. Nitrate uptake systems of the ATP-dependent periplasmic binding protein-dependent (ABC transporter) type have been shown to be involved in nitrate assimilation in *K. pneumoniae* and the cyanobacterium *Synechococcus* sp. PCC7942 [18,325,400] (Section 5.2).

Whatever the mechanism of respiratory nitrate uptake, it is clear that in the presence of oxygen the movement of nitrate across the cytoplasmic membranes of several organisms is restricted [12,226,393]. The observations, discussed in Section 3.2, that both hexacyanoferrate and nitrous oxide mimic this effect and that inhibition is relieved in the presence of antimycin A [13,309] suggest that the inhibition is redox-mediated. An obvious candidate for a redox sensor is the QH_2/Q ratio and evidence in favour of this has arisen from fluorescence probe studies [14]. This could allow the operation of the nitrate transporter under aerobic conditions in intact cells in which the poise of the quinol/quinone pool is perturbed. This would facilitate aerobic nitrate respiration in the absence of a periplasmic nitrate reductase.

7. Nitrite reductases

7.1. Energy-conserving bacterial nitrite reductase systems

Three classes of enzyme can couple the reduction of nitrite to energy conserving electron transport pathways. As ascertained by cell fractionation and the presence of signal peptides in the preproteins, all three classes are periplasmically located in Gram-negative bacteria. The cytochrome *c* respiratory nitrite reductase (Section 7.4) reduces nitrite to ammonia, while the other two classes can take part in denitrification. One of the denitrifying enzymes contains two types of haem (the cytochrome cd_1 nitrite reductase; Section 7.2), the other contains copper (the copper nitrite reductase; Section 7.3). In vivo the denitrifying nitrite reductases reduce nitrite to nitric oxide (7.1.2). The copper and cytochrome cd_1 nitrite reductases have apparently similar roles in denitrifying bacteria and no organism has yet been identified which can express both types of the enzyme. The presence of the copper nitrite reductase in an organism can be determined in intact cells and cell extracts by assessing the sensitivity of nitrite reduction to the copper chelator DDC [485]. The validity of this approach relies on the use of artificial electron donors. In at least one case this technique has been applied to intact cells respiring nitrite using physiological electron donors [447]. This practice can lead to erroneous conclusions, since DDC could also chelate copper from cupredoxins which may be serving as electron donors to the nitrite reductase (see, for example, the reassessment of the work of Robertson and Kuenen [447] by Moir and coworkers [382]). Polyclonal antibodies and gene probes have also been used to determined the type of nitrite reductase in a particular denitrifying bacteria [128,129,606]. The cytochrome cd_1 nitrite reductase has been found most commonly in environment isolates, but it should be noted that these culturable bacteria may not be typical of bacteria

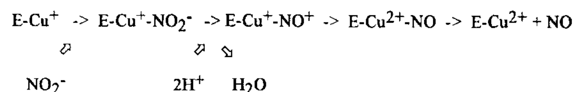
in the environment and that the copper nitrite reductase appears to be distributed over a more diverse range of bacteria which includes the Archaeon *Halobacterium denitrificans* [128,606].

It has been suggested that aerobic nitrite reduction might be restricted to bacteria that express the copper nitrite reductase [443,447]. As copper nitrite reductases reduce oxygen to hydrogen peroxide resulting in inactivation of the enzyme [283], the suggestion is unlikely. In fact a number of bacteria that reduce nitrite using the cd_1 enzyme have been shown to reduce nitrite aerobically [14,141,150,151,333,380,382]. While the cytochrome cd_1 nitrite reductase will reduce oxygen to molecular water at rates approaching that of nitrite reduction, the K_m of the enzyme for oxygen is much higher than for nitrite and it has been demonstrated that cytochrome cd_1 will reduce nitrite and oxygen when both are present [14,545].

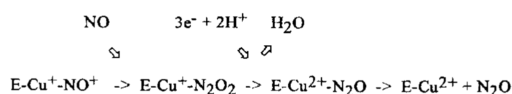
A fourth type of nitrite reductase has been identified in bacteria; the cytoplasmic NADH-dependent sirohaem nitrite reductase which reduces nitrite to ammonium [120]. This nitrite reductase is probably a homo-dimer of 90–100 kDa polypeptides. The prosthetic groups are FAD, an iron-sulfur cluster(s) and sirohaem, an isobacteriochlorin, which is the site of nitrite reduction. The sirohaem nitrite reductase is structurally quite similar to *E. coli* NADPH-dependent sulfite reductase and can also reduce sulfite to sulfide. Although the sirohaem nitrite reductase is only expressed under anaerobic conditions in *E. coli*, nitrite reduction via this enzyme is not coupled to energy conservation. In this organism, the main role of the sirohaem nitrite reductase may be in the removal of nitrite, which is toxic in the cell at high levels (Section 4.3) [405]. The enzyme may also play a role in the recycling of reduced pyridine nucleotide generated during fermentative metabolism. Both processes would also generate potentially useful ammonia for assimilation. In *E. coli* and *S. typhimurium* the operon containing the sirohaem nitrite structural gene (*nirB*) also contains the gene for a putative nitrite transporter (*nirC*; Section 6.1) [413,598]. In other bacteria, the sirohaem nitrite reductase structural gene is linked to genes coding for nitrate uptake system and the assimilatory nitrate reductase (Section 5.2).

7.1.1. The product of copper and cytochrome cd_1 nitrite reductases

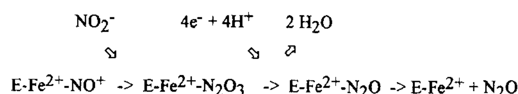
Both nitric oxide and nitrous oxide have been detected as reaction products from the cytochrome cd_1 and copper nitrite reductases using physiological electron donors (cupredoxins and *c*-type cytochromes) or non-physiological electron donors such as ascorbate plus PMS. With dithionite-reduced viologens as electron donors, the product is invariably nitrous oxide. This may be due simply to the direct reduction by dithionite or the reduced viologen of any nitric oxide produced by the enzyme. With the other more electropositive electron donors the reaction product can be dependent on the assay condition. A combination of isotope exchange experiments, using $H_2^{18}O$, and chemical trapping experiments have led to the view that the reduction of nitrite, by both types of enzyme, proceeds via an enzyme bound nitrosyl intermediate [7,249,261,488,582]. The formation of this intermediate probably proceeds via the protonation of E-NO_2^-, followed by dehydration to the unstable E-NO^+. If we consider nitric oxide to be the nitrite reaction product for copper nitrite reductase, this could be represented as:



However, the reaction product of the copper nitrite reductase is affected by the accumulation of nitric oxide in, or addition of nitric oxide to, the assay system [261]. Thus, in the presence of nitric oxide and nitrite, nitrous oxide accumulates as a reaction product, but if a nitric oxide trapping agent, such as deoxyhaemoglobin, is included in the reaction assay no accumulation of nitrous oxide is observed [261]. It is probable that nitrous oxide production occurs through a nitric oxide rebinding mechanism:



Some evidence for a similar nitric oxide rebinding mechanism during turnover of the cytochrome cd_1 nitrite reductase is apparent when considering the data of Wharton and Weintraub [588], which show that nitrous oxide production only commences after an initial period of nitric oxide formation. However, Jackson and co-workers showed that only 0.5% of the reaction product of cytochrome cd_1 was nitrous oxide and that the removal or addition of nitric oxide did not affect this ratio [261]. A nitrite rebinding mechanism in which nitrite makes a nucleophilic attack on E-NO^+ could explain these data. Such a mechanism is advocated by Aerssens and co-workers [7,582] and may be as outlined below:



The major product of nitrite reduction by the cytochrome cd_1 nitrite reductase in vivo is nitric oxide and it appears that the side-reaction of nitrous oxide production has little physiological relevance. A nitric oxide reductase deficient mutant of *Pseudomonas stutzeri* ZoBell is unable to grow anaerobically on nitrate or nitrite [84]. This strain contains the cytochrome cd_1 nitrite reductase. Nitric oxide

is normally only present at very low levels in denitrifying cultures of bacteria and the lethality of the mutation is probably due to accumulation of nitric oxide to toxic levels, rather than to insufficient energy transduction. In view of the capacity of copper nitrite reductases to rebind nitric oxide and form nitrous oxide, it will be of interest to determine whether the deletion of nitric oxide reduction from a denitrifier with a copper nitrite reductase is also lethal.

A widely documented property of the copper nitrite reductase is the capacity to produce nitrous oxide from nitrite using hydroxylamine as a reductant [260,261]. The physiological significance of this reaction remains uncertain, but it is notable that a copper nitrite reductase has been isolated from *Nitrosomonas europeae* [376]. This organism emits nitrous oxide during nitrification. Since ammonia oxidation produces hydroxylamine and nitrite, the two substrates for production of nitrous oxide by the copper nitrite reductase are both present. Production of nitrous oxide from nitrite and hydroxylamine is not apparent in cytochrome cd_1 nitrite reductases, which are sensitive to inhibition by hydroxylamine [314]. This may be due to competitive inhibition, since some reports suggest that the enzyme can also serve as a hydroxylamine reductase [310].

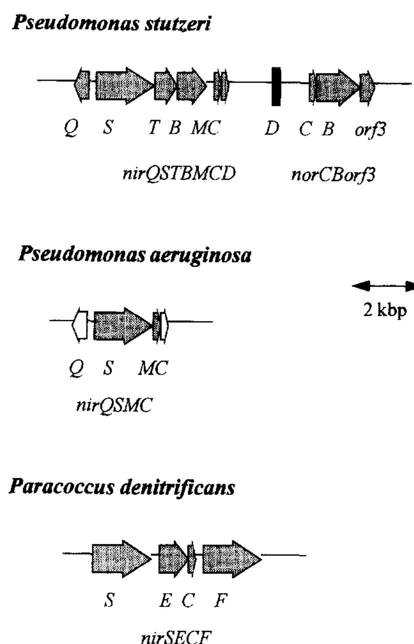


Fig. 15. Cytochrome cd_1 nitrite reductase and nitric oxide reductase gene clusters. The *nir*D locus of *P. stutzeri* has not been analysed in detail but may be required for cytochrome d_1 synthesis [275,276]. The *orf*3 gene is predicted to encode a soluble cytoplasmic protein of currently unknown function [625]. Unfilled arrows represent genes that are currently incompletely sequenced.

7.2. Cytochrome cd_1 nitrite reductase

Cytochrome cd_1 nitrite reductases have been isolated from a number of denitrifying organisms including *Pseudomonas aeruginosa* [243,215], *P. denitrificans* [314,388], *P. stutzeri* [622], *Alcaligenes faecalis* [259], *T. pantotropha* [382], *R. denitrificans* [151], *Paracoccus halodenitrificans* [343], *A. eutrophus* [464] and *Thiobacillus denitrificans* [251,473]. The corresponding structural gene (*nirS*) has been cloned and sequenced from *P. aeruginosa* [499], two strains of *P. stutzeri* [85,275,276,501] and two strains of *P. denitrificans* [142,398]. The enzyme has a subunit molecular mass of around 60 kDa. As judged by gel permeation chromatography, sedimentation velocity [39] and cystallographic analysis [180], the enzyme is a functional homodimer. Each subunit contains one covalently bound *c*-type haem and one non-covalently bound molecule of the unique d_1 'haem' prosthetic group. The d_1 haem can be extracted from the enzyme with organic solvents, resulting in an inactive semi-apoprotein. Activity can be restored to the semi-apoprotein by reconstitution with haem d_1 or, to a much lesser extent with haem *a* [230].

In *P. stutzeri*, *P. aeruginosa* and *P. denitrificans*, *nirS* clusters with genes encoding electron transport proteins and enzymes that may be involved in biosynthesis of the d_1 haem. In *P. stutzeri* (Fig. 15) *nirS* is followed, in order, by genes for NirT, a member of the tetra-*c*-haem family of electron transport proteins that includes NapC of the periplasmic nitrate reductase system (Section 5.1.3), di-haem cytochrome *c*-552 (*nirB*), mono-haem cytochrome *c*-551 (*nirM*) and a small monohaem *c*-type cytochrome (*nirC*) [276]. A non-polar transposon T*n5*-insertion in *nirT* results in expression of a functional cytochrome cd_1, but loss of in vivo activity, a phenotype consistent with an electron transfer role for NirT [276,622]. *nirT* and *nirB* are not found at the nitrite reductase locus of *P. aeruginosa* [21,395,499] or of *P. denitrificans* [142]. In *P. denitrificans* the gene cluster contains the additional genes *nirE*, predicted to encode uroporphyrinogen III methyl transferase, and *nirF*, a 60 kDa cytoplasmic protein of unknown function. Insertion mutagenesis in any of *P. denitrificans nirECF* results in an inactive nitrite reductase, apparently due to a failure either to synthesise or insert the d_1 haem [142]. *nirC* has also been identified and shown to affect cytochrome cd_1 biosynthesis in *P. fluorescens* [604].

7.2.1. The molecular structure

The X-ray structure of oxidised *T. pantotropha* cytochrome cd_1 nitrite reductase crystallised at pH 7, has recently been determined at a resolution of 1.55 Å [179,180]. The crystal space group contains a dimer of cytochrome cd_1 with each monomer organised into two domains (Fig. 16). The smaller (residues 1–134), predominantly α-helical, domain binds the *c*-type haem. This domain superficially resembles the folding of Class I *c*-type cytochromes [383], but the axial haem ligands and the threading and connectivities of the helices are different.

The c-type haem is liganded by His-17 (distal) and His-69 (proximal). This was unexpected, since *bis*-His ligated cytochromes normally have low redox potentials [383], while the c-type haem of cytochrome cd_1 is likely to have a reduction potential of +200 to +300 mV in order to accept electrons from cytochrome c-550 and pseudoazurin [381,382].

The larger domain (residues 135–567) forms an eight-bladed β-propeller structure. This fold is also found in the periplasmic enzyme methanol dehydrogenase [600]. Seven-bladed propeller structures are found in galactose oxidase [256] and periplasmic methylamine dehydrogenase [568], while neuraminidase [567] has a six-bladed propeller. In the nitrite reductase, the d_1 haem is centrally located in the large domain. The haem iron is ligated by conserved His200 originating from the β-propeller domain, and Tyr-25 on a 'finger' from the α-helical c-type haem domain that extends into the d_1-haem domain. A tyrosine residue is conserved at or near position 25 in the published sequences of cytochrome cd_1 from *P. denitrificans* [142,398] and *P. aeruginosa* [499]. However, in *P. stutzeri* there is a long deletion in this region, indicating that the small domain ligand to d_1 may be different in this nitrite reductase. In addition to the Tyr-25 ligand, the two

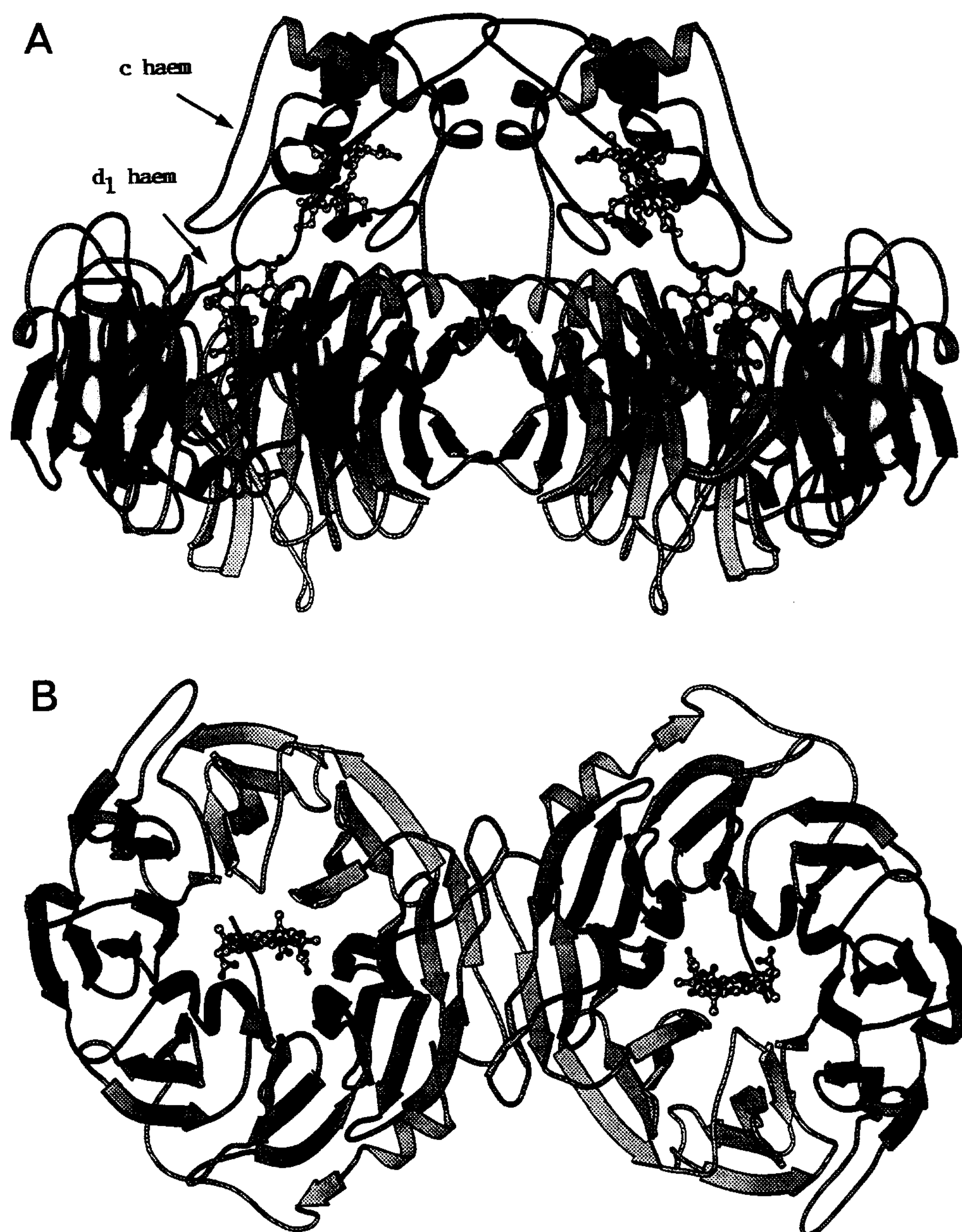


Fig. 16. The structure of the cytochrome cd_1 nitrite reductase showing the secondary structural elements and the positions and relative orientations of the c-type and d_1-type haems. (a) The entire dimeric molecule with the domain containing the c-type haem to the top (b) An end-on view of the eight-bladed β propeller structure that binds the d_1 haem at which reduction of nitrite is catalysed.

domains are connected by approximately 20 hydrogen bonds at the domain interface. The shortest edge-to-edge distance between the *c*-type haem and d_1-haem within a monomer of cd_1 is 11.0 Å.

Crystals of *P. aeruginosa* cytochrome cd_1 nitrite reductase were reported in 1963 by Yamanaka and Okunuki [603]. Akey et al. [9] and Takano et al. [539] obtained crystal of the same enzyme which diffracted to 12 Å and 2.5 Å resolution, respectively, but no three-dimensional structure has resulted from this work. Secondary structure predictions for the cytochrome cd_1 nitrite reductase of *P. aeruginosa* indicate that, in agreement with the structure from *T. pantotropha* [180], the N-terminal portion of the polypeptide, which contains the *c*-type haem binding site, would be predominantly α-helical in structure, while the remainder of the molecule is more likely to contain predominantly β sheet structure [497]. Also, proteolytic digestion of the native enzyme with subtilisin releases an 11 kDa N-terminal *c*-type haem-containing fragment, indicating that the region that binds *c*-type haem constitutes a distinct domain structure [245]. In spite of these gross similarities between the two enzymes, MCD of the enzyme from *P. aeruginosa* unambiguously shows that the *c*-type haem is His-Met ligated [580]. The inference from this and the deletion of the region containing the Tyr-25 residue in the cd_1 from *P. stutzeri* is that there is structural diversity between the members of the cd_1 nitrite reductase family.

Cytochrome cd_1 nitrite reductase may undergo conformational change during the catalytic cycle. As assessed by circular dichroism, then 'β-sheet' content of the enzyme decreases from 48% to 35% on reduction [546]. Additionally, Berger and Wharton [65] found by small-angle X-ray scattering that the radius of gyration of the nitrite reductase decreased on reduction. These results may underlie the observations that cytochrome cd_1 crystals crack on reduction [9] and that the thermal stability of the reduced enzyme is lower than that of the oxidised enzyme [378]. Ligand binding may also cause gross conformational changes. Cyanide (which can ligand the *c*-type and d_1 haems) has the effect of rendering the cd_1 nitrite reductase susceptible to the proteolytic fragmentation by subtilisin described above [245].

The possibility of conformational changes is also supported by spectroscopic analysis of the enzyme from *P. aeruginosa*. Greenwood and co-workers have investigated the haem ligation in *P. aeruginosa* nitrite reductase [268,580]. The form of the temperature dependencies of the MCD spectra together with EPR measurements show that reduced haem d_1 is high spin, whereas the oxidised forms of both haems are low spin. A high spin state for a cytochrome is 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. Tyr-25 is a ligand to the oxidised form of haem d_1 in the enzyme from *T. pantotropha*, but it has been proposed that this ligand becomes dissociated from the haem d_1 on reduction, leading to the conformational changes described above [180].

7.2.2. The roles of the c-type and d_1 haems

The current consensus is that the d_1 haem is the site of nitrite reduction, while the *c*-type haem is thought to transfer electrons from the electron donor protein to the d_1 haem. Detailed kinetic studies with the cytochrome cd_1 from *P. aeruginosa* have revealed that cytochrome *c*-551 and azurin can act as electron donors [243,409,498,560, 602,603], azurin via a hydrophobic interaction [560] and *c*-551 via an exposed haem edge, possibly through electrostatic interactions [498]. It has been proposed that the cytochrome *c*-551 is the physiological electron donor in this organism [545]. This judgement is based on the finding that the cytochrome *c*-551 has a higher affinity for cytochrome cd_1 than azurin. However, since both cytochrome *c*-551 and azurin donate electrons to the nitrite reductase at a rate faster than the rate-determining step of nitrite reductase, this may not be a critical factor. The concentration of these electron donors in the periplasm are probably far higher than their respective K_m values; concentrations of periplasmic proteins have been calculated to be around 1 mM [46,350,382]. Similar considerations also apply when considering these proteins as electron donors to the copper nitrite reductase (Section 7.3.3) and the nitrous oxide reductase (Sections 2.1 and 2.3).

In the reaction scheme outlined in section 7.1.2, nitric oxide is both a product and an enzyme-bound intermediate. In the reduced enzyme the d_1 haem binds exogenous nitric oxide over the entire pH range (4–9) that the enzyme is active, while the *c*-type haem only binds nitric oxide at pH values less than 7 [497] indicating that only the d_1 haem can be the site of nitrite reduction. The affinity of the d_1 haem for nitric oxide is higher than that of the *c*-type haem [497]. Rapid mixing experiments indicate that the nitric oxide bound form of the d_1 haem is a reaction intermediate and that the reduction of the NO-bound form of the oxidised d_1 haem to the NO-bound reduced form could be a significant rate limiting step in the overall reaction [500]. In these latter experiments, carried out at pH 8, there was no indication of nitric oxide binding to the *c*-type haem.

The structure of d_1 proposed by Chang et al. [105], and shown in Fig. 17, has been confirmed by complete chemical synthesis and reconstitution of the synthetic material with a semi-apo form of the nitrite reductase [583,599]. Two of the adjacent pyrroles of 'haem' d_1 are partially saturated and possess oxo and carboxylate functions. Thus the molecule is a dioxoisobacteriochlorin, or dionehaem, rather than a haem [106,529,599]. Compared with *b*-haem, d_1-haem is bulkier and does not possess the vinyl groups (Fig. 17). The latter are involved in the covalent bonding of the haem to the polypeptide in *c*-type cytochromes.

The importance of the novel features of d_1 haem is not clear. However, Ozawa et al. [403] report that the oxidised

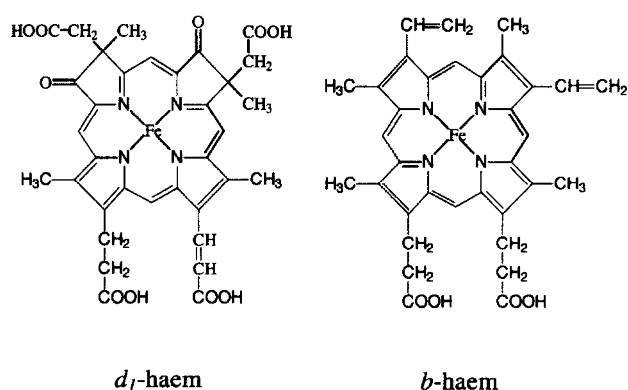


Fig. 17. Structure of the d_1-type haem and its comparison with *b*-type (proto) haem.

state of nitrosyl-bound d_1 model compounds is stabilised by the formation of a π-cation radical with accompanying reduction of iron to the 2^+ oxidation state. Sirohaem (Section 7.1.2) is an isobacteriochlorin and is also stabilised in the oxidised form by the formation of a π-cation [106]. The reaction products of the sirohaem and cytochrome cd_1 nitrite reductases are different but in each case an early intermediate may be the NO-bound form of the oxidised isobacteriochlorin. In cytochrome cd_1 nitric oxide is the reaction product and is released from the enzyme, whereas in the sirohaem nitrite reductase nitric oxide is further reduced to ammonia.

7.2.3. The mechanism of nitrite reduction

Garber and Hollocher [184] have demonstrated by $H_2^{18}O$ exchange studies that the first step of nitrite reduction is a protonation/dehydration. Nitrite bound to reduced haem d_1 (E-NO_2^-) is protonated and then dehydrated to give an enzyme bound nitrosyl intermediate (E-NO^+) (Fig. 18). These two intermediates can freely exchange so that the ^{18}O from the water becomes incorporated into the reaction product. The crystal structure of the *T. pantotropha* enzyme has revealed a possible mechanism for the protonation/dehydration of nitrite to nitric oxide. Two histidine residues, His-345 and His-388, are found close to the d_1 haem on the same side as the ligand Tyr-25. These residues are proposed to bind a water molecule in the resting state of the enzyme and to be involved in the protonation of nitrite during the turnover of the enzyme [180].

Stopped flow studies by Silvestrini et al. [500] show that the first detectable species in the reaction mechanism starting from fully reduced enzyme has the *c*-type haem reduced and nitric oxide bound to oxidised haem d_1 ($c^{2+}d_1^{3+}$-NO). This species is produced in the dead time of the experiment (4 ms). Therefore, the protonation/dehydration must occur very rapidly and be followed by a second rapid step in which the d_1 haem is oxidised and bound NO^+ is reduced to NO (Fig. 18). The next, slow, step is probably release of NO from the d_1^{3+} (Fig. 18). Nitric oxide generally binds less tightly to Fe^{3+} than Fe^{2+} [489] and thus the stabilisation of the oxidised state of the d_1 haem by π-cation radical formation (Section 7.2.2) is expected to assist this step by driving the reaction sequence $d_1^{2+}\text{-}NO^+ \leftrightarrow d_1^{3+}\text{-}NO \leftrightarrow d_1^{3+} + NO$ to the right (in the sirohaem nitrite reductase this stabilisation will also be useful if, as seems likely, enzyme-bound NO rather than NO^+ is required in the next step in the reduction reactions).

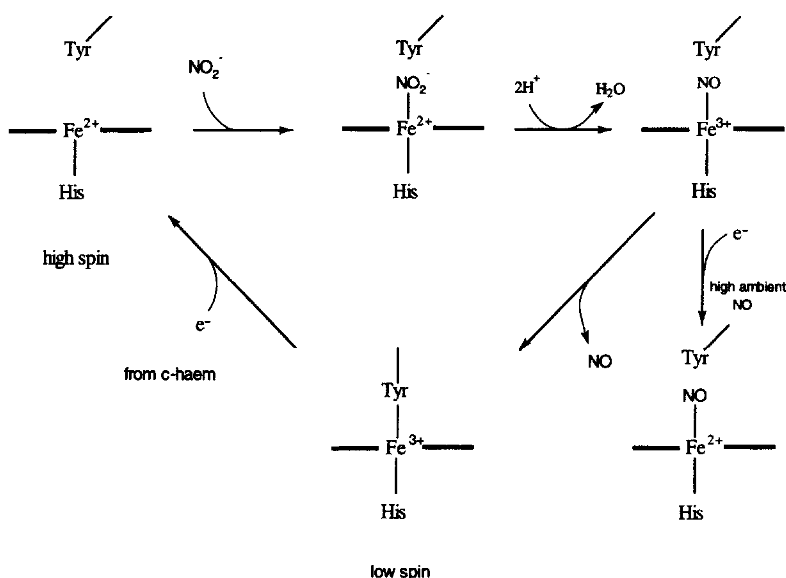


Fig. 18. A reaction scheme for the cytochrome cd_1 nitrite reductase. As described in the text it is proposed that the tyrosine d_1 haem coordinates the haem iron only in the oxidised state and that nitrite binds to reduced haem d_1. The depiction of the nitrite and nitric oxide d_1 haem ligands is not meant to distinguish between Fe-N or Fe-O bonding, although Fe-N bonding is more probable. The formation of a species in which nitric oxide is bound to the reduced form of the d_1 haem [498] is suggested not to be on the catalytic cycle. Rather it is proposed to occur as a result of electron transfer from the *c*-type haem when the ambient nitric oxide concentration is high enough to favour nitric oxide ligation to the oxidised d_1 haem.

Relative to the histidine or methionine ligation normally found in haemoproteins, coordination of the d_1 haem iron by the more anionic phenolate function of the tyrosine ligand should also stabilise the iron in the oxidised state. This may account for the release of this ligand from reduced haem d_1, while the rebinding of the tyrosine to the oxidised haem may provide some of the thermodynamic driving force for NO release (Fig. 18).

Silvestrini et al. [500] showed the reduction of d_1^{3+}-NO by the *c*-haem contains the rate-limiting step in the nitrite reductase reaction (starting with the fully reduced enzyme). We suggest that this is most likely due to a slow rate of NO release from the haem and/or the conformational changes required for rebinding of the tyrosine ligand. Implicit in this model is the idea that the electron transfer is rapid when tyrosine is coordinated to the d_1 haem but much slower than the rate-limiting step when the tyrosine is released. The distance between the haems observed in the crystal structure of the oxidised state is around 1.1 nm (Section 7.2.1), allowing for very rapid electron transfer [385]. The abolition of electron transfer in the NO-bound oxidised state may be ascribed to a number of factors: (i) the reorganisation energy associated with the need for conformational change to attain the reduced state; (ii) binding of NO stabilises the oxidised state and therefore decreases the driving force for the electron transfer; (iii) if the tyrosine is no longer coordinated to the haem iron, this may mean that the tyrosine and the associated electron transfer pathway from the *c*-haem have moved further away from the d_1-haem and therefore that the electron

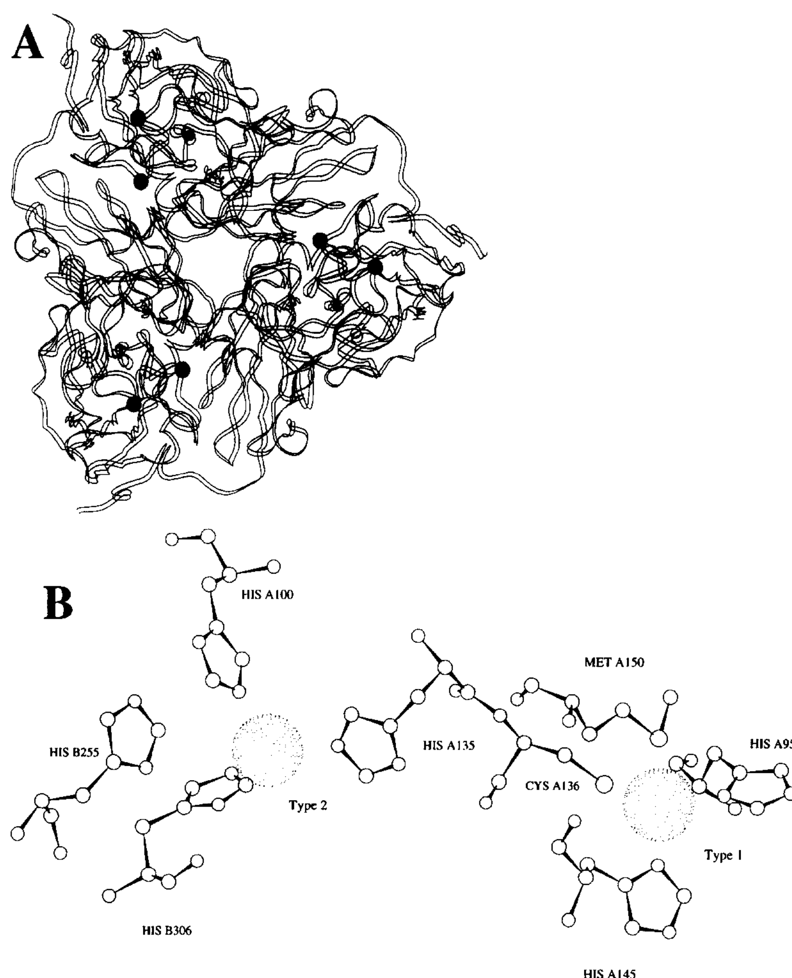


Fig. 19. Structure of the trimeric copper nitrite reductase from *A. faecalis* (oxidised state). (A) Ribbon diagram showing the trimeric structure and the positions of the type 1 and type 2 coppers. The three type 2 coppers (innermost copper atoms) are located at the subunit interfaces. (B) The coordination environment of the type 1 and type 2 coppers. A and B indicate that the amino acids are from different subunits. The adjacent type 1 copper and type 2 copper ligands Cys-136 and His-135 could provide a route for through-bond electron transfer (see text and Fig. 20). While His-255 is not a copper ligand (see text and Fig. 20) the ϵ nitrogen can move freely to within 3.1 Å of the copper without rearrangement of the backbone chain (our analysis). Thus during the catalytic cycle this residue could act as a transient copper ligand or act as a proton donor to the active site. The PDB structure coordinates file is 1AFN.

transfer distance has been increased; (iv) through-space electron transfer is very much slower than through-bound transfer [58] – thus, breaking the iron-tyrosine bond will markedly decrease electron transfer rate unless alternative pathways are available. Examination of the crystal structure suggests that alternative pathways are unlikely; the

A

```
Ascorbate oxidase
                   2   3                                3 3
D1   56 G V V I H W H G I        98 A G T Y F Y H G H L G M Q R S A G L
D2  226 S L N F A I G N H       272 P - L E N Y W V S I G V R A R(5) G L
D3  444 I H P W ■ L H G H       500 P G V W A F H C H I E P H L H M G M
          1     2   3                       3 1 3       1         1

A. cycloclastes Nir
          1           2                     2 1         1         1
D1   94 L H N I D F H A A       129 P G V F V Y H C A (5) P W H V T S G M
D2  251 D T R P ■ L I G G       298 P G V Y A Y V N H N L I (2) F E L G A
                *                                  2

N. gonorrhoeae Nir
D1  133 P H N V D F H A A       168 P G L Y I Y H C A (4) G M H I A N G M
D2  276 L V S S ■ V I G E       321 P G S Y T L V D H S I F(2) F N K G A
                *
```

B

Ascorbate oxidase

Cu2

H-O — Cu4

OH

^{2}His60

^{3}His508

Cu3

^{3}His62

^{3}His104

Cys507 — Cu (type 1)

Nitrite reductase

Cu

H_2O

Cys136 — Cu (type 1)

Fig. 20. Alignment of copper binding domains of the copper nitrite reductase from *A. cycloclastes* with the *Cucurbita pepo medullosa* ascorbate oxidase and the *N. gonorrhoeae* AniA (Nir) protein. (A) Amino acid alignment based on that of Fenderson et al. [165]. Copper ligands established by crystallography are numbered 1, 2 or 3 to indicate ligation to type 1, 2 or 3 copper. D1 to D3 refer to domains numbered from the polypeptide N-terminus. Histidines that can be superimposed in structural alignments of ascorbate oxidase and nitrite reductase are shaded. * *H* is conserved between copper nitrite reductases. It is not a copper ligand but is close to the active site (Fig. 19). The corresponding histidine in ascorbate oxidase is a type 2 copper ligand (Fig. 19; Fig. 20B). (B) Schematic comparison of the coordination environment of the active site copper atoms of oxidised ascorbate oxidase and nitrite reductase. In ascorbate oxidase Cu2 and Cu3 are type 3 copper atoms and Cu4 is a type 2 copper atom. Cu2 is the binding site of peroxide (and by implication oxygen) and azide. Ligand binding or reduction leads to structural rearrangement of the trinuclear centre mainly around Cu2. The hydroxyl bridge is broken, His-106 shifts position considerably and Cu2 moves closer to all three of its histidine ligands increasing the Cu2–Cu3 distance [366]. Structural alignments [366] demonstrate that the nitrite reductase type 2 copper and ligands can be closely superimposed onto the Cu2 copper of ascorbate oxidase. One of the ascorbate oxidase type 2 (Cu4) copper ligands is also conserved in the nitrite reductases (dark shading in both this figure and part (A)) and may play a role during the catalytic cycle in proton conduction or as a transient copper ligand (see text). Electrons from the type 1 copper may reach the active site in both enzymes by through-bond electron transfer via the type 1 copper cysteine ligand and the adjacent type 3/type 2 copper histidine ligands (see text).

tyrosine finger of the *c*-haem domain is bound by the d_1 domain principally by the haem–tyrosine interaction.

7.3. The copper nitrite reductase

Copper nitrite reductases have been identified in a number of bacterial genera and purified from several Gram negative bacteria, including *Achromobacter cycloclastes* [260,332], *A. faecalis* Strain S-6 [282], *R. sphaeroides* f.sp. *denitrificans* [373,471], *Achromobacter xylosoxidans* (formerly *Alcaligenes* Sp. NCIB11015) [3,349], *Pseudomonas aureofaciens* [621] and *N. europeae* [149] and one Gram-positive organism, *B. halodenitrificans* [146]. The enzymes are probably all homotrimers of 36 kDa subunits in which each subunit binds one type I and one type II copper centre. The structural gene coding for the copper type nitrite reductases has been sequenced from *A. faecalis* [392], *Pseudomonas* species G179 [606] and *P. aureofaciens* [201]. The primary structure of the enzyme from *A. cycloclastes* has also been determined by protein sequencing [165] (Fig. 20).

In addition to those proteins outlined above, our analysis of the *aniA* gene product of *Neisseria gonorrhoeae* suggests that it is also a copper nitrite reductase. This gene is induced anaerobically following growth in the presence of nitrite [234–236]. The *aniA* gene product has been localised to the outer membrane, suggesting that nitrite may be reduced extracellularly. The AniA protein can be detected immunologically in patient sera, indicating that adaptation to anaerobiosis with associated nitrite reduction is important is successful gonococcal infection. The AniA sequence contains N- and C-terminal motifs characteristic of gonococcal lipoproteins [236]. The region between these motifs reveals significant similarity (30% identity including all type I and type II copper ligands) to copper nitrite reductases (Fig. 20). We suggest that an azurin-like protein, which also possesses a lipoprotein signal peptide and may therefore be located in the outer membrane [206a], is the electron donor to AniA. The primary structures of the copper nitrite reductases from *A. cycloclastes*, *A. faecalis*, *Pseudomonas* species G179 and *P. aureofaciens* share 60% to 80% identity, making identification of functionally important residues from sequence conservation difficult. The addition of the highly divergent *N. gonorrhoeae* sequence makes such comparisons of much greater utility.

7.3.1. Biological copper centres

The structures, functions and spectroscopic character of copper proteins have each been extensively reviewed and the reader is directed to the following references for a detailed treatment of the subject [4,5,96,97,220,508–510,519,536]. The redox states of copper found in biological system are Cu (II) (d^9 and paramagnetic), which favours tetragonal ligand fields, and Cu(I) (d^{10}, diamagnetic and optically inactive) which prefers a tetrahedral arrangement of ligands. On the basis of spectroscopic properties Malkin and Malmstrom [340] divided copper centres into three classes, types I to III. Copper binding domains of known structure contain predominantly β-sheet structure arranged in a 'Greek Key' fold and may in many cases share an evolutionary origin [5].

'Normal copper', or type II copper, centres contain a single copper atom with spectroscopic properties similar to those of typical inorganic copper complexes. Functional type II sites are invariably involved in catalysis at the active site. The structures of type II copper sites are very diverse, but ligation of the copper is predominantly by histidine residues with at least one water-derived ligand that can be displaced by substrate or other exogenous ligands. Cysteine ligation is absent.

'Blue copper' or type I copper centres are mononuclear centres with cysteine ligation. They are characterised by an intense blue colour ($\varepsilon_{600nm} > 1$ mM^{-1} cm^{-1}), relatively high reduction potentials (200–700 mV) and an axial or rhombically distorted EPR signal with unusually small copper parallel hyperfine splitting ($A_{\parallel} < 7 \cdot 10^{-3}$ cm^{-1}). Two histidines and one cysteine form strong bonding interactions with the copper atom. The copper atom is positioned slightly out of the plane of these ligands with distortion in the direction of the single, weak, usually methionine, axial ligand [449,508]. In some cases there is electrostatic interaction between the copper atom and an axially located main chain carbonyl group [97]. The copper ligands are found on two of the loops at one end of a Greek Key β-barrel structure; the buried histidine on the upstream loop, the other three ligands closely spaced on the second loop. A 107° cysteine-copper C–S–Cu bond angle enforced by the protein chain leads to two σ and one π interactions between the cysteine thiolate and the copper atom. A consequence of this unusual geometry is that there is substantial overlap of the S_{cys} and Cu $d_{x^2-y^2}$ orbitals, allowing extensive charge delocalisation onto the cysteine sulfur ligand. This covalency is the origin of the small EPR $g_{\parallel}$ hyperfine splitting [483]. The intense optical (blue) absorption at approx. 600 nm is assigned to an $S_{cys}\pi \rightarrow$ Cu $d_{x^2-y^2}$ charge transfer band. The strength of this transition is another consequence of the large orbital overlap. In certain type I copper centres, including those of a number of copper nitrite reductases, a second $S_{cys} \rightarrow$ Cu transition at around 460 nm is more intense than the 600 nm transition. These centres are green rather than blue in appearance. The intensity of the 460 nm band correlates with increased rhombic distortion of the EPR signal and appears to reflect a distorted tetrahedral rather than trigonal planar arrangement of copper ligands. This can arise from a shortening of the axial (Met) interaction [221]. Type I copper centres are always involved in electron transfer rather than catalytic reactions. The rigid geometry of the centre minimises the reorganisation energy associated with change in oxidation state of the copper. Electron

transfer to or from the centre appears to involve predominantly the cysteine ligand and the solvent-exposed histidine.

Dinuclear type III copper centres are characterised by an EPR-silent Cu(II)-Cu(II) state. The diamagnetism of this state arises from antiferromagnetic exchange coupling of the cupric centres. This coupling proceeds by a superexchange pathway mediated by bridging ligand(s). Type III copper centres are involved in ligand binding and/or catalysis. Three structurally distinct classes of this centre are known. In haemocyanin and tyrosinase each copper atom has three histidine ligands, the centre being bridged by various oxygen, water or substrate derivatives. In the multicopper oxidases (including ascorbate oxidase) the dinuclear type III centre (three histidine ligands for each copper atom) is probably bridged by a hydroxide ion (Fig. 20B) [366–370]. The final class of type III centre is found in nitrous oxide reductase. This centre probably contains a cysteine sulfur bridge and is considered in more detail in Section 9.4.

A fourth type of copper centre, the Cu_A centre, which has so far been detected only in cytochrome *c* oxidase and nitrous oxide reductase, is discussed in detail in Section 9.3.

7.3.2. The molecular structure

The copper nitrite reductase from *A. cycloclastes* was originally crystallised at pH 5.8 and the amino acid sequence fitted to a 2.3 Å resolution electron-density map [202]. More recently, crystals have been obtained at pH 6.2 and pH 6.8 and X-ray diffraction data collected at 2.5 Å and 2.8 Å, respectively [6]. The structure of the *A. faecalis* S-6 enzyme crystallised at pH 4.6 has also been determined [313]. The copper nitrite reductase from *A. cycloclaste* was originally described as a dimer containing two atoms of copper per subunit [260]. However, both crystallised enzymes are homotrimers and bind three type I and three type II copper centres (Fig. 19). Each monomer forms two β barrel domains (domain I, residues 8 to 160 and domain II, residues 161 to 340). The domains are stacked on top of one another so that the β sheets form a four-layered sandwich. The interaction between domains I and II may be stabilised by a helical region in each domain. The domain I 141–149 helix provides two ligands for the type I copper centre (His-145 and Met-150) with His-95 and Cys-136 also in domain I providing the other ligands. The type II copper is liganded by His-100 and His-135 of domain I, a solvent molecule (presumably either water or hydroxyl) and His-306 from domain II of a different subunit (Figs. 19 and 20). The type II copper ligands are, unusually, arranged as a regular tetrahedron. The two copper molecules are spaced 12 Å apart and are coordinated by adjacent amino acids: the type II copper ligand His-135 and the type I copper ligand Cys-136 (Fig. 19). All the type I and type II copper ligands are conserved in the sequenced copper nitrite reductases.

The three-dimensional structures of the two domains of the copper nitrite reductase can be superimposed and it is probable that the monomer arose from a gene duplication of a single domain [165]. Comparison of the three-dimensional structure of the nitrite reductase with those of other copper proteins reveals structural similarities that are not readily apparent from sequence alignments [165]. Apart from a similarity of the type I copper centre structure to that found in cupredoxins, there is extensive structural similarity to domains one and three of the multi-copper enzyme ascorbate oxidase (and by implication to domains of laccase and ceruloplasmin that have sequence similarity to ascorbate oxidase; Fig. 20) [165,202,366,370,458]. In ascorbate oxidase domain 3 (C-terminal) provides ligands to a type 1 copper centre. Domain 1 (N-terminal) and domain 3 bind a trinuclear copper centre, in which a pair of type III copper atoms and a type II copper atom are bound by eight histidine ligands, four from each of the two domains (Fig. 20). Features of structural alignments of the ascorbate oxidase and nitrite reductase domains include (Fig. 20): (i) domain 1 (N-terminal) of nitrite reductase can be superimposed on domain 3 (C-terminal) of ascorbate oxidase such that the type I copper ligands and copper atom are coincident; (ii) the type II copper ligands in nitrite reductase domain 1 correspond to type III copper ligands of ascorbate oxidase domains 1 and 3; (iii) the type II copper ligand in nitrite reductase domain 2 aligns with ascorbate oxidase type III copper ligands in domains 1 and 3; (iv) a type II copper ligand in ascorbate oxidase domain 2 is conserved in nitrite reductase domain 2; (v) in structural alignments of the trinuclear and mononuclear centres the nitrite reductase type I copper site can be superimposed onto the ascorbate oxidase Cu2 site (Fig. 20B).

Before the determination of the crystal structure of the copper nitrite reductase from *A. cycloclastes*, a consensus quaternary structure for this class of enzyme had not emerged. As the trimeric arrangement is the minimal structure that satisfies the liganding requirements of the type II coppers other oligomers can be excluded. The trimeric structures of the *A. cycloclastes* and *A. xylosoxidans* enzymes has been confirmed by sedimentation equilibrium analysis [202] and solution X-ray scattering [212]. The sedimentation analyses did, however, suggest that at low protein concentration the enzyme could exist in a dimeric state, with the trimeric state predominating at high protein concentrations. Formation of the dimeric state would result in loss of type II copper, explaining the low copper stoichiometries and low specific activities reported for some preparations of copper nitrite reductases [349,621].

7.3.3. Rôles of the type I and the type II copper centres

The type I copper centre serves to transfer electrons from donor proteins (cupredoxins and cytochromes) to the type II centre [535b]. Such a role would be analogous to that of the *c*-type haem in the cytochrome cd_1 nitrite reductase, which mediates electron transfer to the d_1-haem

catalytic site (Section 7.2). Type II copper centres commonly form catalytic sites. The type II copper centres in nitrite reductase are therefore probably the site of nitrite reduction. Accordingly, the activity of the enzyme containing one type I copper atom per subunit is proportional to the type II copper content, with zero activity at zero type II copper content [322] (see also the copper contents and enzyme activities reported in [3]). The integrity of the type II copper site is clearly important for nitrite reduction, since substitution of the type II copper ligand His-135 with lysine abolishes activity, even though the enzyme retains type I and type II copper [313].

In the model outlined in Section 7.1.2, nitrite binds to the reduced form of the enzyme, for which it has a low K_m (5.6 μM for the enzyme of *A. cyclolastes* [260]). Density difference maps of oxidised nitrite reductase crystallised in the absence and presence of nitrite indicate that nitrite is bound at the type II copper centre [202], probably at the expense of the solvent ligand. Addition of nitrite to the oxidised copper nitrite reductase does not affect the visible spectrum but alters the type II ENDOR and EPR signals, again consistent with the type II centre being the site of the nitrite binding [29,30,247,373]; Hulse et al. (unpublished data, cited in [322]). Copper nitrite reductases are sensitive to inhibition by cyanide and carbon monoxide. Studies using EPR and visible spectroscopy suggest that cyanide binds to the type II, rather than type I copper [373]. Unlike the cytochrome cd_1 nitrite reductase, azide does not inhibit the copper enzyme. The suggestion that nitrite displaces the solvent ligand of the type II copper during the catalytic cycle is supported by studies on a mononuclear derivative of haemocyanin (Cu(II)-Hc) and a nitrite-bound form (Cu(II)-Hc-NO_2^-) [93]. ESEEM studies indicate that the ligand structure of Cu(II)-Hc is similar to that of the copper nitrite reductase type II copper centre and that a water molecule is displaced by nitrite in the Cu(II)-Hc-NO_2^- form [93a].

Nitric oxide binds reductively to the type II copper, presumably forming the Cu^+-NO^+ species that is a putative intermediate in nitrite reduction [29,30]. Nitric oxide addition also results in the partial reduction of the type I centre. While some workers have taken this as evidence that the E-Cu^+-NO^+ intermediate is formed at the type I copper, this interpretation should be treated with caution, since nitric oxide will form a Cu^+-NO^+ complex with the clearly electro-transferring type I copper of cupredoxins [206] and because binding of nitric oxide to the type I centre inhibits nitric oxide production from nitrite [261].

Two conserved amino acids that lie close to the type II copper, but are not ligands at any of the pH values studied, are His-255 and Asp-98. The position of His-255 relative to the copper molecule exhibits some pH dependence and the possibility has been raised that at high pH this histidine could replace the type II centre solvent ligand [202]. In this event, nitrite binding would be precluded, perhaps accounting for the rapid loss of activity of nitrite reductases at pH > 7. It is notable that His-255 aligns with a type II copper ligand in ascorbate oxidase, laccase and ceruloplasmin (Fig. 20). Possibly, transient ligation of the type II copper by His-255 during catalysis serves to displace the reaction product, nitric oxide. His-255 might also function as the proton donor to the active site or stabilise the E-Cu^+-NO^+ intermediate (Section 7.1.2) [6].

In the absence of exogenous ligands, the two copper centres of nitrite reductase are approximately isopotential ($E^{0\prime} \approx 240$ mV) [29,30,293]. As described in Section 7.3.2, the type I and type II copper centres are spaced 1.2 nm apart and liganded by adjacent amino acids (Cys-136 and His-135). Studies with the enzyme from *R. sphaeroides* f. sp. *denitrificans* suggest that electrons cannot be passed to the type II copper in the absence of the type I copper, since all activity using reduced viologens as electron donors was lost if type I copper was specifically removed to form the type II copper semi-apo-protein [373]. Similarly, substitution of the type I copper ligand Met-150 with Glu in the *A. faecalis* S-6 enzyme results in a type I copper deficient protein that loses 95% of the wild-type viologen-linked activity [313]. In cupredoxins, electron egress from the type I copper can involve the cysteine ligand and a surface-exposed tyrosine (at the 'acidic patch') [5,109]. By analogy, in the copper nitrite reductases electrons may egress type I copper through the bonds of Cys-136 and enter type II copper via His-135 (Fig. 20B). A similar arrangement of ligands is found between the type I copper and both type III coppers in the ascorbate oxidase (Fig. 20B).

In addition to donating electrons to the type II copper, the type I copper must also interact closely with, and accept electrons from, the redox centre of the electron donor protein(s). Loss of the type I centre in the *A. faecalis* Met-150 → Glu enzyme reduces by 1000-fold electron transfer from pseudoazurin to the type II centre [313]. In cupredoxins, electron transfer into the type I copper is through a surface-exposed histidine copper ligand (at the 'hydrophobic patch' [5,109]). In the nitrite reductase, the type I centre is buried 0.4 nm from the surface of the protein. However, a depression in the protein surface at the closest approach is likely to be filled by a solvent molecule, limiting accessibility of a redox partner. A close association will be further precluded by a protein loop arising from domain II [202]. The physiological electron donor to the type I centre is likely to be pseudoazurin in *A. cycloclastes* and *A. faecalis* [278,285,294,330], azurin in *P. aureofaciens* [621], cytochrome c_2 in *R. sphaeroides* f. sp. *denitrificans* [471,556] and cytochrome *c*-552 in *N. europeaea* [149]. However, a multiplicity of electron donors is probable in most species. Glockner and co-workers have expressed the copper nitrite reductase of *P. aureofaciens* in a *P. stutzeri* mutant deficient in cytochrome cd_1 [201]. *P. stutzeri* does not express a cupredoxin, but can still provide an electron transport pathway to the copper nitrite reductase. Holo-

copper nitrite reductase was also expressed in *E. coli*, but this organism could not provide an electron transfer pathway to the enzyme [201].

7.4. The cytochrome c nitrite reductase

7.4.1. Molecular, spectroscopic and catalytic properties

The molecular and spectroscopic properties of the cytochrome *c* or 'hexahaem' nitrite reductases have recently been reviewed in some detail [91]. A major development since that work has been the suggestion that the enzyme binds four, rather than six, *c*-type haems [138,478a]. This has major implications for the interpretation of spectroscopic signals and the development of catalytic models.

The cytochrome *c* nitrite reductase has been purified from a number of sources, including *E. coli* [281,328], *Wolinella succinogenes* [70,329], *Vibrio fisheri* [331], *Desulfovibrio desulfuricans* [124,326], *Vibrio alginolyticus* [430] and *S. deleyianum* [478a]. A single polypeptide of 50–70 kDa is present. The enzymes are either soluble periplasmic proteins or bound to the periplasmic face of the cytoplasmic membrane. The DNA-derived amino acid sequence of the *E. coli* enzyme has a putative signal peptide consistent with a periplasmic location for the mature protein [138]. Biochemical studies have placed the stoichiometry of *c*-type haem per polypeptide at between four and six [281,326–331,478a]. The view that the *c*-type cytochrome nitrite reductase was a hexa-haem enzyme was attractive because the reduction of nitrite to ammonia requires six electrons. The strongest spectroscopic support for the presence of six haems comes from Mossbauer studies on enzyme from *D. desulfuricans* [124], which indicate the presence of five low-spin and one high-spin haem. In attempting to fit spectroscopic data to the presence of six haems, the complex EPR spectra have been interpreted as indicating two isolated haems and two pairs of coupled haems [71,72,91].

The derived amino acid sequence of the *E. coli* cytochrome *c* nitrite reductase (NrfA) structural gene corresponds to a 50 kDa protein (rather than the 69 kDa protein indicated by SDS-PAGE analysis) [138,281]. Four consensus *c*-type haem binding motifs (Cys-Xaa-Xaa-Cys-His), with His as axial ligand, are present [138]. A fifth cysteine-rich sequence, Cys-Xaa-Xaa-Cys-Lys, is also present. Although this sequence is not the site of covalent haem binding in any known *c*-type cytochrome, lysine can replace the distal methionine haem ligand in horse-heart cytochrome *c* at high pH [383]. It is also notable that cyt *f* (a *c*-type cytochrome) is ligated by an amino group provided by the polypeptide N-terminus [348]. Thus, the possibility that the Cys-Xaa-Xaa-Cys-Lys motif defines a fifth haem binding site should not be excluded, especially as one of the haem centres presumably has to be quite distinct from the others to provide a site for catalysis.

The UV-visible, EPR and MCD spectra of cytochrome *c* nitrite reductases have features that indicate the presence of high-spin and *bis*-His ligated low spin *c*-type haem [71,72,326] (note that it can be difficult to resolve *bis*-His ligation from His-Lys ligation using MCD spectroscopy). Comparisons of spectra of the dithionite-reduced enzyme with those of ascorbate-reduced enzyme indicate that some haem is only fully reducible with dithionite. Thus, distinct haems of low and high reduction potentials are present. Only the fully reduced form of the enzyme is reactive towards nitrite [327]. The EPR spectra of the cytochrome *c* nitrite reductases of *E. coli*, *D. desulfuricans* and *W. succinogenes* [71,327], are very similar. In the oxidised state, signals at around $g = 2.9$, 2.3 and 1.5 are ascribed to low-spin haem, signals at around $g = 9.7$ and 3.7 to a high-spin haem [327] or to a weakly magnetically exchange-coupled high spin/low spin haem pair [71] and signals at around $g = 4.8$ and 3.2 to a weakly magnetically exchange coupled low-spin haem pair [71]. Following reduction and subsequent reoxidation by nitrite, some EPR studies have detected signals assigned as nitric oxide bound to high-spin iron [327,330], leading to suggestions that the proposed high-spin/low-spin haem pair 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 the penta-coordinate high spin haem with the bound nitrogen intermediates providing the sixth haem ligand. As an alternative, Blackmore et al. [72] have suggested that the high spin/low spin haem pair 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. This raises the possibility, already discussed for the cytochrome cd_1 and copper nitrite reductases (Section 7.2.2), that transient binding of a side chain of an amino acid, in this case histidine, to the metal during the catalytic cycle could serve to displace reaction products.

The cytochrome *c* nitrite reductase can reduce both nitrite and hydroxylamine to ammonia. The V_{max} values for nitrite and hydroxylamine are similar but the K_m for hydroxylamine is much higher than for nitrite [281]. The enzyme can also reduce nitric oxide to nitrous oxide at a rate comparable to that of nitric oxide reductase [123]. Thus, it is likely that nitric oxide and hydroxylamine are enzyme-bound intermediates in the reduction of nitrite to ammonia. The full reaction mechanism remains to be determined but it is conceivable that the six-electron reduction proceeds via nitrosyl (NO^+), one electron reduced nitric oxide (NO), two electrons reduced nitroxyl (NOH) and four electrons reduced hydroxylamine (NH_2OH) intermediates. During these transitions electrons could be transferred, in single or concerted two-electron steps, from two (or more) electron carrying haems to the haem or haem-haem pair at which the reaction intermediates bind. The reduction of nitrite to ammonia has been achieved using the cationic iron porphyrin complex $[Fe^{III}(H_2O)$-$(TMPyP]^{5+}$ [42]. In an electrochemical study it was possible to show that at low potential (−400 mV) this complex

was competent in reducing nitrite to either nitrous oxide or ammonia. The reduction to ammonia proceeded via the bound intermediates NO^+, NOH and NH_2OH, consistent with postulated intermediates outlined above.

7.4.2. Electron transport to E. coli cytochrome c nitrite reductase

The *E. coli* cytochrome *c* nitrite reductase operon has recently been sequenced [77,250]. In addition to the nitrite reductase structural gene *nrfA*, the operon encodes three electron transfer proteins which we propose form a membrane-bound quinol oxidase complex (NrfBCD)(Fig. 3) and three proteins that are probably involved in attachment of *c*-type haem to apo-cytochromes *c* (*nrfEFG*; Table 1). *nrfB*, *nrfC*, and *nrfD* have overlapping stop and start codons suggestive of translational coupling and equimolar synthesis of the polypeptides. *nrfEFG* overlap in a similar manner.

NrfB is predicted to be a soluble periplasmic protein (18 kDa mature apoprotein) as it contains an obvious signal peptide but no other potential membrane-spanning regions. Five *c*-type haem binding motifs are present. NrfB exhibits no significant sequence similarity with other *c*-type cytochromes. The cytochrome *c* nitrite reductases from *S. deleyianum* and *W. succinogens* can be copurified with a 22 kDa *c*-type cytochrome (putative NrfB homolog) with an apparent 4:1 NrfA to NrfB stoichiometry [478a]. NrfC (22 kDa mature apoprotein) has a possible signal sequence but is otherwise predicted to be a soluble protein. By sequence similarity NrfC is a member of the '16Fe' ferredoxin family discussed in Section 5.4.3 (see alignment in Fig. 13) and is thus expected to bind four $[4Fe\text{-}4S]^{2+,1+}$ clusters. Sequence similarity is highest to the PsrB subunit of the periplasm-facing membrane-bound MGD-dependent polysulfide reductase of *W. succinogenes* [298]. NrfD (35 kDa) shows high sequence similarity to the integral membrane subunit, PsrC, of the poly sulfide reductase. NrfD and PsrC have seven potential membrane-spanning helices with a probable topology placing the N-terminus of the polypeptide in the periplasm.

As NrfB, C and D are likely present at equimolar stoichiometry, and as the NrfCD homologues PsrBC are thought to form part of a membrane-associated complex, we speculate that NrfB, C and D constitute a periplasm-facing cytoplasmic membrane quinol oxidase complex that is the electron donor to the cytochrome *c* nitrite reductase (Fig. 3). NrfD would contain the site of (mena)quinol [1,2] oxidation, electrons being transferred to NrfD via NrfC (Fig. 3). There is only one histidine residue conserved between NrfD and PsrB and this is in a region of the polypeptide not predicted to be membrane-spanning. It is thus unlikely that NrfD or PsrC binds *b*-haem, as this normally requires *bis*-His ligation. This suggests that menaquinol is oxidised at the periplasmic face of the membrane as in the absence of *b*-haem groups electrons cannot be carried from the cytoplasmic side of the membrane. Whether a Δp is generated in the span menaquinol to nitrite is unclear, but if this does occur the lack of *b*-haems would exclude transmembrane electron transfer as a mechanism.

8. Nitric oxide reductase

8.1. Nitric oxide as a freely diffusible intermediate in denitrification

For many years there was debate as to whether nitric oxide is a freely diffusible intermediate in denitrification. As discussed in Section 7.1.2, it is now clear that both the cytochrome cd_1 and copper nitrite reductases catalyse formation of nitric oxide rather than nitrous oxide as the principal reaction product. Several lines of evidence obtained in the late 1980's (reviewed in detail in [170]) substantiate a role for nitric oxide as a free intermediate in denitrification. These include: (i) trapping of extracellular nitric oxide, formed from nitrate or nitrite, in suspensions of bacterial cells by either binding to haemoglobin or reduction through the action of the NADH-nitric oxide oxidoreductase activity of inside-out cytoplasmic membrane vesicles prepared from *P. denitrificans* [98,204]; (ii) displacement of the low steady-state concentration of nitric oxide from a suspension of cells by blowing a stream of argon though the suspension [205]; (iii) use of very sensitive approaches for measuring steady-state nitric oxide concentrations in suspensions of denitrifying cells with the outcome that nitric oxide is estimated as varying between 10^{-9} M and 10^{-7} M [205].

Formation of nitric oxide requires in turn an enzyme to reduce it to nitrous oxide. Clear evidence that such a nitric oxide reductase is a membrane-bound enzyme, and distinct from the periplasmic water-soluble nitrite reductase, 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 nitric oxide [622], as well as studies of inside-out vesicles from *P. denitrificans* which possessed NADH-nitric oxide oxidoreductase activity, but in which nitrite reductase was absent [99]. Later it was found that deletion of the copper-type nitrite reductase from *Pseudomonas* sp. strain G-179 also did not abolish nitric oxide reductase activity [605], thus establishing that a distinct nitric oxide reductase is present irrespective of the type of nitrite reductase. Confirmation that this nitric oxide reductase is involved in denitrification has come from the work of Braun and Zumft [84], who deleted the gene for this enzyme from *P. stutzeri* and showed that the resulting mutant was unable to denitrify owing to the accumulation of toxic nitric oxide from the reduction of nitrite.

8.2. The molecular properties of the nitric oxide reductase

Nitric oxide reductase has been purified from *P. stutzeri* [225], *P. denitrificans* [99,147] and *A. cycloclastes* [269b].

The enzyme contains two types of subunit, a 17 kDa *c*-type cytochrome and a 53 kDa (apparent molecular mass 37 kDa by SDS-PAGE) polypeptide that binds haem *b*. As well as haem, the enzyme may also bind non-haem iron [225]. The enzyme purified from *P. stutzeri* had a K_m(nitric oxide) of 40 μM when PMS was the electron donor [225], but the K_m(nitric oxide) of the enzyme from *P. denitrificans* assayed with cytochrome *c*-550 is sub-micromolar [99]. In view of the evident success of the nitric oxide reductase in whole cells in keeping the concentration of nitric oxide down to 10^{-7} M or less [205], the latter value seems more reasonable.

The genes for the 17 kDa and 53 kDa subunits (*norC* and *norB*) have been cloned from *P. stutzeri*, *P. aeruginosa* and *P. denitrificans* [24,142,562,625]. In *P. stutzeri* they are transcribed on a single transcript in the order *NorCB* and are separated by about 40 bases (Fig. 15). A putative Fnr-binding motif is located upstream of the transcription start site. NorC is predicted to have a single N-terminal transmembrane helix that anchors a periplasmic domain to the cytoplasmic membrane. A single Cys-Xaa-Xaa-Cys-His *c*-type haem binding motif has been identified in this domain. This is consistent with biochemical analysis and redox titrations that reveal a single *c*-type haem component ($E_{m,7}$ = 225 or 280 mV for the enzymes from *P. denitrificans* (Chan and Ferguson, unpublished data) and *P. stutzeri* [286]).

Analysis of the primary structure of NorB has revealed sequence similarity with the catalytic subunits of the haem-copper oxidase family [103,467,468,562], in particular those of the so-called 'cytochrome cbb_3' sub-group [143,185,207,278,344,345,421,477,543]. The oxidase polypeptides are thought to form 12 transmembrane helices. Six invariant histidines have been identified in the haem-copper oxidases. These provide two ligands to a low-spin haem, one ligand to a high-spin haem and three ligands to a copper ion that, together with the high-spin haem, forms the dinuclear centre at which oxygen is reduced (Fig. 21). These histidine metal ligands are also conserved in NorB and raise the possibility that a similar arrangement of redox centres is present. Helices II and X would provide histidine ligands to a low-spin haem, helix X to a high-spin haem and helices VI, VII and VIII to a metal ion. By analogy to the haem-copper oxidases the low-spin haem would be involved in mediating electron transfer (presumably from NorC) to the dinuclear centre which would be the site of substrate (nitric oxide) reduction.

At present, the experimental evidence does not completely support the haem-copper oxidase model of nitric oxide reductase. Signals attributable to high-spin and low-spin haem have been detected in EPR studies [225,286]. However, biochemical analysis and redox titrations of *b*-haem have not resolved more than one redox component (E_m = 305 mV or 322 mV for *P. denitrificans* and *P. stutzeri* (Chan and Ferguson, unpublished data; [286]) and most studies have reported a stoichiometry of two rather than three haems per NorCB complex [99,225]. Copper has not been detected in any analyses to date, but the presence of non-haem iron in the enzyme [225] raises the possibility that, if present, the dinuclear centre in NorB is haem/non-haem iron rather than haem/copper.

The copper ion in the haem-copper oxidases is situated between helices VI, VII and VIII, positioned in the membrane near the periplasmic face. Two of the ligating histidine residues derive from helix VII and one from helix VI (Fig. 21). These are the third, fourth and fifth residues from the periplasmic face of the proposed membrane-spanning helices, indicating that the histidines could form a plane nearly perpendicular to the membrane-spanning helices. Assuming that the copper ion also ligates a solvent molecule or reaction intermediate, the four ligands comprise a suitable ligand field for copper. However, if a non-haem iron is bound by the nitric oxide reductases in place of copper this ligand field would not be appropriate since iron prefers octahedral ligation. From the two published nitric oxide reductase sequences, in addition to the three conserved histidine residues a glutamate eight residues from the predicted periplasmic end of helix VIII

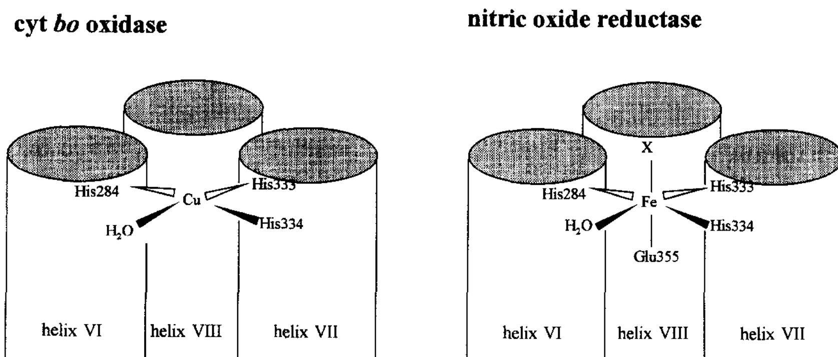


Fig. 21. A possible mononuclear iron site in nitric oxide reductase. The structure is based on the proposed coordination site of copper in the *E. coli* cytochrome *bo* oxidase. The conserved ligand Glu355 is found in both nitric oxide reductase primary structures currently available but is absent from the oxidases. This ligand is proposed to create an octahedral coordination site that favours iron rather than copper insertion. Ligand X could be an amino acid or a solvent molecule. The amino acid numbering is taken from the *E. coli* cytochrome *bo* oxidase.

is conserved. This glutamate is the only charged residue in helix VIII. We propose that this glutamate may ligate non-haem iron with the bond parallel to the transmembrane helices and on the cytoplasmic side of the plane formed by the ligating histidines (Fig. 21). A fifth protein ligand for the non-haem iron may derive from one of the loops between membrane-spanning helices, but currently there are too few nitric oxide reductase sequences for a sensible candidate to be suggested.

The similarity of nitric oxide reductases to haem-copper oxidases has implications for identification of nitric reductase activities in bacteria. We have previously suggested that nitric oxide reductase may be widely distributed in bacteria that do not denitrify [52]. For example, the enzyme has been found in several strains of *R. capsulatus*, including one that has no other enzymes of the denitrification pathway [52]. However, the identification of cytochrome cbb_3 in *R. capsulatus* [207,543] and the demonstration of nitric oxide reductase activity in cytochrome aa_3 oxidase raises the possibility that nitric oxide reduction could sometimes be misascribed to the nitric oxide reductase when identified at low rates. It remains to be determined whether the cytochrome cbb_3 oxidase has nitric oxide reductase activity, but in the case of *R. capsulatus* a strain (M5) completely deficient in oxidase activity could still reduce nitric oxide at wild-type rates [52].

8.3. The catalytic mechanism

The similarity in structure between the nitric oxide reductases and members of the haem-copper oxidase family could shed light on the catalytic mechanism of the former. The capacity of cytochrome aa_3 oxidase to reduce nitric oxide to nitrous oxide was reported in 1980 [93] and a number of the intermediates were identified using electronic and EPR spectroscopy. When the ascorbate-reduced enzyme (in which the low-spin haem *a*, Cu_A, high-spin haem a_3 and Cu_B were all reduced) was incubated with low concentrations of nitric oxide, optical and EPR signals attributable to cyt a_3 Fe(II)-NO were detected. At higher concentrations, the EPR signals disappeared, but the optical signals remained. It was argued that a second nitric oxide molecule had bound Cu_B (the copper of the dinuclear catalytic site) and was antiferromagnetically coupled to the cyt a_3 Fe(II)-NO site so that the dinuclear site was EPR-silent. Thus the reduced oxidase appears to bind two molecules of nitric oxide in close proximity at the catalytic site as Fe(II)-NO and Cu(I)-NO. These nitric oxide molecules were proposed to be reduced by electrons originating from the ferrous and cuprous metal centres, resulting in the formation of two metal-nitroxyl ($M\text{-}NO^-$) species; nitrous oxide and water could then arise from a reaction between the two nitroxyls and two protons.

Another dinuclear centre that is able to reduce nitric oxide at a high rate and by a similar mechanism is found in haemocyanin [568a]. Nitric oxide reacts rapidly with the deoxy form [Cu(I)Cu(I)] converting it to the met form [Cu(II)Cu(II)] and yielding nitrous oxide [568a]. Two nitric oxide molecules bind per dinuclear site [568a]. It is likely that electrons from the two cuprous molecules of the deoxy form reduce each bound nitric oxide to nitroxyl. These copper bound nitroxyls could then dimerise to form nitrous oxide as proposed for the cyt aa_3 oxidase.

The examples above give a new significance to the claim that nitroxyl can be trapped during turnover of the nitric oxide reductase in the presence of dithiothreitol [552].

8.4. Is the nitric oxide reductase a proton pump?

The location of nitric oxide reductase as an integral membrane protein raises the question of whether the site of nitric oxide reduction is at the cytoplasmic or periplasmic side of the membrane and whether the enzyme might, in common with cytochrome aa_3 oxidase, act as a proton pump. Although this issue cannot be regarded as settled, three pieces of evidence point to a periplasmic site of nitric oxide reduction and absence of proton pumping. First, Shapleigh and Payne [486] showed that proton uptake associated with reduction of nitric oxide appeared to be from the periplasm rather than the cytoplasm. Second, Bell et al. [52] demonstrated that in chromatophores of *R. capsulatus* there was no generation of membrane potential when electrons were fed into the electron transfer system at the level of periplasmic *c*-type cytochrome. This observation rules out both a cytoplasmic site of nitric oxide reduction (i.e., with protons taken from the cytoplasm) and a proton pumping activity of the nitric oxide reductase. Third, Carr et al. [99] have demonstrated that the ATP:$2e^-$ ratio is identical for nitrate and nitric oxide reduction. Since two charges are translocated per $2e^-$ by the membrane-bound nitrate reductase and a similar charge translocation accompanies electron transport via the cytochrome bc_1 complex, it follows that the reactions catalysed by the nitric oxide reductase are not associated with charge movement. The implication of these findings is that the catalytic site is accessible to protons originating from the periplasm but not from the cytoplasm.

The suggestion that the nitric oxide reductase is not a proton pump and takes up protons from the periplasm rather than the cytoplasm should be considered in the light of the similarity in structure to the haem-copper oxidases. In oxidases, the reduction of oxygen to water consumes one proton per electron at the catalytic site but, in addition, cytochrome aa_3 oxidase and cytochrome *bo* oxidase both translocate one proton per electron from the cytoplasm to the periplasm [246]. There is currently some debate as to whether the cytochrome cbb_3 oxidases are proton pumping. Analysis in *P. denitrificans* of proton translocation in whole cell suspensions of cytochrome aa_3-deficient and cytochrome aa_3- plus cytochrome bb_3-(ubiquinol oxidase) deficient mutants (i.e., cells in which cytochrome cbb_3 is

the only cytochrome *c* oxidase) has yielded values that vary between 0 and $2H^+ \uparrow :2e^-$ depending on the electron donor and experimental system [143,424]. An impure preparation of what is almost certainly [543] a cytochrome cbb_3 oxidase from *R. capsulatus* 37b4 has been reconstituted into liposomes [247a]. Using ferrocytochrome *c* as electron donor, DCCD-sensitive proton translocation was estimated to be approximately $2H^+ \uparrow :2e^-$. However, it is notable that some conserved residues that are thought to be important in proton translocation in the cytochrome *bo* oxidase are not conserved in either the cytochrome cbb_3 oxidase or the nitric oxide reductase. These include a tyrosine in helix VI and two threonines and a lysine in helix VIII [246,592a]. A helical loop between Helices III and IV is also thought to be important in proton translocation [246,592a]. In particular, an aspartate residue is conserved in this region in cytochrome *bo* and aa_3 oxidases, substitution of which decouples oxygen reduction and proton translocation. Although not conserved in the same position in primary structure alignments, both cytochrome cbb_3 and nitric oxide reductase have an aspartate in this loop. It is known that the exact position of the aspartate in cyt *bo* oxidase is not absolutely critical (Puustinen, A. and Garcia-Horsman, A., unpublished data, cited in [592a]). Thus, the significance of the aspartate in the Helix III–Helix IV loop in the nitric oxide reductase and the cytochrome cbb_3 oxidase remains unclear. In conclusion, the uncertainty of whether the cytochrome cbb_3 oxidase is a proton pump makes it difficult to draw concrete conclusions on the significance of amino residues implicated in proton translocation in cytochrome *bo* oxidase being substituted in the nitric oxide reductase. However, in the light of the bulk of the biochemical evidence, we take the absence of some of these residues to support the view that the nitric oxide reductase is not a proton pump.

9. Nitrous oxide reductase

9.1. Molecular properties

Nitrous oxide reductase (previously reviewed in [305,618]) has been purified from *P. stutzeri* [127], *R. capsulatus* [358], *R. sphaeroides* f.sp. *denitrificans* [374], *P. denitrificans* [502], *W. succinogenes* [540], *A. cycloclastes* [248], *P. aeruginosa* P2 [512], *T. pantotropha* [61], *A. faecalis* IAM1015 [352] and *Alcaligenes* sp.NCIB 11015 [353]. In all cases the enzymes are of essentially the same type. The subunit molecular mass is 66–68 kDa when determined by mass spectrometry or when predicted from the gene sequence. SDS-PAGE estimates are consistently slightly larger at 70–75 kDa. On the basis of gel-permeation chromatography it would appear that the enzyme is a homodimer. Purified nitrous oxide reductase is a water-soluble enzyme. It has been found to be periplasmic in all cases where cellular localisation has been investigated ([14,297,358,377,558]; signal peptides are present in the precursor proteins [237,570,624]; Section 9.7).

Copper is the only metal consistently found in nitrous oxide reductase. There is no evidence that the enzyme contains modified amino acids or other organic cofactors, although these possibilities cannot yet be totally excluded [163,439]. Analytical determinations of the copper content of the enzyme from a number of sources are generally agreed upon a stoichiometry of four copper atoms per polypeptide, a number consistent with recent spectroscopic interpretations. These models assume that two types of copper site are present, a Cu_A site structurally related to the Cu_A centre of cytochrome *c* oxidase that is thought to mediate electron transfer between the external electron donor and the catalytic site, and the catalytic site itself, variously termed a Cu_B, Cu_C or Cu_Z site with Cu_Z the term adopted here.

Unusual nitrous oxide reductases have been reported from two organisms. The nitrous oxide reductase of *W. succinogenes* is reported to copurify with a *c*-type cytochrome [540]. There is some evidence from proteolytic degradation experiments [540] and mass spectrometry [613] that this cytochrome is a domain of the nitrous oxide reductase polypeptide. A small, basic, soluble, *c*-type cytochrome purified from *W. succinogenes* can act as an electron donor to this nitrous oxide reductase in vitro [612]. Jones and co-workers report [270,271] that the nitrous oxide reductase of *Flexibacter canadensis* is membrane-bound, insensitive to inhibition by acetylene (at least in the whole organism when grown in the presence of sulfide) and (Knowles, R., quoted in [104]) does not contain copper. Its activity is highly unstable to oxygen. The *P. stutzeri* nitrous oxide reductase structural gene (*nosZ*) is reported not to hybridise with the DNA of *F. canadensis*. Further characterisation of the nitrous oxide reductases of *W. succinogenes* and *F. canadensis* would be welcome.

The primary structures of the nitrous oxide reductases of *P. stutzeri*, *P. aeruginosa*, *A. eutrophus* and *P. denitrificans* have been determined from the sequences of the corresponding structural genes (*nosZ*) [237,570,624]. Apart from a strong amino-acid sequence similarity between the extreme C-terminus of nitrous oxide reductase and part of cytochrome *c* oxidase subunit II (discussed in Section 9.3), there is no significant sequence similarity to other proteins. In *P. stutzeri nosZ* is part of a gene cluster that includes operons involved in synthesis of the nitrous oxide reductase catalytic centre and implicated in regulation of *nosZ* expression (Fig. 22; Sections 3.1, 5.1.4, 9.7). Preliminary evidence suggests a similar gene organisation in *P. aeruginosa* [624] and *P. denitrificans* [237]. In contrast, in *A. eutrophus* there is no evidence for the presence of these accessory genes adjacent to *nosZ* [624]. In *P. stutzeri* the nitrous oxide reductase gene cluster is linked to clusters coding for the dissimilatory nitrite and nitric oxide reductases and associated functions [275]. In *A. eutrophus* and

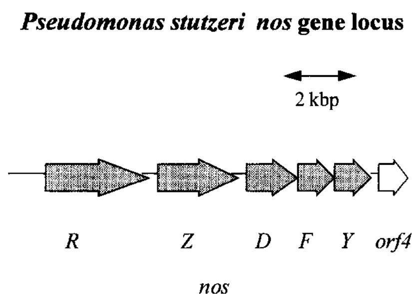


Fig. 22. The *nos* gene cluster of *P. stutzeri*. *nosZ* is the structural gene for nitrous oxide reductase, *nosR* codes for an apparent *trans*-activator of *nosZ* expression. *nosDFY* code for proteins involved in assembly of the catalytic (Cu_Z) site of the nitrous oxide reductase [137,570,623].

Rhizobium meliloti nosZ is encoded on megaplasmid DNA [104,624]. The *A. eutrophus* megaplasmid also codes for a periplasmic nitrate reductase (Section 5.1) [495,581]. The *R. meliloti* megaplasmid carries the *nod* genes involved in symbiotic nitrogen fixation. At first sight it seems odd that a denitrification gene is present on a plasmid encoding genes involved in nitrogen fixation. However, nitrous oxide is an alternative substrate for nitrogenase (Section 9.5). The high solubility of nitrous oxide (25 mM at 25°C) relative to dinitrogen (0.6 mM at 25°C), together with the preferential reduction of nitrous oxide by nitrogenase when dinitrogen is also present [321] means that nitrogen fixation could be abolished in environments containing nitrous oxide. We suggest that the function of nitrous oxide reductase in *R. meliloti* may be to lower intracellular nitrous oxide concentrations in order to prevent this inhibition of nitrogen fixation. Alternatively, as nitrous oxide is not a source of directly assimilable nitrogen, there is no reason why it should not act as a terminal respiratory oxidant under anaerobic conditions generating ATP (and dinitrogen) for the nitrogen fixation reactions.

9.2. Catalytic properties

Purified nitrous oxide reductases exhibit highest activities when reduced viologens ($E_{m,7} < -350$ mV) are used as the electron donor [300]. Electron donors with reduction potentials that more nearly match those of the presumed physiological electron donors (*c*-type cytochromes or cupredoxins with $E_{m,7}$ of around +200 to +300 mV; Sections 2.1, 2.3) support lesser activities. A turnover-dependent inactivation of the enzyme has been noted in many assay systems (e.g., [61,127,374]). This may be due to the use of electron donors of potentials very much lower than the enzyme would be exposed to in vivo. The nitrous oxide reductases of *P. stutzeri* and *P. aeruginosa* can be activated by up to 30-fold by dialysis against a high pH buffer [127,512]. This is a permanent chemical change and not related to any pH maximum of the enzyme activity. At high pH the *T. pantotropha* enzyme exhibits turnover-dependent activation [61]. Activation of the enzymes of *P. denitrificans* and *T. pantotropha* can also be achieved by incubation with reduced viologens [61,502]. An apparently allosteric activation of the nitrous oxide reductase from *W. succinogenes* has been reported and was interpreted as being due to interaction of the two homodimer subunits [613].

The most specific inhibitor of nitrous oxide reductase is acetylene which is isoelectronic with nitrous oxide. Other inhibitors include azide anion and thiocyanate, both also isoelectronic with nitrous oxide, as well as nitric oxide, cyanide, nitrite and fluoride which presumably act as general copper ligands [177,440,502]. Prolonged exposure of the oxidised enzyme to high concentrations of nitrous oxide is also inhibitory [439].

Different spectral forms of nitrous oxide reductase are isolated depending upon whether purification is carried out anaerobically or aerobically. These are referred to as the 'purple' or N_2ORI form (anaerobic purification) and the 'pink' or N_2ORII (oxygen-affected) form [440]. Purified *P. stutzeri* N_2ORI can be converted to N_2ORII by extended aerobic incubation, by dithionite reduction followed by oxidation in the presence of molecular oxygen, or by extended incubation with nitrous oxide [127,439,440]. Only the enzyme from *P. stutzeri* has been isolated in both forms. For this enzyme the anaerobically purified form has, on average, 2.5-times the activity of the aerobically isolated form [127]. A correlation between absolute specific activities of preparations of enzyme from different species and anaerobic/aerobic form is not established. Riester [439] has, however, shown an approximate correlation between enzyme activity and copper content in the *P. stutzeri* enzyme. Oxygen is not inhibitory to the nitrous oxide reduction reaction itself, at least in so far as it does not inhibit the reaction of the oxygen-affected enzyme from *R. capsulatus*, *T. pantotropha* or *W. succinogenes* when assayed with a *c*-type cytochrome as electron donor [61,436,612]. In general, the 'oxygen-inactivation effect' can be ascribed to oxidation by molecular oxygen, although this reaction may be potentiated by some, possibly cytoplasmic, component(s) present during early stages of cell fractionation and enzyme purification [61,127,619]. The question of whether formation of the oxygen-affected nitrous oxide reductase occurs in vivo is so far unresolved. In the aerobic denitrifier *T. pantotropha*, the same type of nitrous oxide reductase is expressed and active under both anaerobic and aerobic growth conditions [50,61,380]. Yet when the enzyme was purified under aerobic conditions the oxygen-affected form was isolated [61].

9.3. The electron transfer (Cu_A) centre

The Cu_A site of nitrous oxide reductase is so named because of its similarity to the Cu_A site of the mitochondrial-type cytochrome *c* oxidase complex (complex IV). Because of its distinctive colour, the Cu_A centre has also sometimes been termed the 'purple copper centre'. There

is now a near consensus that the Cu_A site in cytochrome *c* oxidase acts solely as an obligate one electron carrier between periplasmically located electron donors and the low spin cytochrome (*a* or its equivalent) and that the Cu_A centre has no role in the proton pumping reactions of the oxidase [110,228,229,292,341,422,466]. The Cu_A centre of nitrous oxide reductase likewise appears to be involved in one-electron transport and not catalysis.

There is amino acid sequence similarity between the C-terminal portion of nitrous oxide reductase and the Cu_A binding site in the periplasmic portion of cytochrome *c* oxidase subunit II. Six amino acids that are absolutely conserved in this region, two histidines, two cysteines, a methionine and an aspartic acid residue, are strong candidates for the copper ligands [126,522,624] (Fig. 23). Weak but significant sequence similarity between nitrous oxide reductases and the cytochrome *c* oxidases can also be detected for the polypeptide region between conserved His-6 and Cys-41(Fig. 23 numbering; [624] and our analysis). From both sequence comparisons, and the results of limited proteolysis and protein engineering studies on the cytochrome oxidases, it appears that the Cu_A region in both enzymes may form a domain structure containing around seventy amino acids [317,561,576]. For two bacterial oxidases, this domain has been expressed as an autonomous soluble fragment [317,576], while the equivalent polypeptide region of a ubiquinol oxidase has been converted into a synthetic 'Cu_A' domain by insertion of the conserved histidine and cysteine residues (Fig. 23) [561]. Removal from the synthetic 'Cu_A' domain of any of the introduced residues leads to gross perturbation of the centre, providing strong experimental support that these residues are copper ligands [288]. Extensive mutagensis experiments using the *P. denitrificans* Cu_A domain confirm that only these conserved cysteine, histidine and methionine residues are essential for formation of the Cu_A centre, i.e., the conserved aspartic acid residue is not required [316]. If the quinol oxidase fragment is mutated towards a type I copper centre consensus sequence (Fig. 23; Section 7.3.1) then a type I copper centre is formed [561]. This has been taken as an indication that the fold of the Cu_A domain may resemble that of the structurally characterised cupredoxins [561]. Secondary structure predictions (e.g., those in [624]) and circular dichroism studies on the unmodified quinol oxidase fragment and the soluble Cu_A fragment of *P. denitrificans* cytochrome *c* oxidase [561,594] support such a predominantly β-sheet structure for the domain.

A form of the *P. stutzeri* nitrous oxide reductase (so-called N_2ORV) which possesses only a Cu_A centre can be purified from a mutant defective in catalytic site biosynthesis (*nosDFY*; Section 9.7). This protein, and the various cytochrome oxidase Cu_A fragments, have been instrumental in identifying the spectroscopic signals that arise from the oxidised Cu_A centres of the native enzymes.

The Cu_A ligands suggested by genetic analysis are supported by spectroscopic studies. Resonance Raman spectra are consistent with cysteine and histidine coordination [16,152,153], while electron nuclear double resonance (ENDOR) experiments on yeast cytochrome *c* oxidase are consistent with one or two cysteine and two histidine ligands [214,238,347,523]. Extended X-ray absorption fine structure (EXAFS) of the N_2ORV protein and *B. subtilis* Cu_A centre [69,153] show (S/Cl) scatterers at 2.2 Å, plausibly from cysteine coordination, (N/O) scatters at 2.0 Å assigned to histidine ligation, and a 2.5 Å scatter that was originally assigned to methionine sulfur. On the basis of their energies, strong intensities and Raman excitation profiles [16,508] the MCD – ([60,163,210,211,479,542]; Farrar, J.A., Berks, B.C., Ferguson, S.J. and Thomson, A.J., unpublished observations) and optically-detected transitions from the paramagnetic Cu_A centre are assigned to cysteine thiolate to copper charge transfer bands. That there are oppositely polarised MCD C-terms (arising from paramagnetic Cu^{2+} centres) of similar intensity means that there are minimally two thiolate-copper transitions at close to 90° to each other.

The oxidised Cu_A centre has a highly unusual EPR spectrum. In anaerobically prepared nitrous oxide reductase a seven-line hyperfine splitting pattern with relative intensities of 1:2:3:4:3:2:1 is clearly resolved at $g_{\parallel} = 2.18$ at X-band [127,502]. The hyperfine lines are equally

```
                                   10         20         30         40         50         60
ubiquinol oxidases             S......... ..P....... .G........ ......Y... .A...G.... ..........
cytochrome c oxidases          ..DV.H.... .......K.. .......... ........G. C.E.CG..H. .M........
nitrous oxide reductases       I.D..HGF.. ......V... PQ.T.S.TF. A..PG..W.Y C..FCHALH. EM..RM.VE.
consensus CuA site             ..D..H.... .......... .......... .......... C...C...H. .M........
E.coli CyoA (168-227)          SNSVMNSFFI PRLGSQIYAM AGMQTRLHLI ANEPGTYDGI SASYSGPGFS GMKFKAIATP
synthetic CuA site             .....H.... .......... .......... .......... C.EIC...H. ..........
synthetic type I Cu site       .....H.... .......... .......... .......... C-----TPHP F.........
```

Fig. 23. Sequence analysis of the Cu_A binding region in nitrous oxide reductase, cytochrome *c* oxidases and related terminal oxidases. The amino acid numbering scheme is arbitrary. (i) Consensus sequence of the Cu_A-binding region of quinol oxidase enzymes that do not possess Cu_A (*E. coli* cytochrome *bo* quinol oxidase subunit II (CyoA) [110]; *Bacillus subtilis* quinol oxidase [199]; *Sulfolobus acidocaldarius* quinol oxidase (SoxA) [336]) (ii) Consensus sequence of the Cu_A binding region of the Cu_A centre-containing terminal oxidases (cytochrome *c* oxidases and caa_3 oxidases). The consensus is that of Zumft et al. [624]. Recent additional sequences do not alter the consensus. (iii) Consensus sequence of the Cu_A binding region of the nitrous oxide reductases [237,570,624]. (iv) Consensus Cu_A binding motif derived from a comparison of (i), (ii) and (iii). (v) Partial sequence of *E. coli* cytochrome *bo* ubiquinol oxidase subunit II (CyoA) corresponding to (i) [110]. This is not the entire CyoA fragment engineered by van der Oost and co-workers [561]. (vi) Changes made to the CyoA fragment sequence (v) by van der Oost et al. [561] in order to produce a synthetic 'Cu_A centre'. (vii) Changes made to the CyoA fragment sequence (v) in order to produce a synthetic 'type I' copper centre [561].

spaced, with the magnitude of the splitting (3.55 mT) even smaller than that exhibited by type I copper centres. Similar, though less clearly resolved spectra, can be identified in aerobically prepared nitrous oxide reductase, the N_2ORV protein, cytochrome *c* oxidase and the Cu_A fragments ([60,127,317,440,561,576]; Farrar, J.A., Berks, B.C., Ferguson, S.J. and Thomson, A.J., unpublished results); other similarities between the EPR properties of nitrous oxide reductase and cytochrome *c*-oxidase Cu_A centres are detailed in [176,264,304,362,440,476].

Kroneck and co-workers [303,304] pointed out that the intensity pattern and even splittings of the seven line signal can only arise from an unpaired electron in an orbital delocalised over two equivalent copper atoms. That is, the electron is split in an identical manner by each of the $I = 3/2$ copper nuclei. The centre is effectively Cu(1.5)-Cu(1.5). Direct biochemical confirmation that the Cu_A centre is indeed dinuclear comes from recent electrospray mass spectrometric measurements of the oxidase Cu_A fragments that show a clear mass difference of 126 Da (Cu = 63 Da) between the holo- and apo-proteins [288,317,576] and from careful metal analyses of cytochrome *c* oxidase [80,159,396,407,521,522,608] and the oxidase Cu_A fragments [288,317]. Strong evidence that the seven-line signal from the dinuclear centre is due to a single electron delocalised over both copper atoms rather than an unpaired electron on each of two spectroscopically inequivalent copper atoms comes from the failure to resolve contributions from more than one signal by varying the microwave frequency (*g*-values vary, but the splitting remains approximately constant as the frequency is changed; [20,303,305,306]) and from observations that only 50% of the copper in the oxidised Cu_A centre is EPR-detectable [317,440,576]. Delocalisation of the electron away from a given copper atom would also explain the very small hyperfine splittings observed.

In the dinuclear centre model, the physiological oxidised form of the centre is the half-reduced state, i.e., a Cu(1.5)-Cu(1.5) centre. Such a centre would act as a one electron acceptor. The $E_{m,7.0}$ of the Cu(1.5)-Cu(1.5)/Cu(1)-Cu(1) couple in *P. stutzeri* nitrous oxide reductase has been measured to be about +260 mV (from data in [127]). This value is similar to those measured for oxidase Cu_A centres (+240 to +260 mV; [561,576,592]) and is appropriate considering the reduction potentials of the proposed electron donor molecules to nitrous oxide reductase (Sections 2.1, 2.3). As expected of a Cu(I)-Cu(I) centre, the reduced state of N_2ORV and oxidase Cu_A fragments is EPR-silent and optically inactive (Cu(I) is d^{10}) [153,440,561,576,620].

The two most recent structural models for the Cu_A centre ligation are shown in Fig. 24. The valence electron of the Cu_A centre remains delocalised, even at temperatures as low as 10 K. Studies on other dinuclear copper systems suggest that extremely high cluster symmetry would be required to obtain two copper atoms with reduction potentials similar enough to maintain delocalisation at these temperatures [223,334,587]; but note that Gubriel and co-workers [214] detect some electronic asymmetry by ENDOR). High cluster symmetry is therefore incorporated in both models. In the model advanced by the Thomson and Saraste groups [316] electron delocalisation is via two bridging cysteine ligands. In the model of Blackburn and co-workers the valence electron is delocalised by means of a direct copper–copper bond [69]. The latter model is based on interpreting the 2.5 Å scatter detected in EXAFS experiments to the second copper atom [69]. Strong arguments that this scatter is not due to methionine sulfur have been advanced [69]. Assignment of the scatter to a second copper atom is plausible on the basis of model compound studies [43,223], but the data fit is not unambiguous [69]. It should also be noted that resonance Raman gave no evidence for a Cu–Cu bond [16].

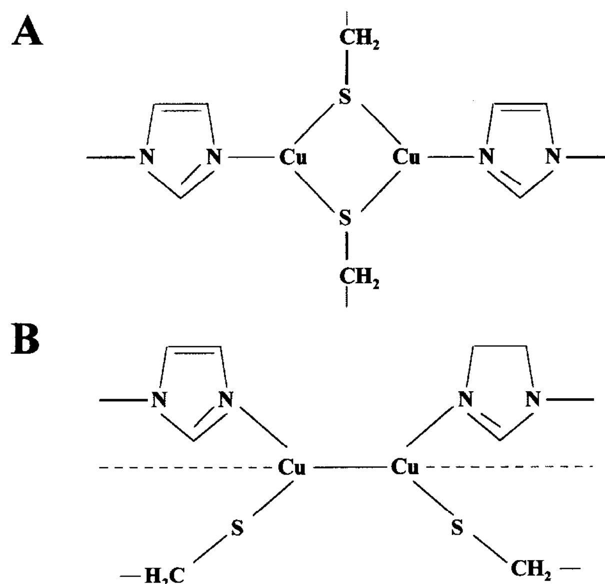


Fig. 24. Models for the ligation of the Cu_A site. Proposals of (A) Saraste, Thomson and co-workers [316] and (B) Blackburn and co-workers [69] slightly modified to incorporate the perpendicular arrangment of the cysteine thiolate-copper bonds suggested by MCD spectroscopy [163].

9.4. *The catalytic site (Cu_Z centre)*

Although not directly demonstrated, it is assumed that the copper atoms not involved in forming the Cu_A centre form the site of nitrous oxide reduction. Currently, the most satisfactory model of this second copper site (Cu_Z) and its redox chemistry is that expounded by Farrar and co-workers [163] and shown in Fig. 25.

The Cu_Z centre is minimally dinuclear. Reduction of *P. stutzeri* nitrous oxide reductase with ascorbate traps the enzyme in a state in which the Cu_A centre is reduced [163] (Fig. 25). Reduction of copper centres (to d^{10} Cu(1)) normally results in the loss of all optical transitions. As the ascorbate-reduced enzyme still exhibits optical transitions this form of the enzyme must retain some copper atom(s)

in a Cu(2) state and this Cu(2) must be associated with an oxidised form of the Cu_Z centre. However, in spite of the presence of oxidised copper, the ascorbate-reduced state is EPR silent, strongly suggesting that this redox state contains a dinuclear antiferromagnetically-coupled (type III; Cu(2)-Cu(2); Section 6.3.1) copper site (Fig. 25). As the oxygen-affected (N_2ORII) and anaerobically-purified (N_2ORI) forms of nitrous oxide reductase have very different absorption spectra but similar paramagnetic (EPR and MCD) properties in the oxidised state ([60,163]; Farrar, J.A., Berks, B.C., Ferguson, S.J. and Thomson, A.J., unpublished results) it appears that the population of these diamagnetic oxidised catalytic sites is lower in the oxygen-affected form of the enzyme.

In addition to the signal from the Cu_A centre, the EPR spectrum of oxidised nitrous oxide reductase exhibits a second, very broad, paramagnetic signal resolvable at Q-band (Kroneck, P.M.H., personal communication quoted in [163]). This signal is absent from the mutant N_2ORV protein, suggesting that it is associated with the catalytic site. Farrar and co-workers [163] propose that the signal arises from an inactive Cu(2)-Cu(1) form of the catalytic centre which they designate Cu_Z^*. The very broad signal is presumably due to rapid spin relaxation and is probably indicative of magnetic coupling of the unpaired spin on the oxidised copper atom with the electrons of the reduced copper atom as seen in the half-met site of haemocyanin. Farrar and co-workers propose that the Cu_Z^* centre can be fully reduced by ascorbate (Fig. 25) [163]. These workers also propose that the Cu_Z^* state arises from binding of exogenous ligands to the catalytic centre resulting in an increase in the reduction potential of the centre. It is possible that the activation of the enzyme by reduction and by high pH discussed in Section 9.2 could arise from conversion of the Cu_Z^* site back to a Cu_Z site in which case the reformation of a thiolate ion (pK_a around 8–9) or a combination of reduction and displacement of an inappropriate ligand by hydroxide are attractive mechanistic possibilities. It should be noted that this mechanism of inactivation of the active site may be unrelated to the inactivation due to oxygen (Section 9.2) accounting for the lack of correlation between activity of enzymes purified from different species and whether purification was aerobic or anaerobic (though it has not been tested adequately, there are suggestions in Ref. [440] that the aerobically purified enzyme has a lower concentration of the Cu_Z^* centre).

Reduction of nitrous oxide reductase by dithionite, or indeed a large number of other reductants (all with $E_{m,7} <$ + 100 mV), generates a form of the enzyme (referred to variously as the 'blue' or N_2ORIII form) in which optical transitions are still present [440]. This indicates that the dithionite-reduced form of the enzyme retains oxidised copper. This oxidised copper is associated with the Cu_Z centre as the mutant N_2OV protein which contains only the Cu_A centre shows no visible absorption bands on reduction with dithionite [440]. The EPR and low-temperature MCD spectra of the dithionite-reduced Cu_Z centre are very similar to those of the oxidised [Cu(2)-Cu(1)] Cu_Z^* site, suggesting similar structures (Fig. 24) [163]. These two

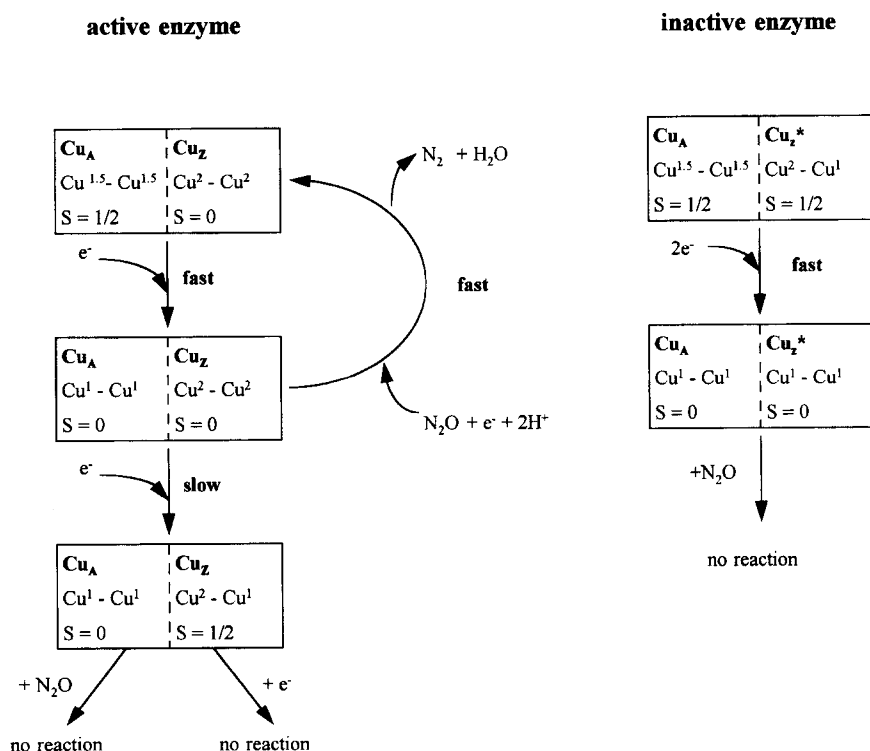


Fig. 25. Model of the spectroscopic states of the Cu_A and Cu_Z centres of nitrous oxide reductase and the possible relation of these states to the catalytic cycle. Based on the model proposed by Farrar and co-workers [163].

forms of the Cu_Z centre must, however, differ in either reduction potential and/or reduction kinetics, presumably as a result of differences in ligation. In the Farrar model, the dithionite-reduced Cu(2)-Cu(1) site arises from reduction of the Cu(2)-Cu(2) form of the Cu_Z site [163] (Fig. 25).

Reduction of nitrous oxide reductase by dithionite [440,502] has an initial rapid phase in which the Cu_A and Cu_Z^* centres are reduced. A second phase in which the oxidised Cu_Z centre is converted to the dithionite-reduced form occurs over a period of about 1 h. This second phase of reduction is too slow for the dithionite-reduced Cu_Z state to be of catalytic relevance. It must instead be considered a dead-end side reaction. From optical redox titrations of the anaerobically prepared nitrous oxide reductase of *P. stutzeri* [127] it can be deduced that reduction of the Cu_Z centre from Cu(2)-Cu(2) to Cu(2)-Cu(1) is, in the absence of nitrous oxide, approximately isopotential with reduction of the Cu_A centre ($E_{m,7.5} = +260$ mV). The dithionite-reduced anaerobically prepared enzyme (N_2ORI) appears to contain more of the Cu(2)-Cu(1) centre than the aerobically prepared enzyme (N_2ORII) [127]. As this centre is proposed to derive from reduction of the Cu(2)-Cu(2) centre this observation is consistent with the suggestion (above) that the aerobically purified enzyme contains less of that diamagnetic oxidised centre.

The Farrar model (summarised in Fig. 25) of the catalytic site, in which the site is dinuclear and exists in two different forms, each with only two accessible reduction states, satisfactorily accounts for the observed spectroscopic features. The proposed classification of a particular form or redox state as catalytically relevant or as 'inactive' should, however, be regarded as tentative.

Possible ligands to the Cu_Z site can be suggested by identifying conserved amino acid residues not involved in binding the Cu_A centre. Of the most likely ligands, eight histidines (one in the putative Cu_A domain) and a single cysteine are absolutely conserved in the known sequences [237,570,624]. Almost all these conserved residues lie in regions strongly predicted to be in β-turn conformation. Most intriguing is a His-His-Xaa-His motif at around amino acid 130 in the mature protein sequence. The presence of only a single conserved cysteine suggests that the proposal of Kroneck and co-workers [305] that the oxidised copper in dithionite-reduced nitrous oxide reductase could arise from oxidation of the reduced Cu_Z centre by a nearby disulfide bridge is probably incorrect.

As with the Cu_A centre, the energies and high intensity of the optical transitions arising from the three optically active Cu_Z states [Cu(2)-Cu(2), dithionite-reduced Cu(2)-Cu(1) and Cu_Z^* Cu(2)-Cu(1)] allow assignment of these transitions to cysteine thiolate-to-copper charge transfer bands. The presence of oppositely polarised C-terms (arising from paramagnetic centres) in the low-temperature MCD spectrum of the dithionite-reduced enzyme and probably also from the oxidised form of the Cu_Z^* centre, suggests, as with the Cu_A centre, the presence of minimally two perpendicularly polarised thiolate-copper transitions [163]. As there is only one conserved cysteine residue outside the Cu_A centre domain, it is likely that all three forms of the Cu_Z centre have a bridging cysteine ligand. On the basis of a general consideration of the copper coordination found in enzyme active sites [4], the remaining protein ligands are most probably some of the conserved histidines.

9.5. Reduction of nitrous oxide

Although the reduction of nitrous oxide to dinitrogen by nitrous oxide reductase using physiological reductants is strongly favoured thermodynamically ($E_{m,7}$ of the N_2O/N_2 couple is around +1.4 V; the proposed direct electron donors have $E_{m,7}$ of approx. +0.25 V), nitrous oxide is kinetically very stable with fully occupied bonding orbitals giving bond orders of 2.73 (N–N) and 1.61 (N–O) [274]. The reactivity of nitrous oxide with transition metals has been reviewed by Kroneck and Zumft [302]. These authors concluded that copper compounds were effective catalysts of the cleavage of the N–O bond and pointed out that the normal reaction of nitrous oxide with transition metals is $M + N_2O \rightarrow O = M^{2+} + N_2$. Although stable complexes of nitrous oxide with transition metals are almost unknown, theoretical calculations suggest that such bonding can occur and that coordination would be via the nitrogen atom [551]. Hyponitrite ($N_2O_2^{2-}$) the conjugate base of hyponitrous acid ($H_2N_2O_2$) is a metal ligand and could possibly be the enzyme substrate.

Reduction of nitrous oxide to dinitrogen is an artifactual reaction of three enzymes that do not contain copper centres. These are the nitrogenase molybdenum-iron protein [263], the nickel-iron carbon monoxide dehydrogenase of the acetogenic bacterium *Clostridium thermoaceticum* [335] and the cobalamin (vitamin B_{12})-dependent enzyme methionine synthase [36]. Cobalamin itself will also reduce nitrous oxide [37,68]. In all cases, the reaction has an overpotential of at least 0.5 V relative to the reaction at the catalytic site of nitrous oxide reductase. Reduction of nitrous oxide by nitrogenase is thought to occur at the dinitrogen-binding step of the normal catalytic cycle. Nitrous oxide binds at the same site as dinitrogen [263]. In the normal catalytic cycle dinitrogen binding is accompanied by release of two hydrogen atoms as dihydrogen. It is thought that during nitrous oxide reduction these hydrogen atoms may be used to reduce the substrate oxygen atom to water [321]. Reduction of nitrous oxide by methionine synthase is thought to be via single electron transfer from Co(I) to nitrous oxide followed by reaction of the nitrous oxide radical anion with a hydrogen atom donor [157]. The involvement of a highly reactive radical species and the very low potential of the radical forming reaction makes this mechanism an unattractive model for that of nitrous oxide reductase.

The apparent difficulty of the chemistry described above suggests that the mechanism of nitrous oxide reduction by nitrous oxide reductase is likely to be unrelated to these examples. Presumably, both the dinuclear nature of the enzyme active site and the tendency of sulfur-liganded copper atoms to covalent bonding are crucial to catalysis. The enzyme is likely to activate the N–O bond of nitrous oxide for cleavage by the formation of bonding interaction between the substrate and both of the Cu_Z copper atoms i.e., nitrous oxide would be bound as a bridging ligand presumably at the expense of some water-derived ligand(s). Upon reductive cleavage, dinitrogen, being a very poor metal ligand, would be efficiently released from the active site, while the oxygen atom plausibly remains bound to one or both of the copper atoms as a hydroxyl ligand.

9.6. The nature of electron transfer between the Cu_A and Cu_Z centres

One of the outstanding questions about the Cu_A centre in both cytochrome-*c* oxidase and nitrous oxide reductase is why this apparently very unusual copper centre is used in preference to the more normal type I centre. In cytochrome-*c* oxidase the Cu_A centre is found in a periplasmic (or intermembrane space) domain, while the electron acceptor, the low-spin haem, is at least somewhat buried in the membrane bilayer. Possibly the extra length of the binuclear centre may assist electron transfer from the protein surface to a more buried centre. That the Cu_A centre in cytochrome-*c* oxidase is found in a separate subunit suggests that, at least in that enzyme, electron transfer from Cu_A centre to electron acceptor is not through a pathway of covalent bonds but rather through hydrogen bonds/through space. This contrasts with the proposed route of internal electron transfer from the type I copper centres of nitrite reductase and the blue copper oxidases which involves covalent bonds (Section 7.3.2). Reversion analysis of non-functional cytochrome-*c* oxidase mutants has suggested that two residues near Cu_A centre ligands, His-6 and Cys-41, may be close to the electron-accepting low-spin haem [371]. Therefore, in this enzyme electrons may be transferred out of the Cu_A centre in the direction of His-6 and Cys-41. In nitrous oxide reductase a histidine residue (His-46) that is not conserved in the cytochrome-*c* oxidases immediately follows the second putative Cu_A centre cysteine ligand (Cys-45; Fig. 23). It has been suggested that this histidine may be a Cu_Z centre ligand providing an electron transfer pathway analogous to that in nitrite reductase or the multicopper oxidases [237]. This proposal would require that a different electron transfer pathway exists in the two Cu_A centre-containing enzymes.

9.7. Biosynthesis of nitrous oxide reductase

In *P. stutzeri* the *nosDFY* operon, located directly downstream of *nosZ* (Fig. 22), encodes proteins whose organisation and structural features resemble those of a periplasmic binding protein-dependent transport system (ABC transporter) [623]. NosD would be predicted to be the periplasmic binding protein, NosY the membrane-spanning transporter, and NosF the cytoplasmic ATP-hydrolysing protein. Transposon Tn*5* insertions in the operon result in the synthesis of a nitrous oxide reductase (N_2ORV) lacking the catalytic (Cu_Z) centre but which still contains a Cu_A centre [153,163,440,620,623]. Both the partially assembled nitrous oxide reductase produced by these *nosDFY* mutants and the apoprotein produced by cells grown on copper-depleted media [297,354,377] are found in the periplasm. This suggests that catalytic centre assembly occurs in the periplasm and that it is not strictly linked to translocation of the apoprotein. At least one further open reading frame (*orf4*), downstream of *nosY* may also be part of the operon, although no mutations in this gene have been reported.

What is the function of the NosDFY system in Cu_Z centre biosynthesis? A rôle in import of copper into the cell across the cytoplasmic membrane seems unlikely if the Cu_Z centre is assembled in the periplasm and if assembly of the other dinuclear centre (Cu_A) is unaffected. Further, *nosDFY* expression is apparently regulated in response to nitrogen oxides and oxygen [623], a pattern that would not be expected of a general copper uptake system. As discussed below, NosD may not be a typical periplasmic binding protein. The involvement of accessory proteins in the synthesis of the Cu_Z centre suggests that a so far unrecognised cofactor or modified amino acid may be present at the active site. NosDFY could thus function in cofactor export or transport linked to the amino acid modification. Alternatively NosDFY could function in transport to the periplasm of copper that is in some way activated (reduced and or chelated), or of a reductant/assembly cofactor. ABC-family transporters involved in export processes do not possess periplasmic binding proteins [227]. The presence of NosD, if it is a binding protein, would thus be highly unusual. Periplasmic binding proteins are generally produced in a large molar excess over the other components of the binding protein-dependent transporter systems. However, the *nosDFY* organisation, in which the start and stop codons of the three reading frames overlap, is suggestive of translational coupling and equimolar synthesis of the polypeptides. It seems most likely that NosD is an enzyme that assembles the Cu_Z centre. It is quite possible that as part of this process NosD binds whatever molecule is transported by NosFY. The suggestion of Zumft and co-workers [623] that NosY couples cytoplasmic ATP hydrolysis by NosF directly to a synthetic activity of NosD in the periplasm cannot be excluded, but would be an unprecedented activity for such a transporter system [227].

Signal peptides in Gram-negative bacteria are generally less than 26 amino acids in length [573]. The weight matrix method of von Heijne [574], however, predicts

unusually long (58–60 amino acid) signal peptides for the NosZ precursor proteins (see also the earlier arguments of Zumft and coworkers for the enzymes from *Pseudomonas* species; [570,624]). For *P. denitrificans* NosZ, this predicted site of signal peptide cleavage matches the N-terminus determined for the mature nitrous oxide reductase of the closely related organism *T. pantotropha* [237]. Apart from their lengths the NosZ signal peptides are also unusual in that there is strong sequence conservation in the N-terminal region with a possible consensus motif of Gly-Xaa-(Ser)-Arg-Arg-Xaa-(Phe/Leu)-Leu-Gly. Similar sequence motifs and long signal peptides are found in other periplasmic proteins that contain complex redox cofactors (B. Berks, manuscript in preparation), for example, the small subunits of hydrogenases [156,577], the small subunit of methylamine dehydrogenase [112,113] and periplasmic MGD-binding proteins including the periplasmic nitrate reductase [64]. This suggests a common specialisation of the pathway by which these proteins are exported.

The Cu_A centre can be reconstituted in vitro into the chemically prepared apoprotein [127]. This observation, together with the biosynthetic experiments discussed above and the in vitro assembly of the engineered oxidase Cu_A centres discussed in Section 9.3, suggests that assembly of the Cu_A centre may be uncatalysed. In contrast, a functional catalytic centre (Cu_Z) is not formed by in vitro reconstitution [127]. The apoprotein does take up the required stoichiometry of copper but if this is inserting into the active site it no longer exhibits sulfur ligation (on the basis of its anonymity in the electronic absorption spectrum of the reconstituted protein) and it is practically EPR-silent. Perhaps the catalytic centre is present in this form as a fully oxidised type III centre (Section 7.3.1) with histidine-only ligation.

In *P. stutzeri* extensive transposon mutagenesis suggests that *nosZ*, *nosR* (Sections 3.1 and 5.1.4) and *nosDFY* are the only genes involved in nitrous oxide reductase synthesis [137,570,620,623]. However, using frame shift mutagens and alternative selection procedures, another gene *nosA* (or a linked gene) involved in nitrous oxide reductase biosynthesis has been identified [379]. NosA binds copper and appears to be an outer-membrane pore with slight selectivity for copper [319]. The synthesis of NosA is repressed by oxygen and exogenous copper [320]. It is not known if *nosA* is linked to the nitrous oxide reductase gene cluster.

10. Concluding remarks

We hope that we have conveyed in this review both the multidisciplinary nature of research in the field of N-oxide respiration and the information to be gained by an integrated approach. We trust we have also demonstrated that significant insight into the enzymes and processes of N-oxide respiration can be gained from comparisons with other systems. A particularly good example is the fresh light thrown on nitric oxide reductase by the recent discovery of a structural relationship to haem-copper oxidases. Workers in other fields should note that the enzymes of N-oxide respiration are now some of the best-characterised metalloproteins and that their properties could inform studies of their systems. For instance, the proposal that the cytochrome oxidase Cu_A centre is dinuclear arose from work on the nitrous oxide reductase.

The need to combat nitrate as a pollutant in waste waters and the recognition that bacteria can make a significant contribution to gaseous N-oxide emissions [447a] have each heightened interest in the biochemistry underlying N-oxide respiration. The last 5 years have seen remarkable advances in our knowledge of respiratory N-oxide reductases. The gene-derived primary structure of each reductase is now available, in all but one case from multiple species. The presence in these reductases of metallocofactors as prosthetic groups has presented problems in most cases for heterologous expression and hence mutagenesis of the enzymes. However, recent and anticipated advances in the genetic tools available in 'non-traditional' bacteria mean that such problems are being rapidly overcome and we are already seeing a movement in the field to a very detailed site-specific mutagenesis-assisted analysis of enzyme structure and mechanism. Determination of high-resolution structures for the copper and cytochrome cd_1 nitrite reductases has revealed quite unexpected features. The trimeric nature of the copper enzyme in which the catalytic copper atoms are bound at subunit interfaces was unforseen, while for the cytochrome cd_1 enzyme the structure showed unanticipated haem iron ligands, lack of sequence conservation in apparently functionally important parts of the protein and tantalising hints of a mechanism for domain movements during the catalytic cycle. We anticipate that the next 5 years will see the determination of three-dimensional structures for all the remaining soluble enzymes and extrinsic membrane portions of the other N-oxide reductases, or at the very least of structurally related proteins. This should ensure an order of magnitude increase in our understanding of these enzymes. We also expect that the next 5 years will produce especially large advances in our understanding of the regulation of N-oxide reduction in organisms other than *E. coli* and of the processes involved in the biogenesis of the N-oxide reductases.

11. Note added in proof

Since submission of this review there have been several very important developments.

The high resolution structures of two cytochrome *c* oxidases have been determined, broadly confirming the structural model for these enzymes inferred from mutagen-

esis and spectroscopic studies [Iwata, S. et al. (1995) Nature 376, 660–669; Tsukihara, T. et al. (1995) Science 269, 1069–1074]. Because nitric oxide reductase is homologous to cytochrome *c* oxidase (Section 7.2.) these structures provide us with an approximate model for the reductase. The cytochrome *c* oxidase amino acids that mutagenesis suggests are involved in proton pumping (discussed in Section 7.4.) do indeed appear to provide portions of a pathway allowing transfer of protons across the membrane. These amino acids are not conserved in either the nitric oxide reductases or the cbb_3 oxidases. Thus, if these enzymes are proton pumps the mechanism differs from that found in cytochrome *c* oxidase. The structure of the Cu_A centre is essentially that proposed by the Thomson and Saraste groups (Fig. 24A; Section 9.3.) though each copper is interpreted as having distorted tetrahedral coordination. The extra ligands are the methionine in the Cu_A consensus sequence and a main chain carbonyl from a glutamate that is conserved in the cytochrome *c* oxidase Cu_A consensus but not found in nitrous oxide reductase (Fig. 23). Although the Cu–Cu distance of 2.5 Å inferred from EXAFS is consistent with the X-ray structure at the current resolution, the double bridging thiolate structure argues against a direct Cu–Cu bond (see also the commentary by Betragnolli, H. and Kaim, W. (1995) Angew. Chem. Int. Ed. Engl. 34, 771–773). Farrar and co-workers have substantially advanced our understanding of the electronic structure of the Cu_A centre in a (pre-oxidase structure) spectroscopic and theoretical analysis [Farrar et al. (1995) Eur. J. Biochem. 232, 294–303].

Chan and coworkers [Chan, M.K. et al. (1995) Science 267, 1463–1469] have solved the structure of the tungsten dipterin-dependent enzyme aldehyde ferredoxin oxidoreductase. While it is currently unclear which structural features can be extrapolated to molybdopterin-dependent enzymes such as the nitrate reductases, the aldehyde oxidase structure does suggest that the proposed pterin cofactor structure shown in Fig. 7a is essentially correct. In particular it can be seen that the metal atom is liganded by both dithiolene sulfur atoms of two adjacent cofactors but by no other part of the pterin cofactor. The pterin cofactor contains a third ring formed by the oxidative closure of the side chain hydroxyl with C-7. Thus the molybdopterin cofactor is potentially able to undergo redox chemistry additional to that shown in Fig. 7b. Each pterin ring system of the dimeric cofactor is nonplanar, indicating that the pterin is not fully oxidized (Fig. 7b). One pterin cofactor lies between the tungsten atom and a proposed electron-accepting [4Fe-4S] cluster suggesting that the pterin is involved in electron transfer between the two metal centres (Section 5.4.2).

Venter and coworkers [Fleischmann, R.D. et al. (1995) Science 269, 496–512] have determined the sequence of the entire genome of the γ-Proteobacterium *Haemophilus influenzae* Rd. The genome codes for periplasmic nitrate reductase (HI0342 to HI0348) and cytochrome *c* nitrite reductase (HI1066 to HI1069) systems. These *H. infuenzae* loci have identical organisation to those of *E. coli* (Fig. 9 and Section 7.4.2.) except that they are not linked to cytochrome *c* biosynthesis genes. The Cys-Xaa-Xaa-Cys-Lys motif that we suggest provides a lysine-liganded *c*-haem binding site (Section 7.4.2) in cytochrome *c* nitrite reductase is conserved in *H. influenzae* NrfA (HI1069). A NapF (Section 5.1.4.) homolog (HI1043) is found in an operon encoding the molybdopterin dependent enzyme membrane-bound dimethylsulfoxide reductase. The availability of additional NapF sequences confirms our tentative conclusion that this protein does not possess a signal peptide (Section 5.1.4.). The *H. influenzae* genome sequence, the purification of a periplasmic nitrate reductase from *Desulfovibrio desulfuricans* [Bursakov, S. et al. (1995) Anaerobe 1, 55–60] and the observations discussed in Section 5.1. all support the idea that a periplasmic, but not a membrane-bound nitrate reductase, is the normal partner for cytochrome *c* nitrite reductase. A detailed analysis of the *E. coli nap* promoter region has been presented [Darwin, A.J. and Stewart, V. (1995) J. Mol. Biol. 251, 15–29]. It has been demonstrated that a constitutively active mutant of the anaerobic regulatory protein Fnr (Section 3.1) binds an iron-sulfur cluster [Khoroshilova, N. et al. (1995) Proc. Natl. Acad. Sci. USA 92, 2499–2503].

Acknowledgements

We would like to thank all members of our laboratories, past and present, for their contribution to our research and the BBSRC, NERC and the Royal Society for funding. We would also like to thank Deepa (BB), Andrea (DJR) and Tina (SJF) for their patience and understanding during the preparation of this review. We are grateful to Stephen Spiro, Brian Bennett, Jaqui Farrar, Nick Watmough, Akber Siddiqui and Andrew Thomson for critical discussions. We also thank Dudley Page, Vilmos Fülöp and Andrew Hemmings for assistance in preparing some of the figures. Sequence analysis methods [15,63,148,161,454,455,575] were broadly as described elsewhere [63].

References

[1] Abou-Jaoudé, A., Chippaux, M. and Pascal, M-C. (1979) Formate-nitrite reduction in *Escherichia coli* K12. Eur. J. Biochem. 95, 309–314.

[2] Abou-Jaoudé, A., Pascal, M.-C. and Chippaux, M. (1979) Formate-nitrite reduction in *Escherichia coli* K12. 2. Identification of components involved in electron transfer. Eur. J. Biochem. 95, 315–321.

[3] Abraham, Z.H.L., Lowe, D.J. and Smith, B.E. (1993) Purification and characterization of the dissimilatory nitrite reductase from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (N.C.I.M.B. 11015): evidence for the presence of both type 1 and type 2 copper centres. Biochem. J. 295, 587–593.

[4] Adman, E.T. (1985) Structure and function of small blue copper

proteins, in Metalloproteins Part I: Metal Proteins with Redox Roles (Harrison, P., ed.), pp. 1–42, Topics in Molecular and Structural Biology, Vol. 6, Macmillan, London.

[5] Adman, E.T. (1991) Copper protein structures. Adv. Protein Chem. 42, 145–197.

[6] Adman, E.T. and Turley, S. (1993) Two crystal forms of *A. cycloclastes* nitrite reductase, in 'Bioinorganic Chemistry of Copper', pp. 397–405, Chapman and Hall, New York.

[7] Aerssens, E., Tiedje, J.M. and Averill, B.A. (1986) Isotope labelling studies on the mechanism of N–N bond formation in denitrification. J. Biol. Chem. 261, 9652–9656.

[8] Ahlers, B., Konig, W., Bock, E. (1990) Nitrite reductase activity in *Nitrobacter vulgaris*. FEMS Microbiol. Lett. 67, 121–126.

[9] Akey, C.W., Moffat, K., Wharton, D.C. and Edelstein, S.J. (1980) Characterisation of crystals of a cytochrome oxidase (nitrite reductase) from *Pseudomonas aeruginosa* by x-ray diffraction and electron microscopy. J. Mol. Biol. 136, 19–43.

[10] Alami, N. and Hallenbeck, P.C. (1995) Cloning and characterization of a gene cluster, *phsBCDEF*, necessary for the production of hydrogen sulfide from thiosulfate by *Salmonella typhimurium*. Gene 156, 53–57.

[11] Alef, K. and Klemme, J-.H. (1979) Assimilatory nitrate reductase of *Rhodopseudomonas capsulata* AD2: a molydoprotein. Z. Naturforsch. 34c, 33–37.

[12] Alefounder, P.R. and Ferguson, S.J. (1980) The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase in *Paracoccus denitrificans*. Biochem. J. 192, 231–240.

[13] Alefounder, P.R., McCarthy, J.E.G. and Ferguson, S.J. (1981) The basis of the control of nitrate reduction by oxygen in *Paracoccus denitrificans*. FEMS Microbiol. Lett. 12, 321–326.

[14] Alefounder, P.R., Greenfield, A.J., McCarthy, J.E.G. and Ferguson, S.J. (1983) Selection and organisation of denitrifying electron transfer pathways in *Paracoccus denitrificans*. Biochim. Biophys. Acta 724, 20–39.

[15] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

[16] Andrew, C.R., Han, J., De Vries, S., Van der Oost, J., Averill, B.A., Loehr, T.M. and Sanders-Loehr, J. (1994) Cu_A of cytochrome *c* oxidase and the A site of N_2O reductase are tetrahedrally distorted type I Cu cysteinates. J. Am. Chem. Soc. 116, 10805–10806.

[17] Andrews, S.C., Harrison, P.M. and Guest, J.R. (1991) A molecular analysis of the 53.3 minute region of the *Escherichia coli* linkage map. J. Gen. Microbiol. 137, 361–367.

[18] Andriesse, A.J. and Bakker, H. (1993) Unpublished sequence, accession number X74597.

[19] Anthamatten, D., Scherb, B. and Hennecke, H. (1992) Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. J. Bacteriol. 174, 2111–2120.

[20] Antholine, W.E., Kastrau, D.H.W., Steffens, G.C.M., Buse, G., Zumft, W.G. and Kroneck, P.M.H. (1992) A comparative EPR investigation of the multicopper proteins nitrous-oxide reductase and cytochrome *c* oxidase. Eur. J. Biochem. 209, 875–881.

[21] Arai, H., Sanbongi, Y., Igarashi, Y. and Kodama, T. (1990) Cloning and sequencing of the gene encoding cytochrome c-551 from *Pseudomonas aeruginosa*. FEBS Lett. 261, 196–198.

[22] Arai, J., Igarashi, Y. and Kodama, T. (1991) Anaerobically induced expression of the nitrite reductase cytochrome *c*-551 operon from *Pseudomonas aeruginosa*. FEBS Lett. 280, 351–353.

[23] Arai, J., Igarashi, Y. and Kodama, T. (1991) Nitrite activates the transcription of the *Pseudomonas aeruginosa* nitrite reductase and cytochrome *c*-551 operon under anaerobic conditions. FEBS Lett. 288, 227–228.

[24] Arai, H., Igarashi, Y. and Kodama, T. (1995) The structural genes for nitric oxide reductase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 1261, 279–284.

[25] Armstrong, F.A. (1992) Dynamic electrochemistry of iron-sulfur proteins. Adv. Inorg. Chem. 38, 117–163.

[26] Augier, V., Guigliarelli, B., Asso, M., Bertrand, P., Frixon, C., Giordano, G., Chippaux, M. and Blasco, F. (1993) Site-directed mutagenesis of conserved cysteine residues within the β subunit of *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of the mutated enzymes. Biochemistry 32, 2013–2023.

[27] Augier, V., Asso, M., Guigliarelli, B., More, C., Bertrand, P., Santini, C.-L., Blasco, F., Chippaux, M. and Giordano, G. (1993) Removal of the high-potential [4Fe-4S] center of the β-subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization of site-directed mutated enzymes. Biochemistry 32, 5099–5108.

[28] Auton, K.A. and Anthony, C. (1989) The role of cytochromes and blue copper proteins in growth of an obligate methylotroph on methanol and methylamine. J. Gen. Microbiol. 135, 1923–1931.

[29] Averill, B.A., Hulse, C.L. and Jackson, M.A. (1991) Properties of the copper-containing nitrite reductase from *Achromobacter cycloclastes* and its anion complexes. J. Inorg. Biochem. 43, 180.

[30] Averill, B.A., Wang, Y., Dailey, G.C., Hulse, C.A., Ye, R.W. and Tiedje, J.M. (1993) Spectroscopic and mechanistic studies of dissimilatory nitrite reductases. J. Inorg. Biochem. 51, 351.

[31] Axley, M.J., Grahame, D.A and Stadtman, T.C. (1990) *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium-dependent dehydrogenase component. J. Biol. Chem. 265, 18213–18218.

[32] Axley, M.J., Böck, A. and Stadtman, T.C. (1991) Catalytic properties of an *Escherichia coli* formate dehydrogenase mutant in which sulfur replaces selenium. Proc. Natl. Acad. Sci. USA 88, 8450–8454.

[33] Ballard, A.L. and Ferguson, S.J. (1987) Molecular properties of the respiratory nitrate reductase of *Paracoccus denitrificans*. Biochem. Soc. Trans. 15, 937–938.

[34] Ballard, A.L. and Ferguson, S.J. (1988) Respiratory nitrate reductase from *Paracoccus denitrificans*. Evidence for two *b*-type haems in the γ subunit and properties of a water-soluble active enzyme containing α and β subunits. Eur. J. Biochem. 174, 207–212.

[35] Ballard, A.L., McEwan, A.G., Richardson, D.J., Jackson, J.B., Ferguson, S.J. (1990) *Rhodobacter capsulatus* strain BK5 possesses a membrane-bound respiratory nitrate reductase rather than the periplasmic enzyme found in other strains. Arch. Microbiol. 154, 301–303.

[36] Banerjee, R.V. and Matthews, R.G. (1990) Cobalamin-dependent methionine synthase. FASEB J. 4, 1450–1459.

[37] Banks, R.G.S., Henderson, R.J. and Pratt, J.M. (1968) Reactions of gases in solution. Part III. Some reactions of nitrous oxide with transition metal complexes. J. Chem. Soc. Ser. A 2886–2889.

[38] Bannan, J.D., Moran, M.J., MacInnes, J.I., Soltes, G.A. and Friedman, R.L. (1993) Cloning and characterization of *btr*, a *Bordetella pertussis* gene encoding an FNR-like transcriptional regulator. J. Bacteriol. 175, 7228–7235.

[39] Barber, D., Parr, S.R. and Greenwood, C. (1976) Some spectral and steady-state kinetic properties of *Pseudomonas* cytochrome oxidase. Biochem. J. 157, 431–438.

[40] Barber, M.J., Siegel, L.M., Schauer, N.L., May, H.D. and Ferry, J.G. (1983) Formate dehydrogenase from *Methanobacterium formicicum*. Electron paramagnetic resonance spectroscopy of the molybdenum and iron-sulfur centers. J. Biol. Chem. 258, 10839–10845.

[41] Barber, M.J., May, H.D. and Ferry, J.G. (1986) Inactivation of formate dehydrogenase from *Methanobacterium formicicum* by cyanide. Biochemistry 25, 8150–8155.

[42] Barley, M.H., Rhodes, M.R., Meyer, T.J. (1987) Electrocatalytic

reduction of nitrite to nitrous oxide and ammonia based on the *N*-methylated, cationic iron porphyrin complex $[Fe(III)(H_2O)(TMPyP)]^{5+}$. Inorg. Chem. 26, 1746–1750.

[43] Barr, M.E., Smith, P.H., Antholine, W.E. and Spencer, B. (1993) Crystallographic, spectroscopic and theoretical studies of an electron-delocalized Cu(1.5)-Cu(1.5) complex. J. Chem. Soc. Chem. Commun. 1649–1652.

[44] Barrett, E.L. and Riggs, D.L. (1982) Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimirium*. J. Bacteriol. 150, 563–571.

[45] Bazylinski, D.A. and Blakemore, R.P. (1983) Denitrification and assimilatory nitrate reduction in *Aquaspirillum magnetaotacticum*. Appl. Environ. Microbiol. 46, 1118–1124.

[46] Beardmore-Gray, M., O'Keeffe, D.T. and Antony, C. (1983) The methanol:cytochrome *c* oxidoreductase activity of methylotrophs. J. Gen. Microbiol. 129, 923–933.

[47] Beckman, D.L. and Kranz, R.G. (1993) Cytochromes *c* biogenesis in a photosynthetic bacterium requires a periplasmic thioredoxin-like protein. Proc. Natl. Acad. Sci. USA 90, 2179–2183.

[48] Beckman, D.L., Trawick, D.R. and Kranz, R.G. (1992) Bacterial cytochromes *c* biogenesis. Genes Devel. 6, 268–283.

[49] Bell, L.C. (1990) Electron transport reactions of denitrification. D.Phil. thesis, University of Oxford.

[50] Bell, L.C. and Ferguson, S.J. (1991) Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*. Biochem. J. 273, 423–427.

[51] Bell, L.C., Richardson, D.J. and Ferguson, S.J. (1990) Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: the periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett. 265, 85–87.

[52] Bell, L.C., Richardson, D.J. and Ferguson, S.J. (1992) Identification of nitric oxide reductase activity in *Rhodobacter capsulatus*: the electron transport pathway can either use or bypass both cytochrome c_2 and the cytochrome bc_1 complex. J. Gen. Microbiol. 138, 437–443.

[53] Bell, L.C., Page, M.D., Berks, B.C., Richardson, D.J. and Ferguson, S.J. (1993) Insertion of transposon Tn*5* into a structural gene of the membrane-bound nitrate reductase of *Thiosphaera pantotropha* results in anaerobic overexpression of periplasmic nitrate reductase activity. J. Gen. Microbiol. 139, 3205–3214.

[54] Bennett, B. and Bray, R.C. (1994) Further studies on redox-related activation and deactivation of *E. coli* nitrate reductase: a possible physiologically relevant role for the low potential [4Fe-4S] centres. Biochem. Soc. Trans. 22, 283S.

[55] Bennett, B., Benson, N., McEwan, A.G., and Bray, R.C. (1994) Multiple states of the molybdenum centre of dimethylsulfoxide reductase from *Rhodobacter capsulatus* revealed by EPR spectroscopy. Eur. J. Biochem. 225, 321–331.

[56] Bennett, B., Berks, B.C., Ferguson, S.J., Thomson, A.J., and Richardson, D.J. (1994) Mo(V) electron paramagnetic resonance signals from the periplasmic nitrate reductase of *Thiosphaera pantotropha*. Eur. J. Biochem. 226, 189–198.

[57] Benson, N., Farrar, J.A., McEwan, A.G. and Thomson, A.J. (1992) Detection of the optical bands of molybdenum(V) in DMSO reductase (*Rhodobacter capsulatus*) by low temperature MCD spectroscopy. FEBS Letts. 307, 169–172.

[58] Beratan, D.N., Betts, J.N. and Onuchic, J.N. (1991) Protein electron transfer rates set by the bridging secondary and tertiary structure. Science 252, 1285–1288.

[59] Berg, B.L., Li, J., Heider, J. and Stewart, V. (1991) Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. I. Nucleotide sequence of the *fdnGHI* operon and evidence that opal (UGA) encodes selenocysteine. J. Biol. Chem. 266, 22380–22385.

[59a] Bergmann, D.J., Arciero, D.M. and Hooper, A.B. (1994) Organization of the *hao* gene cluster of *Nitrosomonas europae*: genes for two tetrahaem *c* cytochromes. J. Bacteriol. 176, 3148–3153.

[60] Berks, B.C. (1991) D. Phil. thesis, University of Oxford.

[61] Berks, B.C., Baratta, D., Richardson, D.J. and Ferguson, S.J. (1993) Purification and characterization of a nitrous oxide reductase from *Thiosphaera pantoropha*. Implications for the mechanism of aerobic nitrous oxide reduction. Eur. J. Biochem. 212, 467–476.

[62] Berks, B.C., Richardson, D.J., Robinson, C., Reilly, A., Aplin, R.T. and Ferguson, S.J. (1994) Purification and characterization of the periplasmic nitrate reductase from *Thiosphaera pantotropha*. Eur. J. Biochem. 220, 117–124.

[63] Berks, B.C., Page, M.D., Richardson, D.J., Reilly, A., Cavill, A., Outen, F. and Ferguson, S.J. (1995) Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron carrying arm of a redox loop. Mol. Microbiol. 15, 319–331.

[64] Berks, B.C., Richardson, D.J., Reilly, A., Willis, A.C. and Ferguson, S.J. (1995) The *napEADBC* gene cluster encoding the periplasmic nitrate reductase system of *Thiosphaera pantotropha*. Biochem. J. 309, 983–992.

[65] Berger, H. and Wharton, D.C. (1980) Small angle X-ray scattering studies of oxidised and reduced cytochrome oxidase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 622, 355–359.

[66] Bertrand, P., More, C., Guigliarelli, B., Fournel, A., Bennett, B. and Howes, B.D. (1994) Biological polynuclear clusters coupled by magnetic interactions: from the point dipole approximation to a local spin model. J. Am. Chem. Soc. 116, 3078–3086.

[67] Bilous, P.T., Cole, S.T., Anderson, W.F. and Weiner, J.H. (1988) Nucleotide sequence of the *dmsABC* operon encoding the anaerobic dimethylsulfoxide reductase of *Escherichia coli*. Mol. Microbiol. 2, 785–795.

[68] Blackburn, R., Kyaw, M. and Swallow, A.J. (1977) Reaction of cob(I)alamin with nitrous oxide and cob(III)alamin. J. Chem. Soc. Faraday Trans. 73, 250–255.

[69] Blackburn, N.J., Barr, M.E., Woodruff, W.H., Van der Oost, J. and De Vries, S. (1994) Metal-metal bonding in biology: EXAFS evidence for a 2.5 Å copper-copper bond in the Cu_A center of cytochrome oxidase. Biochemistry 33, 10401–10407.

[70] Blackmore, R., Robertson, A.M. and Brittain, T. (1986) The purification and some equilibrium properties of the nitrite reductase of the bacterium *Wollinella succinogenes*. Biochem J. 233, 547–552.

[71] Blackmore, R.S., Brittain T., Gadsby, P.M.A., Greenwood, C. and Thomson, A.J. (1987) Electron paramagnetic resonance and magnetic circular dichroism studies of a hexa-heme nitrite reductase from *Wolinella succinogenes*. FEBS Lett. 219, 244–248.

[72] Blackmore R.S., Gadsby, P.M.A., Greenwood, C. and Thomson, A.J. (1990) Spectroscopic studies of partially reduced forms of *Wollinella succinogenes* nitrite reductase. FEBS Lett. 264, 257–262.

[73] Blasco, F., Iobbi, C., Giordano, G., Chippaux, M. and Bonnefoy, V. (1989) Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the α and β subunits in iron binding and electron transfer. Mol. Gen. Genet. 218, 249–256.

[74] Blasco, F., Iobbi, C., Ratouchniak, J., Bonnefoy, V. and Chippaux, M. (1990) Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by the *narGHJI* operon. Mol. Gen. Genet. 222, 104–111.

[75] Blasco, F., Nunzi, F., Pommier, J., Brasseur, R., Chippaux, M. and Giordano, G. (1992) Formation of active heterologous nitrate reductases between nitrate reductases A and Z of *Escherichia coli*. Mol. Microbiol. 6, 209–219.

[76] Blasco, F., Pommier, J., Augier, V., Chippaux, M. and Giordano, G. (1992) Involvement of the *narJ* or *narW* gene product in formation of active nitrate reductase in *Escherichia coli*. Mol. Microbiol. 6, 221–230.

[77] Blattner, F.R., Burland, V.D., Plunkett, G. III, Sofia, H.J. and

Daniels, D.L. (1993) Analysis of the *Escherichia coli* genome. IV. DNA sequence of the region from 89.2 to 92.8 minutes. Nucleic Acids Res. 21, 5408–5417.

[77a] Bleakley, B.H. and Tiedje, J.M. (1982) Nitrous oxide production by organisms other than nitrifiers and denitrifiers. Appl. Environ. Microbiol. 44, 1342–1348.

[77b] Bock, E, Schmidt, I, Stuven, R. and Zart, D. (1995) Nitrogen loss caused by denitrifying Nitrosomonas cells using ammonium of hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. 165, 16–20.

[78] Böhm, R., Sauter, M. and Böck, A. (1990) Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenlyase components. Mol. Microbiol. 4, 231–243.

[79] Bokranz, M., Gutmann, M., Körtner, C., Kojro, E., Fahrenholz, F., Lauterbach, F. and Kröger, A. (1991) Cloning and nucleotide sequence of the structural genes encoding the formate dehydrogenase of *Wolinella succinogenes*. Arch. Microbiol. 156, 119–128.

[80] Bombelka, E., Richter, F.-W., Stroh, A. and Kadenbach, B. (1986) Analysis of the Cu, Fe and Zn contents in cytochrome *c* oxidase from different species and tissues by proton-induced X-ray emission (PIXE). Biochem. Biophys. Res. Commun. 140, 1007–1014.

[81] Boogerd, F.C., Van Verseveld, H.W. and Stouthamer, A.H. (1983) Dissimilatory nitrate uptake in *Paracoccus denitrificans* via a $\Delta\tilde{\mu}_{H^+}$-dependent system and a nitrate-nitrite antiport system. Biochim. Biophys. Acta 723, 415–427.

[82] Bouvier, J. and Stragier, P. (1991) Nucleotide sequence of the *lsp-dapB* interval in *Escherichia coli*. Nucleic. Acids Res. 19, 180.

[83] Boxer, D.H. (1989) Involvement of the chlorate resistance loci and the molybdenum cofactor in the biosynthesis of the *Escherichia coli* respiratory nitrate reductase, in Molecular and Genetic Aspects of Nitrate Assimilation (Wray, J.C. and Kinghorn, J.R., eds.), pp. 27–39, Oxford Scientific Publications, Oxford.

[84] Braun, C. and Zumft, W.G. (1991) Marker exchange of the structural genes for nitric oxide reductase blocks the denitrification pathway of *Pseudomonas stutzeri*. J. Biol. Chem. 266, 22785–22788.

[85] Braun, C. and Zumft, W.G. (1992) The structural genes of the nitric oxide reductase complex from *Pseudomonas stutzeri* are part of a 30-kilobase gene cluster for denitrification. J. Bacteriol. 174, 2394–2397.

[86] Bray, R.C. (1988) The inorganic biochemistry of molybdoenzymes. Quart. Rev. Biochem. 21, 299–329.

[87] Bray, R.C., Gutteridge, S., Lamy, M.T. and Wilkinson, T. (1983) Equilibria amongst different molybdenum (V)-containing species from sulfite oxidase. Evidence for a halide ligand of molybdenum in the low-pH species. Biochem. J. 211, 227–236.

[88] Breton, J., Berks, B.C., Reilly, A., Thomson, A.J., Ferguson, S.J. and Richardson, D.J. (1994) Characterization of the paramagnetic iron-containing redox centres of *Thiosphaera pantotropha* periplasmic nitrate reductase. FEBS Lett. 345, 76–80.

[89] Brettar, I. and Höfle, M.G. (1993) Nitrous oxide producing heterotrophic bacteria from the water column of the central Baltic: abundance and molecular identification. Mar. Ecol. Prog. Ser. 94, 253–265.

[90] Brito, F., Hsu, P.-Y. and DeMoss, J.A. (1993) Menaquinone-9: a new cofactor for the nitrate reductase of *Escherichia coli*? FASEB J. 7, Abstract number 1312.

[91] Brittain, T., Blackmore, R., Greenwood, C. and Thomson, A.J. (1992) Bacterial nitrite-reducing enzymes. Eur. J. Biochem. 209, 793-802.

[91a] Brons. H.J., Hagen, W.R. and Zehnder, A.J.B. (1991) Ferrous iron dependent nitric oxide production in nitrate reducing cultures of *Eschichia coli*. Arch. Microbiol. 155, 341–347.

[92] Bruschi, M. and Guerlesquin, F. (1988) Structure, function and evolution of bacterial ferredoxins. FEMS Microbiol. Rev. 54, 155–176.

[93] Brudwig, G.W., Steven, T.H. and Chan, S.I. (1980) Reactions of nitric oxide with cytochrome *c* oxidase. Biochemistry 19, 5275–5285.

[93a] Bubacco, L., Maglozzo, R.S., Wirt, M.D., Beltramini, M., Salvato, B. and Peisach, J. (1995) Structural Characterisation of Mononuclear Cu(II) and its nitrite complex in the active site of *Carcinus maenas* hemocyanin. Biochemistry 34, 1524–1533.

[94] Byrne, M.D. and Nicholas, D.J.D. (1987) A membrane-bound dissimilatory nitrate reductase from *Rhodobacter sphaeroides* f.sp. *denitrificans*. Biochim. Biophys. Acta 915, 120–124.

[95] Cammack, R. (1992) Iron-sulfur clusters in enzymes: themes and variations. Adv. Inorg. Chem. 38, 281–322.

[96] Canters, G.W. and Van de Kamp, M. (1992) Protein-mediated electron transfer. Current Opinion in Structural Biology 2, 859–869.

[97] Canters, G.W. and Gilardi, G. (1993) Engineering type 1 copper sites in proteins. FEBS Lett. 325, 39–48.

[98] Carr, G.J., Page, M.D. and Ferguson, S.J. (1989) The energy conserving nitric-oxide-reductase system in *Paracoccus denitrificans*: distinction from the nitrite reductase that catalyses synthesis of nitric oxide and evidence from trapping experiments for nitric oxide as a free intermediate during denitrification. Eur. J. Biochem. 179, 683–692.

[99] Carr, G.J. and Ferguson, S.J. (1990) The nitric oxide reductase of *Paracoccus denitrificans*. Biochem. J. 269, 423–429.

[100] Carter, J.P., Richardson, D.J. and Spiro, S. (1995) The isolation and characterisation of a strain of *Pseudomonas putida* that can express a periplasmic nitrate reductase. Arch. Microbiol. 163, 159–166.

[100a] Carter, J.P., Hsia, Y.H., Spiro, S. and Richardson, D.J. (1995) Soil and sediment bacteria capable of aerobic nitrate respiration. Appl. Environ. Microbiol. 61, 2852–2858.

[101] Cassella, S., Shapleigh J.A. and Payne W.J. (1986) Nitrite reduction in *Rhizobium hedysari* strain HCNT-1. Arch. Microbiol 146, 233–238.

[102] Cassella, S., Shapleigh, J.A., Lupi, F. and Payne, W.J. (1988) Nitrite reduction in bacteroids of *Rhizobium Hedysari* strain HCNT-1. Arch. Microbiol. 149, 384–388.

[103] Castresana, J., Lübben, M., Saraste, M., and Higgins, D.G. (1994) Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. EMBO J. 13, 2516–2525.

[104] Chan, Y.-K. and Wheatcroft, R. (1993) Detection of a nitrous oxide reductase structural gene in *Rhizobium meliloti* strains and its location on the *nod* megaplasmid of JJ1c10 and SU47. J. Bacteriol. 175, 19–26.

[105] Chang, C.K., Timkovich, R. and Wu, W. (1986) Evidence that heme d_1 is a 1,3-porphyrindione. Biochemistry 25, 8447–8453.

[106] Chang, C.K., Hanson, L.K., Richardson, P.F., Young, R. and Fajer, J. (1981) π cation radicals of ferrous and free base isobacteriochlorins: models for siroheme and sirohydrochlorin. Proc. Natl. Acad. Sci. USA 78, 2652–2656.

[107] Chaudry, G.R. and MacGregor, C.H. (1983) *Escherichia coli* nitrate reductase subunit A: its role as the catalytic site and evidence for its modification. J.Bacteriol. 154, 387–394.

[108] Chaudhry, G.R. and MacGregor, C.H. (1983) An activity from *Escherichia coli* membranes responsible for the modification of nitrate reductase to its precursor form. J. Biol. Chem. 258, 5828–5833.

[109] Chen, L., Durley, R., Poliks, B.J., Hamada, K., Chen, Z., Mathews, F.S., Davidson, V.L., Satow, Y., Huizinga, E., Vellieux, F.M.D. and Hol, W.G.J. (1992) Crystal structure of an electron-transfer complex between methylamine dehydrogenase and amicyanin. Biochemistry 31, 4959–4964.

[109a] Cheng, H., Xia, B., Reed, G.H. and Markley, J.L. (1994) Optical,

EPR, and ^{1}H NMR spectroscopy of serine-ligated [2Fe-2S] ferredoxins produced by site-directed mutagenesis of cysteine residues in recombinant *Anabaena* 7120 vegetative ferredoxin. Biochemistry 33, 3155–3164.

[110] Chepuri, V., Lemieux, L., Au, D.C.-T. and Gennis, R.B. (1990) The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of cytochrome *c* oxidases. J. Biol. Chem. 265, 11185–11192.

[111] Chiang, R.C., Cavicchioli, R. and Gunsalus, R.P. (1994) Accession number L34011.

[112] Chistoserdov, A.Y. and Lidstrom, M.E. (1991) The small-subunit polypeptide of methylamine dehydrogenase from *Methylobacterium extorquens* AM1 has an unusual leader sequence. J. Bacteriol. 173, 5909–5913.

[113] Chistoserdov, A.Y., Boyd, J., Mathews, F.S., and Lidstrom, M.E. (1992) The genetic organisation of the *mau* gene cluster of the faculative autotroph *Paracoccus denitrificans*. Biochem. Biophys. Res. Commun. 184, 1226–1234.

[114] Christoserdov, A.Y., Christoserdova, L.V., McIntire, W.S. and Lidstrom, M.E. (1994) Genetic organisation of the *mau* gene cluster in *Methylobacterium extorquens* AM1: complete nucleotide sequence and generation and characteristics of *mau* mutants. J. Bacteriol. 176, 4052–4065.

[115] Christoserdov, A.Y., McIntire, W.S., Mathews, F.S. and Lidstrom, M.E. (1994) Organisation and methylamine utilization (*mau*) genes in *Methylophilus methylotrophus* W3A1-NS. J. Bacteriol. 176, 4073–4080.

[116] Choe, M. and Reznikoff, W.S. (1991) Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. J. Bacteriol. 173, 6139–6146.

[117] Choe, M. and Reznikoff, W.S. (1993) Identification of the regulatory gene sequence of anaerobically expressed locus *aeg-46.5*. J. Bacteriol. 175, 1165–1172.

[118] Chow, W., Ragan, I. and Robinson, B.H. (1991) Determination of the cDNA sequence for the human mitochondrial 75-kDa Fe-S protein of NADH-coenzyme Q reductase. Eur. J. Biochem. 201, 547–550.

[119] Cobley, J.G. (1976) Reduction of cytochromes by nitrite in electron-transport particles from *Nitrobacter winogradski*. Biochem. J. 156, 493–498.

[120] Cole, J.A. and Ferguson, S.J. (eds.) (1988) 'The Nitrogen and Sulfur Cycles', SGM Symposia No. 42, Cambridge University Press, Cambridge.

[121] Cole, J.A. (1990) Physiology, biochemistry and genetics of nitrate dissimilation to ammonia, in 'Denitrification in Soil and Sediment', ed. Revsbech, N.P. and Sorenson, J., FEMS Symposium No. 56.

[122] Cole, S.T. (1987) Nucleotide sequence and comparative analysis of the *frd* operon encoding the fumarate reductase of *Proteus vulgaris*. Extensive sequence divergence of the membrane anchors and abscence of an *frd*-linked *ampC* cephalosporinase gene. Eur. J. Biochem. 167, 481–488.

[123] Costa, C., Macedo, A., Moura, I., Moura, J.J.G., Le Gall, J., Berlier, Y., Liu, M-Y. and Payne, W.J. (1990) Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*. FEBS Lett. 276, 67–70.

[124] Costa, C., Moura, J.J.G., Moura,I., Liu, M-Y., Peck, H.D., Le Gall, J., Wang, Y. and Huynh, B.H. (1990) Hexaheme nitrite reductase from *Desulfovibrio desulfuricans*. J. Biol. Chem. 265, 14382–14387.

[125] Coughlan, M.P. Ed. (1980) 'Molybdenum and Molybdenum-Containing Enzymes', Pergamon, Oxford.

[126] Covello, P.S. and Gray, M.W. (1990) RNA sequence and the nature of the Cu_A site in cytochrome *c* oxidase. FEBS Lett. 268, 5–7.

[127] Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Körner, H. and Jakob, W. (1985) Nitrous oxide reductase from denitrifying *Pseudomonas perfectomarina*: purification and properties of a novel multicopper enzyme. Eur. J. Biochem. 153, 459-467.

[128] Coyne, M.S., Arunakumari, A., Averill, B.A. and Tiedje, J.M. (1989) Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. 55, 2924–2931.

[129] Coyne, M.S., Arunakumari, Pankratz, H.S. and Tiedje, J.M. (1990) Localisation of the cytochrome cd_1 and copper nitrite reductases in denitrifying bacteria. J. Bacteriol. 172, 2558–2562.

[130] Coyne, M.S. and Tiedje, J.M. (1990) Induction of denitrifying enzymes in oxygen-limited *Achromobacter cycloclastes* continuous culture. FEMS Microbiol. Ecol. 73, 263–270.

[131] Craig, J.A. and Holm, R.H. (1989) Reduction of nitrate to nitrite by molybdenum-mediated atom transfer: a nitrate reductase analogue reaction system. J. Am. Chem. Soc. 111, 2111–2115.

[132] Cramer, S.P. (1983) Molybdenum enzymes: a survey of structural information from EXAFS and EPR spectroscopy. Adv. Inorg. Bioinorg. Mech. 2, 259–316.

[133] Craske, A. and Ferguson, S.J. (1986) The respiratory nitrate reductase from *Paracoccus denitrificans*. Molecular characterisation and kinetic properties. Eur.J.Biochem. 158, 429–436.

[134] Crofts, A., Hacker, B., Barquera, B., Yun, C.-H. and Gennis, R. (1992) Structure and function of the *bc*-complex of *Rhodobacter sphaeroides*. Biochim.Biophys. Acta 1101, 162–165.

[135] Cuypers, H. and Zumft, W.G. (1992) Regulatory components of the denitrification gene cluster of *Pseudomonas stutzeri*, in *Pseudomonas*, Molecular Biology and Biotechnology, Eds. Galli, E., Silver, S., and Witholt, B., pp. 188–197, American Society for Microbiology, Washington, DC.

[136] Cuypers, H. and Zumft, W.G. (1993) Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an *fnr*-like gene. J.Bacteriol. 175, 7236–7246.

[137] Cuypers, H., Viebrock-Sambale, A. and Zumft, W.G. (1992) NosR, a membrane-bound regulatory component necessary for expression of nitrous oxide reductase in denitrifying *Pseudomonas stutzeri*. J.Bacteriol. 174, 5332–5339.

[138] Darwin, A., Hussain, H., Griffiths, L., Grove, J., Sambongi, Y., Busby, S. and Cole, J. (1993) Regulation and sequence of the structural gene for cytochrome c_{552} from *Escherichia coli*: not a hexahaem but a 50 kDa tetrahaem nitrite reductase. Mol. Microbiol. 9, 1255–1265.

[139] Darwin, A., Tormay, P., Page, L., Griffiths, L. and Cole, J. (1993) Identification of the formate dehydrogenases and genetic determinants of formate-dependent nitrite reduction in *Escherichia coli* K12. J.Gen. Microbiol. 139, 1829–1840.

[140] Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E. and Daldal, F. (1992) Potential ligands to the [2Fe-2S] Rieske cluster of the cytochrome bc_1 complex of *Rhodobacter capsulatus* probed by site-directed mutagenesis. Biochemistry 31, 3342–3351.

[141] Davies, K.J.P., Lloyd, D. and Boddy, L. (1989) The effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeruginosa*. J. Gen. Microbiol. 135, 2445–2451.

[142] De Boer, A.P.N., Reijnders, W.N.M., Kuenen, J.G., Stouthamer, A.H. and Van Spanning, R.J.M. (1994) Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in *Paracoccus denitrificans*. Antonie van Leeuwenhoek 66, 111–127.

[143] De Gier, J.-W.L., Lübben, M., Reijnders, W.N.M., Tipker, C.A., Slotboom, D.-J., Van Spanning, R.J.M., Stouthamer, A.H. and Van der Oost, J. (1994) The terminal oxidases of *Paracoccus denitrificans*. Mol. Microbiol. 13, 183–196.

[144] DeMoss, J.A. and Hsu, P.-Y. (1991) NarK enhances nitrate uptake and nitrite excretion in *Escherichia coli*. J.Bacteriol. 173, 3303–3310.

[145] DeMoss, J.A., Fan, T.Y. and Scott, R.H. (1981) Characterization

of subunit structural alterations which occur during purification of nitrate reductase from *Escherichia coli*. Arch. Biochem. Biophys. 206, 54–64.

[146] Denariaz, G., Payne, W.J. and LeGall, J. (1991) The denitrifying nitrite reductase of *Bacillus halodenitrificans*. Biochim. Biophys. Acta 1056, 225–232.

[147] Dermastia, M., Turk, T. and Hollocher, T.C. (1991) Nitric oxide reductase. Purification from *Paracoccus denitrificans* with use of a single column and some characteristics. J. Biol. Chem. 266, 10899–10905.

[148] Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.

[149] DiSpirito, A.A., Taaffe, L.R., Lipscomb, J.D. and Hooper, A.B. (1985) A blue copper oxidase from *Nitrosomonas europaea*. Biochim. Biophys. Acta 827, 320–326.

[150] Doi, M. and Shioi, Y. (1991) Enhancement of denitrifying activity in cells of *Roseobacter denitrificans* grown aerobically in the light. Plant Cell Physiol. 32, 356–370.

[151] Doi, M., Shioi, Y., Morita, M. and Takamiya, K.-I. (1989) Two types of cytochrome cd_1 in the aerobic photosynthetic bacterium *Erytherobacter* sp. OCh 114. Eur. J. Biochem. 184, 521–527.

[152] Dooley, D.M., Moog, R.S. and Zumft, W.G. (1987) Characterization of the copper sites in *Pseudomonas perfectomarina* nitrous oxide reductase by resonance Raman spectroscopy. J. Am. Chem. Soc. 109, 6730–6735.

[153] Dooley, D.M., McGuirl, M.A., Rosenzweig, A.C., Landin, J.A., Scott, R.A., Zumft, W.G., Devlin, F. and Stephens, P.J. (1991) Spectroscopic studies of the copper sites in wild-type *Pseudomonas stutzeri* N_2O reductase and in an inactive protein isolated from a mutant deficient in copper-site biosynthesis. Inorg. Chem. 30, 3006–3011.[154] Reference deleted.

[155] Dracheva, S.M., Drachev, L.A., Konstantinov, A.A., Semenov, A.Y., Skulachev, V.P., Arutjunjan, A.A., Shuvalov, V.A. and Zaberezhnaya, S.M. (1988) Electrogenic steps in the redox reactions catalyzed by photosynthetic reaction-centre complex from *Rhodopseudomonas viridis*. Eur. J. Biochem. 171, 253–264.

[156] Dross, F., Geisler, V., Lenger, R., Theis, F., Krafft, T., Fahrenholz, F., Kojro, E., Duchêne, A., Tripier, D, Juvenal, K. and Kröger, A. (1992) The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. Eur. J. Biochem. 206, 93–102.

[157] Drummond, J.T. and Mathews, R.G. (1994) Nitrous oxide degradation by cobalamin-dependent methionine synthase: characterization of the reactants and products in the inactivation reaction. Biochemistry 33, 3732–3741.

[158] Dubourdieu, M. and DeMoss, J.A. (1992) The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*. J.Bacteriol. 174, 867–872.

[159] Einarsdottir, O. and Caughey, W.S. (1985) Bovine heart cytochrome *c* oxidase preparations contain high affinity binding sites for magnesium as well as for zinc, copper, and heme iron. Biochem. Biophys. Res. Commun. 129, 840–847.

[160] Enemark, J.H. and Young, C.G. (1993) Bioinorganic chemistry of pterin-containing molybdenum and tungsten enzymes. Adv. Inorg. Chem. 40, 1–88.

[161] Engelman, D.M., Steitz, T.A. and Goldman, A. (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Biophys. Chem. 15, 321–353.

[162] Enoch, H.G. and Lester (1974) The role of a novel cytochrome *b*-containing nitrate reductase and quinone in the in vitro reconstitution of formate-nitrate reductase activity of *E.coli*. Biochim. Biophys. Res. Commun. 61, 1234–1241.

[163] Farrar, J.A., Thomson, A.J., Cheesman, M.R., Dooley, D.M. and Zumft, W.G. (1991) A model of the copper centres of nitrous oxide reductase (*Pseudomonas stutzeri*). Evidence from optical, EPR and MCD spectroscopy, FEBS Lett. 294, 11–15.

[164] Fearnley, I.M. and Walker, J.E. (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. Biochim. Biophys. Acta 1140, 105–134.

[165] Fenderson, F.F., Kumar, S., Adman, E.T., Liu, M.-Y., Payne, W.J. and LeGall, J. (1991) Amino acid sequence of nitrite reductase: a copper protein from *Achromobacter cycloclastes*. Biochemistry 30, 7180–7185.

[166] Ferguson, S.J. (1982) Is a proton-pumping cytochrome oxidase essential for energy conservation in *Nitrobacter*? FEBS Lett. 146, 239–243.

[167] Ferguson, S.J. (1988) Periplasmic electron transport reactions, in Bacterial Energy Transduction, Ed. Anthony, C., pp 151–182, Academic Press, London.

[168] Ferguson, S.J. (1988) The redox reactions of the nitrogen and sulfur cycles, in The Nitrogen and Sulfur Cycles, 42nd SGM Symposium, pp 1–29, Ed. Cole, J.A. and Ferguson, S.J., Cambridge University Press, Cambridge.

[169] Ferguson, S.J. (1992) The periplasm, in Prokaryotic Structure and Function–a New Perspective, Ed. Cole, J.A., pp 297–315. Society for General Microbiology Symposium 47.

[170] Ferguson, S.J. (1994) Denitrification and its control. Antonie van Leeuwenhoek 66, 89–110.

[170a] Ferguson, S.J., Jackson, J.B. and McEwan, A.G. (1987) Anaerobic respiration in the Rhodospirillaceae: characterization of pathways and evaluation of roles in redox balancing during photosynthesis. FEMS Microbiol. Rev. 46, 117–143.

[171] Finlay, B.J., Span, A.S.W., Harman, J.M.P. (1983) Nitrate respiration in primitive eukaryotes. Nature 303, 333–336.

[172] Finnegan, M.G., Hilton, J., Rajagopalan, K.V. and Johnson, M.K. (1993) Optical transitions of molybdenum(V) in glycerol-inhibited DMSO reductase from *Rhodobacter sphaeroides*. Inorg. Chem. 32, 2616–2617.

[173] Fraústo da Silva, J.J.R. and Williams, R.J.P. (1991) 'The Biological Chemistry of the Elements: the Inorganic Chemistry of Life', Oxford University Press, Oxford.

[174] Freitag, A., Rudert, M. and Bock, E. (1987) Growth of *Nitrobacter* by dissimilatory nitrate reduction. FEMS Microbiol. Lett. 48, 105–109.

[175] Friedebold, J. and Bowien, B. (1993) Physiological and biochemical characterization of the soluble formate dehydrogenase, a molybdoenzyme from *Alcaligenes eutrophus*. J. Bacteriol. 175, 4719–4728.

[176] Froncisz, W., Scholes, C.P., Hyde, J.S., Wei, Y.-H., King, T.E., Shaw, R.W. and Beinert, H. (1979) Hyperfine structure resolved by 2 to 4 GHz EPR of cytochrome *c* oxidase. J. Biol. Chem. 254, 7482–7484.

[177] Frunzke, K. and Zumft, W.G. (1986) Inhibition of nitrous-oxide respiration by nitric oxide in the denitrifying bacterium *Pseudomonas perfectomarina*. Biochim. Biophys. Acta 852, 119–125.

[178] Fu, W., Jack, R.F., Morgan, T.V., Dean, D.R. and Johnson, M.K. (1994) *nifU* gene product from *Azotobacter vinelandii* is a homodimer that contains two identical [2Fe-2S] clusters. Biochemistry 33, 13455–13463.

[178a] Fukusaki, E., Panbangred, W., Shinmyo, A. and Okada, H. (1984) The complete nucleotide sequence of the xylanase gene (*xynA*) of *Bacillus pumilus*. FEBS Lett. 171, 197–201.

[179] Fülöp, V., Moir, J.W.B., Ferguson, S.J. and Hajdu, J. (1993) Crystallization and preliminary crystallographic study of cytochrome cd_1 nitrite reductase from *Thiosphaera pantotropha*. J. Mol. Biol. 232, 1211–1212.

[180] Fülöp, V., Moir, J.W.B., Ferguson, S.J. and Hajdu, J. (1995) The anatomy of a bifunctional enzyme: structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome cd_1. Cell 81, 369–377.

[181] Galimand, M., Gamper, M., Zimmermann, A. and Haas, D. (1991) Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. J. Bacteriol. 173, 1598–1606.

[182] Gangeswaran, R., Lowe, D.J. and Eady, R.R. (1993) Purification and characterization of the assimilatory nitrate reductase of *Azotobacter vinelandii*. Biochem. J. 289, 335–342.

[183] Gao, J.-L., Shopes, R.J. and Wraight, C.A. (1990) Charge recombination between the oxidized high-potential *c*-type cytochromes and Q_A^- in reaction centers from *Rhodopseudomonas viridis*. Biochim. Biophys. Acta 1015, 96–108.

[184] Garber, E.A.E. and Hollocher, T.C. (1982) Positional equivalence of nitrogen in N_2O produced by the denitrifying bacterium *Pseudomonas stutzeri*. J. Biol. Chem. 257, 4705–4708.

[185] García-Horsman, J.A., Berry, E., Shapleigh, J.P., Alben, J.O. and Gennis, R.B. (1994) A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* that lacks Cu_A. Biochemistry 33, 3113–3119.

[186] Gardlik, S. and Rajagopalan, K.V. (1990) The state of reduction of molybdopterin in xanthine oxidase and sulfite oxidase. J.Biol. Chem. 265, 13047–13054.

[187] Gardlik, S. and Rajagopalan, K.V. (1991) Oxidation of molybdopterin in sulfite oxidase by ferricyanide. Effect on electron transfer activities. J. Biol. Chem. 266, 4889–4895.

[188] Garland, P.B., Downie, A.J. and Haddock, B.A. (1975) Proton translocation and respiratory nitrate reductase of *Escherichia coli*. Biochem.J. 152, 549–559.

[189] Garner, C.D. and Bristow, S. (1985) in Molybdenum Enzymes, Metal Ions in Biology 7, (Spiro, T.G., ed.), pp. 343–410, Wiley Interscience, New York.

[190] Gennis, R.B., Barquera, B., Hacker, B., Van Doren, S.R., Arnaud, S., Crofts, A.R., Davidson, E., Gray, K.A. and Daldal, F. (1993) The bc_1 complexes of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. J. Bioenerg. Biomemb. 25, 195–209.

[191] George, G.N. (1985) The proton spin-flip lines of Mo(V) EPR signals from sulfite oxidase and xanthine oxidase. J.Magn. Reson. 64, 384–394.

[191a] George, G.N. and Bray, R.C. (1983) Reaction of arsenite ions with the molybdenum center of milk xanthine oxidase. Biochemistry 22, 1013–1021.

[191b] George, G.N. and Bray, R.C. (1983) Formation of the inhibitory complex of *p*-mercuribenzoate with xanthine oxidase, evaluation of hyperfine and quadroplole couplings of mercury to molybdenum(V) from the electron paramagnetic resonance spectrum and structure of the complex. Biochemistry 22, 5443–5452.

[192] George, G.N., Bray, R.C., Morpeth, F.F. and Boxer, D.H. (1985) Complexes with halide and other anions of the molybdenum centre of nitrate reductase from *Escherichia coli*. Biochem. J. 227, 925–931.

[193] George, G.N., Turner, N.A., Bray, R.C., Morpeth, F.F., Boxer,D.H. and Cramer, S.J. (1989) X-ray-absorption and electron-paramagnetic-resonance spectroscopic studies of the environment of molybdenum in high-pH and low-pH forms of *Escherichia coli* nitrate reductase. Biochem. J. 259, 693–700.

[194] George, G.N., Kipke, C.A., Prince, R.C., Sunde, R.A., Enemark, J.H. and Cramer, S.P. (1989) Structure of the active site of sulfite oxidase. X-ray absorption spectroscopy of the Mo(IV), Mo(V), and Mo(VI) oxidation states. Biochemistry 28, 5075–5080.

[195] Gheller, S.F., Schultz, B.E., Scott, M.J. and Holm, R.H. (1992) A broad-substrate analogue reaction system of the molybdenum oxotransferases. J.Am. Chem. Soc. 114, 6934–6935.

[196] Gilles-Gonzales, M.A., Gonzalez, G., Perutz, M., Kiger, L., Marden, M.C. and Poyart, C. (1994) Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autoxidation. Biochemistry 33, 8067–8073.

[197] Giordano, G., Boxer, D.H. and Pommier, J. (1990) Molybdenum cofactor requirement for in vitro activation of apo-molybdoenzymes of *Escherichia coli*. Mol. Microbiol. 4, 645–650.

[198] Gladyshev, V.N., Khangulov, S.V., Axley, M.J. and Stadtman, T.C. (1994) Coordination of selenium to molybdenum in formate dehydrogenase H from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 91, 7708–7711.

[199] Glaser, P., Kunst, F., Arnaud, M., Coudart, M.-P., Gonzales, W., Hullo, M.-F., Ionescu, M., Lubochinsky, B., Marcelino, Moszer, I., Presecan, E., Santana, M., Schneider, E., Schweizer, J., Vertes, A., Rapoport, G. and Danchin, A. (1993) *Bacillus subtilis* genome project: cloning and sequencing of the 97 kb region from 325° to 333°. Mol. Microbiol. 10, 371–384.

[200] Glaser, P., Danchin, A., Kunst, F., Zuber, P. and Nakano, M.M. (1994) Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*. J. Bacteriol. 177, 1112–1115.

[201] Glöckner, A.B., Jüngst, A. and Zumft, W.G. (1993) Copper-containing nitrite reductase from *Pseudomonas aureofaciens* is functional in a mutationally cytochrome cd_1-free background ($NirS^-$) of *Pseudomonas stutzeri*. Arch. Microbiol. 160, 18–26.

[202] Godden, J.W., Turley, S., Teller, D.C., Adman, E.T., Liu, M.Y., Payne, W.J. and LeGall, J. (1991) The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. Science 253, 438–442.

[203] Goldman, B.S., Lin, J.T. and Stewart, V. (1994) Identification and structure of the *nasR* gene encoding a nitrate- and nitrite-responsive positive regulator of *nasFEDCBA* (nitrate assimilation) operon expression in *Klebsiella pneumoniae* M5a1. J. Bacteriol. 176, 5077–5085.

[204] Goretski, J. and Hollocher, T.C. (1988) Trapping of nitric oxide produced during denitrification by extracellular hemoglobin. J. Biol. Chem. 263, 2316–2323.

[205] Goretski, J., Zafiriou, O.C. and Hollocher, T.C. (1990) Steady-state nitric oxide concentrations during denitrification. J.Biol. Chem. 265, 11535–11538.

[206] Gorren, A.C.F., De Boer, E., Wever, R. (1987) The reaction of nitric oxide with copper proteins and the photodissociation of copper-NO complexes. Biochim. Biophys. Acta 916, 38–47.

[206a] Gotschlich, E.C. and Seiff, M.E. (1987) Identification and gene structure of an azurin-like protein with a lipoprotein signal peptide in *Neisseria gonorrhoeae*. FEMS Microbiol Lett. 43, 253–255.

[207] Gray, K.A., Grooms, M., Myllyykallio, H., Moomaw, C., Slaughter, C. and Daldal, F. (1994) *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a Cu_A center. Biochemistry 33, 3120–3127.

[208] Green, J. and Guest, J.R. (1993) A role for iron in transcriptional activation by FNR. FEBS Lett. 329, 55–58.

[209] Green, J. and Guest, J.R. (1993) Activation of FNR-dependent transcription by iron: an in vitro switch for FNR. FEMS Microbiol. Lett. 113, 219–222.

[210] Greenwood, C., Hill, B.C., Barber, D., Eglinton, D.G. and Thomson, A.J. (1983) The optical properties of Cu_A in bovine cytochrome *c* oxidase determined by low-temperature magnetic circular dichroism spectroscopy. Biochem. J. 215, 303–316.

[211] Greenwood, C., Thomson, A.J., Barrett, C.P., Peterson, J., George, G.N., Fee, J.A. and Reichardt, J. (1988) Some spectroscopic views of the Cu_A site in cytochrome *c* oxidase preparations. Ann. New York Acad. Sci. 550, 47–52.

[212] Grossmann, J.G., Abraham, Z.H.L., Adman, E.T., Neu, M., Eady, R.R., Smith, B.E. and Hasnain, S.S. (1993) X-ray scattering using synchotron radiation shows nitrite reductase from *Achromobacter xylosoxidans* to be a trimer in solution. Biochemistry 32, 7360–7366.

[213] Gruber, S., Kilpatrick, L.T., Bastian, N.R., Rajagopalan, K.V. and Spiro, T.G. (1990) Dithiolene coordination in the molybdopterin cofactor of DMSO reductase: in situ evidence from resonance Raman spectroscopy. J.Am. Chem. Soc. 112, 8179–8180.

[214] Gubriel, R.J., Fann, Y.-C., Surerus, K.K., Werst, M.M., Musser, S.M., Doan, P.E., Chan, S.I., Fee, J.A. and Hoffman, B.M. (1993) Detection of two histidyl ligands to Cu_A of cytochrome oxidase by 35-GHz ENDOR: $^{14,15}N$ and $^{63,65}Cu$ ENDOR studies of the

Cu_A site in bovine heart cytochrome aa_3 and cytochromes caa_3 and ba_3 from *Thermus thermophilus*. J. Am. Chem. Soc. 115, 10888–10894.

[215] Gudat, J.C., Singh, J. and Wharton, D.C. (1973) Cytochrome oxidase from *Pseudomonas aeruginosa*. Purification and some properties. Biochim. Biophys. Acta 292, 376–390.

[216] Guest, J.R. (1992) Oxygen-regulated gene expression in *Escherichia coli*. J. Gen. Microbiol. 138, 2253–263.

[217] Guigliarelli, B., Asso, M., More, C., Augier, V., Blasco, F., Pommier, J., Giordano, G. and Bertrand, P. (1992) EPR and redox characterization of iron-sulfur centers in nitrate reductases A and Z from *Escherichia coli*. Evidence for a high-potential and a low-potential class and their relevance in the electron-transfer mechanism. Eur. J. Biochem. 207, 61–68.

[218] Gunsalus, R.P. (1992) Control of the electron flow in *Escherichia coli*: coordinated transcription of respiratory pathway genes. J. Bacteriol. 174, 7069–7074.

[218a] Gutteridge, S., Tanner, S.J. and Bray, R.C. (1978) Comparison of the molybdenum centres of native and desulfo xanthine oxidase: the nature of the cyanide-labile sulfur atom and the nature of the proton-accepting group. Biochem. J. 175, 887–897.

[219] Hackett, N.R. and Bragg, P.D. (1982) The association of two distinct *b* cytochromes with the respiratory nitrate reductase of *Escherichia coli*. FEMS Microbiol. Lett. 13, 213–217.

[220] Han, J., Adman, E.T., Beppu, T., Codd, R., Freeman, H.C., Huq, L., Loehr, T.M. and Sanders-Loehr, J. (1991) Resonance raman spectra of plastocyanin and pseudoazurin: evidence for conserved cysteine ligand conformations in cupredoxins (blue copper proteins). Biochemistry 30, 10904–10913.

[221] Han J., Loehr, T.M., Lu Y., Selverstone J., Averill B.A. and Sanders-Loehr, J. (1993) Resonance raman excitation profiles indicate multiple cys-Cu charge transfer transitions in type 1 copper proteins. J. Am. Chem. Soc. 115, 4256–4263.

[222] Harborne, N.R., Griffiths, L., Busby, S.J.W. and Cole, J.A. (1992) Transcriptional control, translation and function of the products of the five open reading frames of the *Escherichia coli nir* operon. Mol. Microbiol. 6, 2805–2813.

[223] Harding, C., McKee, V. and Nelson, J. (1991) Highly delocalized Cu(I)/Cu(II): a copper–copper bond? J. Am. Chem. Soc. 113, 9684–9685.

[224] Heider, J. and Böck, A. (1993) Selenium metabolism in microorganisms. Adv. Microbiol. Physiol. 35, 71–109.

[225] Heiss, B., Frunzke, K. and Zumft, W.G. (1989) Formation of the N–N bond from nitric oxide by a membrane-bound cytochrome bc complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri*. J. Bacteriol. 171, 3288–3297.

[226] Hernandez, D. and Rowe, J.J. (1988) Oxygen inhibition of nitrate uptake is a general regulatory mechanism in nitrate respiration. J. Biol. Chem. 263, 7937–7939.

[227] Higgins, C.F. (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell. Biol. 8, 67–113.

[228] Hill, B.C. (1991) The reaction of the electrostatic cytochrome *c*-cytochrome oxidase complex with oxygen. J. Biol. Chem. 266, 2219–2226.

[229] Hill, B.C. (1993) The sequence of electron carriers in the reaction of cytochrome *c* oxidase with oxygen. J. Bioenerg. Biomemb. 25, 115–120.

[230] Hill, K.E. and Wharton, D.C. (1977) Reconstitution of the apoenzyme of cytochrome oxidase from *Pseudomonas aeruginosa* with heme d_1 and other heme groups. J. Biol. Chem. 253, 489–495.

[231] Hille, R. (1994) The reaction mechanism of oxymolybdenum enzymes. Biochim. Biophys. Acta 1184, 143–169.

[232] Hinton, S.M. and Dean, D. (1990) Biogenesis of molybdenum cofactors. CRC Crit. Rev. Microbiol. 17, 169–188.

[233] Hochstein, L.I. and Tomlinson, G.A. (1988) The enzymes associated with denitrification. Annu. Rev. Microbiol. 42, 231–261.

[234] Hoehn, G.T. and Clark, V.L. (1990) Distribution of a protein antigenically related to the major anaerobically induced gonococcal outer membrane protein among other *Neisseria* species. Infect. Immun. 58, 3929–3933.

[235] Hoehn, G.T. and Clark, V.L. (1992) Isolation and nucleotide sequence of the gene (*aniA*) encoding the major anerobically induced outer membrane protein of *Neisseria gonorrhoeae*. Infect. Immun. 60, 4695–4703.

[236] Hoehn, G.T. and Clark, V.L. (1992) The major anaerobically induced outer membrane protein of *Neisseria gonorrhoeae*, Pan 1, is a lipoprotein. Infect. Immun. 60, 4704–4708.

[237] Hoeren, F.U., Berks, B.C., Ferguson, S.J. and McCarthy, J.E.G. (1993) Sequence and expression of the gene encoding the respiratory nitrous-oxide reductase from *Paracoccus denitrificans*. New and conserved structural and regulatory motifs. Eur. J. Biochem. Eur. J. Biochem. 218, 49–57.

[238] Hoffman, B.M., Roberts, J.E., Swanson, M., Speck, S.H. and Margoliash, E. (1980) Copper electron-nuclear double resonance of cytochrome *c* oxidase. Proc. Natl. Acad. Sci. USA 77, 1452–1456.

[238a] Hoffmann, T., Szabo, A., Troup, B., Hungerer, C. and Jahn, D. (1995) The anaerobic life of *Bacillus subtilis*: cloning of the genes encoding the respiratory nitrate reductase system. FEMS Microbiol. Lett. 131, 219–225.

[239] Hongo, E., Morimyo, M., Mita, K., Machida, I., Hama-Inaba, H. and Ichimura, S. (1994) Accession number D26057.

[240] Hoitink, C.W.G., Woudt, L.P., Turenhout, J.C.M., Van de Kamp, M. and Canters, G.W. (1990) Isolation and sequencing of the *Alicaligenes denitrificans* azurin-encoding gene: comparison with the genes encoding the blue copper proteins from *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. Gene 90, 15–20.

[241] Holm, R.H. (1990) The biologically relevant oxygen atom transfer chemistry of molybdenum: from synthetic analogue systems to enzymes. Coord. Chem. Rev. 100, 183–221.

[242] Holm, R.H. (1992) Trinuclear cuboidal and heterometallic cubane-type iron-sulfur clusters: new structural and reactivity themes in chemistry and biology. Adv. Inorg. Chem. 38, 1–71.

[243] Horio, T. (1958) Terminal oxidation system in bacteria. J. Biochem. 45, 195–205.[244] Reference deleted.

[245] Horowitz, P.M. Falksen, K., Muhoberac, B.B. and Wharton, D.C. (1982) Activation of *Pseudomonas* cytochrome oxidase by limited proteolysis with subtilisin. J. Biol. Chem. 257, 9258–9260.

[246] Hösler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L.J., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenberg, M.M.J., Babcock, G.J. and Gennis, R.B. (1992) Insight into the active site structure and function of cytochrome oxidase by analysis of site-directed mutants of bacterial cytochrome aa_3 and cytochrome *bo*. J. Bioenerg. Biomembr. 25, 121–136.

[247] Howes, B.D., Abraham, Z.H.L., Lowe, D.J., Brüser, T., Eady, R.R. and Smith, B.E. (1994) EPR and electron nuclear double resonance (ENDOR) studies show nitrite binding to the type 2 copper centers of the dissimilatory nitrite reductase of *Alcaligenes xylosoxidans* (NCIMB 11015). Biochemistry 33, 3171–3177.

[247a] Hüdig, H. and Drew, G. (1984) Reconstitution of *b*-type cytochrome oxidase from *Rhodopseudomonas capsulata* in liposomes and turnover studies of proton translocation. Biochim. Biophys. Acta 765, 171–177.

[248] Hulse, C.L. and Averill, B.A. (1990) Isolation of a high specific activity pink, monomeric nitrous oxide reductase from *Achromobacter cycloclastes*. Biochem. Biophys. Res. Commun. 166, 729–735.

[249] Hulse, C.L., Averill, B.A., Tiedje, J.M. (1989) Evidence for a Copper nitrosyl intermediate in denitrification by the copper-containing nitrite reductase of *Achromobacter cycloclastes*. J. Am. Chem. Soc. 111, 2322–2323.

[250] Hussain, H., Grove, J., Griffiths, L., Busby, S. and Cole, J. (1994)

A seven-gene operon essential for formate-dependent nitrite reduction to ammonia by enteric bacteria. Mol. Microbiol. 12, 153–163.

[251] Huynh, B.H., Lui, M.C., Moura, J.J.G., Moura, I., Ljungdahl, P.O., Munck, E., Payne, W.J., Peck, H.D., DerVartanian, D.V. and LeGall, J. (1982) Mossbauer and EPR studies on the nitrite reductase from *Thiobacillus denitrificans*. J. Biol. Chem. 257, 9576–9581.

[252] Ikebukuro, K., Nishio, M., Yano, K., Tomiyama, M., Tamiya, E. and Karube, I. (1993) Unpublished sequence, accession number D14422.

[253] Ingledew, W.J. and Halling, P.J. (1976) Paramagnetic centers of the nitrite oxidizing bacterium *Nitrobacter*. FEBS Lett. 67, 90–93.

[254] Iobbi-Nivol, C., Santini, C.-L., Blasco, F. and Giordano, G. (1990) Purification and further characterization of the second nitrate reductase of *Escherichia coli* K12. Eur. J. Biochem. 188, 679–687.

[255] Isaki, L., Beers, R. and Wu, H.C. (1990) Nucleotide sequence of the *Pseudomonas flourescens* signal peptidase II gene (*lsp*) and flanking genes. J. Bacteriol. 172, 6512–6517.

[256] Ito, N., Phillips, S.E.V., Yadav, K.D.S. and Knowles, P.F. (1994) Crystal structure of a free radical enzyme, galactose oxidase. J. Mol. Biol. 238, 794–814.

[257] Iuchi, S. and Lin, E.C.C. (1993) Adaptation of *Escherichia coli* to redox environments by gene expression. Mol.Microbiol. 9, 9–15.

[258] Iuci, S., Aristarkhov, S., Dong, J.M., Taylor, J.S., and Lin, E.C.C. (1994) Effects of nitrate respiration on expression of the Arc-controlled operons encoding succinate dehydrogenase and flavin-linked *L*-lactate dehydrogenase. J.Bacteriol. 176, 1695–1701.

[259] Iwasaki, H. and Matsubara, T. (1971) Cytochrome *c*577 (551) and cytochrome *cd* of *Alcaligenes faecalis*. J.Biochem. 69, 847–857.

[260] Iwasaki, H. and Matsubara, T. (1972) A Nitrite reductase from *Achromobacter cycloclastes*. J.Biochem. 71, 645–652.

[261] Jackson, M.A., Tiedje, J.M. and Averill, B.A. (1992) Evidence for an NO-rebound mechanism for the production of N_2O from nitrite by the copper-containing nitrite reductase from *Achromobacter cycloclastes*. FEBS Lett. 291, 41–44.

[262] Jenney, F.E. and Daldal, F. (1993) A novel membrane-associated *c*-type cytochrome, cyt c_y, can mediate the photosynthetic growth of *Rhodobacter sphaeroides*. EMBO J. 12, 1283–1292.

[263] Jensen, B.B. and Burris, R.H. (1986) N_2O as a substrate and as a competitive inhibitor of nitrogenase. Biochemistry 25, 1083–1088.

[263a] Ji, X-B. and Hollocher, T.C. (1988) Reduction of nitrite to nitric oxide by enteric bacteria. Biochem Biophys. Res. Commun. 157, 106–108.

[264] Jin, H., Thomann, H., Coyle, C.L. and Zumft, W.G. (1989) Copper coordination in nitrous oxide reductase from *Pseudomonas stutzeri*. J.Am.Chem.Soc. 111, 4262–4269.

[265] John, P. (1977) Aerobic and anaerobic bacterial respiration monitored by electrodes. J. Gen. Microbiol. 98, 231–238.

[266] Johnson, J.L., Bastian, N.R. and Rajagopalan, K.V. (1990) Molybdopterin guanine dinucleotide: a modified form of molybdopterin identified in the molybdenum cofactor of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* forma specialis *denitrificans*. Proc. Natl. Acad. Sci. USA 87, 3190–3194.

[267] Johnson, J.L. Indermaur, L.W. and Rajagopalan, K.V. (1991) Molybdenum cofactor biosynthesis in *Escherichia coli*. Requirement of the *chlB* gene product for the formation of molybdopterin guanine dinucleotide. J. Biol. Chem. 266, 12140–12145.

[268] Johnson, M.K., Thomson, A.J., Walsh, T.A., Barber, D. and Greenwood, C. (1980) Electron paramagnetic resonance studies on *Pseudomonas* nitrosyl nitrite reductase. Evidence for multiple species in the electron paramagnetic resonance spectra of nitrosyl haemoproteins. Biochem. J. 189, 285–294.

[269] Johnson, M.K., Bennett, D.E., Morningstar, J.E., Adams, M.W.W. and Mortenson, L.E. (1985) The iron-sulfur cluster composition of *Escherichia coli* nitrate reductase. J. Biol. Chem. 260, 5456–5463.

[269a] Johnston, M. *et al.* (1994) product of reading frame YHL008c in U11581.

[269b] Jones, A.M. and Hollocher, T.C. (1993) Nitric oxide reductase of *Achromobacter cycloclastes*. Biochim. Biophys. Acta 1144, 359–366.

[270] Jones, A.M., Adkins, A.M., Knowles, R. and Rayat, G.R. (1990) Identification of a denitrifying gliding bacterium, isolated from soil and able to reduce nitrous oxide in the presence of sulfide and acetylene, as *Flexibacter canadensis*. Can. J. Microbiol. 36, 765–770.

[271] Jones, A.M., Hollocher, T.C. and Knowles, R. (1992) Nitrous oxide reductase of *Flexibacter canadensis*: a unique membrane-bound enzyme. FEMS Microbiol. Lett. 92, 205–210.

[272] Jones, M.R., McEwan, A.G. and Jackson, J.B. (1990) The role of *c*-type cytochromes in the photosynthetic electron transport pathway of *Rhodobacter capsulatus* Biochim. Biophys. Acta 1019, 59–66.

[273] Jones, M.R., Richardson, D.J., McEwan, A.G., Ferguson, S.J. and Jackson, J.B. (1990) In vivo redox poising of the cyclic electron transport system of *Rhodobacter capsulatus* and the effects of the auxiliary oxidants, nitrate, nitrous oxide and trimethylamine *N*-oxide, as revealed by multiple flash excitation. Biochim. Biophys. Acta 1017, 209–216.

[273a] Jones, R.W., Lamont, A. and Garland, P.B. (1980) The mechanism of proton translocation driven by the respiratory nitrate reductase complex of *Escherichia coli*. Biochem. J. 190, 79–94.

[274] Jug, K. (1978) Bond order orbitals and eigenvalues. J. Am. Chem. Soc. 100, 6581–6586.

[275] Jüngst, A., Braun, C. and Zumft, W.G. (1991) Close linkage in *Pseudomonas stutzeri* of the structural genes for respiratory nitrite reductase and nitrous oxide reductase, and other essential genes for denitrification. Mol. Gen. Genet. 225, 241–248.

[276] Jüngst, Wakabayashi, S., Matsubara, H. and Zumft, W.G. (1991) The *nirSTBM* region coding for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. FEBS Lett. 279, 205–209.

[277] Jüngst, A. and Zumft, W.G. (1992) Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of *nirQ*, a novel gene in the denitrification cluster of *Pseudomonas stutzeri*. FEBS Lett. 314, 308–314.

[278] Kahn, D.D. (1994) Accession number Z21854.

[279] Kahn, D., David, M., Domergue, O., Daveran, M.-L., Ghai, J., Hirsch, P.R. and Batut, J. (1989) *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J. Bacteriol. 171, 929–939.

[280] Kaiser, M. and Sawers, G. (1994) Pyruvate formate-lyase is not essential for nitrate respiration by *Escherichia coli*. FEMS Microbiol. Letts. 117, 163–168.

[281] Kajie, S. and Anraku, Y. (1986) Purification of a hexaheme nitrite reductase c_{552} from *Escherichia coli* K12 and its properties as a nitrite reductase. Eur. J. Biochem. 154, 457–463.

[282] Kakutani, T., Watanabe, H., Arima, K. and Beppu, T. (1981) Purification and properties of a copper-containing nitrite reductase from a denitrifying bacterium *Alcaligenes faecalis* strain S6. J. Biochem. 89, 453–461.

[283] Kakutani, T., Watanabe, H., Arima, K. and Beppu, T. (1981) A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis* strain S-6. J. Biochem 89, 463–472.

[283a] Kaldorf, M., Von Berg, K.-H., Meier, U., Servos, U. and Bothe, H. (1993) The reduction of nitrous oxide to dinitrogen by *Escherichia coli*. Arch. Microbiol. 169, 432–439.

[284] Karzanov, V.V., Bogatsky, Y.A., Tischkov, V.I. and Egorov, A.M. (1989) Evidence for the presence of a new NAD-dependent formate dehydrogenase in *Pseudomonas* sp 101 cells grown on a

molybdenum-containing medium. FEMS Microbiol. Lett. 60, 197–200.

[285] Kashem, M.A., Dunford, H.B., Liu, M.Y., Payne, W.J. and LeGall, J. (1987) Kinetic studies of the copper nitrite reductase from *Achromobacter cycloclastes* and its interaction with a blue copper protein. Biochem. Biophys. Res. Commun. 145, 563–568.

[286] Kastrau, D.H.W., Heiss, B., Kroneck, P.M.H. and Zumft, W.G. (1994) Nitric oxide reductase from *Pseudomonas stutzeri*, a novel cytochrome *bc* complex. Phospholipid requirement, electron paramagnetic resonance and redox properties. Eur. J. Biochem. 222, 293–303.

[287] Kay, C.J. and Barber, M.J. (1989) EPR and kinetic analysis of the interaction of halides and phosphate with nitrate reductase. Biochemistry 28, 5750–5758.

[288] Kelly, M., Lappalainen, P., Talbo, G., Haltia, T., Van der Oost, J. and Saraste, M. (1993) Two cysteines, two histidines, and one methionine are ligands of a binuclear purple copper centre. J. Biol. Chem. 268, 16781–16787.

[289] Kerby, R.L., Hong, S.S., Ensign, S.A., Coppoc, L.J., Ludden, P.W. and Roberts, G.P. (1992) Genetic and physiological characterisation of the *Rhodospirillum rubrum* carbon monoxide dehydrogenase system. J. Bacteriol. 174, 5284–5294.

[290] Kirstein, K. and Bock, E. (1993) Close genetic relationship between *Nitrobacter hamburgensis* nitrite oxidoreductase and *Escherichia coli* nitrate reductases. Arch. Microbiol. 160, 447–453.

[291] Knaff, D.B. (1993) The cytochrome bc_1 complex of photosynthetic purple bacteria. Photosynth. Res. 35, 117–133.

[292] Kobayashi, K., Une, H., and Hayashi, K. (1989) Electron transfer process in cytochrome oxidase after pulse radiolysis. J. Biol. Chem. 264, 7976–7980.

[293] Kohzuma, T., Shidara, S. and Suzuki, S. (1993) Direct electrochemistry of copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM1013. J. Inorg. Biochem. 51, 368.

[294] Kohzuma, T., Takase, S., Shidara, S. and Suzuki, S. (1993) Electrochemical properties of copper proteins, pseudoazurin and nitrite reductase from *Achromobacter cycloclastes* IAM 1013. Chem. Lett. 1, 149–152.

[295] Körner, H. (1993) Anaerobic expression of nitric oxide reductase from denitrifying *Pseudomonas stutzeri*. Arch. Microbiol. 159, 410–416.

[296] Körner, H. and Zumft, W.G. (1989) Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. 55, 1670–1676.

[297] Körner, H. and Mayer, F. (1992) Periplasmic location of nitrous oxide reductase and its apoform in denitrifying *Pseudomonas stutzeri*. Arch. Microbiol. 157, 218–222.

[298] Krafft, T., Bokranz, M., Klimmek, O., Schröder, I., Fahrenholz, F., Kojro, E. and Kröger, A. (1992) Cloning and nucleotide sequence of the *psrA* gene of *Wolinella succinogenes* polysulfide reductase. Eur. J. Biochem. 206, 503–510.

[299] Kramer, S.P., Johnson, J.L., Ribeiro, A.A., Millington, D.S. and Rajagopalan, K.V. (1987) The structure of the molybdenum cofactor. Characterisation of di-(carboxyamidomethyl)molybdopterin from sulfite oxidase and xanthine oxidase. J. Biol. Chem. 262, 16357–16363.

[300] Kristjansson, J.K. and Hollocher, T.C. (1980) First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterisation. J. Biol. Chem. 255, 704–707.

[301] Kröger,A., Winkler, E., Innerhofer, A., Hackenberg, H. and Schägger (1979) The formate dehydrogenase involved in electron transport from formate to fumarate in *Vibrio succinogenes*. Eur. J. Biochem. 94, 465–475.

[302] Kroneck, P.M.H. and Zumft, W.G. (1990) Bio-inorganic aspects of denitrification: structures and reactions of N_xO_y compounds and their interaction with iron and copper proteins, in Denitrification in Soil and Sediment (Revsbech, N.P. and Sfrensen, J., eds.), pp. 37–55, Plenum Press, New York.

[303] Kroneck, P.M.H., Antholine, W.A., Riester, J. and Zumft, W.G. (1988) The cupric site in nitrous oxide reductase contains a mixed-valence [Cu(II),Cu(I)] binuclear centre: a multifrequency electron paramagnetic resonance investigation. FEBS Lett. 242, 70–74.

[304] Kroneck, P.M.H., Antholine, W.A., Riester, J. and Zumft, W.G. (1989) The nature of the cupric site in nitrous oxide reductase and of Cu_A in cytochrome *c* oxidase. FEBS Lett. 248, 212–213.

[305] Kroneck, P.M.H., Riester, J., Zumft, W. and Antholine, W.E. (1990) The copper site in nitrous oxide reductase. Biol. Metals 3, 103–109.

[306] Kroneck, P.M.H., Antholine, W.A., Kastrau, D.H.W., Buse, G., Steffens, G.C.M. and Zumft, W.G. (1990) Multifrequency EPR evidence for a bimetallic centre at the Cu_A site in Cytochrome *c* oxidase. FEBS Lett. 268, 274–276.

[307] Kroneck, P.M.H., Beuerle, J. and Schumacher, W. (1992) Metal-dependent conversion of inorganic nitrogen and sulfur compounds. Metal ions in Biological Systems 28, (eds. H. Sigel and A. Sigel) pp. 455–505.

[308] Krüger, B., Meyer, O., Nagel, M., Andreesen, J.R., Meincke, M., Bock, E., Blümle, S., Zumft, W.G. (1987) Evidence for the presence of bactopterin in the eubacterial molybdoenzymes nicotinic acid dehydrogenase, nitrite oxidoreductase, and respiratory nitrate reductase. FEMS Microbiol. Lett. 48, 225–227.

[309] Kucera, I., Karlovsky, P. and Dadak, V. (1981) Control of nitrate respiration in *Paracoccus denitrificans* by oxygen. FEMS Microbiol. Lett. 12, 391–394.

[310] Kucera, I. and Skládal, P. (1990) Hydroxylamine as an inhibitor and terminal acceptor in the respiratory chain of the bacterium *Paracoccus denitrificans*. Gen. Physiol. Biophys. 9, 501–518.

[311] Kucera, I., Hedbavny, R. & Dadak, V. (1988) Separate binding sites for antimycin and mucidin in the respiratory chain of the bacterium *Paracoccus denitrificans* and their occurrence in other denitrifying bacteria. Biochem. J. 252, 905–908.

[312] Kuenen, J.G. and Robertson, L.A. (1988) Ecology of nitrification and denitrification, in The Nitrogen and Sulfur Cycles, (SGM Symposium No. 42, Cole, J.A. and Ferguson, S.J., eds.), pp. 161–218, Cambridge University Press, Cambridge.

[313] Kukimoto, M., Nishiyama, M., Murphy, M.E.P., Turley, S., Adman, E.T., Horinouchi, S. and Beppu, T. (1994) X-ray structure and site-directed mutagenesis of a nitrite reductase from *Alcaligenes faecalis* S-6: roles of two copper atoms in nitrite reduction. Biochemistry 33, 5246–5252.

[314] Lam, Y. and Nicholas, D.J.D. (1969) A nitrite reductase with cytochrome oxidase activity from *Micrococcus denitrificans*. Biochim. Biophys. Acta 180, 459–472.

[315] Lamy, M.T., Gutteridge, S. and Bray, R.C. (1980) Electron-paramagnetic-resonance parameters of molybdenum(V) in sulfite oxidase from chicken liver. Biochem. J. 185, 397–403.

[316] Lappalainen, P. and Saraste, M. (1994) The binuclear Cu_A centre of cytochrome oxidase. Biochim. Biophys. Acta 1187, 222–225.

[317] Lappalainen, P., Aasa, R., Malmström, B.G. and Saraste, M. (1993) Soluble Cu_A-binding domain from the *Paracoccus* cytochrome *c* oxidase. J. Biol. Chem. 268, 26416–26421.

[318] Lazazzera, B.A., Bates, D.M. and Kiley, P.J. (1993) The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state. Genes Dev. 7, 1993–2005.

[319] Lee, H.S., Hancock, R.E.W. and Ingraham, J.L. (1989) Properties of a *Pseudomonas stutzeri* outer membrane channel-forming protein (NosA) required for production of copper-containing N_2O reductase. J. Bacteriol. 171, 2096–2100.

[320] Lee, H.S., Abdelal, A.H.T., Clark, M.A. and Ingraham, J.L. (1991) Molecular characterization of *nosA*, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to

make copper-containing N_2O reductase. J. Bacteriol. 173, 5406–5413.

[321] Liang, J. and Burris, R.H. (1988) Interactions among N_2, N_2O, and C_2H_2 as substrates and inhibitors of nitrogenase from *Azotobacter vinelandii*. Biochemistry 27, 6726–6732.

[322] Libby, E. and Averill, B.A. (1992) Evidence that the type 2 copper centers are the site of nitrite reduction by *Achromobacter cycloclastes* nitrite reductase. Biochem. Biophys. Res. Commun. 187, 1529–1535.

[323] Lin, E.C.C. and Iuchi, S. (1991) Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. Annu. Rev. Genet. 25, 361–387.

[324] Lin, J.T., Goldman, B.S. and Stewart, V. (1993) Structures of genes *nasA* and *nasB*, encoding assimilatory nitrate and nitrite reductases in *Klebsiella pneumoniae* M5a1. J. Bacteriol. 175, 2370–2378.

[325] Lin, J.T., Goldman, B.S. and Stewart, V. (1994) The *nasFEDCBA* operon for nitrate and nitrite assimilation in *Klebsiella pneumoniae* M5a1. J. Bacteriol. 176, 2551–2559.

[326] Liu, M-C. and Peck, H.D. (1981) The isolation of a hexaheme cytochrome from *Desulfovibrio desulfuricans* and its identification as a new type of nitrite reductase. J. Biol. Chem. 256, 13159–13164.

[327] Liu, M-C., DerVartianian, D.V. and Peck, H.D. (1980) On the nature of the oxidation-reduction properties of nitrite reductase from *Desulfovibrio desulfuricans*. Biochem. Biophys. Res. Commun. 96, 278–285.

[328] Liu, M-C., Peck, H.D., Abou-Jaoude, A., Chippaux, M. and Le Gall, J. (1981) A reappraisal of the role of the low potential *c*-type cytochrome (cytochrome c_{552}) in NADH-dependent nitrite reduction and its relationship with a co-purified NADH oxidase in *Escherichia coli* K-12. FEMS Microbiol. Lett. 10, 333–337.

[329] Liu, M-C., Liu, M-Y., Payne, W.J., Peck, H.D., and Le Gall, J. (1983) *Wolinella succinogenes* nitrite reductase: purification and properties. FEMS Microbiol. Lett. 19, 201–206.

[330] Liu, M-C., Huynh, B-H., Payne, W.J, Peck, H.D., DerVartanian, D.V. and LeGall, J. (1987) Optical, EPR and Mossbauer spectroscopic studies on the NO derivatives of cytochrome cd1 from *Thiobacillus denitrificans*. Eur. J. Biochem. 169, 253–258.

[331] Liu, M-C., Bakel, B.W., Liu, M-Y. and Dao, T.N. (1988) Purification of *Vibrio fischeri* nitrite reductase and its characterisation as a hexaheme *c*-type cytochrome. Arch. Biochem. Biophys. 262, 259–265.

[332] Liu M.-Y., Liu, M.-C., Payne, W.J. and LeGall, J. (1986) Properties and electron transfer specificity of copper proteins from the denitrifier *Achromobacter cycloclastes*. J. Bacteriol. 166, 604–608.

[333] Lloyd, D., Boddy, L., Davies, K.J.P. (1987) Persistance of bacterial denitrification under aerobic conditions: the rule rather than the exception. FEMS Microbiol. Ecol. 45, 185–190.

[334] Long, R.C. and Hendrickson, D.N. (1983) Intramolecular electron transfer in a series of mixed-valence copper(II)-copper(I) complexes. J.Am.Chem.Soc. 105, 1513–1521.

[334a] Loveley, D.R. (1991) Dissimilatory iron (III) and Mn (IV) reduction. Microbiol. Rev. 55, 259–287.

[335] Lu, W-.P. and Ragsdale, S.W. (1991) Reductive activation of the coenzyme A/acetyl-CoA isotopic exchange reaction catalyzed by carbon monoxide dehydrogenase from *Clostridium thermoaceticum* and its inhibition by nitrous oxide and carbon monoxide. J. Biol. Chem. 266, 3554–3564.

[336] Lübben, M., Kolmerer, B. and Saraste, M. (1992) An archaebacterial terminal oxidase complex combines core structures of two mitochondrial respiratory complexes. EMBO J. 11, 805–812.

[336a] Ludwig, W., Mittenhuber, G. and Friedrich, C.G. (1993) Transfer of *Thiosphaera pantotropha* to *Paracoccus denitrificans*. Int. J. Sys. Bacteriol. 43, 363–367.

[337] Luque, F., Mitchenall, L.A., Chapman, M., Christine, R. and Pau, R.N. (1993) Characterization of genes involved in molybdenum transport in *Azotobacter vinelandii*. Mol. Microbiol. 7, 447–459.

[338] MacGregor, C.H. (1975) Anaerobic cytochrome b_1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. 121, 1111–1116.

[339] MacGregor, C.H. (1976) Biosynthesis of membrane-bound nitrate reductase in *Escherichia coli*: evidence for a soluble precursor. J. Bacteriol. 126, 122–131.

[340] Malkin, R. and Malmström (1970) The state and function of copper in biological systems. Adv. Enzymol. 35, 177–244.

[341] Malmström, B.G. and Aasa, R. (1993) The nature of the Cu_A center in cytochrome *c* oxidase. FEBS Lett. 325, 49–52.

[342] Maloy, S. and Stewart, V. (1993) Autogenous regulation of gene expression. J. Bacteriol. 175, 307–316.

[343] Mancinelli, R.L., Cronin, S. and Hochstein, L.I. (1986) The purification and properties of a *cd*-cytochrome nitrite reductase from *Paracoccus halodenitrificans*. Arch. Microbiol. 145, 202–208.

[344] Mandon, K., Kaminski, P.A., Mougel, C., Desnoues, N., Dreyfus, B. and Elmerich, C. (1993) Role of the *fixGHI* region of *Azorhizobium caulinodans* in free-living and symbiotic nitrogen fixation. FEMS Microbiol. Lett. 114, 185–190.

[345] Mandon, K., Kaminski, P.A. and Elmerich, C. (1994) Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. 176, 2560–2568.

[346] Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R.P., Werth, M.T. and Johnson, M.K. (1990) [3Fe-4S] to [4Fe-4S] cluster conversion in *Escherichia coli* fumarate reductase. Biochemistry 31, 2703–2712.

[347] Martin, C.T., Scholes, C.P. and Chan, S.I. (1988) On the nature of cysteine coordination to Cu_A in cytochrome *c* oxidase. J. Biol. Chem. 263, 8420–8429.

[348] Martinez, S.E., Huang, D., Szczepaniak, Cramer, W.A. and Smith, J.L. (1994) Crystal structure of chloroplast cytochrome *f* reveals a novel cytochrome fold and unexpected heme ligation. Structure 2, 95–105.

[349] Masuko, M., Iwasaki, H., Sakurai, T., Suzuki, S. and Nakahara, A. (1984) Chracterisation of nitrite reductase from a denitrifier *Alcaligenes* sp. NCIB 11015. A novel copper protein. J. Biochem. 96, 447–454.

[350] Matchová, I. and Kucera, I. (1991) Evidence for the role of soluble cytochrome *c* in the dissimilatory reduction of nitrite and nitrous oxide by cells of *Paracoccus denitrificans*. Biochim. Biophys. Acta 1058, 256–260.

[351] Matsubara, H. and Saeki, K. (1992) Structural and functional diversity of ferredoxins and related proteins. Adv. Inorg. Chem. 38, 223–280.

[352] Matsubara, T. and Iwasaki, H. (1972). A new type of copper-protein from *Alcaligenes faecalis*. J. Biochem. 71, 747–750.

[353] Matsubara, T. and Sano, M. (1985) Isolation and some properties of a novel violet copper protein from a denitrifying bacterium, *Alcaligenes* sp. Chem. Lett. 1053–1056.

[354] Matsubara, T., Frunzke, K. and Zumft, W.G. (1982) Modulation by copper of the products of nitrite respiration in *Pseudomonas perfectomarinus*. J. Bacteriol. 149, 816–823.

[355] McEwan, A.G., Jackson, J.B. and Ferguson, S.J. (1984) Rationalisation of the properties of nitrate reductase in *Rhodopseudomonas capsulata*. Arch. Microbiol. 137, 344–349.

[356] McEwan, A.G., George, C.L., Ferguson S.J. and Jackson J.B. (1982) A nitrate reductase activity in *Rhodopseudomonas capsulata* linked to electron transfer and generation of membrane potential. FEBS Lett. 150, 277–280.

[357] McEwan, A.G., Cotton N.P.J., Ferguson, S.J. and Jackson, J.B. (1983) The inhibition of nitrate reduction by light in *Rhodopseudomonas capsulata* is mediated by membrane potential, but inhibition by oxygen is not, in 'Proceedings of the Sixth International Congress on Photosynthesis; Brussels', (Ed. Symbesma, C.), pp.

449–452, Mattinus Nijhoff/Dr.W.Junk Publishers, Dordrecht, The Netherlands.

[358] McEwan, A.G., Greenfield, A.J., Wetzstein, H.G., Jackson, J.B. and Ferguson, S.J. (1985) Nitrous oxide reduction by members of the family *Rhodospirillaceae* and the nitrous oxide reductase of *Rhodopseudomonas capsulata*. J. Bacteriol. 164, 823–830.

[359] McEwan, A.G., Cotton, N.P.J., Ferguson, S.J. and Jackson, J.B. (1985) The role of auxilliary oxidants in the maintenance of a balanced redox poise for photosynthesis in bacteria. Biochim. Biophys. Acta 810, 140–147.

[360] McEwan, A.G., Wetzstein, H.G., Meyer, O., Jackson, J.B. and Ferguson, S.J. (1987) The periplasmic nitrate reductase of *Rhodobacter capsulatus;* purification, characterisation and disticntion from a single reductase for trimethylamine-N-oxide, dimethylsulfoxide and chlorate. Arch. Microbiol. 147, 340–345.

[361] McEwan, A.G., Richardson, D.J., Jones, M.R., Jackson, J.B. and Ferguson, S.J. (1990) The functions and components of the anaerobic respiratory electron transport systems in *Rhodobacter capsulatus* in Molecular Biology of Membrane-Bound Complexes in Phototrophic Bacteria, FEMS Symp. 53, pp 433–442, Ed. Drews, G. and Dawes, E.A., Plenum, New York.

[362] Mchaourab, H.S., Pfenninger, S., Antholine, W.E., Felix, C.C., Hyde, J.S. and Kroneck, P.M.H. (1993) Multiquantum EPR of the mixed valence copper site in nitrous oxide reductase. Biophys. J. 64, 1576–1579.

[363] Meincke, M., Bock, E., Kastrau, D. and Kroneck, P.M.H. (1992) Nitrite oxidoreductase from *Nitrobacter hamburgensis*: redox centres and their catalytic role. Arch. Microbiol. 158, 127–131.

[364] Méjean, V., Iobbi-Nivol, C., Lepelletier, M., Giordano, G., Chippaux, M. and Pascal, M.-C. (1994) TMAO anaerobic respiration in *Escherichia coli*: involvement of the *tor* operon. Mol. Microbiol. 11, 1169–1179.

[365] Menon, N.K., Chatelus, C.Y., DerVartanian, M., Wendt, J.C., Shanmugam, K.T., Peck, H.D. Jr., and Przybyla, A.E. (1994) Cloning, sequencing, and mutational analysis of the *hyb* operon encoding *Escherichia coli* hydrogenase 2. J. Bacteriol. 176, 4416–4423.

[366] Messerschmidt, A. (1993) Blue copper oxidases. Adv. Inorg. Chem. 40, 121–185.

[367] Messerschmidt, A. and Huber, R. (1990) The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin: modelling and structural relationships. Eur. J. Biochem. 187, 341–352.

[368] Messerschmidt, A., Rossi, A., Landenstein, R., Huber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R. and Finazzi-Agró, A. (1989) X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini: analysis of the polypeptide fold and a model of the copper sites and ligands. J. Mol. Biol. 206, 513–529.

[369] Messerschmidt, A., Landenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. and Finazzi-Agró, A. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. J. Mol. Biol. 224, 179–205.

[370] Messerschmidt, A., Luecke, H. and Huber, R. (1993) X-ray structures and mechanistic implications of three functional derivatives of ascorbate oxidase from zucchini. Reduced, peroxide and azide forms. J. Mol. Biol. 230, 997–1014.

[371] Meunier, B. and Colson, A.-M. (1993) Insight into the interactions between subunits I and II of the cytochrome *c* oxidase of the yeast *Saccharomyces cerevisiae* by means of extragenic complementation. FEBS Lett. 335, 338–340.

[372] Meyer, J., Fujinaga, J., Gaillard, J. and Lutz, M. (1994) Mutated forms of the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* with noncysteinyl ligands to the iron-sulfur cluster. Biochemistry 33, 13642–13650.

[373] Michalski, W.P. and Nicholas, D.J.D. (1985) Molecular characterisation of a copper containing nitrite reductase from *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. Biochim. Biophys. Acta 828, 130–137.

[374] Michalski, W.P., Hein, D.H. and Nicholas, D.J.D. (1986) Purification and characterization of nitrous oxide reductase from *Rhodopseudomonas sphaeroides* f.sp. *denitrificans*. Biochim. Biophys. Acta 872, 50–60.

[375] Mikami, B. and Ida, S. (1984) Purification and properties of ferredoxin-nitrate reductase from the cyanobacterium *Plectonema boryanum*. Biochim. Biophys. Acta 791, 294–304.

[376] Miller, D.J. and Wood, P.M. (1983) The soluble oxidase of *Nitrosomonas europeae*. J. Gen. Microbiol. 129, 1645–1650.

[377] Minagawa, N. and Zumft, W.G. (1988) Cadmium-copper antagonism in the activation of periplasmic nitrous oxide reductase of copper-deficient cells from *Pseudomonas stutzeri*. Biol. Metals 1, 117–122.

[378] Mitra, S., Donovan, J.W. and Bersohn, R. (1981) Heat stabilization dependence on redox state of cytochrome cd_1 oxidase from *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. 98, 140–146.

[379] Moekhele, K., Tang, Y.J., Clark, M.A. and Ingraham, J.L. (1987) A *Pseudomonas stutzeri* outer membrane protein inserts copper into N_2O reductase. J. Bacteriol. 169, 5721–5726.

[380] Moir, J.W.B. (1993) 'Aspects of Electron Transport in *Thiosphaera pantotropha* and *Paracoccus denitrificans*', D.Phil. thesis, University of Oxford.

[381] Moir, J.W.B. and Ferguson, S.J. (1994) Properties of a *Paracoccus denitrificans* mutant deleted in cytochrome c_{550} indicate that a copper protein can substitute for this cytochrome in electron transport to nitrite, nitric oxide and nitrous oxide. Microbiology 140, 389–397.

[382] Moir, J.W.B., Baratta, D., Richardson, D.J. and Ferguson, S.J. (1993) The purification of a cd_1-type nitrite reductase from, and the absence of a copper-type nitrite reductase from, the aerobic denitrifier *Thiosphaera pantotropha*; the role of pseudoazurin as an electron donor. Eur. J. Biochem. 212, 377–385.

[383] Moore. G.R. and Pettigrew, G.W. (1990) Cytochromes *c*: Evolutionary, Structural and Physicochemical Aspects, Springer, Berlin.

[384] Morpeth, F.F. and Boxer, D.H. (1985) Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12 Biochemistry 24, 40–46.

[385] Moser, C.C., Keske, J.M., Warncke, K., Farid, R.S. and Dutton, P.L. (1992) Nature of biological electron transfer. Nature 355, 796–802.

[386] Nakahara, K., Tanimoto, T., Hatano, K.-i., Usuda, K. and Shoun, H. (1993) Cytochrome P-450 55A1 (P-450dNIR) acts as nitric oxide reductase employing NADH as the direct electron donor. J. Biol. Chem. 268, 8350–8355.

[387] Neidle, E.L. and Kaplan, S. (1992) *Rhodobacter sphaeroides rdxA*, a homolog of *Rhizobium meliloti fixG*, encodes a membrane protein which may bind cytoplasmic [4Fe-4S] clusters. J. Bacteriol. 174, 6444–6454.

[388] Newton, N. (1969) The two haem nitrite reductase of *Micrococcus denitrificans*. Biochim. Biophys. Acta 185, 316–331.

[389] Nicholas, D.J.D. and Wilson, P.J. (1964) A dissimilatory nitrate reductase from *Neurospora crassa*. Biochim. Biophys. Acta 86, 466–476.

[390] Nicholls, D.G. and Ferguson, S.J. (1992) Bioenergetics 2, Academic Press, London.

[391] Niehaus, F., Hantke, K. and Unden, G. (1991) Iron content and FNR-dependent gene regulation in *Escherichia coli*. FEMS Microbiol. Lett. 84, 319–324.

[392] Nishiyama, M., Suzuki, J., Kukimoto, M., Ohnuki, T., Horinouchi, S. and Beppu, T. (1993) Cloning and characterisation of a nitrite reductase gene from *Alcaligenes faecalis* and its expression in *Escherichia coli*. J. Gen. Microbiol. 139, 725–733.

[392a] Nohno, T., Noji, S., Taniguchi, S. and Saito, T. (1989) The *narX*

and *narL* genes endoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two-component regulatory genes. Nucleic Acids Res. 17, 2947–2957.

[393] Noji, S. and Taniguchi, S. (1987) Molecular oxygen controls nitrate transport of *Escherichia coli* nitrate-respiring cells. J. Biol. Chem. 262, 9441–9443.

[394] Noji, S., Nohno, T., Saito, T. and Taniguchi, S. (1989) The *narK* gene product participates in nitrate transport in *Escherichia coli* nitrate-respiring cells. FEBS Lett. 252, 139–143.

[395] Nordling, M., Young, S., Karlsson, B.G. and Lundberg, L.G. (1990) The structural gene for cytochrome c_{551} from *Pseudomonas aeruginosa* – the nucleotide sequence shows a location downstream of the nitrite reductase gene. FEBS Lett. 259, 230–232.

[396] Öblad, M., Selin, E., Malmström, B., Strid, L., Assa, R. and Malmström, B.G. (1989) Analytical characterization of cytochrome oxidase preparations with regard to metal and phopholipid contents, peptide composition and catalytic activity. Biochim. Biophys. Acta 975, 267–270.

[397] Ogasawara, N., Nakai, S. and Yoshikawa, H. (1994) Systematic sequencing of the 180 kilobase region of the *Bacills subtilis* chromosome containing the replication origin. DNA Res. 1, 1–14.

[397a] Ogawa, K.-I., Akagawa, E., Yamane, K., Sun, Z.-W., Lacelle, M., Zuber, P. and Nakano, M.M. (1995) The *nasB* operon and the *nasA* gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. J. Bacteriol. 177, 1409–1413.

[398] Ohshima, T., Sugiyama, M., Uozumi, N., Iijima, S. and Kobayashi, T. (1993) Cloning and sequencing of a gene encoding nitrite reductase from *Paracoccus denitrificans* and expression of the gene in *Escherichia coli*. J. Ferment. Bioeng. 76, 82–88.

[399] Okamura, K., Takamiya, K. and Nishimura, M. (1985) Photosynthetic electron transport is inoperative in anaerobic cells of *Erythrobacter species* strain OCH-114. Arch. Microbiol 129, 94–99.

[400] Omata, T., Andriesse, X. and Hirano, A. (1993) Idenitification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* sp. PCC7942. Mol. Gen. Genet. 236, 193–202.

[401] Oppenheim, D.S. and Yanofsky, C. (1980) Translational coupling during expression of the tryptophan operon in *Escherichia coli*. Genetics 95, 785–795.

[402] Ouzounis, C., Bork, P. and Sander, C. (1994) The modular structure of NifU proteins. Trends Biochem. Sci. 19, 199–200.

[403] Ozawa, S., Fujii, H. and Morishima, I. (1992) NMR studies of iron (II) nitrosyl π-cation radicals of octaethylchlorin and octaethylisobacteriochlorin as models for reation intermediate of nitrate reductase. J. Am. Chem. Soc. 114, 1548–1554.[404] Reference deleted.

[405] Page, L., Griffiths, L. and Cole, J.A. (1990) Different physiological roles of two independent pathways for nitrite reduction to ammonia by enteric bacteria. Arch. Microbiol. 154, 349–354.

[405a] Page, M.D. and Ferguson, S.J. (1995) Cloning and sequence analysis of *cycH* gene from *Paracoccus denitrificans*: the *cycH* gene product is required for assembly of all *c*-type cytochromes, including cytochrome c_1. Mol. Microbiol. 15, 307–318.

[406] Palmer, T., Vasishta, A., Whitty, P.W. and Boxer, D.H. (1994) Isolation of protein FA, a product of the *mob* locus required for molytbdenum cofactor biosynthesis in *Escherichia coli*. Eur. J. Biochem. 222, 687–692.

[407] Pan, L.P., Li, Z., Larsen, R. and Chan, S.I. (1991) The nature of Cu_x in cytochrome *c* oxidase. J. Biol. Chem. 266, 1367–1370.

[408] Parkinson, J.S. and Kofoid, E.C. (1992) Communication modules in bacterial signaling proteins. Annu. Rev. Genet. 26, 71–112.

[409] Parr, S.R., Barber, D. and Greenwood, C. (1977) The electron-transfer reaction between azurin and the cytochrome *c* oxidase from *Pseudomonas aeruginosa*. Biochem. J. 167, 447–455.

[410] Parsonage, D. and Ferguson, S.J. (1983) Reassessment of pathways of electron flow to nitrate reductase that are coupled to energy conservation in *Paracoccus denitrificans*. FEBS Lett. 153, 108–112.

[411] Parsonage, D., Greenfield, A.J. and Ferguson, S.J. (1985) The high affinity of *Paracoccus denitrificans* cells for nitrate as an electron acceptor. Analysis of possible mechanisms of nitrate and nitrite movement across the plasma membrane and the basis for inhibition by added nitrite of oxidase activity in permeabilised cells. Biochim. Biophys. Acta 807, 81–95.

[412] Parsonage, D., Greenfield, A.J. and Ferguson, S.J. (1986) Evidence that the energy conserving electron transport pathways to nitrate and cytochrome *o* branch at ubiquinone in *Paracoccus denitrificans*. Arch. Microbiol. 145, 191–196.

[413] Peakman, T., Crouzet, J., Mayaux, J., Busby, S., Mohan, S., Harborne, N., Wootton, J., Nicolson, R. and Cole, J.A. (1990) Nucleotide sequence, organisation and structural analysis of the products of genes in the *nirB-cysG* region of the *Escherichia coli* chromosome. Eur. J. Biochem. 191, 315–323.

[414] Pettigrew, G.W. and Moore, G.R. (1987) Cytochromes *c*: Biological Aspects, Springer-Verlag, Berlin.

[414a] Pierson, D.E. and Campbell, A. (1990) Cloning and nucleotide sequence of *bisC*, the structural gene for biotin sulfoxide reductase in *Escherichia coli*. J. Bacteriol. 172, 2194–2198.

[415] Pilato, R.S., Eriksen, K.A., Greaney, M.A., Steifel, E.I., Goswami, S., Kilpatrick, L., Spiro, T.G., Taylor, E.C. and Rheingold, A.L. (1991) Model complexes for molybdopterin-containing enzymes: preparation and crystallographic characterization of a molybdenum-ene-1-perthiolate (trithiolate) complex. J. Am. Chem. Soc. 113, 9372–9374.

[416] Pilato, R.S., Steifel, E.I., Eriksen, K., Greaney, M.A., Gea, Y., Taylor, E.C., Goswami, S., Kilpatrick, L., Spiro, T.G. and Rheingold, A.C. (1992)Pterins, quinoxalines, and metallo-ene-dithiolates; a synthetic approach to the molybdenum cofactor (Moco). Abs. Am. Chem. Soc. 204, INOR 175.

[417] Plunkett, G., Burland, V., Daniels, D.L. and Blattner, F.R. (1993) Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. Nucleic Acids Res. 21, 3391–3398.

[418] Pommier, J., Mandrand, M.A., Holt, S.E., Boxer, D.H. and Giordano, G. (1992) A second phenazine methosulfate-linked formate dehydrogenase enzyme in *Escherichia coli* Biochim. Biophys. Acta 1107, 305–313.

[419] Poth, M (1986) Dinitrogen production from nitrite by a *Nitrosomonas* isolate. Appl. Env. Microbiol. 52, 957–959.

[420] Poth, M. and Focht, D.D. (1985) ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*. An examination of nitrifier denitrification. Appl. Environ. Microbiol. 49, 1134–1141.

[420a] Preis, D., Weidner, U., Conzen, C., Azevedo, J.E., Nehls, U., Röhlen, D., Van der Pas, J., Sackmann, U., Schneider, R., Werner, S. and Weiss, H. (1991) Primary structures of two subunits of NADH:ubiquinone reductase from *N. crassa* concerned with NADH-oxidation. Relationsip to a soluble NAD-reducing hydrogenase of *Alcaligenes eutrophus*. Biochim. Biophys. Acta 1090, 133–138.

[421] Preisig, O., Anthamatten, D. and Hennecke, H.(1993) Genes for a novel, microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. USA 90, 3309–3313.

[422] Puustinen, A., Finel, M., Haltia, T., Gennis R.B. and Wikström, M. (1991) Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry 30, 3936–3942.

[423] Rabin, R.S. and Stewart, V. (1993) Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. 175, 3259–3268.

[424] Raito, M. and Wikström, M. (1994) An alternative cytochrome oxidase of *Paracoccus denitrificans* functions as a proton pump. Biochim. Biophys. Acta 1186, 100–106.

[425] Rajagopalan, K.V. (1988) Molybdopterin — problems and perspectives. Biofactors. 1, 273–278.

[426] Rajagopalan, K.V. (1991) Novel aspects of the biochemistry of the molybdenum cofactor. Adv. Enzymol. Rel. Areas Mol. Biol. 64, 215–290.

[427] Rajagopalan, K.V. and Johnson, J.L. (1992) The pterin molybdenum cofactors. J. Biol. Chem. 267, 10199–10202.

[428] Ramseier, T.M., Winteler, H.V. and Hennecke, H. (1991) Discovery and sequence analysis of bacterial genes involved in the biogenesis of *c*-type cytochromes. J. Biol. Chem. 266, 7793–7803.

[429] Rees, D.C. and Farrelly, D. (1990) Biological electron transfer, in The Enzymes (third edition): Mechanisms of Catalysis (Sigman, D.S. and Boyer, P.D., eds.), Vol. 19, pp. 37–97, Academic Press, San Diego, CA.

[430] Rehr, B. and Klemme, J.-H. (1986) Metabolic role and properties of nitrite reductase of nitrate-ammonifying marine *Vibrio* species. FEMS Microbiol. Lett. 35, 325–328.

[431] Richardson, D.J. (1988) Anaerobic Respiration in *Rhodobacter capsulatus*, Ph.D. thesis, University of Birmingham.

[432] Richardson, D.J., Kelly, D.K., Jackson, J.B., Ferguson, S.J. and Alef, K. (1986) Inhibitory effects of myxothiazol and 2-n-heptyl-4-hydroxyquinoline *N*-oxide on the auxiliary electron transport pathways of *Rhodobacter capsulatus*. Arch. Microbiol. 146, 159-165.

[433] Richardson, D.J., King, G.F., Kelly, D.K., McEwan, A.G., Ferguson, S.J. and Jackson, J.B. (1988) The role of auxilliary oxidants in maintaining redox balance during phototrophic growth of *Rhodobacter capsualtus* on propionate or butyrate. Arch. Microbiol. 150, 131–137.

[434] Richardson, D.J., McEwan, A.G., Jackson, J.B. and Ferguson, S.J. (1989) Electron transport pathways to nitrous oxide in *Rhodobacter* species. Eur. J. Biochem. 185, 659–669.

[435] Richardson, D.J., McEwan, A.G., Page, M.L.D., Jackson, J.B. and Ferguson, S.J. (1990) The identification of cytochromes involved in the transfer of electrons to the periplasmic NO_3^- reductase of *Rhodobacter capsulatus* and resolution of a soluble NO_3^--cytochrome c_{552} redox complex. Eur. J. Biochem. 194, 263–270.

[435a] Richardson, D.J., Bell, L.C., Moir, J.W.B. and Ferguson, S.J. (1994) A denitrifying strain of *Rhodobacter capsulatus*. FEMS Microbiol. Lett. 120, 323–328.

[436] Richardson, D.J., Bell, L.C., McEwan, A.G., Jackson, J.B. and Ferguson, S.J. (1991) Cytochrome c_2 is essential for electron transfer to nitrous-oxide reductase from physiological substrates in *Rhodobacter capsulatus* and can act as an electron donor to the reductase in vitro: correlation with photoinhibition studies. Eur. J. Biochem. 199, 677–683.

[437] Richardson, D.J. and Ferguson, S.J. (1992) The influence of carbon substrate on the activity of the periplasmic nitrate reductase in aerobically grown *Thiosphaera pantotropha*. Arch. Microbiol. 157, 535–537.

[438] Richterich, P., Lakey, N., Gryan, G., Jaehn, L., Mintz, L., Robison, K. and Church, G.M. (1993) Accession number U00008.

[439] Riester, J. (1989) Dictickstoffoxid-reduktase, ein Multikupferenzym in Denitrifizierenden Bakterien: Physikochemische Untersuchungen zur Struktur und Katalytischen Funktion, PhD thesis, University of Konstanz, Germany.

[440] Riester, J., Zumft, W.G. and Kroneck, P.M.H. (1989) Nitrous oxide reductase from *Pseudomonas stutzeri*: redox properties and spectroscopic characterization of different forms of the multicopper enzyme. Eur. J. Biochem. 178, 751–762.

[441] Ritz, D., Bott, M. and Hennecke, H. (1993) Formation of several bacterial *c*-type cytochromes requires a novel membrane-anchored protein that faces the periplasm. Mol. Microbiol. 9, 729–740.

[442] Rivers, S.L., McNairn, E., Blasco, F., Giordano, G. and Boxer, D.H. (1993) Molecular genetic analysis of the *moa* operon of *Escherichia coli* K-12 required for molybdenum cofactor biosynthesis. Mol. Micro. 8, 1071–1081.

[443] Robertson, L. (1988) Aerobic Denitrification and Heterotrophic Nitrification in *Thiosphaera pantotropha* and Other Bacteria, PhD thesis, Delft University of Technology, The Netherlands.

[444] Robertson, L.A. and Kuenen, J.G. (1983) *Thiosphaera pantotropha* gen. nov. sp. nov., a faculatively anaerobic, faculatively autotrophic sulfur bacterium. J.Gen.Microbiol. 129, 2847–2855.

[445] Robertson, L.A. and Kuenen, J.G. (1984) Aerobic denitrification: a controversy revived. Arch. Microbiol. 139, 351–354.

[446] Robertson, L.A. and Kuenen, J.G. (1988) Heterotrophic nitrification in *Thiosphaera pantotropha*: oxygen uptake and enzyme studies. J. Gen. Microbiol. 134, 857–863.

[447] Robertson, L.A. and Kuenen, J.G. (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria. Antonie van Leeuwenhoek. 57, 139–152.

[447a] Robertson, L.A. and Kuenen, J.G. (1992) Nitrogen removal from water and waste. Microbial control of pollution (Society for General Microbiology Symposium 48; Fry, J.C., Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson-Craik, I.A., eds.), Cambridge University Press.

[448] Robertson, L.A., Van Niel, E.W.J., Torremans, R.A.M. and Kuenen, J.G. (1988) Simultaneous nitrification and denitrification in aerobic chemostat cultures of *Thiosphaera pantotropha*. Appl. Env. Microbiol. 54, 2812–2818.

[449] Romero, A., Hoitink, C.W.G., Nar, H., Huber, R., Messerschmidt, A. and Canters, G.W. (1993) X-ray analysis and spectroscopic characterization of M121Q azurin. A copper site model for stellacyanin. J. Mol. Biol. 229, 1007–1021.

[450] Römermann, D. and Friedrich, B. (1985) Denitrification by *Alcaligenes eutrophus* H16 is plasmid dependent. J. Bacteriol. 162, 852–854.

[451] Römermann, D., Lohmeyer, M., Friedrich, C.G. and Friedrich, B. (1988) Pleiotropic mutants from *Alcaligenes eutrophus* defective in the metabolism of hydrogen, nitrate, urea, and fumarate. Arch. Microbiol. 149, 471–475.

[452] Roof, W.D., Horne, S.M., Young, K.D. and Young, R.(1994) *slyD*, a host gene required for fX174 lysis, is related to the FK506-binding protein family of peptidyl-prolyl *cis-trans*-isomerases. J. Biol. Chem. 269, 2902–2910.

[453] Rossi, M., Pollock, W.B.R., Reij, M.W., Keon, R.G., Fu, R. and Voordouw, G. (1993) The *hmc* operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough encodes a potential transmembrane redox protein complex. J. Bacteriol. 175, 4699–4711.

[453a] Rossmann, R., Sawers, G. and Böck, A. (1991) Mechanism of regulation of the formate-hydrogen lyase pathway by oxygen, nitrate and pH: definition of the formate regulon. Mol. Microbiol. 5, 2807–2814.

[454] Rost, B. and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. J. Mol. Biol. 232, 584–599.

[455] Rost, B. and Sander, C. (1994) Combining evolutionary information and neural networks to predict protein secondary structure. Proteins 19, 55–72.

[456] Rothery, R.A. and Weiner, J.E. (1991) Alteration of the iron-sulfur cluster composition of *Escherichia coli* dimethyl sulfoxide reductase by site-directed mutagenesis. Biochemistry 30, 8296–8305.

[457] Rowe, J.J., Ubbink-Kok, T., Molenaar, D., Konings, W.N. and Driessen, A.J.M. (1994) NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. Mol. Microbiol. 12, 579–586.

[457a] Rudd, K.E., Sofia, H.J., Koonin, E.V., Plunkett, G., III, Lazar, S. and Rouviere, P.E. (1995) A new family of peptidyl-prolyl isomerases. Trends Biochem. Sci. 20, 12–14.

[457b] Runswick, M.J., Gennis, R.B., Fearnley, I.M. and Walker, J.E. (1989) Mitochondrial NADH:ubiquinone reductase: complementary DNA sequence of the import precursor of the bovine 75 kDa subunit. Biochemistry 28, 9452–9459.

[458] Rydén, L.G. and Hunt, L.T. (1993) Evolution of protein complexity: the blue copper-containing oxidases and related proteins. J. Mol. Evol. 36, 41–66.

[459] Sabaty, M., Gans, P. and Verméglio, A. (1993) Inhibition of nitrate reduction by light and oxygen in *Rhodobacter sphaeroides* forma sp. *denitrificans*. Arch. Microbiol. 159, 153–159.

[460] Sabaty, M., Gagnon, J., and Verméglio, A. (1994) Induction by nitrate of cytoplasmic and periplasmic proteins in the photodenitrifier *Rhodobacter sphaeroides* forma sp. *denitrificans* under anaerobic or aerobic conditions. Arch. Microbiol. 162, 335–343.

[461] Saffarini, D.A. and Nealson, K.H. (1993) Sequence and genetic characterization of *etrA*, an *fnr* analog that regulates anaerobic respiration in *Shewanella putrefaciens* MR-1. J. Bacteriol. 175, 7938–7944.

[462] Sambasivarao, D. and Weiner, J.H. (1991) Dimethyl sulfoxide reductase of *Escherichia coli*: an investigation of function and assembly by use of in vivo complementation. J. Bacteriol. 173, 5935–5943.

[463] Samyn, B., Berks, B.C., Page, M.D., Ferguson, S.J. and Van Beeumen, J.J. (1994) Characterization and amino acid sequence of cytochrome *c*-550 from *Thiosphaera pantotropha*. Eur. J. Biochem. 219, 585–594.

[464] Sann, R., Kostka, S. and Friedrich, B. (1994) A cytochrome cd_1-type nitrite reductase mediates the first step of denitrification in *Alcaligenes eutrophus*. Arch. Microbiol. 161, 453–459.

[465] Santini, C.-L., Iobbi-Nivol, C., Romane, C., Boxer, D.H. and Giordano, G. (1992) Molybdoenzyme biosynthesis in *Escherichia coli*: in vitro activation of purified nitrate reductase from a *chlB* mutant. J. Bacteriol. 174, 7934–7940.

[466] Saraste, M. (1990) Structural features of cytochrome oxidase. Q. Rev. Biophys. 23, 331–366.

[467] Saraste, M. and Castresana, J. (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. FEBS Lett. 341, 1–4.

[468] Saraste, M., Holm, L., Lemieux, L., Lübben and Van der Oost, J. (1991) The happy family of cytochrome oxidases. Biochem. Soc. Trans. 19, 608–612.

[469] Satoh, T. (1981) Soluble dissimilatory nitrate reductase containing cytochrome *c* from a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. Plant Cell Physiol. 22, 443–452.

[470] Satoh, T., Hoshino, Y. and Kitamura, H. (1976) *Rhodopseudomonas sphaeroides* sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseodomonas sphaeroides*. Archiv. Microbiol. 108, 265–269.

[470a] Satoh, T., Hom, S.S.M. and Shanmugam, K.T. (1983) Production of nitrous oxide from nitrite in *Klebsiella pneumoniae*: mutants altered in nitrogen metabolism. J. Bacteriol. 155, 454–458.

[471] Sawada, E., Satoh, T. and Kitmura, M. (1978) Purification and properties of a dissimilatory nitrite reductase of a denitrifying phototrophic bacterium. Plant Cell Physiol. 19, 1339–1351.[472] Reference deleted.

[473] Sawhney, V. and Nicholas, D.J.D. (1978) Sulfide linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: purification and properties. J. Gen. Microbiol. 106, 119–128.

[474] Schauer, N.L. and Ferry, J.G. (1986) Composition of the coenzyme F_{420}-dependent formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. 165, 405–411.

[475] Schneider, B., Nies, A. and Freidrich, B. (1988) Transfer and expression of lithoautotrophy and denitrification to a host lacking these abilities. Appl. Environ. Microbiol. 54, 3173–3176.

[476] Scholes, C.P., Janakiraman, R. and Taylor, H. (1984) Temperature dependence of the electron spin–lattice relaxation rate from pulsed EPR of Cu_A and heme *a* in cytochrome *c* oxidase. Biophys. J. 45, 1027–1030.

[477] Schueler, A., Rueberg, S., Kraemer, M., Weidner, S. and Priefer, U. (1994) Accession number Z46239.

[478] Schumacher, W. and Kroneck, P.M.H. (1992) Anaerobic energy metabolism of the sulfur-reducing bacterium "Spirillum" 5175 during dissimilatory nitrate reduction to ammonia. Arch. Microbiol. 157, 464–470.

[478a] Schumacher, W., Hole, U. and Kroneck, P.M.H. (1994) Ammonia-forming cytochrome *c* nitrite reductase from *Sulfurospirillum deleyianum* is a tetraheme protein: new aspects of the molecular composition and spectroscopic properties. Biochem. Biophys. Res. Commun. 205, 911–916.

[479] Scott, R.A., Zumft, W.G., Coyle, C.L. and Dooley, D.M. (1989) *Pseudomonas stutzeri* N_2O reductase contains Cu_A-type sites. Proc. Natl. Acad. Sci. USA 86, 4082–4086.

[480] Sears, H. (1995) Ph.D. thesis, University of East Anglia, Norwich, England.

[481] Sears H.J., Ferguson, S.J., Richardson, D.J. and Spiro, S. (1993) The identification of a periplasmic nitrate reductase in *Paracoccus denitrificans*. FEMS Microbiol. Lett. 113, 107–112.

[482] Seki, S., Hattori, Y., Hasegawa, T., Haraguchi, H. and Ishimoto, M. (1987) Studies on nitrate reductase of *Clostridium perfringens*. IV. Identification of metals, molybdenum cofactor, and iron-sulfur cluster. J. Biochem. 101, 503–509.

[483] Shadle, S.E., Penner-Hahn, J.E., Schugar, H.J., Hedman, B., Hodgson, K.O. and Solomon, E.I. (1993) X-ray absorption spectroscopic studies of the blue copper site: metal and ligand K-edge studies to probe the origin of the EPR hyperfine splitting in plastocyanin. J. Am. Chem. Soc. 115, 767–776.

[484] Shanmugan, K.T., Stewart, V., Gunsalus, R.P., Boxer, D.H., Cole, J.A., Chippaux, M., DeMoss, J.A., Giordano, G., Lin, E.C.C. and Rajagopalan, K.V. (1992) Proposed nomenclature for the genes involved in molybdenum metabolism in *Escherichia coli* and *Salmonella typhimurium*. Mol. Microbiol. 8, 3452–3454.

[485] Shapleigh, J.P. and Payne W.J. (1985) Differentiation of cd_1 cytochrome and copper nitrite reductase production in denitrifiers. FEMS Lett. 26, 275–279.

[486] Shapleigh, J.P. and Payne. W.J. (1985) Nitric oxide-dependent proton translocation in various denitrifiers. J. Bacteriol. 163, 837–840.

[487] Sharrocks, A.D., Green, J. and Guest, J.R. (1990) In vivo and in vitro mutants of FNR, the anaerobic transcriptional regulator of *E.coli*. FEBS Lett. 270, 119–122.

[488] Shearer, G. and Kohl, D.H. (1988) Nitrogen isotopic fractionation and ^{18}O exchange in relation to the mechanism of denitrification of nitrite by *Pseudomonas stutzeri*. J. Biol. Chem. 263, 13231–13245.

[489] Shimada, H. and Orii, Y. (1975) The nitric oxide compounds of *Pseudomonas aeruginosa* nitrite reductase and their participation in nitrite reduction. FEBS Lett. 54, 237–240.

[490] Short, K.A. and Blakemore, R.P. (1989) The periplasmic superoxide dismutase of *Aquaspirrillum magnetacticum*. Arch. Microbiol. 152, 342–346.

[491] Shoun, H. and Tanimoto, T. (1991) Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrite reductase. J. Biol. Chem. 266, 11078–11082.

[492] Shoun, H., Suyama, W. and Yasui, T. (1989) Soluble, nitrate/nitrite-inducible cytochrome P-450 of the fungus, *Fusarium oxysporum*. FEBS Lett. 244, 11–14.

[493] Shoun, H., Kim, D-H., Uchiyama, H. & Sugiyama, J. (1992) Denitrification by fungi. FEMS Microbiol. Lett. 94, 277–282.

[494] Shuber, A.P., Orr, E.C., Recny, M.A., Schendel, P.F., May, H.D., Schauer, N.L. and Ferry, J.G. (1986) Cloning, expression and nucleotide sequence of the formate dehydrogenase genes from *Methanobacterium formicicum*. J. Biol. Chem. 261, 12942–12947.

[495] Siddiqui, R.A., Warnecke-Eberz, U., Hengsberger, A., Schneider,

B., Kostka, S. and Friedrich, B. (1993) Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. J. Bacteriol. 175, 5867–5876.
[496] Silver, S. and Walderhaug, M. (1992) Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56, 195–228.
[497] Silvestrini, M.C., Colosimo, A., Brunori, M., Walsh, T.A., Barber, D. and Greenwood, C. (1979) A re-evaluation of some basic structural and functional properties of *Pseudomonas* cytochrome oxidase. Biochem J. 183, 701–709.
[498] Silvestrini, M.C., Tordi, M.G. Colosimo, A., Antonino, E. and Brunori, M. (1982) The kinetics of electron transfer between *Pseudomonas aeruginosa* cytochrome c_{551} and its oxidase. Biochem. J. 203, 445–451.
[499] Silvestrini, M.C., Galeotti, C.L., Gervais, M., Schinina, E., Barra, D., Bossa, F. and Brunori, M. (1989) Nitrite reductase from *Pseudomonas aeruginosa*: sequence of the gene and the protein. FEBS Lett. 254, 33–38.
[500] Silvestrini, M.C., Tordi, M.G., Musci, G. and Brunori, M. (1990) The reaction mechanism of *Pseudomonas* nitrite reductase and nitrite. A stopped-flow and EPR study. J. Biol. Chem. 265, 11783–11787.
[500a] Smith, M.S. (1983) Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. Appl. Environ. Microbiol. 45, 1545–1547.
[501] Smith, G.B. and Tiedje, J.M. (1992) Isolation and characterisation of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Environ. Microbiol. 58, 376–384.
[501a] Snedecor, B., Chu, H. and Chen, E.Y. (1991) Selection, expression, and nucleotide sequencing of the glutamate dehydrogenase gene of *Peptostreptococcus asaccharolyticus*. J. Bacteriol. 173, 6162–6167.
[502] Snyder, S.W. and Hollocher, T.C. (1987) Purification and some characteristics of nitrous oxide reductase from *Paracoccus denitrificans*. J. Biol. Chem. 262, 6515–6525.[503]Snyder, S.W., Bayzylinski, D.A. and Hollocher, T.C. (1987) Loss of N_2O reductase activity as an explanation for poor growth of *Pseudomonas aeruginosa* on N_2O. Appl. Environ. Microbiol. 53, 387–391.
[504] Sodegren, E.J. and DeMoss, J.A. (1988) *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. J. Bacteriol. 170, 1721–1729.
[505] Sodergren, E.J., Hsu, P.-Y. and DeMoss, J.A. (1988) Roles of the *narJ* and *narI* gene products in the expression of nitrate reductase in *Escherichia coli*. J. Biol. Chem. 263, 16156–16162.
[506] Sofia, H.J., Burland, V., Daniels, D.L., Plunkett, G., III and Blattner, F.R. (1994) Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. Nucleic Acids Res. 22, 2576–2586.
[507] Solioz, M., Odermatt, A. and Krapf, R. (1994) Copper pumping ATPases: common concepts in bacteria and man. FEBS Lett. 346, 44–47.
[508] Solomon, E.I., Penfield, K.W. and Wilcox, D.E. (1983) Active sites in copper proteins: an electronic structure overview. Structure Bonding 53, 1–57.
[509] Solomon, E.I., Baldwin, M.J. and Lowery, M.D. (1992) Electronic structures of active sites in copper proteins: contributions to reactivity. Chem. Rev. 92, 521–542.
[510] Solomon, E.I. and Lowery, M.D. (1993) Electronic structure contributions to function in bioinorganic chemistry. Science 259, 1575–1581.
[511] Solomonson, L.P. and Barber, M.J. (1990) Assimilatory nitrate reductase: fuctional properties and regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 225–253.
[512] SooHoo, C.K. and Hollocher, T.C. (1991) Purification and characterization of nitrous oxide reductase from *Pseudomonas aeruginosa* strain P2. J. Biol. Chem. 266, 2203–2209.
[513] Spence, J.T., Barber, M.J. and Siegel, L.M. (1982) Determination of the stoichiometry of electron uptake and the midpoint reduction potentials of milk xanthine oxidase at 25°C by microcoulometry. Biochemistry 21, 1656–1661.
[514] Spence, J.T., Barber, M.J. and Solomonson, L.P. (1988) Stoichiometry of electron uptake and oxidation-reduction midpoint potentials of NADH:nitrate reductase. Biochem. J. 250, 921–923.
[515] Spence, J.T., Kipke, C.A., Enemark, J.H. and Sunde, R.A. (1991) Stoichiometry of electron uptake and the effect of anions and pH on the molybdenum and heme reduction potentials of sulfite oxidase. Inorg.Chem. 30, 3011–3015.
[516] Spiro, S. (1994) The FNR family of transcriptional regulators. Antonie van Leeuwenhoek 66, 23–36.
[517] Spiro, S. and Guest, J.R. (1990) FNR and its role in oxygen-regulated gene expresssion in *Escherichia coli* FEMS Microbiol. Lett. 75, 399–428.
[518] Spiro, S. and Guest, J.R. (1991) Adaptive responses to oxygen limitation in *Escherichia coli*. Trends Biochem. Sci. 16, 310–314.
[519] Spiro, T.G., ed. (1981) Copper Proteins, Metal Ions in Biology, Vol. 3, Wiley-Interscience, New York.
[520] Spiro, T.G., ed. (1985) Molybdenum Enzymes, Metal Ions in Biology, Vol. 7, Wiley Interscience, New York.
[521] Steffens, G.C.M., Biewald, R., and Buse, G. (1987) Cytochrome *c* oxidase is a three-copper, two haem *a* protein. Eur. J. Biochem. 164, 295–300.
[522] Steffens, G.C.M., Soulimane, T., Wolff, G. and Buse, G. (1993) Stoichiometry and redox behaviour of metals in cytochrome-*c* oxidase. Eur. J. Biochem. 213, 1149–1157.
[523] Stevens, T.H., Martin, C.T., Wang, H., Brudvig, G.W., Scholes, C.P. and Chan, S.I. (1982) The nature of the Cu_A site in cytochrome *c* oxidase. J. Biol. Chem. 257, 12106–12113.
[524] Stewart, V. (1988) Nitrate respiration in relation to faculative metabolism in enterobacteria. Microbiol. Rev. 52, 190–232.
[525] Stewart, V. (1993) Nitrate regulation of anaerobic respiratory gene expression in *Escherichia coli*. Mol. Microbiol. 9, 425–434.
[526] Stewart, V. and Cali, B.M. (1990) Genetic evidence that NarL function is not required for nitrate regulation of nitrate assimilation in *Klebsiella pneumoniae* M5a1. J. Bacteriol. 172, 4482–4488.
[527] Stewart, V. and MacGregor, C.H. (1982) Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, *chlG* loci. J. Bacteriol. 151, 788–799.
[528] Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Protein phosophorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. 53, 450–490.
[529] Stoltzenberg, A.M., Strauss, S.H. and Holm, R.H. (1981) Iron (II,III) -chlorin and -isobacteriochlorin complexes. Models of the heme prosthetic groups in nitrite and sulfite reductases: means of formation and spectroscopic and redox properties. J. Am. Chem. Soc. 103, 4763–4778.
[530] Stouthamer, A.H. (1988) Dissimilatory reduction of oxidized nitrogen compounds, in Biology of Anaerobic Microorganisms (Zehnder, A.J.B., ed.), pp. 245–303, Wiley, New York.
[531] Stouthamer, A.H. (1991) Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. J. Bioenerg. Biomembr. 23, 163–185.
[532] Stouthamer, A.H. (1992) Metabolic pathways in *Paracoccus denitrificans* and closely related bacteria in relation to phylogeny of prokaryotes. Antonie van Leewenhoek 61, 1–33.
[533] Sucheta, A., Ackrell, B.A.C., Cochran, B. and Armstrong, F.A. (1992) Diode-like behaviour of a mitochondrial electron-transport enzyme. Nature 356, 361–362.
[534] Sucheta, A., Cammack, R., Weiner, J. and Armstrong, F.A. (1993) Reversible electrochemistry of fumarate reductase immobilized on an electrode surface. Direct voltammetric observations of redox cnters and their participation in rapid catalytic electron transport. Biochemistry 32, 5455–5465.

[535] Sundermeyer-Klinger, H., Meyer, W., Warninghoff, B. and Bock, E. (1984) Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrate reductase system. Arch. Microbiol. 149, 153–158.

[535a] Suppmann, B. and Sawers, G. (1994) Isolation and characterization of hypophosphite-resistant mutants of *Escherichia coli*: idenitification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. Mol. Microbiol. 11, 965–982.

[535b] Suzuki, S., Kohzuma, T., Deligeer, Yamaguchi, K., Nakamura, N., Shidara, S., Kobayashi, K. and Tagawa, S. (1994) Pulse radiolysis studies on nitrite reductase from *Achromobacter cycloclastes* IAM 1013: evidence for intramolecular electron transfer from type I Cu to type 2 Cu. J. Am. Chem. Soc. 116, 11145–11146.

[536] Sykes, A.G. (1991) Active-site properties of the blue copper proteins. Adv. Inorg. Chem. 36, 377–407.

[537] Tanaka, Y., Fukomori, Y. and Yamanaka, T. (1983) Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium. Arch. Microbiol. 135, 265–271.

[538] Takamiya, K., Arata, H., Shioi, Y. and Doi, M. (1988) Restoration of the optimal redox state for the photosynthetic electron transfer system by auxiliary oxidants in an aerobic photosynthetic bacterium, *Erythrobacter* sp. OCh 114. Biochim. Biophys. Acta 935, 26–33.

[539] Takano, T., Dickerson, R., Schichman, S.A. and Meyer, T.E. (1979) Crystal data, molecular dimensions and molecular symmetry in cytochrome oxidase from *Pseudomonas aeruginosa*. J. Mol. Biol. 133, 185–188.

[540] Teraguchi, S. and Hollocher, T.C. (1989) Purification and some characteristics of a cytochrome *c*-containing nitrous oxide reductase from *Wolinella succinogenes*. J. Biol. Chem. 264, 1972–1979.

[541] Thomsen, J.K., Lønsmann Iversen, J.J. and Cox, R.P. (1993) Interactions between respiration and denitrification during growth of *Thiosphaera pantotropha* in continuous culture. FEMS Microbiol. Lett. 110, 319–324.

[542] Thomson, A.J., Greenwood, C., Peterson, J. and Barrett, C.P. (1986) Determination of the optical properties of Cu_A(II) in bovine cytochrome *c* oxidase using magnetic circular dichroism as an optical detector of paramagnetic resonance. J. Inorg. Biochem. 28, 195–205.

[543] Thöny-Meyer, L., Beck, C., Preisig, O. and Hennecke, H. (1994) The *ccoNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. Mol. Microbiol. 14, 705–716.

[543a] Thöny-Meyer, L., Ritz, D. and Hennecke, H. (1994) Cytochrome *c* biogenesis in bacteria: a possible pathway begins to emerge. Mol. Microbiol. 12, 1–9.

[544] Tiedje, J.M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium, in Biology of Anaerobic Microorganisms (Zehnder, A.J.B., ed.), pp. 178–244, Wiley-Interscience, New York.

[545] Timkovich, R., Dhesi, R., Martinkus, K.J., Robinson, M.K. and Rea, T.M. (1982) Isolation of *Paracoccus denitrificans* cytochrome cd_1: comparative kinetics with other nitrite reductases. Arch. Biochem. Biophys. 215, 47–58.

[546] Tordi, M.G., Silvestrini, M.C., Colosimo, A., Provencher, S. and Brunori, M. (1984) Circular dichroic properties and secondary structure of *Pseudomonas aeruginosa* soluble cytochrome c oxidase. Biochem. J. 218, 907–912.

[547] Trageser, M. and Unden, G. (1988) Role of cysteine residues and of metal ions in the regulatory functioning of FNR, the transcriptional regulator of anaerobic respiration in *Escherichia coli*. Mol. Microbiol. 3, 593–599.

[548] Trandinh, C.C., Pao, G.M. and Saier, M.H., Jr. (1992) Structural and evolutionary relationships among the immunophilins: two ubiquitous families of peptidyl-prolyl *cis–trans* isomerases. FASEB J. 6, 3410–3420.

[549] Trieber, C.A., Rothery, R.A. and Weiner, J.H. (1994) Multiple pathways of electron transfer in dimethyl sulfoxide reductase of *Escherichia coli*. J. Biol. Chem. 269, 7103–7109.

[550] Tsay, Y.-F., Schroeder, J.I., Feldmann, K.A. and Crawford, N.M. (1993) The herbicide sensitivity gene *CHL1* of arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72, 705–713.

[551] Tuan, D.F.-T and Hoffmann, R. (1985) N vs. O linkage and σ vs. π bonding in transition-metal complexes of N_2O and NCO^-. J. Am. Chem. Soc. 24, 871–876.

[552] Turk, T. and Hollocher, T.C. (1992) Oxidation of dithiothreitol during turnover of nitric oxide reductase: evidence for generation of nitroxyl with the enzyme from *Paracoccus denitrificans*. Biochem. Biophys. Res. Commun. 183, 983–988.

[553] Tyson, K.L., Bell, A.I., Cole, J.A. and Busby, S.J.W. (1993) Definition of nitrite and nitrate response elements at the anaerobically inducible *Escherichia coli nirB* promoter: interactions between FNR and NarL. Mol. Microbiol. 7, 151–157.

[553a] Tyson, K.L., Cole, J.A. and Busby, S.J.W. (1994) Nitrite and nitrate regulation at the promoters of two *Escherichia coli* operons encoding nitrite reductase: identification of common target heptamers for both NarP- and NarL-dependent regulation. Mol. Microbiol. 13, 1045–1055.

[554] Unden, G. and Trageser, M. (1991) Oxygen regulated gene expression in *Escherichia coli*: control of anaerobic respiration by the FNR protein. Antonie van Leeuwenhoek 59, 65–76.

[554a] Unden, G., Trageser, M. and Duchêne, A. (1990) Effect of positive redox potentials (> +400 mV) on the expression of anaerobic respiratory enzymes of *Escherichia coli*. Mol. Microbiol. 4, 315–319.

[555] Unkles, S.E., Hawker, K.L., Grieve, C., Campbell, E.I., Montague, P. and Kinghorn, J.R. (1991) *crnA* encodes a nitrate transporter in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 88, 204–208.

[556] Urata, K. and Satoh, T. (1985) Mechanism of nitrite reduction to nitrous oxide in a photodenitrifier, *Rhodopseudomonas sphaeroides* f.sp. *denitrificans*. Biochim. Biophys. Acta 841, 201–207.

[557] Urata, K. and Satoh, T. (1987) Specific inhibition of electron transfer for nitrate reduction by a low concentration of cyanide in *Rhodobacter sphaeroides* f.sp. *denitrificans*. Plant Cell Physiol. 28, 1351–1355.

[558] Urata, K., Shimada, K. and Satoh, T. (1982) Periplasmic location of nitrous oxide reductase in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma. sp. *denitrificans*. Plant Cell Physiol. 23, 1121–1124.

[559] Urata, K. and Satoh, T. (1991) Enzyme localization and orientation of the active site of dissimilatory nitrite reductase from *Bacillus firmus*. Arch. Microbiol. 156, 24–27.

[560] Van de Kamp, M., Silvestrini, M.C., Brunori, M., Beeuman, J.V. Hali, F.C. and Canters, G.W. (1990) Involvement of the hydrophobic patch of azurin in the electron-transfer reactions with c_{551} and nitrite reductase. Eur. J. Biochem. 194, 109–118.

[561] Van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R.B., Aasa, R., Pascher, T., Malmström, B.G. and Saraste, M. (1992) Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the *E.coli* cytochrome *o* quinol oxidase complex. EMBO J. 11, 3209–3217.

[562] Van der Oost, J., De Boer, A.P.N., Gier, J.-W.L., Zumft, W.G., Stouthamer, A.H. and Van Spanning, R.J.M. (1994) The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. FEMS Microbiol. Lett. 121, 1–10.

[563] Van der Palen, C.J.N.M., Slotboom, D.-J., Jongejan, L., Reijn-

ders, W.N.M., Harms, N., Duine, J.A. and Van Spanning, R.J.M. (1995) Mutational analysis of *mau* genes involved in methylamine metabolism in *Paracoccus denitrificans*. Eur. J. Biochem. 230, 860–871.

[564] Van Spanning, R.J.M., Wansell, C., Harms, N., Oltman, L.F. and Stouthamer, A. (1990) Mutagenesis of the gene encoding cytochrome c_{550} of *Paracoccus denitrificans* and analysis of the resultant physiological effects. J.Bacteriol. 172, 986–996. A correction to the amino acid sequence of cytochrome c_{550} reported in this paper can be found in J. Bacteriol. 172, 3534.[565] Reference deleted.

[566] Van't Riet, J., Wientjes, F.B., Van Doorn, J. and Planta, R.J. (1979) Purification and characterization of the respiratory nitrate reductase of *Bacillus licheniformis*. Biochim. Biophys. Acta 576, 347–360.

[567] Varghese, J.N., Laver, W.G. and Colman, P.M. (1983) Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. Nature 303, 35–40.

[568] Vellieux, F.M.D., Huitema, F., Groendijk, H., Kalk, K.H., Frank, J., Jongejan, J.A., Duine, J., Petratos, K., Drenth, J. and Hol, W.G.J. (1989) Structure of quinoprotein methylamine dehydrogenase at 2.25 Å resolution. EMBO J. 8, 2171–2178.

[568a] Verplaetse, J., Van Tornhout, P., Defreyn, G., Witters, R. and Lontie, R. (1979) The reaction of nitrogen monoxide and of nitrite with deoxyhaemocyanin and methaemocyanin of Helix pomatia. Eur. J. Biochem. 95, 327–331.

[569] Viebrock, A. and Zumft, W.G. (1987) Physical mapping of transposon Tn*5* insertions defines a gene cluster functional in nitrous oxide respiration by *Pseudomonas stutzeri*. J. Bacteriol. 169, 4577–4580.

[570] Viebrock, A. and Zumft, W.G. (1988) Molecular cloning, heterologous expresion, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. J. Bacteriol. 170, 4658–4668.

[571] Vincent, S.P. (1979) Oxidaton-reduction potentials of molybdenum and iron-sulfur centres in nitrate reductase from *Escherichia coli*. Biochem. J. 177, 757–759.

[571a] Vincent, S.P. and Bray, R.C. (1978) Electron-paramagnetic-resonance studies on nitrate reductase from *Escherichia coli* K12. Biochem.J. 171, 639–647.

[572] Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E.C., Frey, M. and Fontecilla-Camps, J.C. (1995) Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. Nature 373, 580–587.

[572a] Völkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A. and Stetter, K.O. (1993) *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic Archaeum. Appl. Environ. Microbiol. 59, 2918–2926.

[573] Von Heijne, G. (1985) Signal sequences. The limits of variation. J. Mol. Biol. 184, 99–105.

[574] Von Heijne, G. (1986) A new method for predicting signal sequence cleavage. Nucleic Acids Res. 14, 4683–4690.

[575] Von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. 225, 487–494.

[576] Von Wachenfeldt, C., De Vries, S. and Van der Oost, J. (1994) The Cu_A site of the *caa*$_3$-type oxidase of *Bacillus subtilis* is a mixed-valence binuclear copper centre. FEBS Lett. 340, 109–113.

[577] Voordouw, G. (1992) Evolution of hydrogenase genes. Adv. Inorg. Chem. 38, 397–422.

[578] Walker, J.E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. Q. Rev. Biophys. 25, 253–324.

[579] Wallace, B.J. and Young, I.G. (1977) Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with a *ubiA*$^-$ *menA*$^-$ double quinone mutant. Biochim. Biophys. Acta 461, 84–100.

[580] Walsh, T.A., Johnson, M.K., Greenwood, C., Barber, D., Springall, J.P. and Thomson, A.J. (1979) Some magnetic properties of *Pseudomonas* cytochrome oxidase. Biochem. J. 177, 29–39.

[581] Warnecke-Eberz, U. and Friedrich, B. (1993) Three nitrate reductase activities in *Alcaligenes eutrophus*. Arch. Microbiol. 159, 405–409.

[582] Weeg–Aerssens, E., Tiedje, J.M. and Averill, B.A. (1988) Evidence from isotope labelling studies for a sequential mechanism of dissimilatory nitrite reduction. J. Am. Chem.Soc. 109, 7214–7215.

[583] Weeg-Aerssens, E., Wu, W., Ye, R.W., Tiedje, J.M. and Chang, C.K. (1991) Purification of cytochrome *cd*$_1$ nitrite reductase from *Pseudomonas stutzeri* JM300 and reconstitution with native and synthetic heme d_1. J. Biol. Chem. 266, 7496–7502.

[584] Wehrfritz, J.-M., Reilly, A., Spiro, S. and Richardson, D.J. (1993) Purification of hydroxylamine oxidase from *Thiosphaera pantotropha*. Identification of electron acceptors that couple heterotrophic nitrification to aerobic denitrification. FEBS Lett. 335, 246–250.

[585] Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H. and Weiss, H. (1993) The gene locus of the proton-translocating NADH:ubiquinone oxideoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I. J. Mol. Biol. 233, 109–122.

[586] Weiner, J.H., Rothery, R.A. Sambasivarao, D. and Trieber, C.A. (1992) Molecular analysis of the dimethylsulfoxide reductase: a complex iron-sulfur molybdoenzyme of *Escherichia coli*. Biochim. Biophys. Acta 1102, 1–18.

[587] Westmoreland, T.D., Wilcox, D.E., Baldwin, M.J., Mims, W.B. and Solomon, E.I. (1989) Detailed spectroscopic analysis of half-met hemocyanins: mixed-valent contributions to electronic properties and structure. J. Am. Chem. Soc. 111, 6106–6123.

[588] Wharton, D.C. and Weintraub, S.T. (1980) Identification of nitric oxide and nitrous oxide as products of nitrite reduction by *Pseudomonas* cytochrome oxidase (nitrite reductase). Biochem. Biophys. Res. Commun. 97, 236–242.

[589] White, W.B. and Ferry, J.G. (1992) Identification of formate dehydrogenase-specific mRNA species and nucleotide sequence of the *fdhC* gene of *Methanobacterium formicicum*. J. Bacteriol. 174, 4997–5004.

[590] White, W.B. and Ferry, J.G. (1992) Unpublished sequence, accession number M64798.

[591] White, W.B. and Ferry, J.G. (1992) Unpublished sequence, accession number M84821.

[592] Wikström, M., Krab, K. and Saraste, M. (1981) 'Cytochrome Oxidase–a Synthesis', Academic Press, London.

[592a] Wikström, M., Bogachev, A., Finel, M., Morgan, J.E., Puustinen, A., Raitio, M., Verkhovskaya, M., and Verkhovsky, M.I. (1994) Mechanism of proton translocation by the respiratory oxidases. The histidine cycle. Biochim. Biophys. Acta 1187, 106–111.

[593] Willison, J.C. (1990) Derivatives of *Rhodobacter capsulatus* strain AD2 cured of their endogenous plasmicd are unable to utilize nitrate. FEMS Microbiol. Lett. 66, 23–28.

[594] Wittung, P., Källebring, B. and Malmström (1994) The cupredoxin fold is found in the soluble Cu_A and CyoA domains of two terminal oxidases. FEBS Lett. 349, 286–288.

[595] Wood, P.M. (1978) Interchangeable copper and iron proteins in algal photosynthesis. Eur. J. Biochem. 87, 9–19.

[596] Wood, P.M. (1986) Nitrification as a bacterial energy source, in, 'Nitrification', pp. 63–78, (Prosser, J., ed.), special publications of the Society for General Microbiology 20, Oxford, IRL Press.

[597] Wootton, J.C., Nicolson, R.E., Cock, J.M., Walters, D.E., Burke, J.F., Doyle, W.A. and Bray, R.C. (1991) Enzymes depending on the pterin molybdenum cofactor: sequence families, spectroscopic properties of molybdenum and possible cofactor-binding domains. Biochim. Biophys. Acta 1057, 157–185.

[598] Wu, J.-Y., Siegel, L.M. and Kredich, N.M. (1991) High-level expression of *Escherichia coli* NADPH-sulfite reductase: requirement for a cloned *cysG* plasmid to overcome limiting siroheme cofactor. J. Bacteriol. 173, 325–333.

[599] Wu, W. and Chang, C.K. (1987) Structure of dioneheme. Total synthesis of the green heme prosthetic group in cytochrome cd_1 dissimilatory nitrite reductase. J. Am. Chem. Soc. 109, 3149–3150.

[600] Xia, Z.X., Dai, W.W., Xiong, J.P., Hao, Z.P., Davidson, V.L., White, S. and Matthews, F.S. (1992). The 3-dimensional structures of methanol dehydrogenase from 2 methylotrophic bacteria at 2.6 Å resolution. J. Biol. Chem. 267, 22289–22297.

[601] Xiao, Z., Young, C.G., Enemark, J.H. and Wedd, A.G. (1992) A single model displaying all the important centers and processes involved in catalysis by molybdoenzymes containing $[Mo^{VI}O_2]^{2+}$ active sites. J. Am. Chem. Soc. 114. 9194–9195.

[601a] Xu, X., Matsuno-Yagi, A. and Yagi, T. (1992) Structural features of the 66-kDa subunit of the energy-transducing NADH-ubiquinone oxidoreductase (NDH-1) of *Paracoccus denitrificans*. Arch. Biochem. Biophys. 296, 40–48.

[602] Yamanaka, T. (1992) The Biochemistry of Bacterial Cytochromes, Japan Scientific Societies Press/Springer, Berlin.

[603] Yamanaka, T., Ota, A. and Okunuki, K. (1961) A nitrite reducing system reconstructed with purified cytochrome components of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 53, 294–308.

[604] Ye, R.W., Arunakumari, A., Averill, B.A. and Tiedje, J.M. (1992) Mutants of *Pseudomonas fluorescens* deficient in dissimilatory nitrite reduction are also altered in nitric oxide reduction. J. Bacteriol. 174, 2560–2564.

[605] Ye, R.W., Averill, B.A. and Tiedje, J.M. (1992) Characterization of Tn5 mutants deficient in dissimilatory nitrite reduction in *Pseudomonas* sp. strain G-179, which contains a copper nitrite reductase. J. Bacteriol. 174, 6653–6658.

[606] Ye, R.W., Fries, M.R., Bezborodnikov, S.G., Averill, B.A. and Tiedje, J.M. (1993) Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. Appl. Environ. Microbiol. 59, 250–254.

[607] Yokota, S., Urata, K. and Satoh, T. (1984) Redox properties of membrane-bound *b*-type cytochromes and a soluble *c*-type cytochrome of nitrate reductase in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. J. Biochem. 95, 1535–1541.

[608] Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T. and Caughey, W.S. (1988) Crystalline cytochrome *c* oxidase of bovine heart mitochondrial membrane: composition and X-ray diffraction studies. Proc. Natl. Acad. Sci. USA 85, 1354–1358.

[609] Yoshimara, T. Iwasaki, H., Shidra, S. Nakahara, A. and Matsubara, J. (1988) Nitric oxide complex of cytochrome *c′* in cells of denitrifying bacteria. J. Biochem. 103, 1016–1019.[610] Reference deleted.

[611] Zambrano, M.M. and Kolter, R. (1993) *Escherichia coli* mutants lacking NADH dehydrogenase I have a competitive disadvantage in stationary phase. J. Bacteriol. 175, 5642–5647.

[612] Zhang, C.-S. and Hollacher, T.C. (1993) The reaction of reduced cytochromes *c* with nitrous oxide reductase of *Wolinella succinogenes*. Biochim. Biophys. Acta 1142, 253–261.

[613] Zhang, C., Jones, A.M., Hollocher, T.C. (1992) An apparently allosteric effect involving N_2O with the nitrous oxide reductase from *Wolinella succinogenes*. Biochem. Biophys. Res. Commun. 187, 135–139.

[614] Zinoni, F., Birkmann, A., Stadtman, T.C. and Böck, A. (1986) Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 83, 4650–4654.

[615] Zumft, W.G. (1992) The denitrifying prokaryotes, in The Prokaryotes, 2nd. Edn. (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., eds.), pp. 554–582, Springer, Berlin.

[616] Zumft, W.G. (1993) The biological role of nitric oxide in bacteria. Arch. Microbiol. 160, 253–264.[617] Reference deleted.

[618] Zumft, W.G. and Kroneck, P.M.H. (1990) Metabolism of nitrous oxide, in Denitrification in Soil and Sediment (Revsbech, N.P. and Sfrensen, J., eds.), pp. 37–55, Plenum Press, New York.

[619] Zumft, W.G., Coyle, C.L. and Frunzke, K. (1985) The effect of oxygen on chromatographic behaviour and properties of nitrous oxide reductase. FEBS Lett. 183, 240–244.

[620] Zumft, W.G., Döhler, K. and Körner, H. (1985) Isolation and characterization of transposon Tn*5*-induced mutants of *Pseudomonas perfectomarina* defective in nitrous oxide respiration. J. Bacteriol. 163, 918–924.

[621] Zumft, W.G., Gotzman, D.J. and Kroneck, P.M.H. (1987) Type 1 blue copper proteins constitute a respiratory nitrite reducing system in *Pseudomonas aureofaciens*. Eur. J. Biochem. 168, 301–307.

[622] Zumft, W.G. Döhler, K., Körner, H., Löchelt, S., Viebrock, A. and Frunzke, K. (1988) Defects in cytochrome cd_1-dependent nitrite respiration of transposon *Tn*5-induced mutants from *Pseudomonas stutzeri*. Arch. Microbiol. 149, 492–498.

[623] Zumft, W.G., Viebrock-Sambale, A. and Braun, C. (1990) Nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*: genes for copper-processing and properties of the deduced products, including a new member of the family of ATP/GTP-binding proteins. Eur. J. Biochem. 192, 591–599.

[624] Zumft, W.G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B. and Schneider, B. (1992) Derived amino acid sequences of the *nos*Z gene (respiratory N_2O reductase) from *Alcaligenes eutrophus, Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper binding residues; implications for the Cu_A site of N_2O reductase and cytochrome *c* oxidase. Eur. J. Biochem. 208, 31–40.

[625] Zumft, W.G., Braun, C. and Cuypers, H. (1994) Nitric oxide reductase from *Pseudomonas stutzeri*. Primary structure and gene organisation of a novel bacterial cytochrome *bc* complex. Eur. J. Biochem. 219, 481–490.