A new assay for nitric oxide reductase reveals two conserved glutamate residues form the entrance to a proton-conducting channel in the bacterial enzyme

Faye H. THORNDYCROFT, Gareth BUTLAND, David J. RICHARDSON and Nicholas J. WATMOUGH¹

Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norfolk NR4 7TJ, U.K.

A specific amperometric assay was developed for the membrane-bound NOR [NO (nitric oxide) reductase] from the model denitrifying bacterium *Paracoccus denitrificans* using its natural electron donor, pseudoazurin, as a co-substrate. The method allows the rapid and specific assay of NO reduction catalysed by recombinant NOR expressed in the cytoplasmic membranes of *Escherichia coli*. The effect on enzyme activity of substituting alanine, aspartate or glutamine for two highly conserved glutamate residues, which lie in a periplasmic facing loop between transmembrane helices III and IV in the catalytic subunit of NOR, was determined using this method. Three of the substitutions (E122A, E125A and E125D) lead to an almost complete loss of NOR activity. Some activity is retained when either Glu¹²²

or Glu¹²⁵ is substituted with a glutamine residue, but only replacement of Glu¹²² with an aspartate residue retains a high level of activity. These results are interpreted in terms of these residues forming the mouth of a channel that conducts substrate protons to the active site of NOR during turnover. This channel is also likely to be that responsible in the coupling of proton movement to electron transfer during the oxidation of fully reduced NOR with oxygen [U. Flock, N. J. Watmough and P. Ådelroth (2005) *Biochemistry* 44, 10711–10719].

Key words: cupredoxin, denitrification, haem-copper oxidase, nitric oxide reductase, nitrous oxide, proton movement.

INTRODUCTION

N₂O (nitrous oxide) along with CO₂ (carbon dioxide) and methane is one of the three most important greenhouse gases. Although the volumes of N₂O discharged into the atmosphere are lower than those of the other two gases, the ability of N₂O to contribute to the global warming process is approx. 300 times greater than that of CO₂ because it is so difficult to break down. On a global scale the largest source of anthropogenic N₂O emissions is agriculture (>70%), because of the increased use in recent decades of artificial fertilizers that contain soluble nitrate (NO₃[−]) and nitrite (NO₂[−]). These are converted by some bacteria and fungi to atmospheric nitrogen in a process known as denitrification. Denitrification requires four specialist enzyme activities to catalyse the sequential conversion of nitrite ions to dinitrogen:

Fungi apparently lack an enzyme that can further reduce N₂O to dinitrogen and under aerobic conditions most denitrifying bacteria produce N₂O instead of dinitrogen partly as a consequence of the oxygen lability of nitrous oxide reductase [1]. Hence organisms that can reduce nitrate to N₂O exert some influence over the process of global warming, and the respiratory NOR (NO reductase) [2,3] which catalyses the final reaction in this pathway in bacteria is the particular focus of the present paper.

Three types of respiratory NOR have been reported in Eubacteria [3–6], but most detailed biochemical and spectroscopic information about NOR has been obtained using the cytochrome *c*-dependent NOR purified from *Paracoccus denitrificans* [7–9].

Complementary biochemical information has been reported for the closely related enzymes isolated from *Pseudomonas stutzeri* [10] and *Paracoccus halodenitrificans* [11]. The cytochrome *c*-dependent NORs are usually purified as a two-subunit complex, NorBC [7,12]. The NorC subunit is a mono-haem *c*-type cytochrome that possesses an N-terminal transmembrane helix that anchors the haem domain to the periplasmic face of the cytoplasmic membrane. In *P. denitrificans* the function of NorC is to serve as the immediate electron acceptor for two periplasmic electron donors: pseudoazurin and cytochrome *c*₅₅₀. The catalytic subunit, NorB, whilst functionally distinct, is structurally homologous with the catalytic subunit (subunit I) of the respiratory haem-copper oxidases (e.g. cytochrome *aa*₃ oxidase) [13,14]. Haemcopper oxidases catalyse the four-electron reduction of dioxygen to water and terminate the aerobic respiratory chains of both bacteria and mitochondria. Much of the energy liberated in this exogonic reaction is conserved by moving protons across the membrane to contribute to the electrochemical proton gradient which is subsequently used to drive ATP synthesis [15,16].

The bioenergetics of succinate-dependent NO respiration in membrane vesicles of *Rhodobacter capsulatus* was studied using a carotenoid bandshift assay to monitor formation of a transmembrane electrochemical potential [17]. Inhibiting the *bc*₁ complex of the main respiratory chain with myxothiazol abolished both succinate-dependent NO reduction and the generation of a transmembrane potential. However using ascorbate and the mediator TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) to donate electrons directly to NOR in membranes inhibited with myxothiazol restored NOR activity, but a transmembrane potential was not generated. These observations led the authors to conclude that NOR is not electrogenic and that NO was reduced close to the periplasmic surface [17]. However the subsequent

Abbreviations used: DAD, 2,3,5,6-tetramethylphenylenediamine; NO, nitric oxide; N₂O, nitrous oxide; NOR, NO reductase; PAWS, pre-antibody wash solution; PMS, phenazine methosulfate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

¹ To whom correspondence should be addressed (email n.watmough@uea.ac.uk).

discovery of the relationship between NorB and subunit I of the haem-copper oxidases requires that this interpretation is re-evaluated.

Subunit I of the haem-copper oxidases comprises a minimum of 12 transmembrane helices that bind three metal centres: a magnetically isolated bis-histidine co-ordinated low-spin haem and an active site, comprised of a high-spin haem and a copper ion (Cu_B) site magnetically coupled to form a dinuclear centre [18,19]. NorB shares the same overall architecture and the seven conserved histidine residues responsible for ligating the metal centres in subunit I of the haem-copper oxidases are completely conserved in NorB [13,14]. The principle difference between NOR and the haem-copper oxidases is in the elemental composition of the dinuclear centre which in NOR contains an iron (Fe_B) rather than a copper (Cu_B) ion.

Movement of protons to the dinuclear centre of haem-copper oxidases, which lies buried in the hydrophobic interior of the membrane, needs to be assisted by pathways formed by ionizable amino acid side-chains. Structural studies of haem-copper oxidases identified two pathways, known as the D-channel and the K-channel, that could conduct protons from the negative face of the membrane towards the dinuclear centre [20–22]. The specific roles of these two channels have been elucidated by site-directed mutagenesis. The K-channel contains a conserved lysine residue (Lys^{362} in the *Rhodobacter sphaeroides* cytochrome *c* oxidase) and appears to be used to transfer protons required for the (re)reduction of the dinuclear centre during turnover [23,24]. The D-channel is named after a conserved aspartate residue (Asp^{132}) at its entrance which when changed to either an alanine residue (D132A) or the corresponding amide (D132N) by site-directed mutagenesis results in a loss of activity [25].

The residues that contribute to the D- and K-channels in subunit I of the haem-copper oxidases are not conserved in NorB [3,26]. This is not surprising as NOR has no requirement to take protons from the cytoplasm for catalysis (see above). However, homology modelling of NorB using the amino acid sequence of NorB from *Pseudomonas stutzeri* and the co-ordinates of the X-ray structure of *P. denitrificans* cytochrome *aa*₃ [27] suggests that the haem: Fe_B dinuclear centre, at which NO is reduced to N_2O , is buried in the lipid bilayer. Consequently NorB is likely to contain structure(s) that allow protons consumed in catalysis to move from the periplasmic face of the enzyme to the buried active site. Consistent with this notion is a recent report that clearly demonstrates proton-coupled electron transfer to the active site of NOR in the reaction of the fully reduced enzyme with molecular oxygen [28].

Multiple sequence alignments of the derived amino acid sequences of NorB from both cytochrome *c*- and quinone-dependent NORs have shown there to be five glutamate residues that are highly conserved in the NorB subunit, but which are not found in the orthodox haem-copper oxidases [3,29]. We have proposed that some of these glutamate residues may form a proton channel leading from the periplasm to the active site. Secondary structure analysis places the glutamate residues at positions 122 and 125 in a periplasmic facing loop between transmembrane helices III and IV and so ideally placed to accept protons from the periplasm. The conserved glutamate residues at positions 198 (helix VI), 202 (helix VI) and 267 (helix VIII) are all candidates for terminating the channel close to the active site. Previous work in this laboratory has shown that substitution of two of these residues, Glu^{125} and Glu^{198} , with alanine gives rise to catalytically inactive NOR [29].

One problem in assessing electron transfer to NOR is that although NOR activity can easily be measured using an amperometric steady-state assay, the assay conditions reported in the literature have used different mediators either singly or in

combination with the chemical electron donor ascorbate. For example PMS (phenazine methosulfate) [30], TMPD [7] and a combination of horse heart cytochrome *c* plus TMPD [31], or DAD (2,3,5,6-tetramethylphenylenediamine) [32] have all been used to mediate electron transfer to NOR. Thus far no single definitive method for assaying NOR using a physiologically relevant electron donor has been reported.

The aim of the work described in the present paper was threefold. First, we wanted to devise a reliable steady-state assay of NOR activity using a physiologically relevant species as the immediate electron donor. The second goal was to optimize the assay to allow assay of recombinant NOR expressed in membranes of *Escherichia coli*, so that site-directed mutants could be rapidly and reliably screened for changes in catalytic activity. Finally, we wished to use this system to determine whether mutations in the two conserved glutamate residues, Glu^{122} and Glu^{125} , that lie in a periplasmic loop between helix III and helix IV, lead to changes in steady-state activity that would be commensurate with them forming the entrance to a channel that might couple the movement of protons from the periplasm to the active site with internal electron transfer during turnover.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth

Bacterial strains and plasmids used in the present paper are summarized in Table 1. The method for the expression of recombinant NOR in *E. coli* has been reported previously along with the plasmids expressing the E125A, E198A and E202A variants of NorB [29]. Plasmids expressing new variants of NorB required by the present paper were constructed from the pNOREX vector by site-directed mutagenesis using the 'Quickchange' method (Stratagene). Silent restriction sites were incorporated into the mutagenic primers (Table 2) to allow rapid screening of the mutants. Restriction analysis followed by sequencing of the inserts confirmed the presence of correct mutations.

Small-scale batch cultures (25 ml) used for growth curves and whole cell assays of *P. denitrificans* 1222 and related strains were routinely grown at 37 °C without aeration on succinate medium [25 mM KNO_3 , 30 mM succinate, 55 mM Na_2HPO_4 , 11 mM KH_2PO_4 , 6 mM NH_4Cl , 0.4 mM MgSO_4 and 2 ml/l Vishniac's trace elements solution (pH 7.6)] [33]. The medium was supplemented with antibiotics as required at the following concentrations: 25 $\mu\text{g}/\mu\text{l}$ kanamycin and 25 $\mu\text{g}/\mu\text{l}$ spectinomycin. Native NOR was purified from *P. denitrificans* 93.11 (ΔctaDI , ΔctaCII *qoxB::kan^R*) grown on minimal medium under anaerobic denitrifying conditions in a 90 litre Bioflow-5000 fermentor (New Brunswick Scientific) as previously described [8].

Strains of *E. coli* expressing recombinant *P. denitrificans* NOR were grown in 15 × 1 litre batch cultures of *E. coli* JM109 transformed with pNOREX and pEC86 as previously described [29]. The structural gene for pseudoazurin from *Paracoccus pantotrophus* (*pazS*) was cloned into a pET-24d expression vector exactly as described by Pauleta and colleagues [34]. The resulting vector (pET-psaz) was used to transform *E. coli* BL21(DE3) and grown aerobically at 37 °C in Luria-Bertani medium supplemented with 30 $\mu\text{g}/\mu\text{l}$ kanamycin and 0.5 mM CuSO_4 for 24 h without induction. Cells were harvested by sedimentation using a Beckman Avanti J-20 centrifuge (JLA 8.1000 rotor) at 5500 g for 20 min.

Preparation of whole cells, membrane vesicles and proteins

Whole cells for assay of NOR activity were harvested from 25 ml of culture by sedimentation (6000 g for 20 min). In order

Table 1 Bacterial strains and plasmids used in this study

	Characteristics	Reference or source
Strain		
<i>E. coli</i> GM119	r_m^- , dam^- , dcm^-	Lab stock
<i>E. coli</i> JM109	<i>RecA endA gyrA thi hsdR supE relA</i> $\Delta(lac-proAB)$ {F ⁺ <i>traD proAB⁺ lacI^q Z</i> Δ M15}	Lab stock
<i>E. coli</i> BL21(DE3)	BF ⁻ <i>dcm ompT hsdS</i> ($r_b^- m_b^-$) <i>gal</i> λ (DE3)	Novagen
<i>P. denitrificans</i> Pd1222	Sp ^r Rif ^r , restriction deficient	[36]
<i>P. denitrificans</i> Pd21.31	Pd1222 <i>cycA</i>	[36]
<i>P. denitrificans</i> IP1013	Pd1222 <i>pazS::Km^r</i>	[36]
<i>P. denitrificans</i> IP1121	Pd21.31 <i>pazS::Km^r</i>	[36]
<i>P. denitrificans</i> GB1	Pd1222 <i>norB::</i> Ω -Km	[29]
Plasmid		
pEC86	Plasmid containing <i>ccm</i> gene cluster	[40]
pJR2	Plasmid containing <i>paz</i> gene	[35]
pET24d	Cloning vector	Novagen
pET-psaz	Pseudoazurin expression vector	The present study
pNOREX	6.1 kb NOR- <i>cycA</i> fusion cloned into pUC18	[29]
pNORxx16	1.6 kb Xba1-Xho1 <i>nor</i> fragment cloned in pBluescript KS ⁺	[29]
pNOR122A	E122A NorB mutation in pNORXX16	The present study
pNOR122D	E122D NorB mutation in pNORXX16	The present study
pNOR122Q	E122Q NorB mutation in pNORXX16	The present study
pNOR125A	E125A NorB mutation in pNORXX16	[29]
pNOR125D	E125D NorB mutation in pNORXX16	The present study
pNOR125Q	E125Q NorB mutation in pNORXX16	The present study
pNOR198A	E198A NorB mutation in pNORXX16	[29]
pNOR202A	E202A NorB mutation in pNORXX16	[29]
pNOR267A	E267A NorB mutation in pNORXX16	[29]
pE122Aex	E122A NorB mutation in pNOREX	The present study
pE122Dex	E122D NorB mutation in pNOREX	The present study
pE122Qex	E122Q NorB mutation in pNOREX	The present study
pE125Aex	(formally p125EX) E125A NorB mutation in pNOREX	[29]
pE125Dex	E125D NorB mutation in pNOREX	The present study
pE125Qex	E125Q NorB mutation in pNOREX	The present study
pE198Aex	(formally p198EX) E198A NorB mutation in pNOREX	[29]
pE202Aex	(formally p202EX) E202A NorB mutation in pNOREX	[29]
pE267Aex	(formally p267EX) E267A NorB mutation in pNOREX	[29]

Table 2 Primer design for site-directed mutagenesis of NorB

	Primers	Restriction enzyme incorporated
E125D	5'-CGCGAGTTCCTTGATCAGCCGAAATGG-3' 3'-GCCGCTCAAGGAAGTAGTCGGCTTTACC-5'	SpeI
E125Q	5'-CGCGAGTTCCTGCAGCAGCCGAAATGG-3' 3'-GCCGCTCAAGGACGTCGTCGGCTTTACC-5'	PstI
E122A	5'-AAGGAGGGGCGCGCGTCTCTGGAACAG-3' 3'-TTCCTCCCGCGCGCAATGACCTTGTC-5'	BssHI
E122D	5'-AAGGAGGGTTCGCGACTTCTGGAACAG-3' 3'-TTCCTCCCGCGCTGAAGGACCTTGTC-5'	NruI
E122Q	5'-AAGGAGGGGCGCAATCTCTGGAACAG-3' 3'-TTCCTCCCGCGGTTAAGGACCTTGTC-5'	NarI

to remove excess nitrate, the cells were then washed three times by re-suspension in 1.5 ml nitrate-free medium and subsequent collection by sedimentation (10 600 *g* for 5 min) in a benchtop micro-centrifuge. After the final wash the cells were resuspended in 1.5 ml nitrate-free medium prior to use. Membrane vesicles were prepared by passing cells twice through a French Pressure Cell (Aminco), at 75.9 MPa, followed by ultracentrifugation on a Beckman Optima XL-100K at 30 000 rev./min (45Ti rotor) for 2 h at 4 °C. Membranes were then resuspended and washed in 50 mM Tris/HCl (pH 7.6), 0.5 M NaCl and sodium-deoxycholate (0.05 % w/v) and sedimented by centrifugation at 180 000 *g* for 2 h at 4 °C. The washed membranes were resuspended in 50 mM

Tris/HCl (pH 7.6), pelleted and resuspended in the same buffer to approx. 20 mg/ml determined by the bicinchoninic acid method with BSA as a standard.

NOR from *P. denitrificans* was purified as previously described by Hendricks et al. [7]. Pseudoazurin was purified as described by Leung et al. [35]. The purity of NOR and pseudoazurin was assessed by SDS/PAGE and UV-visible spectra which were recorded using a Hitachi U3100 spectrophotometer. The concentrations of oxidized NOR and pseudoazurin were calculated on the basis of their molar absorption coefficients: NOR, $\epsilon_{411} = 3.11 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$; pseudoazurin, $\epsilon_{590} = 1360 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Whole cells, membrane vesicles and purified protein were stored at -80 °C until needed.

Determination of NOR concentrations in whole cells and membranes

Proteins were separated by SDS/PAGE, blotted onto nitrocellulose and blocked overnight at 4 °C in PAWS [pre-antibody wash solution; 10 mM Tris/HCl, 150 mM NaCl (pH 7.5) supplemented with 5 % (w/v) non-fat dried milk powder]. The nitrocellulose was probed for 3 h at 4 °C with an antibody (1:10000 dilution in PAWS) raised in rabbit and directed against a synthetic peptide corresponding to the NorB sequence. Excess primary antibody was removed by washing the membrane three times with PAWS buffer. The nitrocellulose was subsequently probed with a goat-anti-rabbit IgG conjugated to alkaline phosphatase (1:7500 in PAWS) for 3 h at 4 °C with agitation. The membrane was then

washed extensively with PAWS buffer followed by distilled water. The blot was developed using the Sigma fast BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (Nitro Blue Tetrazolium) system. Known amounts of purified NOR from *P. denitrificans* were run as standards alongside samples of membranes and cells. The intensity of the standards on the developed blots was analysed using Kodak ID 3.5 software and used to construct a standard curve from which the concentration of NOR in the membrane could be calculated.

Amperometric assays of NO consumption

The consumption of NO was measured anaerobically using a modified oxygen electrode (Oxytherm, Hansatech Instruments). The platinum cathode was polarized at -0.8 V with respect to the Ag:AgCl reference electrode in order to increase sensitivity towards NO. The electrolyte was 1 M KCl and a PTFE (polytetrafluoroethylene) membrane ($12.5\text{ }\mu\text{m}$ thickness) was used as a gas-permeable barrier to protect the electrodes from the reactants in the chamber.

NO stock solutions were prepared by addition of 8 ml NO gas to 3 ml of nitrogen-sparged water (pH 3.0). The concentration of NO was confirmed to be 1.8 mM by titration against reduced myoglobin: $\epsilon_{421\text{nm}} = 114\text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 20°C .

Assay of NOR activity in whole cells

The reaction chamber was filled with 2 ml of nitrate-free succinate medium [30 mM succinate, 55 mM Na_2HPO_4 , 11 mM KH_2PO_4 , 6 mM NH_4Cl , 0.4 mM MgSO_4 and 2 ml/l of Vishniac's trace element solution (pH 7.6)] [33] and warmed to 30°C with stirring. The lid of the chamber was added and approx. 0.1 mg of pre-prepared whole cells were added using a Hamilton syringe. The cells were allowed to respire and consume the oxygen in the media. Once the conditions in the chamber were anaerobic, NO was added to a final concentration of $100\text{ }\mu\text{M}$ and an immediate increase in the signal was observed with the subsequent decay being due to the succinate-dependent NOR activity of the cells. The initial rate of NO consumption was calculated from the time taken for 20% of the NO to be consumed. Note that the response of the electrode in the presence of whole cells to the addition of $100\text{ }\mu\text{M}$ NO is less than in the presence of purified enzyme which we attribute to non-enzymic consumption of NO.

Assay of NOR activity in *P. denitrificans* membranes

Resuspended membrane vesicles ($100\text{ }\mu\text{l}$) containing approx. 0.2 mg total protein were added to 2 ml of the same reaction medium in a stirred reaction chamber thermostatically maintained at 30°C . The lid was secured and oxygen removed from the chamber by the glucose (16 mM)/glucose oxidase (4 units/ml)/catalase (20 units/ml) system. Once the system was anaerobic, 5 mM nitrogen-sparged ascorbate and $60\text{ }\mu\text{M}$ pseudoazurin were added. When a steady baseline was observed, $100\text{ }\mu\text{M}$ NO was added. The initial rate of reaction was calculated by the time taken for 20% of the NO to be consumed.

Assay of NOR activity in *E. coli* membranes

The reaction chamber was filled with 2 ml of 20 mM phosphate buffer [50 mM KCl (pH 7.6)] at 30°C and the lid attached. Subsequent additions were made using Hamilton syringes. Resuspended membrane vesicles ($100\text{ }\mu\text{l}$) containing approx. 400 ng of NOR were added to the stirred reaction chamber. Anaerobic conditions were achieved in the chamber by a glucose (16 mM)/glucose oxidase (4 units/ml)/catalase (20 units/ml) sys-

tem. Once the system was anaerobic, 5 mM nitrogen-sparged ascorbate and $1.85\text{--}200\text{ }\mu\text{M}$ pseudoazurin were added. When a steady baseline was observed, $100\text{ }\mu\text{M}$ of NO was added. Again the initial rate of consumption of NO was measured by the time taken for 20% of the NO to be consumed.

Assay of purified NOR

NOR (25 nM), in 2 ml of 20 mM phosphate buffer [50 mM KCl (pH 7.6)] supplemented with 0.02% (w/v) dodecyl- β -D-maltoside, was added to the 30°C stirred reaction chamber. The lid was fastened and oxygen removed from the chamber by a glucose (16 mM)/glucose oxidase (4 units/ml)/catalase (20 units/ml) system. Once the system was anaerobic, 5 mM ascorbate and $5\text{--}200\text{ }\mu\text{M}$ pseudoazurin were added. Once a steady baseline was observed, $100\text{ }\mu\text{M}$ of saturated NO was added to initiate the reaction. The rate of NO consumption was measured over the initial part of the reaction.

RESULTS

NOR activity in strains in which the gene encoding either pseudoazurin or cytochrome c_{550} has been deleted

At the outset we wished to compare two natural electron donors with NOR, pseudoazurin and cytochrome c_{550} , in order to determine which might be the most appropriate mediator to use with ascorbate for *in vitro* assays of NOR activity. Strains of *P. denitrificans* in which the genes that encode cytochrome c_{550} and pseudoazurin had been disrupted, either individually or both together, have been described previously [36]. Comparison of these strains with the parent *P. denitrificans* strain and the ΔNorB strain [37] showed that disruption of either the gene encoding cytochrome c_{550} (*cycA*[−]) or the gene encoding pseudoazurin (*pazS*[−]) had little effect on anaerobic growth (Figure 1A), and these mutations only slightly attenuated succinate-dependent NOR activity (Figure 1B) when compared with the parent strain (Pd1222). Interestingly, using the same criteria, a strain (*cycA*[−] *pazS*[−]) in which the genes encoding both cytochrome c_{550} and pseudoazurin had been disrupted gave a phenotype that was indistinguishable from that of ΔNorB (Figures 1A and 1B).

Analysis of protein expression by SDS/PAGE followed by immunostaining using a primary antibody directed against NorB confirmed the presence of NorB in the parent strain as well as in the strains lacking a single electron donor (Figure 1C), but not in the double mutant or ΔNorB strains. These data are consistent with the proposal that pseudoazurin and cytochrome c_{550} act as alternative electron donors to NOR. However, the absence of antigen in the Western blot of the double mutant may arise because electron donation to cytochrome *cd*₁ (nitrite reductase) is not possible, preventing NO production. Transcription of the *nor* genes is activated by NNR (nitrite reductase and nitric oxide reductase regulator) under anaerobic growth conditions in response to NO or a related species. If NO is not produced by cytochrome *cd*₁ the NNR genes will not be activated and NOR will, in turn, not be expressed [38,39].

In the present paper we elected to use pseudoazurin as the electron donor to NOR for two reasons. First, unlike cytochrome c_{550} , pseudoazurin can be expressed at high levels in *E. coli* [35] without the need for a helper plasmid containing genes expressing proteins required for cofactor maturation [40]. The subsequent purification of recombinant pseudoazurin is both well established and straightforward [35]. Secondly, the UV-visible spectrum of pseudoazurin does not overlay that of NOR, which will greatly

Figure 1 Succinate-dependent NOR activity in whole cells

(A) Growth curves for batch cultures of *P. denitrificans*; Pd1222 (—) and mutant strains *cycA*⁻ (---), *pazS*⁻ (.....), *cycA*⁻ *pazS*⁻ (-.-.-) and Δ NorB (- - -). Each point is the mean of three replicate observations. (B) Succinate-dependent NOR activity measured using whole cells. NO consumption was measured anaerobically using a Clark-type platinum electrode polarized at -0.8 V with respect to a silver reference electrode. In a 2 ml reaction volume, approx. 120 mg of whole cells was added to 2.0 ml of a 20 mM Hepes buffer containing 20 mM succinate (pH 7.2 at 30 °C). Once cells had consumed all of the oxygen in the reaction, NO was added to a final concentration of 100 μ M. Pd1222 (—) and mutant strains *cycA*⁻ (---), *pazS*⁻ (---), *cycA*⁻ *pazS*⁻ (-.-.-) and Δ NorB (- - -). (C) Western blot of prepared membranes probed with an anti-NorB antibody. Lane 1, loading control (1.4 μ g of purified NOR); lane 2, molecular mass markers; lane 3, Pd1222; lane 4, *cycA*⁻; lane 5, *pazS*⁻; lane 6, *cycA*⁻ *pazS*⁻; lane 7, Δ NorB.

simplify interpretation of any future experiments in which the progress of the reaction is monitored optically.

A pseudoazurin-dependent amperometric assay of purified NOR

We decided to develop an amperometric assay of NOR using ascorbate as the bulk electron donor and pseudoazurin as the mediator. Clark-type oxygen electrodes have been used to detect

Figure 2 Ascorbate/pseudoazurin-dependent amperometric assay of purified NOR

(A) NO consumption by purified NOR was measured anaerobically using a Clark-type platinum electrode polarized at -0.8 V with respect to a silver reference electrode. The reaction mixture (2.0 ml) comprised of 50 mM Hepes and 0.02 % (w/v) dodecyl- β -D-maltoside (pH 7.6). The system was made anaerobic by the addition of 16 mM glucose, 4 units/ml of glucose oxidase and 20 units/ml of catalase. Ascorbate (2 mM), NO (100 μ M), NOR (25 nM) and pseudoazurin (5–200 μ M) were added as indicated. (B) The effect of varying the concentration of pseudoazurin on the initial rate of NO consumption. Measurements were made in triplicate and the data points represent the means $\pm$ S.E.M. A line of best fit to the Michaelis-Menten equation yielded an apparent K_m of 27 μ M and a V_{max} of 904 min⁻¹.

NO consumption in a number of studies of NOR. Typically the Pt cathode is polarized at -800 mV with respect to the Ag:AgCl reference electrode rather than -700 mV used for measurements of dioxygen consumption to increase the sensitivity of the system to NO. Moreover anaerobic conditions are required so that the electrode only responds to NO and not O₂. These were achieved by the use of degassed buffers from which the final traces of oxygen were removed using glucose/glucose oxidase/catalase scrubbing system. The reduction of NO by ascorbate was absolutely dependent upon the presence of both NOR and pseudoazurin in the assay medium (Figure 2A).

Assays were done at a range of different concentrations of pseudoazurin and an excess of ascorbate using purified NOR from *P. denitrificans*. The apparent K_m for pseudoazurin is estimated to be 27 μ M (Figure 2B) and the maximum turnover of the enzyme 904 min⁻¹ (15 s⁻¹). The data presented in Table 3 show that the maximum turnover observed with pseudoazurin is comparable

Table 3 A comparison of the NOR activity of NorBC purified from *P. denitrificans* measured using different combinations of mediators in the presence of 5 mM ascorbate

Mediator	Maximum turnover number (min^{-1})
Pseudoazurin (200 μM)	801 ± 20 ($n=3$)
TMPD (2.3 mM)	190 ± 21 ($n=3$)
Horse heart cytochrome <i>c</i> (50 μM)	106 ± 5 ($n=5$)
TMPD (2 mM) + horse heart cytochrome <i>c</i> (50 μM)	170 ± 15 ($n=3$)
Hexa-aminoruthenium-(III)-chloride (4 mM)	316 ± 31 ($n=6$)
Phenazine ethylsulfate (20 μM)	812 ± 138 ($n=3$)
Phenazine methosulfate (10 μM)	640 ± 24 ($n=3$)

with rates observed with the chemical mediators used in previous studies.

NOR activity in *P. denitrificans* and *E. coli* membrane vesicles

Purifying NOR from membrane vesicles is an expensive and labour-intensive process, not least for the recombinant enzyme expressed in *E. coli*. Moreover, yields of purified enzyme may fall if a particular mutation destabilizes the enzyme outside its membrane environment. A convenient alternative to purifying the enzyme is specifically to measure NOR activity in membrane vesicles using the ascorbate/pseudoazurin amperometric assay. Such an assay requires any endogenous NOR activity in the membranes to be inhibited, and pseudoazurin to donate electrons to NOR as if it were an isolated system. In membrane vesicles isolated from *P. denitrificans*, succinate-dependent NOR activity is halted in the presence of myxathiazol, a specific inhibitor of the bc_1 complex (Figure 3A). Subsequent addition of ascorbate/pseudoazurin to the system restores NOR activity (Figure 3A).

The next step was to apply this assay to measure NOR activity in membrane vesicles isolated from strains of *E. coli* expressing recombinant NOR. In principle this is straightforward because *E. coli* does not possess either a bc_1 complex or a membrane-bound respiratory NOR. The electrons for its terminal respiratory complexes are derived directly from the quinone pool. Figure 3(B) shows that NOR activity in *E. coli* membranes is absolutely dependent upon heterologous expression of NOR by the pNOREX plasmid. To ascertain that pseudoazurin was interacting with recombinant NOR in the membrane vesicles in a similar fashion to the purified enzyme, we investigated the relative NOR activity as a function of pseudoazurin concentration. The apparent K_m of the membrane-associated recombinant NOR for pseudoazurin was estimated to be 35 μM (Figure 3B inset) suggesting a similar mode of interaction to that we established between the purified native NOR and pseudoazurin (Figure 2B). Interestingly the estimated V_{max} of 4877 min^{-1} is approx. 5-fold higher than the value obtained with purified enzyme, suggesting some loss of activity associated with removal of the enzyme from its membrane environment.

Alanine scanning mutagenesis of five conserved glutamate residues in NorB

Each of the five conserved glutamate residues in NorB was systematically replaced by alanine residues and the variant NORs expressed in *E. coli*. The level of expression of NorC was assessed by SDS/PAGE followed by staining for covalently bound haem [41] (Figure 4A) and the level of expression of NorB evaluated

Figure 3 NOR activity of cytoplasmic membranes of *P. denitrificans* and *E. coli* expressing NOR

(A) Myxathiazol inhibits succinate-dependent NO reduction in *P. denitrificans* membranes and the use of ascorbate/pseudoazurin restores activity by donating electrons directly to NOR. (B) NOR activity can be measured directly in *E. coli* membranes and depends on the expression of NOR from pNOREX. Dotted line, JM109(pEC86)pUC18; solid line, JM109(pEC86)pNOREX. The inset of (B) shows the effect of varying the concentration of pseudoazurin on the initial rate of NO consumption. Each data point represents the means $\pm$ S.E.M. of at least three independent observations. A line of best fit to the Michaelis–Menten equation yielded an apparent K_m value of 35 μM and a V_{max} of 4877 min^{-1} .

by SDS/PAGE followed by immunostaining (Figure 4B). In each case, levels of expression in the membranes of both subunits of the variant NOR was comparable with that of the wild-type enzyme.

The mutation essentially abolished activity in each of the variants except E202A which retained approx. 50 % of the activity of the wild-type enzyme. Since the reduction of NO to N_2O is a proton-consuming process it was proposed that lowering the pH of the reaction would increase the rate of reaction of NOR. Decreasing the pH of the medium to see whether activity could be recovered had little or no effect in all of the mutants except for the E125A variant in which the specific activity at pH 8.5 was 0.5 nmol NO per mg of protein (± 0.07 , $n=3$) compared with 0.9 nmol NO per mg of protein (± 0.09 , $n=3$) at pH 6.0. This contrasts strongly with the enhanced rate determined for recombinant wild-type NOR expressed in *E. coli* membranes [19.42 nmol NO per mg of protein (± 2.22 , $n=3$) at pH 8.5, compared with 31.92 nmol NO per mg of protein (± 1.06 , $n=3$) at pH 6.0].

Figure 4 SDS/PAGE analysis of *E. coli* cytoplasmic membranes expressing recombinant wild-type and mutant NOR

(A) Haem staining confirms the presence of normal amounts of NorC. (B) Western blot of prepared membranes probed with an anti-NorB antibody confirms the presence of NorB in the membranes. For each sample 10 μ l of resuspended membranes were added to 90 μ l of sample buffer [6 M urea, 5% (w/v) SDS, 5% (w/v) glycerol and 0.05% Bromophenol Blue]. These were incubated at room temperature (22 °C) for 30 min and aliquots analysed by SDS/PAGE. Each lane contained approximately 100 μ g of total protein for haem staining (A) or 20 μ g of total protein for immunostaining (B). Lane 1, purified *P. denitrificans* NOR; lane 2, wild-type recombinant NOR; lane 3 E122A; lane 4 E122D; lane 5 E122Q; lane 6 E125A; lane 7 E125D; lane 8 E125Q; lane 9 E198A; lane 10 E202A; lane 11 E267A.

Further analysis of the surface glutamate residues Glu¹²² and Glu¹²⁵

The periplasmic facing loop between transmembrane helices III and IV is far removed from the active site, and so these glutamates are unlikely to serve as ligands to the cofactors and the loss of activity associated with alanine residue substitution is likely to be related to their proposed function in proton uptake. Consequently it might be expected that replacement of these residues with an amino acid with a functionally related side-chain might lead to some recovery of activity. Again, analysis by SDS/PAGE followed by staining for covalently bound haems showed that NorC was present in the membranes of *E. coli* strains expressing the E125D/Q and E122D/Q variants (Figure 4A). Parallel experiments in which NorB was visualized by immunostaining showed that NorB was present in each of the membrane vesicle samples (Figure 4B). Assaying NOR activity at pH 7.2 with pseudoazurin (200 μ M) and ascorbate (5 mM) showed that substitution of both Glu¹²² and Glu¹²⁵ with glutamine yielded a form of the enzyme that had approx. 10% of the specific activity of the wild-type enzyme. Substitution of Glu¹²² with an aspartate residue gave an enzyme that had approx. 80% of the specific activity of the wild-type enzyme, but rather surprisingly the E125D variant showed virtually no residual activity (Table 4).

DISCUSSION

Initial experiments in which succinate-dependent NOR activity was measured in whole cells showed that the ability of NOR to reduce NO was only slightly attenuated in strains lacking either pseudoazurin or cytochrome *c*₅₅₀. This observation is consistent with an earlier proposal that in *P. denitrificans* both of these periplasmic proteins can transfer electrons between

Table 4 The effects of amino acid substitutions in NorB on the ascorbate/pseudoazurin-dependent NOR activity of *P. denitrificans* NOR expressed in *E. coli*

	Turnover number (min ⁻¹ ; means $\pm$ S.E., <i>n</i> = 3)	Activity relative to wild-type NOR (%)
Wild-type	4223 $\pm$ 120	100
E122A	612 $\pm$ 68	14.5
E122D	3511 $\pm$ 363	83.2
E122Q	53 $\pm$ 3	1.3
E125A	311 $\pm$ 29	7.4
E125D	76 $\pm$ 17	1.8
E125Q	566 $\pm$ 71	13.4
E198A	61 $\pm$ 39	1.5
E202A	1660 $\pm$ 134	39.3
E267A	155 $\pm$ 6	3.6

the *bc*₁ complex and three respiratory enzymes involved in denitrification: cytochrome *cd*₁ nitrite reductase, NOR and nitrous oxide reductase [36].

Further evidence for the participation of pseudoazurin in the transfer of electrons between the *bc*₁ complex and NOR comes from experiments with membrane vesicles prepared from *P. denitrificans* grown under anaerobic denitrifying conditions. In these vesicles, which contain an intact electron-transfer chain, it is possible to measure succinate-dependent NOR activity (Figure 3A). Electrons are transferred from succinate dehydrogenase to NOR via the ubiquinone pool, the *bc*₁ complex and a periplasmic electron-transfer protein (cytochrome *c*₅₅₀ or pseudoazurin). The preparation of membrane vesicles inevitably leads to the loss of periplasmic proteins. Consequently it was not surprising that supplementing the assay medium with 60 μ M pseudoazurin further increased the rate of NO reduction (results not shown).

The current view of intra-molecular electron transfer in NorBC based on both measurement of the reduction potentials of the metal centres [8] and kinetic measurements of electron backflow [42] is that electrons move from the *c*-type haem in NorC to the dinuclear centre via the low-spin *b* haem in NorB. The presence of the *c*-type haem in a periplasmic domain of NorC suggests that this centre serves to accept electrons from pseudoazurin/cytochrome *c*₅₅₀, and that interaction of pseudoazurin is primarily with NorC. The interaction of pseudoazurin with cytochrome *c* peroxidase from *P. denitrificans* is believed to occur through ionic interactions between a patch of acidic residues on the surface of the peroxidase and a group of positively charged lysine residues on pseudoazurin [34]. Experiments using purified NOR in the pseudoazurin-dependent steady-state assay showed that NOR activity is also dependent on the ionic strength of the medium with the optimal buffer conditions containing 50 mM KCl (results not shown). Under these conditions we established an apparent *K*_m for pseudoazurin of 27 μ M. This is similar to the apparent *K*_m for pseudoazurin reported for two other *Paracoccus* respiratory enzymes, the di-haem cytochrome *c* peroxidase (13 μ M [34]) and cytochrome *cd*₁ nitrite reductase (9.7 μ M [43]).

The participation of cupredoxins as electron carriers is well documented in both photosynthetic and bacterial respiratory electron-transfer chains [44]. The question as to why *P. denitrificans* appears to require two periplasmic electron carriers remains unresolved, but the work in the present paper provides a sound basis for our use *in vitro* of pseudoazurin as a physiologically relevant and specific electron donor to NOR expressed in *E. coli* membranes.

Previously reported amperometric methods to measure the NOR activity of NorBC have relied upon the use of small molecules to mediate electron transfer between a chemical reductant, typically ascorbate, and the enzyme. Different workers have used PMS [30], TMPD [7] or a combination of horse heart cytochrome *c* plus DAD [32], TMPD [31] or TMPD and PMS [9] to mediate electron transfer from ascorbate to NOR. At the outset of this study we had some concerns about the use of PMS, because ascorbate/PMS represents an efficient system for donating electrons to the qNOR from *Ralstonia eutropha* [45]. This enzyme lacks a *c*-type haem and derives electrons for NO reduction directly from the ubiquinone pool. Consequently it is conceivable that in NorBC, PMS can donate electrons directly to NorB, bypassing the haem *c* of NorC. Thus although 10 μ M PMS as a mediator gave rates of NO reduction comparable to 200 μ M pseudoazurin (Table 3), it may not be an appropriate choice of mediator.

Another consideration in the choice of electron donors arises from a study of the qCu(A)NOR from *Bacillus azotoformans*, a Gram-positive denitrifying soil bacterium. This NOR is apparently able to receive electrons directly from the quinone pool via menaquinol and also from a membrane-bound cytochrome *c*₅₅₁. It is proposed the menaquinol pathway, which has a 4-fold greater maximal activity than the pathway via cytochrome *c*₅₅₁, is used for NO detoxification, whereas electron donation via the endogenous cytochrome *c* involves the cytochrome *bf*₆ complex to serve the bioenergetic needs of the organism [46]. These observations raise the question as to whether the *P. denitrificans* NorBC can use quinols as alternate electron donors. Recombinant NOR expressed in *E. coli* inner membranes does not support NADH- or succinate-dependent NO-respiration. Since *E. coli* does not possess a *bc*₁ complex this clearly demonstrates that the recombinant NOR is unable to accept electrons directly from the ubiquinol pool. Moreover purified NOR is unable to catalyse NO reduction in the presence of either dithiothreitol plus ubiquinone or dithiothreitol plus menaquinone as an electron-donating system.

E. coli does not possess a membrane-bound respiratory NOR, instead it uses a number of soluble enzymes including NrfA, a pentahaem nitrite reductase [47], NorVW, a flavorubredoxin [48] and flavohaemoglobin [49] to detoxify NO. As already noted, *E. coli* does not possess a *bc*₁-dependent respiratory chain and the terminal respiratory complexes derive electrons directly from the quinone pool. Aerobically grown *E. coli* expresses two quinol-dependent terminal oxidases, cytochrome *bo*₃ and cytochrome *bd*, and although cytochrome *bo*₃ can reduce NO very slowly [50], it is unable to accept electrons from pseudoazurin. Consequently recombinant NOR expressed in *E. coli* membranes can be specifically assayed with ascorbate/pseudoazurin without the need of additional inhibitors.

Two previous studies have shown that NOR is not electrogenic and that the two substrate protons required for NO reduction are taken up from the periplasm [17,42]. In addition it has recently been reported that in the reaction of the fully reduced enzyme with molecular oxygen, electron transfer to the active site of NOR is coupled to proton uptake [28]. The combination of the conservation of both Glu¹²⁵ and Glu¹²² amongst NorB sequences and their predicted projection into the periplasm on a loop region between transmembrane helices III and IV, suggests a possible role in accepting protons at the entrance to a channel that conducts substrate protons to the dinuclear centre. To investigate this proposition further we constructed variants in which each of these two residues was replaced by an aspartate residue (E122D and E125D) or the equivalent amide (E122Q and E125Q).

The E122Q variant has a level of residual activity similar to the E122A variant. In contrast the E122D variant exhibits a high level

of activity. This pattern of results is reminiscent of those obtained from variants of cytochrome *c* oxidase from *R. sphaerioides* in which the critical aspartate (Asp¹³²) at the entrance to the D-channel has been mutated. Substitution of this aspartate residue with anything else, other than a glutamate residue, leads to very low levels of oxidase activity, whilst the glutamate mutant has levels of activity similar to the wild-type enzyme [25].

In contrast, substitution of either an aspartate or glutamine residue for the glutamate residue at position 125 in NorB yields an enzyme that has less than 15 % of the activity of the wild-type enzyme. In this context it is interesting to note that the glutamate residue (Glu¹⁰¹ in subunit II) at the mouth of the K-channel in cytochrome *c* oxidases will not tolerate substitution with any other residue [51]. In the absence of a structure it is not possible to say which of the two glutamate residues (Glu¹²² or Glu¹²⁵) in NorB is the primary proton acceptor, but we speculate that it is Glu¹²² and that the role of Glu¹²⁵ in the proposed channel might be structural. This would account for the observation that the length of the amino acid side-chain (e.g. E125Q) seems to be a more important determinant of activity than retaining a carboxylate in that position.

In conclusion this study demonstrates the usefulness of using pseudoazurin as a specific electron donor in experiments involving NorBC. Using recombinant pseudoazurin we measured changes in the steady-state activity of recombinant NOR expressed in *E. coli* membranes brought about by substitutions to the glutamate residues at positions 122 and 125 of NorB. The results are consistent with these residues forming the entrance to a proton-conducting channel, which we refer to as the 'E-channel', whose proposed function is to conduct protons from the periplasm to the active site of the enzyme during turnover.

We thank Dr Myles Cheesman (School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, U.K.), Dr Sarah Field (School of Biological Sciences, University of East Anglia, Norwich, U.K.) and Dr Pia Adelroth (Department of Biochemistry, University of Stockholm, Stockholm, Sweden) for many useful discussions about this work, and Hansatech Instruments for kindly modifying the electrode to allow reliable measurement of NO. F.H.T. is funded by a U.K. Medical Research Council Priority Area Studentship. The work was supported by grants from the John and Pamela Salter Trust to N.J.W. and the U.K. BBSRC (Biotechnology and Biological Sciences Research Council) to D.J.R. (B19851) and N.J.W./D.J.R. (BBC0077191).

REFERENCES

- 1 Takaya, N., Catalan-Sakairi, M. A., Sakaguchi, Y., Kato, I., Zhou, Z. and Shoun, H. (2003) Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Appl. Environ. Microbiol.* **69**, 3152–3157.
- 2 Richardson, D. J. and Watmough, N. J. (1999) Inorganic nitrogen metabolism in bacteria. *Curr. Opin. Chem. Biol.* **3**, 207–219.
- 3 Watmough, N. J., Butland, G., Cheesman, M. R., Moir, J. W., Richardson, D. J. and Spiro, S. (1999) Nitric oxide in bacteria: synthesis and consumption. *Biochim. Biophys. Acta* **1411**, 456–474.
- 4 Hendriks, J., Gohlke, U. and Saraste, M. (1998) From NO to O₂: nitric oxide and dioxygen in bacterial respiration. *J. Bioenerg. Biomembr.* **30**, 15–24.
- 5 Busch, A., Friedrich, B. and Cramm, R. (2002) Characterization of the *norB* gene, encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. strain PCC6803. *Appl. Environ. Microbiol.* **68**, 668–672.
- 6 Suharti, Stramprecht, M. J., Schroder, I. and de Vries, S. (2001) A novel copper A containing menaquinol NO reductase from *Bacillus azotoformans*. *Biochemistry* **40**, 2632–2639.
- 7 Hendriks, J., Warne, A., Gohlke, U., Haltia, T., Ludovici, C., Lubben, M. and Saraste, M. (1998) The active site of the bacterial nitric oxide reductase is a dinuclear iron center. *Biochemistry* **37**, 13102–13109.
- 8 Grönberg, K. L. C., Roldán, M. D., Prior, L., Butland, G., Cheesman, M. R., Richardson, D. J., Spiro, S., Thomson, A. J. and Watmough, N. J. (1999) A low-redox potential heme in the dinuclear center of bacterial nitric oxide reductase: implications for the evolution of energy-conserving heme-copper oxidases. *Biochemistry* **38**, 13780–13786.

- 9 Girsch, P. and de Vries, S. (1997) Purification and initial kinetic and spectroscopic characterization of NO reductase from *Paracoccus denitrificans*. *Biochim. Biophys. Acta* **1318**, 202–216
- 10 Cheesman, M. R., Zumft, W. G. and Thomson, A. J. (1998) The MCD and EPR of the heme centers of nitric oxide reductase from *Pseudomonas stutzeri*: evidence that the enzyme is structurally related to the heme-copper oxidases. *Biochemistry* **37**, 3994–4000
- 11 Sakurai, N. and Sakurai, T. (1997) Isolation and characterization of nitric oxide reductase from *Paracoccus halodenitrificans*. *Biochemistry* **36**, 13809–13815
- 12 Kastrau, D. H. W., Heiss, B., Kroneck, P. M. H. and Zumft, W. G. (1994) Nitric oxide reductase from *Pseudomonas stutzeri*: Phospholipid requirement, electron paramagnetic resonance and redox properties. *Eur. J. Biochem.* **222**, 293–303
- 13 van der Oost, J., deBoer, A. P. N., deGier, 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**, 1–9
- 14 Saraste, M. and Castresana, J. (1994) Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.* **341**, 1–4
- 15 Babcock, G. T. and Wikström, M. (1992) Oxygen activation and the conservation of energy in cell respiration. *Nature* **356**, 301–309
- 16 Ferguson-Miller, S. and Babcock, G. T. (1996) Heme/copper terminal oxidases. *Chem. Rev.* **96**, 2889–2908
- 17 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
- 18 Van Gelder, B. F. and Beinert, H. (1969) Studies of the heme components of cytochrome *c* oxidase by EPR spectroscopy. *Biochim. Biophys. Acta* **189**, 1–24
- 19 Cheesman, M. R., Oganessian, V. S., Watmough, N. J., Butler, C. S. and Thomson, A. J. (2004) The nature of the exchange coupling between high-spin Fe(III) heme O₃ and Cu_B(II) in *Escherichia coli* quinol oxidase, cytochrome *bo*₃: MCD and EPR studies. *J. Am. Chem. Soc.* **126**, 4157–4166
- 20 Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* **376**, 660–669
- 21 Svensson-Ek, M., Abramson, J., Larsson, G., Tornroth, S., Brzezinski, P. and Iwata, S. (2002) The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*. *J. Mol. Biol.* **321**, 329–339
- 22 Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science* **272**, 1136–1144
- 23 Jünemann, S., Meunier, B., Gennis, R. B. and Rich, P. R. (1997) Effects of mutation of the conserved lysine-362 in cytochrome *c* oxidase from *Rhodobacter sphaeroides*. *Biochemistry* **36**, 14456–14464
- 24 Vygodina, T. V., Pecoraro, C., Mitchell, D., Gennis, R. and Konstantinov, A. A. (1998) Mechanism of inhibition of electron transfer by amino acid replacement K362M in a proton channel of *Rhodobacter sphaeroides* cytochrome *c* oxidase. *Biochemistry* **37**, 3053–3061
- 25 Mills, D. A., Florens, L., Hiser, C., Qian, J. and Ferguson-Miller, S. (2000) Where is 'outside' in cytochrome *c* oxidase and how and when do protons get there? *Biochim. Biophys. Acta* **1458**, 180–187
- 26 Pereira, M. M., Santana, M. and Teixeira, M. (2001) A novel scenario for the evolution of haem-copper oxygen reductases. *Biochim. Biophys. Acta* **1505**, 185–208
- 27 Zumft, W. G. (2005) Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J. Inorg. Biochem.* **99**, 194–215
- 28 Flock, U., Watmough, N. J. and Ädelroth, P. (2005) Electron/proton coupling in bacterial nitric oxide reductase during reduction of oxygen. *Biochemistry* **44**, 10711–10719
- 29 Butland, G., Spiro, S., Watmough, N. J. and Richardson, D. J. (2001) Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme. *J. Bacteriol.* **183**, 189–199
- 30 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
- 31 Matsuda, Y., Inamori, K., Osaki, T., Eguchi, A., Watanabe, A., Kawabata, S., Iba, K. and Arata, H. (2002) Nitric oxide-reductase homologue that contains a copper atom and has cytochrome *c*-oxidase activity from an aerobic phototrophic bacterium *Roseobacter denitrificans*. *J. Biochem.* **131**, 791–800
- 32 Carr, G. J. and Ferguson, S. J. (1990) The nitric oxide reductase of *Paracoccus denitrificans*. *Biochem. J.* **269**, 423–429
- 33 Burnell, J. N., John, P. and Whatley, F. R. (1975) The reversibility of active sulphate transport in membrane vesicles of *Paracoccus denitrificans*. *Biochem. J.* **150**, 527–536
- 34 Pauleta, S. R., Guerlesquin, F., Goodhew, C. F., Devreese, B., Van Beeumen, J., Pereira, A. S., Moura, I. and Pettigrew, G. W. (2004) *Paracoccus pantotrophus* pseudoazurin is an electron donor to cytochrome *c* peroxidase. *Biochemistry* **43**, 11214–11225
- 35 Leung, Y. C., Chan, C., Reader, J. S., Willis, A. C., van Spanning, R. J., Ferguson, S. J. and Radford, S. E. (1997) The pseudoazurin gene from *Thiosphaera pantotropha*: analysis of upstream putative regulatory sequences and overexpression in *Escherichia coli*. *Biochem. J.* **321**, 699–705
- 36 Pearson, I. V., Page, M. D., van Spanning, R. J. and Ferguson, S. J. (2003) A mutant of *Paracoccus denitrificans* with disrupted genes coding for cytochrome *c*₅₅₀ and pseudoazurin establishes these two proteins as the *in vivo* electron donors to cytochrome *cd*₁ nitrite reductase. *J. Bacteriol.* **185**, 6308–6315
- 37 de Boer, A. P., van der Oost, J., Reijnders, W. N., Westerhoff, H. V., Stouthamer, A. H. and van Spanning, R. J. (1996) Mutational analysis of the *nor* gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *Eur. J. Biochem.* **242**, 592–600
- 38 van Spanning, R. J., Houben, E., Reijnders, W. N., Spiro, S., Westerhoff, H. V. and Saunders, N. (1999) Nitric oxide is a signal for NNR-mediated transcription activation in *Paracoccus denitrificans*. *J. Bacteriol.* **181**, 4129–4132
- 39 Hutchings, M. I. and Spiro, S. (2000) The nitric oxide regulated *nor* promoter of *Paracoccus denitrificans*. *Microbiology* **146**, 2635–2641
- 40 Arslan, E., Schulz, H., Zufferey, R., Kunzler, P. and Thöny-Meyer, L. (1998) Overproduction of the *Bradyrhizobium japonicum* *c*-type cytochrome subunits of the *cbb*₃ oxidase in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **251**, 744–747
- 41 Goodhew, C., Brown, K. and Pettigrew, G. W. (1986) Haem staining in gels, a useful tool in the study of bacterial *c*-type cytochromes. *Biochim. Biophys. Acta* **852**, 288–294
- 42 Hendriks, J. H., Jasaitis, A., Saraste, M. and Verkhovsky, M. I. (2002) Proton and electron pathways in the bacterial nitric oxide reductase. *Biochemistry* **41**, 2331–2340
- 43 Koutny, M., Kucera, I., Tesarik, R., Turanek, J. and VanSpanning, R. J. M. (1999) Pseudoazurin mediates periplasmic electron flow in a mutant strain of *Paracoccus denitrificans* lacking cytochrome *c*₅₅₀. *FEBS Lett.* **448**, 157–159
- 44 Gough, J. and Chothia, C. (2004) The linked conservation of structure and function in a family of high diversity: the monomeric cupredoxins. *Structure* **12**, 917–925
- 45 Cramm, R., Pohlmann, A. and Friedrich, B. (1999) Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16. *FEBS Lett.* **460**, 6–10
- 46 Suharti, S. and de Vries, S. (2005) Membrane-bound denitrification in the Gram-positive bacterium *Bacillus azotoformans*. *Biochem. Soc. Trans.* **33**, 130–133
- 47 Poock, S. R., Leach, E. R., Moir, J. W., Cole, J. A. and Richardson, D. J. (2002) Respiratory detoxification of nitric oxide by the cytochrome *c* nitrite reductase of *Escherichia coli*. *J. Biol. Chem.* **277**, 23664–23669
- 48 Gardner, A. M., Helmick, R. A. and Gardner, P. R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J. Biol. Chem.* **277**, 8172–8177
- 49 Gardner, P. R., Gardner, A. M., Martin, L. A. and Salzman, A. L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10378–10383
- 50 Butler, C., Forte, E., Maria Scandurra, F., Arese, M., Giuffrè, A., Greenwood, C. and Sarti, P. (2002) Cytochrome *bo*₃ from *Escherichia coli*: the binding and turnover of nitric oxide. *Biochem. Biophys. Res. Commun.* **296**, 1272–1278
- 51 Tomson, F. L., Morgan, J. E., Gu, G., Barquera, B., Vygodina, T. V. and Gennis, R. B. (2003) Substitutions for glutamate 101 in subunit II of cytochrome *c* oxidase from *Rhodobacter sphaeroides* result in blocking the proton-conducting K-channel. *Biochemistry* **42**, 1711–1717

Received 8 June 2006/6 September 2006; accepted 8 September 2006

Published as BJ Immediate Publication 8 September 2006, doi:10.1042/BJ20060856