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Nitric Oxide sensing by the transcription factor DNR from *Pseudomonas aeruginosa*: a novel heme dependent mechanism

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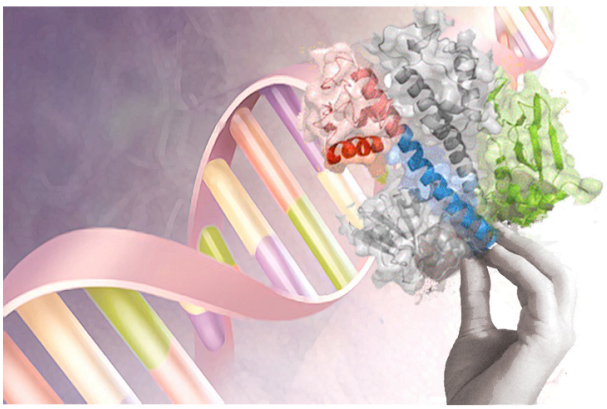

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INTRODUCTION

1.1 ***Pseudomonas aeruginosa* denitrification: involvment of this pathway in the biofilm formation**

P. aeruginosa is one of the most studied among Gram-negative bacteria due to its metabolic versatility, its ability to thrive in many different environments, and its important role as a pathogen. It is an important cause of both community- and hospital-acquired infections, accounting for 11-13% of all nosocomial infections. In addition, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis (CF) patients (Driscoll *et al*., 2007; Buchanan *et al.,* 2009). In the thick CF airway mucus, *P.aeruginosa* grows forming antibiotic- and phagocyte-resistant multicellular assemblies, named biofilm, which is a highly organized structure consisting of cells embedded within a matrix of extracellular polymeric substance, attached to a surface (Hassett *et al*., 2008; Drago I. *et al.,* 2009) (Fig. 1.1).

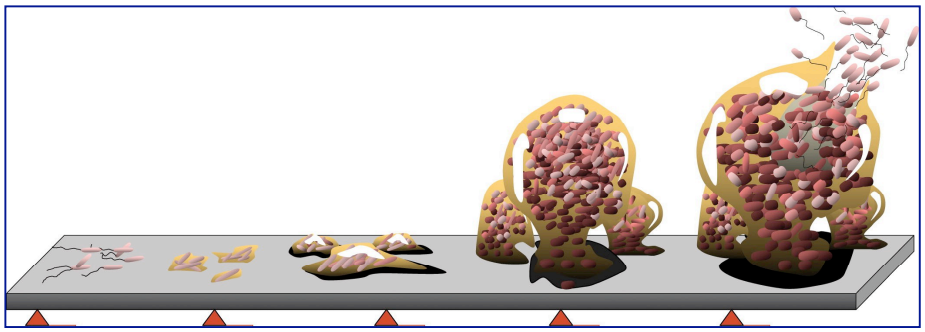

Figure 1.1. Biofilm developmental cycle

The stagnant mucus overlaying the CF lung epithelium constitutes a nitrate-rich microaerobic/anaerobic environment; nitrate in CF mucus is generated in part by the host inflammatory response to infection via nitric oxide. In this environment, *P. aeruginosa*, which is a facultative anaerobe, can use

denitrification as the anaerobic energy producing pathway (Moreau-Marquis S *et al.*, 2008; Hassett *et al.*, 2002; Hoboth *et al.*, 2009).
The complete denitrification pathway (Fig. 1.2) involves four enzymes: the nitrate reductase (NAR), the nitrite reductase (NIR), the nitric oxide reductase (NOR) and the nitrous oxide reductase (NOS), operating sequentially to reduce nitrate to dinitrogen gas via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997).

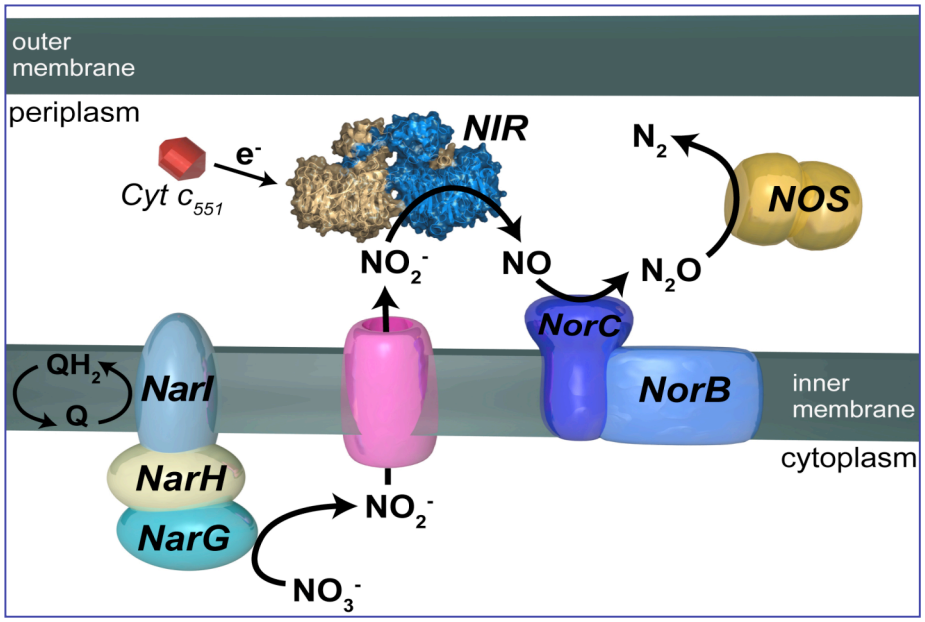

Figure 1.2 Schematic representation of the denitrification in *Pseudomonas aeruginosa*. Q/QH2 indicates the quinol mediated electron transfer; cyt is cytochrome.

The activity of the four enzymes is tightly controlled both kinetically and transcriptionally because it is mandatory for the bacteria to keep the concentration of intracellular NO below cytotoxic levels, to limit nitrosative stress (Hassett *et al.*, 2002).
At low concentration (nM) NO functions as a signalling molecule whereas at high concentration (above μM) NO is toxic: mutations which inactivate the

NOR protein are lethal for the bacterium but this effect could be abolished by inactivating the NIR protein (Braun & Zumft 1991).

The toxicity of the NO molecule is due to its chemical properties: the presence of an unpaired electron, a long half–life, the capability to permeate through the membranes confer to this gas high reactivity (Feelisch & Stamler, 1996). The NO gas can alter biological macromolecules both directly or indirectly through NO-derived species.

At high concentration, the gas reacts mainly with heme centres and labile 4Fe–4S clusters and thus inhibits terminal oxidases and aerobic respiration (Poole and Hughes, 2000; Wink and Mitchell, 1998). NO can also react both with molecular oxygen or superoxide (O_2^-) to produce nitration/nitrosation modifications or peroxynitrite ($OONO^-$, Huie and Padmaja, 1993) respectively.

Moreover the NO molecule has also mutagenic effect on bacterial DNA (Richardson, *et al.,* 2009) with complex effects escaping from chromosomal repair system.

Eukaryotic cells use high concentrations of NO to fight invading prokaryotic pathogens and parasites (Bastian and Hibbs, 1994; Nathan and Hibbs, 1991).

The NO released by macrophages is not the only source of NO that microbes need to deal with, because this compound is also produced abiotically (*e.g.* by decomposition of nitrite) and biotically by denitrifiers (Zumft, 1997) and as a product of side reactions in ammonification and nitrate assimilation/respiration (Corker and Poole, 2003).

It has been also described that NO can be cytoprotective because it's involved in adaptation to oxidative stress in bacteria (Gusarov and Nudler, 2005). Recently, the NO produced by the bacterial nitric oxide synthases (bNOS) has been shown to increase the resistance of bacteria to a broad spectrum of antibiotics, enabling the bacteria to survive and share habitats with antibiotic-producing microorganisms (Gusarov *et al.,* 2009).

Recent findings suggest that in *P. aeruginosa* the regulation of the NO levels is crucial also for the formation and survival of persistent biofilms and thus in pathogenesis; it has been, ideed, demonstrated that the exposure to non-toxic concentrations of NO causes biofilm dispersal and restores *P.aeruginosa* vulnerability to conventional antimicrobials (Barraud *et al.,* 2006). Moreover, the NO concentration affects the two important systems involved in the

biofilm development: the quorum sensing (Yoon *et al.,* 2002), which is a communication system which optimizes the metabolic and behavioral activities of bacterial communities towards changes in environmental conditions (Williams and Cámara, 2009) and the turnover of the c-di-GMP (3', 5'-cyclic diguanylic) (Barraud *et al.,* 2009) which is a novel intracellular second messenger controlling an array of cellular processes linking environmental sensing with the sessile-motile transition (Camilli and Bassler, 2006).
Hassett et al., (2002) demonstrated that under low oxygen tension and the presence of N-oxides produced by the host defence mechanism there is high levels of expression of *nir-nor* operons. Moreover Van Alst *et al.,* (2007) described that genetic mutants lacking *nar* and *nir* genes show swarming defects and reduced virulence.
These findings suggest that denitrification, NO regulation and pathogenesis are therefore strictly related but the molecular basis of these complex regulation system is yet to be revealed.

1.2 ***Regulation of NO homeostasis in Pseudomonas aeruginosa***

In *P. aeruginosa* the expression of the denitrification gene clusters is controlled by redox signalling through a cascade of oxygen-responsive regulators activating the N-oxides-responsive ones.
These regulators belong to the CRP-FNR (cAMP receptor protein/fumarate and nitrate reductase regulator) superfamily of bacterial regulators which play a crucial role on the control of the nitric oxide (NO) homeostasis, maintaining the steady-state concentration of nitrite and NO below cytotoxic levels; as a consequence, free NO concentration is in the nanomolar range (Spiro, 2007).
In particular, the global oxygen-sensing regulator ANR (anaerobic regulation of arginine deaminase and nitrate reduction) (Galimand et al., 1991), an homologue of the *Escherichia coli* oxygen sensor FNR (fumarate and nitrate reductase regulator) activates, under anaerobic conditions, the gene coding for the transcription factor DNR (dissimilatory nitrate respiration regulator); this

protein, in the presence of N-oxides, promotes the expression of the *nir*, the *nor* and the *nos* genes (Arai et al., 1995, 1997, 1999, 2003) (Figure 1.3).

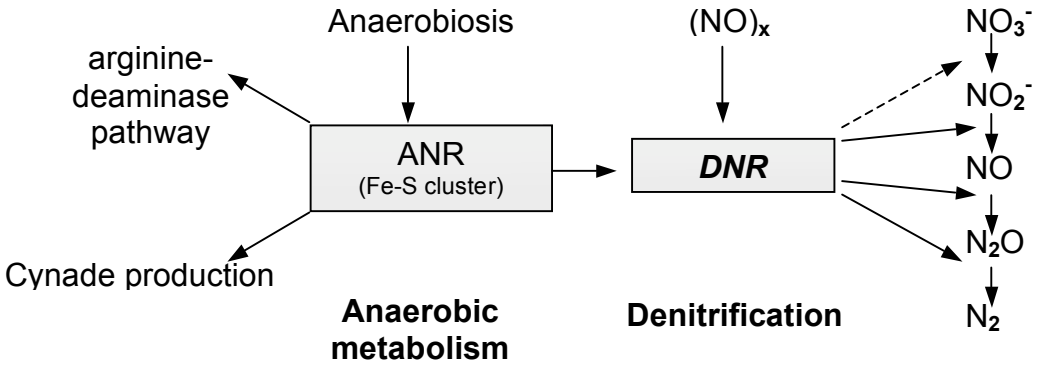

Figura 1.3 Signals and components involved the regulation of the denitrification in *P. aeruginosa*.

P. aeruginosa mutants lacking the *anr* (*anr*-) or *dnr* (*dnr*-) genes are not able to activate the *nir* and *nor* promoters under growth conditions where denitrification should be active (Arai et al., 1995). The *anr*- strain transformed with a plasmid carrying the *dnr* gene (*anr*-pDNR) recovers the promoters activation (Arai et al., 1997). DNR-mediated transcriptional activation of denitrification depends on endogenous NO concentration (Arai et al., 1999, 2003); the transcriptional activation analysis is summarized in Table 1

strains	*dnr*	*nir*	*nor*
anr-	-	-	-
dnr-		-	-
anr-pDNR		+	+

Table 1 Transcriptional activation analysis of the *dnr, nir* and *nor* promoters carried out by using reporter gene systems. *P. aeruginosa* mutants lacking the anr (*anr*-) or dnr (*dnr*-) genes are not able to activate these promoters. The *anr*- strain transformed with a plasmid carrying the *dnr* gene (*anr*-pDNR) recovers the *nir* and *nor* promoters activation.

DNR is a member of a large superfamily of regulators, the CRP-FNR superfamily of transcription factors; this transcription factor will be described in more detail below.

1.4 The CRP-FNR superfamily of transcription factors

The CRP-FNR regulators are DNA-binding proteins that mainly function as positive transcription factors, even if an additional role as repressors cannot be ruled out. CRP-FNR superfamily classification based on a phylogenetic relationship results in the assembly of the regulators in the following distinct clusters or subgroups: ArcR, CooA, CprK, Crp, Dnr, FixK, Flp, Fnr, FnrN, MalR, NnrR, NtcA, PrfA, and YeiL (Figure 1.4, A). Several other groups consist of sequence-derived proteins of unknown physiological roles; some of them are tight clusters of highly similar members (Körner et al., 2003).

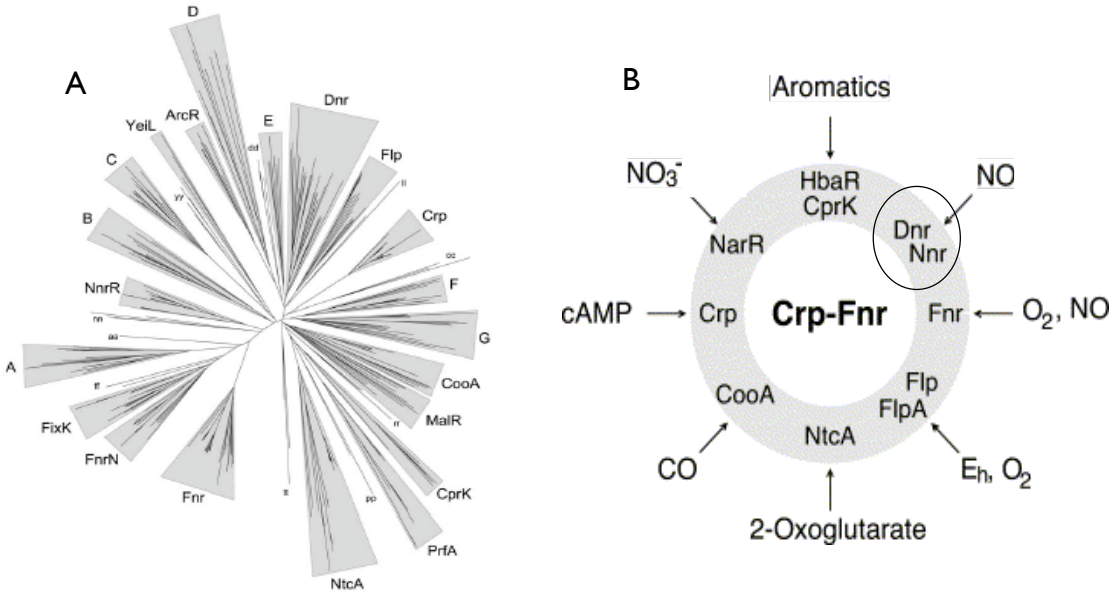

Figure 1.4 A) The main branches of the CRP-FNR superfamily of transcription factors (modified from Körner *et al.*, 2003). B) Signals processed by regulators of the CRP-FNR family. (Körner *et al.*, 2003; Joyce et al., 2006).

The CRP-FNR regulators are involved in the regulation of many enzymatic pathways including oxygen respiration, denitrification, nitrogen fixation, methanogenesis, CO-respiration, halo-respiration and virulence activation.
Therefore they respond to a broad spectrum of intracellular and exogenous signals such as cAMP, anoxia, the redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate, or temperature (Figure 1.4, B) (Aono, 2003; Eiting et al., 2005; Joyce et al., 2006). To accomplish their roles, CRP-FNR members have intrinsic sensory modules allowing direct binding of allosteric effector molecules, or have prosthetic groups which interact with the signalling molecule(s).
The CRP-FNR proteins are constant in size with 230-250 amino acid residues, the first 150-170 residues corresponding to the effector domain (Korner et al., 2003). They are usually homodimers, each monomer being formed by three domains (McKay & Steitz, 1981): (i) an N-terminal sensing domain (also referred to as the effector domain) with the typical fold of the cAMP binding domain of CRP; (ii) a long dimerization α-helix recruited to form the dimer interface; and (iii) a C-terminal DNA binding domain that contains a helix–turn–helix (HTH) motif (fig. 1.5). These transcription factors present an high plasticity, in fact the binding with the signal molecule entail a large conformational change of the protein from an "OFF" conformation to a "ON" conformation able to bind the DNA; an example of the ON conformation is shown in fig. 1.5 which represent the **CRP** or CAP (Catabolite Activator Protein) protein structure from *E. coli* crystallized bound to the DNA.

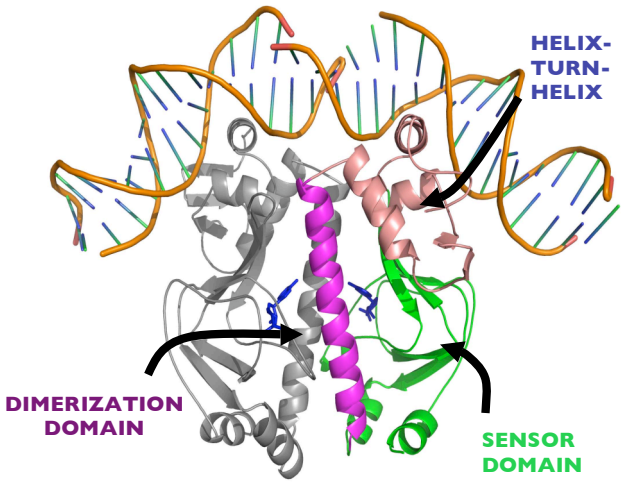

Figure 1.5 (CAP) from *E. coli*. B) The fold of these proteins consists of three conserved domains: a N-terminal sensor domain, a dimerization helix and a DNA binding domain (helix-turn-helix).

This protein is very well known as it was the first transcription activator to be discovered and to have its X-ray structure solved. The relatively simple activation mechanism of a target gene, requiring in the basic case besides CRP and RNA polymerase no additional protein factor, has made CRP a prime object of structural, biochemical, and genetic research (Schultz et al., 1991; Botsford et al., 1992; Busby et al., 1999). Being CRP the first member of the superfamily, all the proteins which share the same domain organization (see below) are classified as CRP-FNR regulators. The mechanism of action of this transcription factor is based on its capability to bind a molecule of cyclic AMP (cAMP). The increase of the cAMP level depends on the concentration of glucose in the growth medium: at low glucose concentration cAMP increases and activates the CRP protein. This entails the consequent transcription of genes coding for enzymes which are able to utilize alternative carbon sources for energy production.

Another CRP-FNR factor well-characterized structurally is **CooA.** The crystal structure of CooA has been determined by Lanzilotta *et al, (*2000) and reveals similarity whit the individual domains of CRP.
It is a homodimeric protein containing one type-b heme for subunit which interacts with the CO gas. In this case the CO molecule binds to the heme iron and triggers a conformational change, thus regulating the expression of the *coo* operon (Lanzilotta et al,. 2000). These genes encode for the CO dehydrogenase and for accessory proteins for the biosynthesis of the Nl-Fe-S center.
A wide group of the CRP/FNR superfamily include the N-oxides sensors which is divided into different subgroups, namely the DNR, NnrR and FNR ones (Rinaldo et al., 2006), which recognize the same consensus sequence in their target promoters, the FNR-box ($TTGATN_4ATCAA$) (Eiglmeier et al., 1989; Hoeren et al., 1993; Hasegawa et al., 1998) (fig. 1.6).

nirS	…atcTTGATtccggTCAAg…
norC	…atcTTGATtgccATCAA…
melR	…aatTTGATgtacATCAA…

Figure 1.6 A FNR-box is present in both the *E.coli* FNR target promoter (*melR*) and in the *P.aeruginosa* DNR target promoters (*nirS* and *norCB*)

All members of the DNR subgroup contain a highly conserved aminoacid sequence motif Glu--SerArg (E--SR), directly involved in the interaction with the FNR-box (Green *et al*., 2001), while most members of the NnrR subgroup contain an histidine instead of a glutamate residue. Another transcription factor belonging to the E subgroup (fig. 1.5) of the CRP/FNR superfamily, i. e. NssR from *Campylobacter jejuni*, has been recently described to act, perhaps indirectly, as a NO sensor (Elvers et al., 2005).
FNR contains an iron-sulphur cluster, and can be considered as the paradigm of an oxygen responsive transcription regulator (Green and Scott, 2001; Spiro, 1994; Guest et al., 1996; Unden, 1998; Kiley and Beinert, 1999). Moreover, it

may be included into the NO-sensor group due to its capability to respond to the NO molecule, which is able to entail the inactivation of the protein by nitrosylation of the iron-sulfur cluster (Creack et al., 2008).
The FNR scaffold must have a high degree of adaptability and flexibility to accommodate interaction with several chemically distinct sensor molecules, and to allow for different DNA-binding and RNA polymerase contacts. The spectrum of regulated functions in which FNR is involved is considerable. Among them are genes coding for alcohol dehydrogenase, aconitase, asparaginase, C4-dicarboxylate transporter, cytochrome d- and o-type oxidases, dimethylsulfoxide reductase, fumarate reductase, molybdenum cofactor biosynthesis, hydrogenase, nickel transport, and NADH and succinate dehydrogenases (Körner et al., 2003).
ANR is the homologue of FNR in *P. aeruginosa*. It was discovered in the context of studying arginine and pyruvate catabolism (Galimand *et al.,* 1991). It has four conserved cystein residues and an iron-sulphur center suggesting that this protein is involved in the oxygen response (Schreiber *et al.,* 2007); when the oxygen levels are low or not sufficient for an aerobic growth, ANR activate the transcription of the DNR regulator as described above. ANR is also involved in the regulation of the transcription of genes for hydrogen cyanide biosynthesis (Cooper *et al.,* 2003).
The **DNR**-type class of regulator will be discusses below in more detail.

1.3 ***DNR-type class of regulators***

DNR was discovered in the context of investigating the expression of the anaerobic respiratory system of nitrate denitrification. DNR does not contain enough cysteines for iron-sulfur clusters formation contrary to FNR, suggesting a different mechanism of N-oxide(s) sensing. In *Pseudomonas aeruginosa* DNR is found to exert the transcriptional regulation in the presence of N-oxide(s) and under low oxygen tension (Arai *et al.* 1999; Hasegawa *et al.,* 1998). In *Paracoccus denitrificans* it was shown, by genetic approach, that the transcriptional regulator designated Nnr (belonging to the DNR subgroup) can

activate the expression of the nitrite and NO reductase genes in response to NO (Van Spanning et al., 1999; Lee *et al.,* 2006).

The members of DNR-type class of regulators found in the *Pseudomonas sp.* (Arai et al., 1997 and Vollack and Zumft, 2001) share a high sequence identity (Figure 1.7) but may not fulfil an identical physiological role. This is not surprising given that Pseudomonads are well known for their metabolic flexibility which reflects the capability of the different species to survive as free living organisms in soil, water and animals, where are often responsible for diseases.

```
Dnr  Reinekea sp.      --MIQDPKVITNLSSHHLFSSLNEVQLSTLLRNATRRTLDEREVLFRQSE 48
Dnr  Moritella sp.     --MITDSHIIDIVSTHHLFSALTDAEFKQLFATAKTFKVEPLENVFHQGD 48
Dnr  P.aeruginosa      --MEFQRVHQQLLQSHHLFEPLSPVQLQELLASSDLVNLDKGAYVFRQGE 48
DnrD P.stutzeri        --MVLHRVHHQILRSHHLFEPLNEEQMEELLNASQLLNLDKGDNLFHQGE 48
DnrS P.stutzeri        --MLTEKTLVAELRRHHLFSRLPDAALQEVCASANLKRLPAGASLFHQGD 48
DnrE P.stutzeri        MAMLTGSAVLNTLRRHHLFSGLAEAALQDIAAHTTVKRLPAGCTLFHQGD 50
                         *         :  ****. *    :. :   :    :  :*:*.:
Dnr  Reinekea sp.      PADRFYYVSHGIIRLYRTSPSGHEKVVDIVREGQCFAEAIMFNAQERYPV 98
Dnr  Moritella sp.     EANRFYLVLRGHLKLYRTSPSGQEKVVEVMRQGNTFAEALMFNNKPFYPV 98
Dnr  P.aeruginosa      PAHAFYYLISGCVKIYRLTPEGQEKILEVTNERNTFAEAMMFMDTPNYVA 98
DnrD P.stutzeri        PAHNFYFVISGAVKVYRLTPDGQEKVFEVIGNRQTFAEAMMLMDTPNYVA 98
DnrS P.stutzeri        KADRFYFLFSGQIKLHRVVCDGQEKLVEVMRAGESFAEALLFKGTPCYPV 98
DnrE P.stutzeri        AAEHFHVLINGQVKLHRVTCDGQEKVIEVVRPGEAFAEAMLFNKLPEHPL 100
                        *. *: :  * ::::*   .*:**:.::    : ****:::     :
Dnr  Reinekea sp.      TADAQVASEVIGIENGSFHGLLKSDNELCMALMRQMSIRLHSQLNEIENL 148
Dnr  Moritella sp.     AAQAVSESELISIDNETYLKILKINPEAGIAIMANMSIRLHHDLNEIEML 148
Dnr  P.aeruginosa      TAQAVVPSQLFRFSNKAYLRQLQDNTPLALALLAKLSTRLHQRIDEIETL 148
DnrD P.stutzeri        SAQAVCPSQVYRFSNAAYMRLLEANQRLTFALLGKLCVRLHQRINEIETL 148
DnrS P.stutzeri        SATALKASLVASLNGPHYRRILEQQPDICLDILATLSIRLHQRMTEIDTL 148
DnrE P.stutzeri        SATTLKESLVLNVQNSHYLRLLETQPQLCMQLLSSLSARLNQRLHQIDSL 150
                       :* :   * :  ...  :   *: :    : ::  :. **:  : :*: *
Dnr  Reinekea sp.      SLQNALHRLVNYLLHDLQSPN---DTLTFDIPKRLIASQLGIQPETFSRL 195
Dnr  Moritella sp.     SVENAKNRLLLFLLKNLQDNNGNEGIIELDIPKRTLASLLSIQPETFSRL 198
Dnr  P.aeruginosa      SLKNATHRVVRYLLTLAAHAPGENCRVEIPVAKQLVAGHLSIQPETFSRI 198
DnrD P.stutzeri        SLKNATHRVVRYLLTQLARVKDGSNSFELPMAKQLVAGHLSIQPETFSRI 198
DnrS P.stutzeri        TLANASHRVVRFLAQSQQDD---SGVVVLDVPKRLIASKLGIQPETFSRI 195
DnrE P.stutzeri        TSSNVSQRVVRYLFQELQAAR--SGVIDLDMPKRLIASQLGIQPETLSRI 198
                       :  *. :*:: :*            . : :.*: :*. *.*****:**:
Dnr  Reinekea sp.      LRKLEDNQLIQVSNRDLKILDRDGLYQLTRD-NQPLRARKP--------- 235
Dnr  Moritella sp.     LKKMTKEGLIEERKGLIRIMDIDALYAASDIPVQSVTGNIPTQFVPGE   246
Dnr  P.aeruginosa      MHRLGDEGIIHLDGREISILDRERLECFE---------------------- 227
DnrD P.stutzeri        IRRLIDEAIITQEGRQIAILDRQRLEQFE---------------------- 227
DnrS P.stutzeri        LHRLIDAGTISVQRRRIEILDNRKLAAYDE--------------------- 225
DnrE P.stutzeri        LHRLTDAGLIAVQRRRIEILDHLSLSAYLDAAA----------------- 231
                       :::: .   *      : *:*   *
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Figure 1.7 Multiple sequence alignment of DNR proteins from *Pseudomonas aeruginosa*, *Moritella sp.*, *Reninekea sp.* (DNR) and *Pseudomonas stutzeri* (DnrD, DnrS and DnrE). Amino acid one-letter code is used. Dashes represent insertions and deletions; numbers indicate absolute sequence numbering. Invariant positions are marked with asterisk; alignment columns displaying amino acid with the same physico-chemical properties are signed with points.

In *Pseudomonas stutzeri* there are at least three regulators (DnrD, DnrE, DnrS) involved in the NO-sensing, activation of the nitrate pathway and possibly in redox sensing, respectively, under anaerobic conditions (Korner *et al.*, 2003). The DnrD transcription factor induces the expression of *nirSTB, norCB*, *nosZ* operons (encoding respectively nitrite, nitric oxide and nitrous oxide reductases) in the presence of NO but not nitrite (the *nos* gene is activated also in presence of high concentration of nitrous oxide). The NO concentration required for the *nir-nor* operons activation is in the range of 5-50 nM (Vollack and Zumft, 2001). DnrD overexpression *per se* is not sufficient for the transcription of the *nir-nor* operons, indicating that additional factors may be required (Vollack and Zumft, 2001).

DNR from *P. aeruginosa*. The gene encoding the DNR transcription factor was revealed upon sequencing the regions downstream of the *nor* operons encoding NO reductase of *P. aeruginosa* (Arai et al., 1995). In this bacterium DNR promotes the expression of the *nir,* the *nor* and the *nos* genes under anaerobiosis in the presence of N-oxides (Arai et al., 1995, 1997, 1999, 2003). In the last years the structural/functional characterization of the DNR protein was undertaken, to gain further insight into the mechanism of NO-sensing. It was recently shown that this transcription factor forms stable complexes with heme, suggesting an involvement of this cofactor in the NO-sensing (Giardina et al., 2008). These studies are described below.
The first structural insight into DNR was obtained by looking at the structure of a truncated version of the protein, in which the DNA binding domain was removed by mutation of Asn152 into a stop codon. The crystal structure of this mutant (named ΔC-DNR) was solved by Dr Giorgio Giardina and is shown in Fig. 1.8.

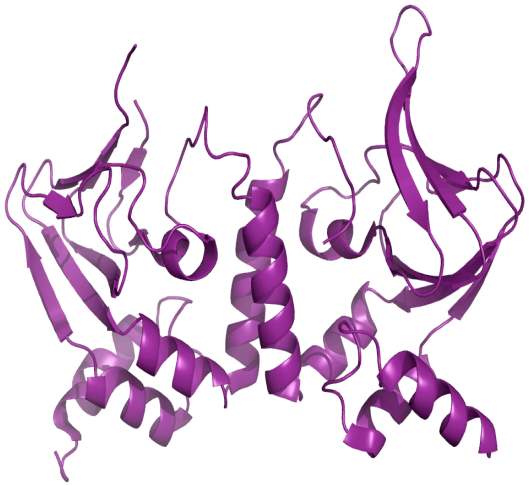

Figure 1.8 Crystal structures of the DNRN152stop mutant (ΔC-DNR), in which the helix-turn helix domain was removed by mutagenesis.

The protein is a homodimer and it crystallized as a apo-protein since no cofactor is bound in the structure. The ΔC-DNR contains a hydrophobic cavity which could be the binding site of a heme and thus form the active site for NO sensing. This hypothesis is supported by the observation that 15 of the 26 residues conserved among the DNR subgroup (DNRD, DNRE and DNRS from *Pseudomonas stutzeri* and DNR regulators from *Reinekea sphaeroides* and *Moritella sphaeroides*) cluster around this cavity (Giardina *et al.,* 2008). Comparing the ΔC-DNR structure with the available structures of other CPR-FNR superfamily proteins it was assumed that the structure of the ΔC-DNR protein is in the "ON" conformation (see discussion).
Moreover, spectral and kinetic characterization was carried out on the purified DNR protein reconstituted with the type-b heme (holo-DNR), to estimate the stability of the heme/protein complex and its reactivity with key ligands such as NO and CO. The spectral properties of the holo-protein are summarized in Fig. 1.9: the ferric derivative shows a peak at 417 nm (A, bold line), suggesting formation of a hexacoordinate hemichrome. This hypothesis is confirmed by looking into the spectrum of the reduced derivative, obtained in the presence of excess of sodium dithionite, which is characterized by a Soret band at 426

nm and two peaks at 530 and 560 nm in the visible region (B, grey line). The ferrous CO-bound derivative of holo-DNR has the typical Soret peak at 421 nm and two bands at 540 and 570 nm (B, dashed line). On the other hand, addition of NO to the reduced protein yields a spectrum with a peak at 389 nm and a broad band in the visible region (Fig. 1.9, B black line), which suggest the possibility of a five-coordinate NO-bound heme, similar to that typical of other NO sensors (Stone *et al.,* 1995; Price *et al.,* 2007). The spectroscopic data are consistent with the hypothesis that DNR binds heme specifically and forms with CO and NO derivatives typical of a heme protein. The two ligands are different in terms of heme coordination, a behaviour typical of other gas sensors (e.g., CooA and H-NOX domains), which highlights the capability of the protein to discriminate between different ligands (Boon *et al.,* 2005).

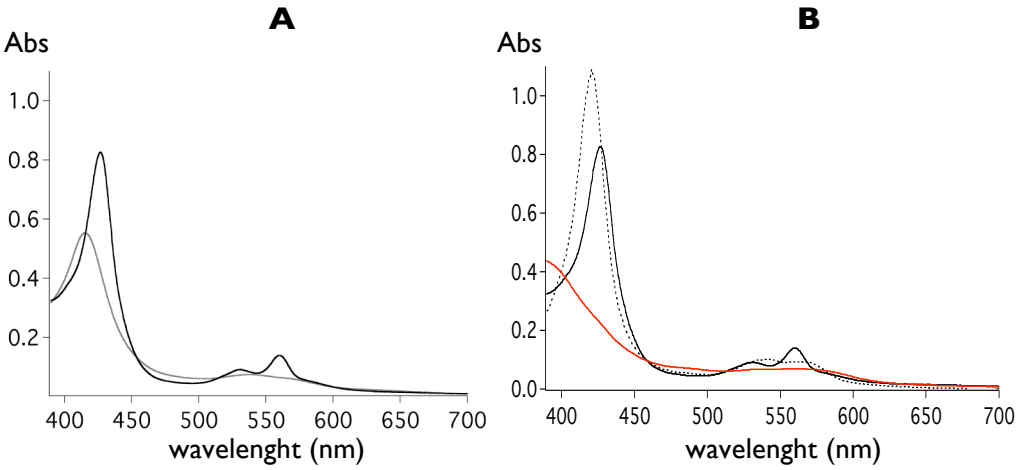

Figure 1.9 DNR-heme complex. (A) Absorbance spectra of the oxidized (gray line) and reduced (black line) forms. (B) Absorbance spectra of reduced DNR-heme complex (black line) bound to NO (red line) or CO (dashed line). NO-bound complex formation is also shown (inset).

Moreover, to assign a role to the heme cofactor in the transcriptional activity of DNR, an *in vitro* functional assay was carried out with the *nir* promoter (Electrophoretic Mobility Shift Assay, EMSA). Recombinant DNR was expressed in the presence of hemin using a modified strain of E. coli harbouring a plasmid which produce ChuA, a protein known to be a heme transporter. DNR purification yields two pools of the protein (holo-protein, pools 1 and apo-protein, pool 2) that were characterized separately. A partial DNR/DNA binding was obtained only in the presence of the holo-protein (fig. 1.10) (Giardina et al., 2008).

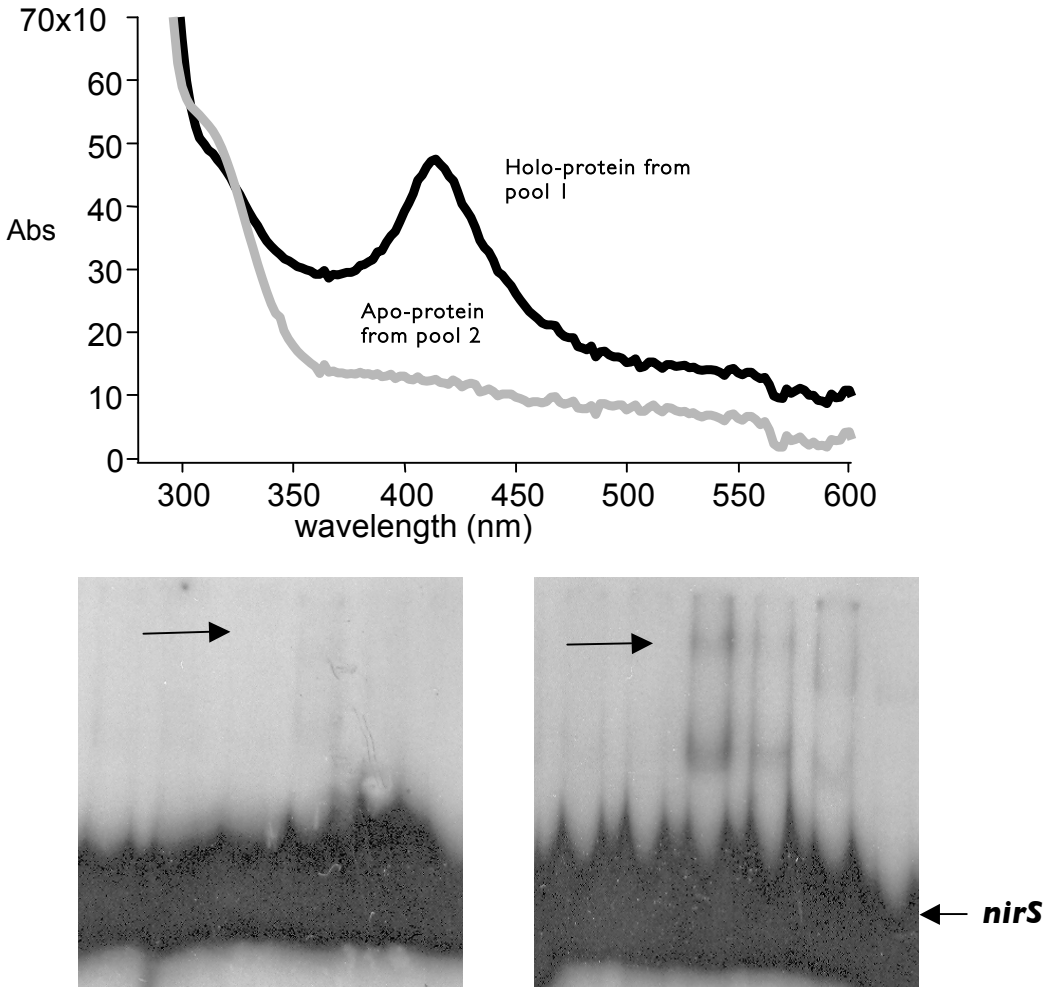

Figure 1.10 Properties of the recombinant DNR expressed in the presence of hemin. Purified DNR yields two pools of the protein (pools 1 and 2) that were characterized separately. In the figure, only selected fractions belonging to each pool are shown: holo protein, bold line, apo protein thin line. (B) EMSA of the DNA binding activity with the radiolabelled nirS promoter: fractions (from 1 to 5) belonging to pool 1 (panel 1) and pool 2 (panel 2), respectively. The arrows show the DNA/DNR complex migration in the acrylamide gel.

AIM OF THE WORK

Understanding the fate of nitric oxide (NO) inside the cell is a major issue in biology, given the large amount of processes controlled by this gas, both physiologically and pathologically.

Denitrifying bacteria, that use denitrification as anaerobic energy producing pathway by reducing NO_3^- to N_2 *via* NO (Moreau-Marquis S *et al.,* 2008; Hassett *et al.,* 2002), can keep the concentration of the intracellular NO below cytotoxic levels thanks to the regulation of the expression and activity of the enzymes nitrite and NO reductases (NIR and NOR, respectively).

Moreover, recent studies indicate that denitrification (Hassett *et al.,* 2002) and in general anaerobiosis (Sarti *et al.,* 2004) are responsible of the NO-resistance of pathogens. Among denitrifiers, *P. aeruginosa* is one of the most studied organisms due to its capability to colonize different environments also as opportunistic pathogen, mainly in cystic fibrosis patients (Driscoll *et al.,* 2007). The lung epithelium of these patients is coated by a mucus layer, due to an altered ion transport which blocks the normal mucociliary clearance; the mucus adheres to the epithelium, and this layer is a barrier to oxygen diffusion.

During colonization of the inflamed tissues this pathogen forms a biofilm which is a highly organized structure consisting of cells embedded within a matrix of extracellular polymeric substance, attached to a surface (Hassett *et al.,*2008). Cells within a biofilm have a number of advantages over their planktonic counterparts, including protection against antimicrobial agents and other stresses (Fux *et al.,* 2005). The mechanisms that permit bacteria to make the transition from an free or planktonic cell to a multicellular biofilm and back have become a subject of intense interest to microbiologists over the past several years (Romeo, 2006).

In this environment, *P. aeruginosa*, which is a facultative anaerobe, can use denitrification as alternative respiration pathway. The expression of the four reductases involved in this process (Zumft, 1997), namely nitrate (NAR), nitrite (NIR), nitric oxide (NOR) and nitrous oxide (NOS) reductases, is tightly regulated, being the intermediate nitric oxide (NO) a cytotoxic compound (Hassett *et al*., 2002).

The regulation of the expression of NIR and NOR enzymes is carried out by the DNR transcription factor which is an NO-sensor belonging to the CRP/FNR superfamily (Rinaldo *et al.,* 2006; Giardina *et al.,* 2008).
Recent studies (Barraud et al., 2006 and 2009) have identified NO, at concentrations (nM) far below the toxic levels for this bacteria, as an elicitor of biofilm dispersal or detachment by *Pseudomonas aeruginosa*. Furthermore, low levels of NO were sufficient to sensitise the cells to at least some bactericidal agents. How these effects of NO are mediated is yet to be determined.
Indeed a deeper insight onto NO-sensing in *P. aeruginosa* could lead to a new therapeutical approach in the treatment of chronic infections based on antipathogenic drugs and/or antibiotic compounds by attenuation of bacterial virulence and inhibition of biofilm formation such that the organism would fail to colonize (Hentzer and Givskov, 2003). One of these new targets could be the DNR-mediated denitrification pathway.
The effect of N-oxides on the expression of the *nir*, *nor* and *nos* genes by DNR has been previously demonstrated in *P. aeruginosa* (Arai *et al*., 1999, 2003).
To date, little structural and biochemical information on the molecular basis of the NO-dependent regulation is available.
Recently a recombinant form of *P. aeruginosa* DNR has been expressed in *E. coli* and its structural and functional properties studied *in vitro:* it has been determined by X-ray crystallography the three-dimensional structure of the NO-sensing domain and it has been shown that DNR binds heme, thus suggesting a possible mechanism for the NO-dependent activity of the protein (Giardina *et al.,* 2008). Moreover partial binding of heme-containing DNR to the target DNA can be obtained *in vitro* by EMSA (Giardina et al., 2008). The latter is one of the few examples of functional assays performed *in vitro* with a CRP/FNR regulator, which underlines the real difficulty in studying *in vitro* the activity of a protein belonging to this superfamily of transcription factors.
The evidence of a direct interaction between DNR proteins and their target promoters was inferred by sequence analysis. All DNR proteins, in fact, share a helix-turn-helix motif in the C- terminal domain assumed to be involved, by sequences comparison, in the DNA binding. Moreover, this domain contains an FNR-box binding motif which is a signature of the FNR-like transcriptional

regulators and the DNR- dependent promoters share an FNR-box (Körner et al., 2003).

Due to the difficutly to characterize the DNR activity *in vitro,* in order to get a deeper insight in the N-oxides mediated transcriptional activity of the protein, we decided to characterize the DNR function *in vivo*. The primary aim of this work was to optimize a NO-dependent DNR transcriptional activity in a heterologous model organism such as *E. coli,* which is unable to carry out denitrification, by using the *P. aeruginosa nor* promoter as a target. Moreover we studied the specificity of the DNR/DNA interaction analyzing both the transcriptional activity of DNR mutants and the effect of mutations in the FNR-box of the *nor* promoter. We have also investigated the importance of heme as the cofactor in the mechanism of DNR-mediated activation of the *nor* promoter in *E.coli*, and analyzed if the protein responds specifically to NO, studing its activity in the presence of the alternative heme ligand carbon monoxide (CO). In parallel we have carried out structural and spectroscopic characterization to better understand the role of heme on DNR activity and to identify which residues were putatively involved in the coordination of the heme cofactor. These studies allowed us to hypothesizes a novel model of activation of the DNR protein.

METHODS

3.1 Plasmids construction

The following oligos (5'-GGAATTCCATATGGAATTCCAGCGCGTCCACC AGC-3', 5'-CCGCTCGAGTCACTCGAAGCACTCCAGGCGTTCGC-3') were used to clone the dnr gene into the pET28b vector in frame with a 5'-sequence encoding for a hisidine tag (his-tag); extra NdeI-XhoI restriction sites at the 5' and 3' ends, respectively, were also introduced.
The dnr gene was linked to the his-tag motif through a sequence encoding a thrombin site, useful to remove the tag from the purified protein. The pET-DNRHIS vector was transformed into BL21(DE3) Escherichia coli strain.
The following oligos (5'-GCGCGATATGTGATAAGAAGGAGATATACCAT GGAATTCCAGCGC -3'; 5'-GCGCGATATCTCACTCGAAGCACTCCAGG CGTTCGCGGTCGAGG -3') were used to clone the dnr gene into the pACYc184 (Biolabs) vector under the constitutive tet promoter; an extra EcoRV restriction site and the ribosome binding site were also added, yielding the pACY-DNR plasmid. The nor-lacZ reporter system was obtained by cloning the nor promoter from P. aeruginosa PAO1 genome first into the EcoRI and BamHI sites of the pUC18 vector, yielding the pUC-NOR plasmid and subsequently into the EcoRI and BamHI sites of the pRS415 vector, yielding the pRS-NOR plasmid. The nor promoter fragment used in this work includes 150 bp upstream of the ATG of the norCB gene (Fig. 1). The pRW50 carrying the FF-pmeIR fragment (Wing et al.,1995) was also used.

3.2 In vivo ß-galactosidase assay.

The pACY-DNR and the pRS-NOR plasmids were transformed into the E. coli strain TOP10 (INVITROGEN) [F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-] or into the C600 *E. coli* strain [F- tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ-(hemA::kan)] (ΔhemA) (a kind gift of

Cécile Wandersman, Institut Pasteur, Paris, France). The rich medium for bacterial growth was Luria-Bertani (LB) broth (tryptone [10 g l -1], yeast extract [5 g l-1], NaCl [10 g l-1]), supplemented with 0,4 % (w/v) glucose (LG broth) and 0,4 mM ALA (5-aminolevulinic acid hydrochloride), as indicated.
DNR activity was tested by the ß-gal assay at least in duplicate according to the method of Miller (Miller, 1972) on at least two independently grown cultures. The aerobic cultures were grown in 50 ml of medium in 250 ml-flasks at 37 °C to O.D.600= 0,4 and were shaken at 200 rpm. Anaerobic growth was carried out by transferring 10 ml of the aerobic samples (O.D.600= 0,4) from the flasks to the filled tubes sealed with a rubber cap. After 30' of growth to remove the residual oxygen, selected samples were induced either with 10 µM aqueous NO from a saturated solution (2 mM) or 2 mM nitrite using a gas-tight syringe or, if indicated, with 5 % (v/v) CO gas; the cultures were then incubated without shaking for 1 h before ß-gal was assayed. For the DNR mutants, the growth temperature has been switched from 37 °C to 20 °C after 1 h of growth. For the ΔhemA strain, the rich medium was supplemented with ALA when indicated.
The O.D. values and the time of ß-gal reaction (Miller, 1972) were inserted into the equation (I) reported below (U.M.=Units Miller):

$$U.M.= 1000 \times (A_{420} - (1,75 \times A_{550})) / (t \times 0,1 \times A_{600}) \qquad (I)$$

3.3 Expression in E. coli and purification of DNR-HIS

Expression of the protein was obtained in Luria Bertani (LB) medium containing 30µg/ml kanamicin. Cultures (0,75 l in 2 l flasks) were shaken at 200 rpm and grown at 37°C for 1h and than at 20°C. DNRHIS protein expression was induced with 1 mM IPTG (isopropyl ß-d-thiogalactoside) when OD600 was 0,4. Cells were then grown for 15 hours.
Cells were resuspended in the lysis buffer: 50 mM Tris-HCl buffer (pH 8.0), 50 mM NaCl, and 1 mM PMSF (Phenylmethylsulfonyl fluoride) for 40' at 4° and

then sonicated. The cell extract was centrifuged 45' at 12000 rpm to remove any insoluble material. The soluble fraction was then applied on a HiTrap™ Chelating HP column (Amersham) containing nickel sulfate salt and equilibrated with 20 mM Tris-HCl pH 7.2, 300 mM NaCl; the protein is eluted with 300mM imidazole, in the same buffer. The purified protein was then loaded on a PD-10 Desalting column (Superdex^{TM} G-25) and eluted with 20 mM Tris-HCl pH 7.2, 300 mM NaCl. Proteolytic digestion was carried out with 25 units of thrombin (Amersham) per mg of tagged-protein, at room Temperature for 15 hours. The sample was then applied a second time on a HiTrap™ Chelating HP column (Amersham) loaded with nickel sulfate and equilibrated with 20 mM Tris-HCl pH 7.2, 300 mM NaCl. Under these experimental conditions, the thrombin enzyme was recovered in the flowthrough, while the his-tag free protein and the his-tag tails eluted in the presence of 100 and 300 mM imidazole, respectively.
To remove the imidazole, the DNR protein was loaded on a HPLC column (Superdex 75 16/30) equilibrated with 20 mM Tris-HCl pH 7.2, 300 mM NaCl; the proteins concentration were calculated spectroscopically according to (Giardina et al., 2008) and the purified protein recovered was then frozen with liquid nitrogen and stored at -70°C.
The extinction coefficient at 280 nm for DNR after digestion was determined by the BCA assay (Sigma) to be: 14.8 mM-1 cm-1 (per monomer).

3.4 Western blot analysis

A goat anti-rabbit IgG (SIGMA) alkaline phosphatase-conjugated was used as secondary antibody.
The Western blot analysis was carried out to detect the DNR protein in the cultures grown for the ß-galactosidase assays and the DNR protein in all the purification steps: cells harvested and disrupted by boiling in the loading buffer (1.50 mM Tris-HCl pH 6.8, 300 mM DTT, 6 % SDS, 0.3 % bromophenol blue, 30 % glycerol) were separated by SDS-PAGE; for the western blot as a primary antibody a polyclonal rabbit antibody (Davids Biotechnologies) was used for the wild type and mutant proteins of the ß-gal assay and for the

digested purified protein; a anti his-tag polyclonal antibodies from rabbit (Santa Cruz Biotechnology, Inc.) was instead used for the DNR-HIS purified protein.
A goat anti-rabbit IgG (SIGMA) alkaline phosphatase-conjugated as secondary antibody.

3.5 Crystallization and data collection

After screening a wide rage of conditions with a crystallization robot (PHOENIX; Art Robbins Instruments), crystals of native DNR were obtained by sitting drop vapor diffusion method mixing 1 µL of protein solution (5 mg/mL in 150 mM NaCl, 20 mM TRIS pH 7.2 buffer) with 1 µL of the 500 µL reservoir solution - 20% (w/v) PEG3350, 0.2M ammonium tartrate. Crystal grew in 2 to 3 days as very thin plates. Before freezing in liquid nitrogen crystals were soaked into a solution of mother liquor and 25% (v/v) ethylene glycol.
Data were collected at 100 K at European Synchrotron Radiation Facility (ESRF) (ID23-1), Grenoble (France). The best crystal diffracted weakly to 3.6 Å resolution and belongs to space group C2. Data collection strategy was calculated with BEST (Popov *et al.,* 2003) Data were integrated using MOSFLM (Leslie *et al.,* 1992) and, due to radiation damage, only the first 220 frames (154°) were scaled with SCALA (Evans *et al.,* 1997) The asymmetric unit (AU) consists of 10 monomers (five noncrystallographic dimers).

3.6 Mutagenesis

Site-directed mutagenesis was done using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions, with pACY-DNR, pET28-DNRHIS or pUC-NOR as a template. Two mutants were obtained in the HTH region of DNR: N152stop, previously described (Giardina et al., 2008), which lacks the entire HTH domain, and E193D, which presents a substitution (Glutamic Acid to Aspartic Acid) in position 193. The mutations introduced in the FNR-box of the nor promoter are: a double mutation in

which the GA bases in position 3 and 4 have been substituted with AG (TTAGTN4ATCAA, named nor1) and a single mutation in which the A base in position 4 has been changed into a G (TTGGTN4ATCAA, named nor2). The nor1 and nor2 fragments were then inserted into the EcoRI and BamHI sites of the pRS415 vector, yielding, respectively, the pRS-NOR1 and pRS-NOR2 plasmids. All the histidines of the protein were mutated in alanines obtaining eight single-mutants and a double mutant (H14AH15A) The desired mutations were confirmed by DNA sequencing.

3.8 Heme reconstitution

The DNR apo-protein was reconstituted with a 1.5 stoichiometric excess of hemin in 20 mM Tris-HCl pH 7.2 and 300 mM NaCl at 16°C. Excess of free hemin was removed by gel filtration on a Sephadex G-25 column (Amersham). The ferric derivative was handled anaerobically in order to obtain the ferrous derivative by adding 1mM of sodium dithionite.
The ferrous protein was incubated in anaerobiosis under either a saturated atmosphere of CO gas or with 100 μl of a 2 mM nitric oxide (NO) solution (20 °C and pH 7,5) to obtain the corresponding derivative.
Spectra of the heme-reconstituted DNR species (4-7 μM solution) were recorded on a JASCO spectrophotometer, using gas tight tonometer containing a 1 cm quartz cuvette.

3.9 Heme dissociation rate

The dissociation rate constant of the heme from the holo- protein was determined according to Giardina *et al.,* (2008) in the presence of excess of sperm whale apo-Mb (prepared according to Antonini and Brunori) as heme scavenger. The reaction has been carried out in 1-cm quartz cuvette in 800 μl of total volume, in the presence of 0.45 M sucrose, 0.15 M sodium phosphate, pH 7.0, 300 mM NaCl, 9 μMapo-Mb and 2.9 μM holo-DNR (final

concentration, added as concentrated solution to the reaction mixture); the process was recorded at 410 nm in a Hewlett Packard spectrophotometer (20 °C).

3.10 Xanes

The Fe K-edge XANES spectra of ferric and ferrous holo-DNR have been collected in fluorescence mode at ESRF-BM30B, Grenoble, by using a 30-elements ultra-pure Ge detector. The spectra were calibrated by assigning the first inflection point of the Fe foil spectrum to 7112. The energy stability of each spectrum was carefully assessed by checking the position of a glitch in the I0 at 7220 eV.
The ferric holo-DNR solution contains 1.6 mM of protein, 32 mM phosphate buffer, pH 7.4, 240 mM NaCl and gly 20%; to obtain a chemically reduced holo-DNR a slight excess of sodium dithionite was added at the protein solution.
The tetra-coordinate FE(II)-protoheme was obtained by dissolving hemin in 10% sodium dodecyl sulphate (SDS) and reducing it by sodium dithionite.

RESULTS

4.1 *In vivo* activation of DNR by N-oxides in the heterologous *E.coli* system

Previous work in our group has shown that partial binding of DNR to the target DNA can be obtained *in vitro* by EMSA (*Giardina et al.,* 2008). In order to get a deeper insight in the N-oxides mediated function of the protein, we decided to characterize the DNR transcriptional activity *in vivo*. We have optimized a reporter system based on the *E.coli* heterologous background using the *P. aeruginosa nor* promoter described in fig. 4.1. The pRS-NOR plasmid, carrying the *lacZ* gene (coding for ß-galactosidase) under the control of the *nor* promoter was transformed into the TOP10 *E. coli* strain together with the pACY-DNR plasmid harbouring the *dnr* gene under the control of a constitutive *tet* promoter (see methods for plasmids construction). DNR activity was tested by the ß-gal assay according to the method of Miller (Miller, 1972) on at least two independently grown cultures. The aerobic cultures were grown in 50 ml of medium in 250 ml-flasks at 37 °C to O.D.600= 0,4 and were shaken at 200 rpm. Anaerobic growth was carried out by transferring 10 ml of the aerobic samples from the flasks to filled tubes sealed with a rubber cap. After 30' of growth to remove the residual oxygen, selected samples were induced either with 10 µM aqueous NO from a saturated solution (2 mM) or 2 mM nitrite using a gas-tight syringe; the cultures were then incubated without shaking for 1 h before ß-gal was assayed. As a control, a ß-gal assay was performed also with the TOP10 *E. coli* strain containing only the pRS-NOR plasmid.

Figure 4.1 Sequence of the *P. aeruginosa nor* promoter fragment used in this study (150 bp). The putative binding site for the DNR protein is boxed and the bases of the consensus FNR-box are highlighted in bold; the initial part of the coding sequence of the *norCB* gene, not included in the PCR fragment, is shown in grey and the translational start point is underlined.

As shown in fig. 4.2, DNR is able to transactivate the *nor* promoter in *E. coli* under growth conditions similar to those found during denitrification in *P. aeruginosa*, i.e. anaerobic conditions and the presence of N-oxides.

The results show that the DNR protein is not active under aerobic conditions, in agreement with its biological function to regulate the denitrification pathway in *P. aeruginosa,* which occurs under anaerobiosis. The low activation observed in air in the presence of 2 mM nitrite (Fig. 4.2) is somewhat unexpected; however it has to be underlined that, after several hours of growth, oxygen consumption by the rapidly growing culture may lead to a microaerobic environment. As a control, the assay was also carried out with a strain containing the sole pRS-NOR plasmid under the same conditions described above; the results show that in *E.coli* the *nor* promoter is not activated in the absence of the DNR protein (Castiglione *et al.,* 2009).

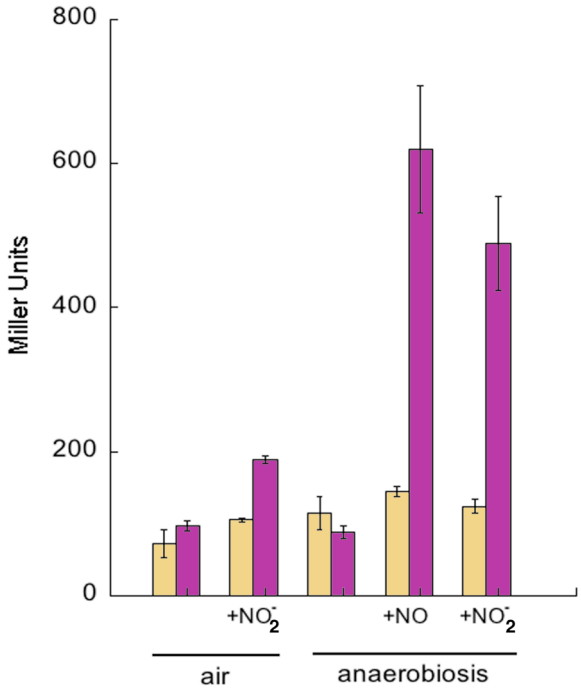

Figure 4.2 Activation of *P.aeruginosa nor* promoter requires DNR and N-oxides. The β-galactosidase activity were reported as Miller units; the yellow bars refer to the control TOP10 *E. coli* strain containing only the pRS-NOR plasmid in which the *nor* promoter is fused to lacZ, while the violet ones refer to the same strain carrying also the pACy-DNR plasmid.

The concentration of NO used in the ß-gal assay was chosen after testing a range of NO concentrations from 5 μM to 100 μM: the maximal activation of the *nor* promoter was observed at 10 μM NO. Increasing the NO concentration from 5 μM to 100 μM has no significant influence on cell growth, at least under the conditions used in this assay (Fig. 4.3).

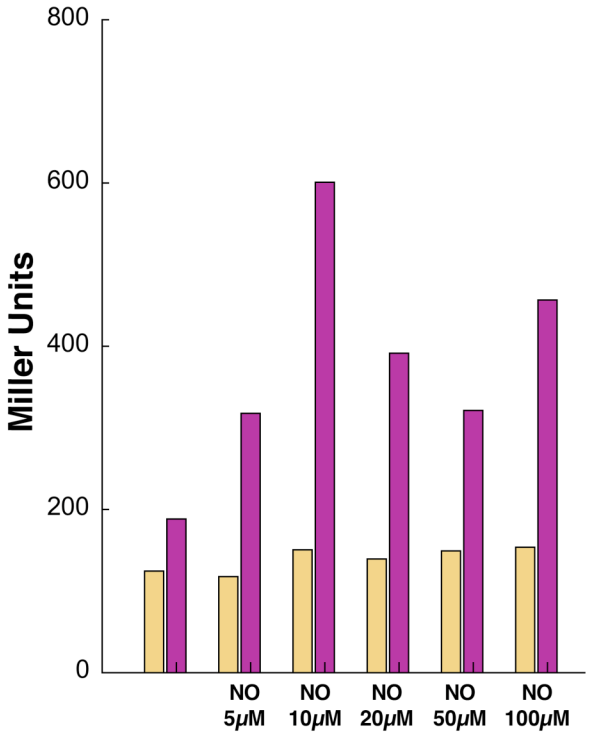

Figure 4.3 NO concentration dependence of promoter activation by *P.aeruginosa* DNR. The yellow bars refer to the control TOP10 *E. coli* strain containing only the pRS-NOR plasmid in which the *nor* promoter is fused to *lacZ*, while the violet ones refer to the same strain carring also the pACy-DNR plasmid. Cultures were grown anaerobically (AN) in LG (see methods), and, as indicated, induced by the addition of 5/10/20/50 or 100 μM NO.

4.2 FNR discriminates between the *nor* promoter and the *melR* one.

The results reported above show that the *nor* promoter is inactive in the absence of the DNR protein, under all the conditions tested. This result was quite surprising, since we expected that the endogenous FNR *E. coli* protein, which recognizes the canonical (-41,5) FNR-box containing *melR* promoter (fig 4.4a) under anaerobic conditions (Barnard *et al.,* 2003), could activate also the *nor* promoter due to the high degree of conservation of the FNR-box target

sequence among the two promoters (Fig 4.4a). To further investigate this aspect we verified if the *E. coli* TOP10 strain used in our study was fenotipically FNR+. Therefore, the *E. coli* pRW50-melR promoter, which contains the *lacZ* gene under the control of the *melR* promoter was transformed in the TOP10 *E.coli* strain and the ß-gal assay was performed under aerobic and anaerobic conditions. The cultures were grown as described above. After 30' of anaerobic growth, the cultures were then incubated without shaking for 1 h before ß-gal was assayed. The results show that in the TOP10 strain the *melR* promoter is very active under anaerobic conditions, whereas a lower level of activity was obtained under aerobic conditions (Fig. 4.4b). The lower (but significant) level of activity of the FNR protein observed under aerobiosis may occur since after several hours of growth, oxygen consumption by the rapidly growing culture leads to a microaerobic environment, responsible for the partial activation of the FNR protein observed in the assay.
The results confirm that the TOP10 strain contains a functional FNR protein, which recognizes the FNR-box of its target promoter. On the other hand, the *E. coli* protein doesn't bind the FNR-box of the *P.aeruginosa nor* promoter; this point will be further analysed in the discussion section.

A

	melR	…aat**TTGAT**gtac**ATCAA**…
	norC	…atc**TTGAT**tgcc**ATCAA**…

B

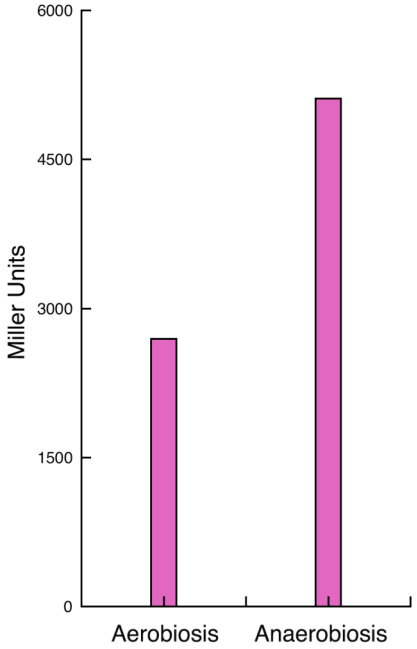

Figure 4.4 The *E. coli mel*R promoter is actively transcribed in the TOP10 strain. (A) comparison between the *E.coli mel*R promoter and the *P.aeruginosa nor* promoter. In bold the consensus sequence (FNR-box). (B) the bars refer to TOP10 *E. coli* strain containing the pRW50-*mel*R plasmid in which the *mel*R promoter is fused to *lac*Z. The β-galactosidase activity was reported as Miller Units;

4.3 DNR binds specifically the *nor* promoter

To demonstrate the specificity of the DNR/*nor* promoter interaction in the *E. coli* system described above, we performed the ß-gal assay with different mutants harbouring mutations in either the HTH DNA recognition motif or in the FNR-box of the *nor* promoter.

Two mutants where produced by site-directed mutagenesis in the HTH domain: DNRN152stop, which lacks the entire HTH domain and DNRE193D which present a substitution of the Glu 193 to an Asp. Glu193 belongs to the Glu-SerArg (E-SR) conserved aminoacid sequence involved in the recognition of the FNR-box consensus (Green *et al.,* 2001).

The pACY-DNRN152stop or the pACY-DNRE193D plasmids were transformed in a TOP10 *E.coli* strain containing the pRS415-NOR plasmid and a ß-gal assay was carried out under anaerobic conditions +/- N-oxides as described above; due to the instability of the N152stop mutant at 37°, the growth temperature for this mutant has been switched from 37 °C to 20 °C after 1 h.

As shown in Fig. 4.5A, the two mutants, compared to the wt protein, are unable to activate the *nor* promoter under any condition tested (Castiglione *et al.,* 2009). This result confirms the specificity of the interaction of DNR with the *nor* promoter. To demonstrate that the lack of activation by the two DNR mutants in the *E. coli* strain was not due to the lack of expression of the mutated proteins, we have verified by Western blot that the proteins were correctly produced in the bacterium (Fig. 4.5B).

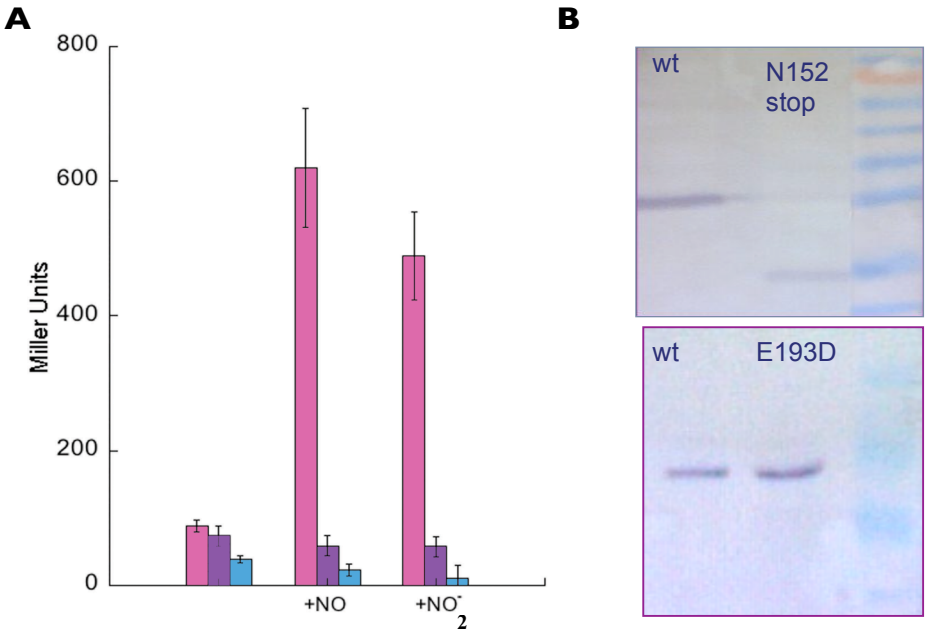

Figure 4.5. Activation of the *nor* promoter requires the direct binding of DNR. (a) The N152stop protein, lacking the DNA binding domain (violet bars), or the E193D protein (blue bars), have been expressed constitutively in the TOP10 *E. coli* strain together with the pRS-NOR plasmid and the β-galactosidase activity was measured. Pink bars refer to the β-galactosidase activity of the wild-type protein. (b) Western blot of cell extracts induced by NO collected 1 h after induction. The two mutants are compared to the wt protein. The β-galactosidase activity were reported as Miller units; the yellow bars refer to the control TOP10 *E. coli* strain containing only the pRS-NOR plasmid in which the *nor* promoter is fused to lacZ, while the violet ones refer to the same strain carrying also the pACy-DNR plasmid.

We have also probed the DNR/DNA interaction by producing specific base substitutions in the FNR-box of the *nor* promoter. Two mutants were produced by site-directed mutagenesis: the first one carries an inversion of two bases in the FNR-box (TT**AG**T-N4-TTGAT) (*nor*1) and the second one carries a single base change (A_4 to G) (TTG**G**T-N4-TTGAT) (*nor*2). The *nor*1 and the *nor*2 fragments were then cloned in the pRS415 vector and the corresponding plasmids (pRS-NOR1 and pRS-NOR2, see methods) were transformed in *E. coli* harbouring the pACY-DNR plasmid. The ß-gal activity under anaerobic conditions with or without NO (10μM) was then measured.

As shown in Fig. 4.6, DNR is unable to activate both mutant promoters under anaerobiosis in the presence of NO (Castiglione *et al.*, 2009).
To control the basal activity of the promoters in the *E. coli* system, the assay was carried out also with a strain containing only the plasmids with the wild type or the mutant promoters.

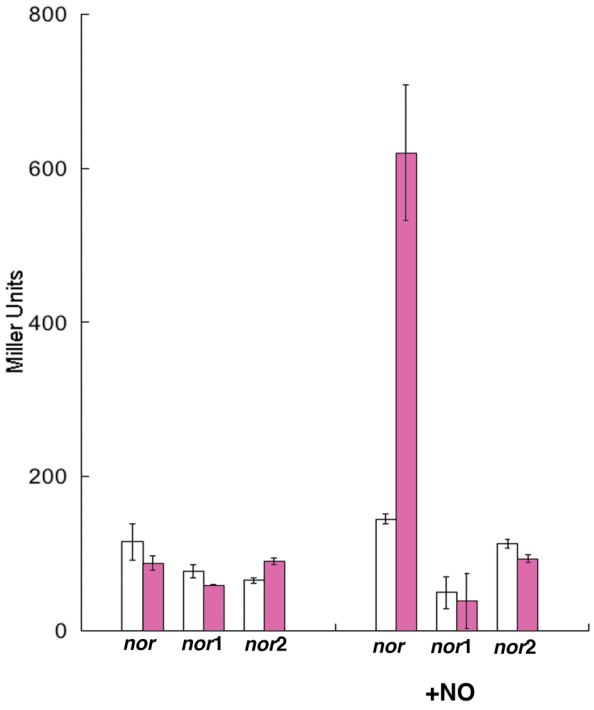

Figure 4.6 Activation of the *nor* promoter requires the direct binding of DNR. The pRS415 plasmids containing the wt *nor* promoter or the mutant promoters *nor*1 (FNR-box: TT**A**G**G**TN$_4$ATCAA) or *nor*2 (FNR-box: TTG**G**TN$_4$ATCAA) were expressed constitutively in the TOP10 *E. coli* strain together with the pACY-DNR plasmid (pink bars). As a control, the β-galactosidase activity was measured under the same experimental conditions in the TOP10 *E. coli* strain containing only the plasmids with the wild type or mutant promoters (white bars).

4.4 *DNR activity requires heme biosynthesis*

The mechanism whereby DNR can sense the signal molecule is not yet fully understood; however, spectroscopic evidence supports the hypothesis that heme is involved in NO sensing (Giardina *et al*., 2008 and see introduction). Therefore, to demonstrate that the heme is required for DNR activity *in vivo*, we have measured the NO-dependent activation of the *nor* promoter in a heme-deficient *E.coli* strain (Δ*hemA* strain), which lacks the HemA protein involved in the production of delta-aminolevulinic acid (ALA) a key precursor of heme (Fig. 4.7).

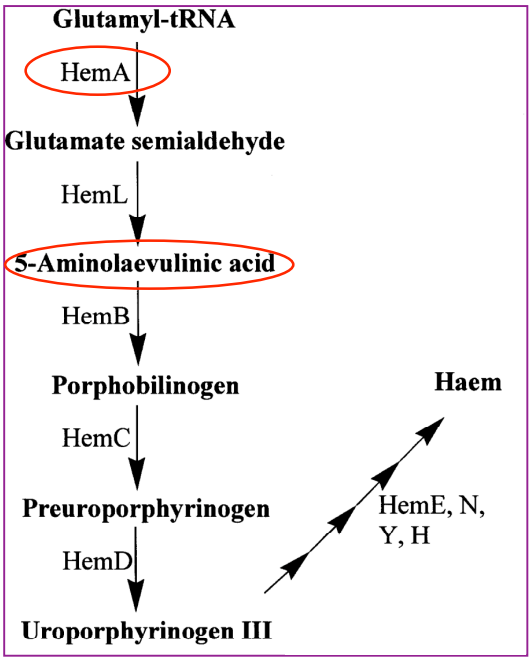

Figure 4.7 Biosynthetic pathway of heme production in *E. coli*. The blue circle indicated the role in the pathway of the HemA protein.

The pRS-NOR plasmid was transformed into the *E. coli* Δ*hemA* strain with or without the pACY-DNR plasmid, and cells were grown in the presence or in the absence of 0,4 mM ALA, under anaerobiosis +/- aqueous NO (final concentration 10μM). The ß-gal activity was measured 1 hour after the

induction time. Moreover, to evaluate the basal activity of the *nor* promoter, we also measured the ß-gal activity in the *E. coli* Δ*hemA* strain containing the sole pRS-NOR plasmid.

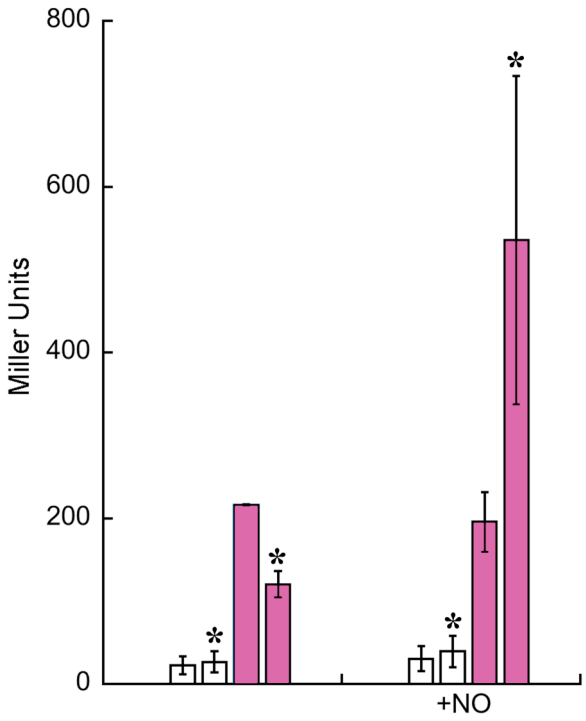

Figure 4.8 Heme dependence of DNR-mediated activation of the *nor* promoter. The DNR protein has been expressed constitutively in the *ΔhemA E. coli* strain together with the pRS-NOR plasmid (pink bars). As a control, the β-galactosidase activity has been assayed under the same experimental conditions in the *ΔhemA E. coli* strain containing only the pRS-NOR vector (white bars). Samples marked with asterisk on the graph refer to cultures grown in the presence of 0,4 mM ALA.

As shown in fig. 4.8, only in the presence of exogenous ALA a NO-dependent DNR activity comparable to that seen in the TOP10 strain is observed, strongly suggesting that heme biosynthesis is required for DNR activity (Castiglione *et al.,* 2009).

4.5 *DNR discriminates between different diatomic gases*

Given that heme is essential for DNR activation we tested if DNR is able to discriminate between different gases during its activity. Therefore, we used the *E. coli* system developed here to compare the activity obtained under anaerobiosis in the presence of NO to that obtained under anaerobiosis in the presence of carbon monoxide (CO).

The pRS-NOR plasmid was transformed into the TOP10 *E. coli* strain with the pACY-DNR plasmid harbouring the *dnr* gene; a ß-gal assay was carried out, under anaerobic conditions and selected samples were induced either with 10 µM aqueous NO or 5 % CO (v/v). The ß-gal activity was measured as described above.

The results (Fig. 4.9) show that DNR is inactive in the presence of CO, suggesting that the protein responds differently to the two diatomic signal molecules, i.e. NO and CO (Castiglione *et al.,* 2009). The *in vivo* result agrees with previous spectroscopic data showing that heme coordination in the CO and NO complexes is different (hexa- vs penta-coordinated).

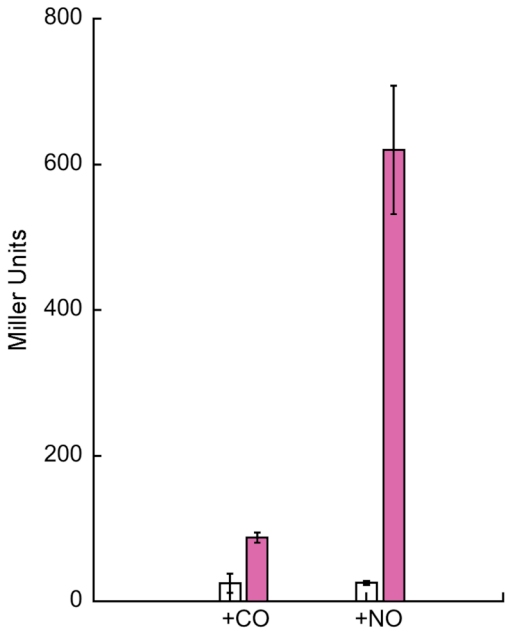

Figure 4.9 DNR is not activated by CO. The DNR protein has been expressed constitutively in the TOP10 *E. coli* strain together with the pRS-NOR plasmid (pink bars). Cultures were grown anaerobically in LG medium (see methods), and, as indicated, induced by the addition of 10 μM NO or 5 % CO (v/v). As a control, the β-galactosidase activity has been assayed under the same experimental conditions in the TOP10 *E. coli* strain containing only the pRS-NOR vector (white bars).

4.6 Expression, purification and 3D crystallographic structure of the wt DNR

To gain a deeper insight about the structure-function relationships of DNR and to clarify the role of the heme in the regulation of the transcriptional activity of the protein, we expressed and purified the protein in order to determine the 3D structure of the protein by X-ray crystallography.

DNR from *P. aeruginosa* has been purified by the heterologous system *E.coli* as described below. The dnr gene has been isolated by PCR from the genomic DNA of *P. aeruginosa* and cloned in the expression vector PET28b (Novagen) in frame with a 6xHistidine tail at the N- terminal of the protein; the

introduction of a tag facilitate the purification of high quantities of protein suitable for crystallization. The pET-DNR-HIS plasmid was transformed into the BL21(DE3) *E. coli* strain and the protein was expressed either in presence of 1 mM IPTG or in the absence of inducers (Figure 4.10 A, lane 1 and 2, respectively). The DNR-HIS protein was detected by western blot, using commercial anti-histag antibodies (Figure 4.10 A, lane 3). The protein was expressed, purified to homogeneity and concentrated as reported in methods. With this method we obtained high amount of purified DNR-HIS (50 mg/l of cell culture). The his-tag tail was then removed by thrombin proteolysis (Fig. 4.10 B).

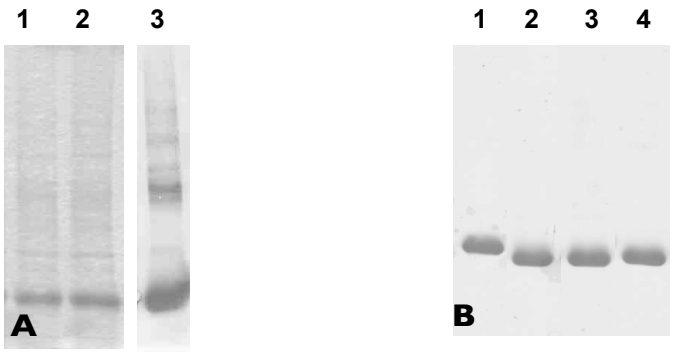

Figure 4.10 Expression and purification of the *P. aeruginosa* DNR-HIS protein from *E. coli*. SDS/PAGE. A) Lane 1: overnight cell extract without IPTG. Lane 2: overnight cell extract after induction with 1 mM IPTG. Lane 3: western blot analysis of overnight cell extract after induction with 1 mM IPTG using anti his-tag antibodies (Santa Cruz Biotechnology, Inc.). B) Lane 1: purified DNR-HIS protein. Lane 2-4: room temperature incubation of DNR-HIS with 20 units of thrombin at different times (2h, 4h and 15 h, respectively).

The purified protein crystallized as a homodimer in the apo form. The structure of wtDNR was solved by Dr Giorgio Giardina, a component of my group, by molecular replacement at 3.6 Å resolution. The 3D structure is shown in Figure 4.11 (Giardina *et al.,* 2009).

As expected, the monomer fold of DNR is similar to that of the other transcription factors of the CRP-FNR superfamily. In particular (i) the sensing domain (N-terminal; residues 1-123) is formed by a β-barrel core (β-sheets 1-

8) and three α-helices (-helix A-C); (ii) α-helix D (residues 124-152) is the dimerization helix; and (iii) the DNA binding domain (C-terminal; residues 153-227) is constituted by three α-helices (α-helix E to G) and two β-sheets (-9 and 10), where -G is the recognition helix of the HTH motif responsible for DNA binding.

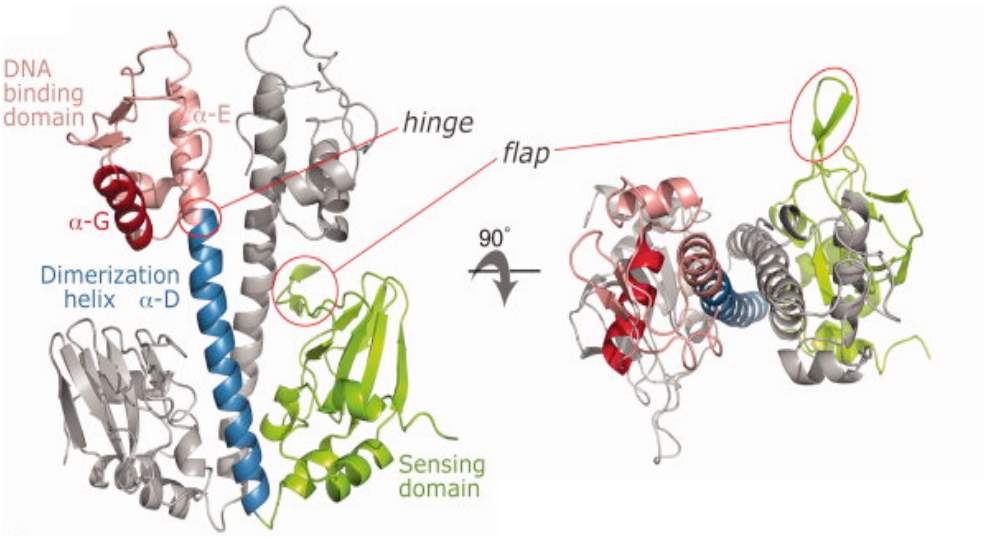

Figure 4.11 Structure of DNR from P. aeruginosa. 3D structure of DNR dimer, side and top views. The monomer domains are highlighted with different colors: green the sensing domain; blue the dimerization helix; salmon the DNA binding domain (the recognition helix -G is shown in red). The structural features hinge and flap are highlighted by red circles. The same color code is used throughout the figure.

4.7 *Identification of the residues involved in the coordination of heme iron*

As described above, the DNR protein has been recently crystallized in the apo form but not yet in the holo form. Therefore, to gain further insight on the Fe-heme environment we have studied the ferric and ferrous holo-DNR in solution by the XANES (X-ray Absorption Near Edge Structure) technique, in collaboration with the group of Prof. Arcovito (University Cattolica of Sacro Cuore of Rome) and Prof. Della Longa (University of L'Aquila).
The Fe K-edge XANES spectra of the ferric and the ferrous holo-DNR (1.6 mM of protein in 32 mM phosphate buffer, pH 7.4, 240 mM NaCl, gly 20%) have been collected in a fluorescence mode at ESRF-BM30B, Grenoble, by using a 30-elements ultra-pure Ge detector. The spectra were calibrated by assigning the first inflection point of the Fe foil spectrum to 7112.
The derivative XANES spectrum of the ferric holo-DNR was compared with different coordination models (Figure 4.12), to gain information on the axial coordination and homogeneity level of heme-iron in DNR. As shown in this Figure, the highest similarities concerning the features are obtained with the spectrum of formally ferric neuroglobin (Ngb): in this case a low-spin heme iron bis-histidine coordination occurs. Unlike other low-spin hexacoordinated species, the ferric Ngb was rapidly photo-reduced under X-ray irradiation, and thus it is a "formally" ferric Ngb. This result confirms the evidence reported in the introduction section about the 6-coordination of the heme iron in the holo-DNR and, moreover, indicates the nature of the residues involved in this coordination.

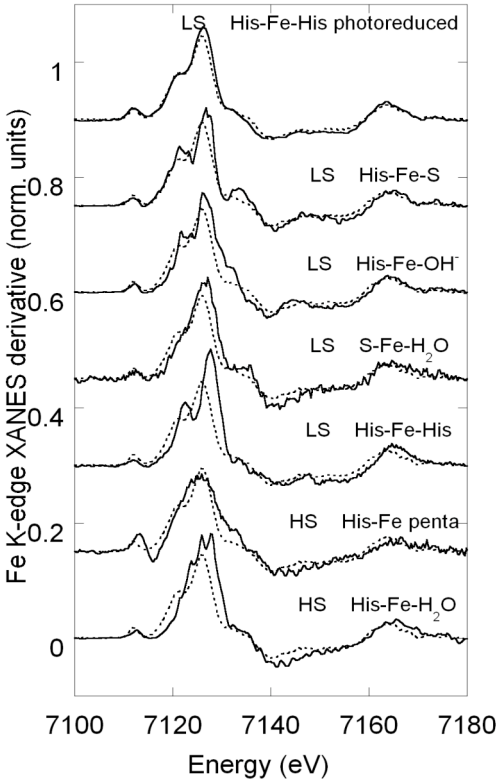

Figure 4.12. The derivative spectrum of DNR (plotted curves) is compared with various high-spin (HS) and low-spin (LS) coordination models of the heme iron in heme models and hemeproteins (solid lines). From top to bottom: neuroglobin (His-Fe(III)-His); Cytochrome C, His-Fe(III)-Met; alcaline LS met-myoglobin, His-Fe(III)-OH^-; P450, Cys-Fe(III)-H_2O; Fe(III)-tetraphenyl-porphryn-$(Imid)_2$, His-Fe-His; 1,2 metil-imidazole (penta-coordinate) hemin dissolved in SDS micelles, His-Fe(III) and acid aquomet-myoglobin, His-Fe(III)-H_2O. The first derivative is fastly photo-reduced under X-ray irradiation.

The holo-DNR protein was chemically reduced by excess of sodium dithionite and the XANES spectrum was obtained. The ferrous DNR compared with the formally ferric one (photoreduced) shows important differences between the two species which suggest the existence of an equilibrium between two different species. Mathematical fit of the data suggests the presence of an hexa- and a tetra-coordinate forms due to a partial destabilization of the axial ligation in ferrous DNR (data not shown).

Finally, the ferrous DNR-NO derivative has been compared with the TPP-NO (tetraphenylporphyrin-NO) and Mb-NO (Myoglobin-NO) derivatives. As shown in figure 4.13, the spectrum of DNR-NO in the ferrous form is similar to that of a 5-coordinate species instead of 6-coordinated species.

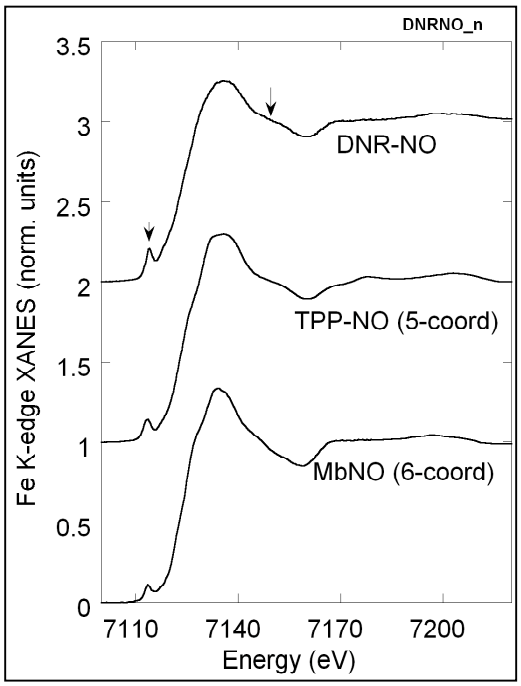

Figure 4.13 XANES derivative spectrum of ferrous DNR-NO (upper curve): the comparison with the TPP-NO derivative (medium curve) and with the Mb-NO derivative (lower curve) suggests that the heme iron of the DNR-NO form is 5-coordinate.

4.8 In vitro* and *in vivo characterizations of DNR histidines mutants

As described in the introduction, spectral and kinetic characterization on the holo-protein was carried out to estimate the stability of the heme/protein complex and its reactivity with key ligands such as NO and CO (see also Giardina *et al.,* 2008); the holo-DNR protein presents a typical spectrum of a hexacoordinate species, both in the ferric and in the ferrous form. Moreover the holo-DNR-NO derivative presents a typical spectrum of a penta-coordinate species; these results are also confirmed by the XANES assay, as described above, which also determined that the residues involved in the coordination of the heme iron are two histidines. For this reason we wanted to investigate which histidines of the protein were involved in this coordination. Therefore, we mutated all the histidines of the protein by site-specific mutagenesis, obtaining 8 single mutants of DNR, which present a substitution of a histidine to alanine and one double mutant (H14A/H15A) which present substitutions of both histidine 14 and histidine 15 to alanines. These mutants were expressed and purifyied, as described in the methods section, and were reconstituted with 1.5 stoichiometric excess of hemin in 20 mM Tris-HCl pH 7.2 and 300 mM NaCl at 16°C. Excess of free hemin was removed by gel filtration on a Sephadex G-25 column. These mutants were characterized spectroscopically: the peaks of both the ferric and the ferrous of heme-reconstituted DNR mutants, compared with the wild-type ones are summarized in the table II.

FERRIC			
WT	417	538	570
H7A	417	538	570
dHis	415	538	568
H51A	413	537	
H139A	415	539	570
H155A	415	538	570
H167A	416	537	
H187A	416 (360)	536	
H200A	415	538	575
H209A	415	537	

FERROUS			
WT	427	530	559
H7A	427	530	560
dHis	426	531	559
H51A	427	530	560
H139A	426	530	559
H155A	425	530	559
H167A	427	530	560
H187A	426(390)	532	560
H200A	425	531	560
H209A	426	530	560

Table II. Spectral properties of wt and mutant DNR proteins. Numbers indicate the wavelenght of the three maxima, in nm.

As shown in Table II, all DNR mutants present the typical peaks of a hexa-coordination of the heme cofactor as for the wild type protein. No HIS-mutation to ALA does significantly change the spectral properties; only the DNRH187A shows some change in the spectrum, as reported in fig. 4.14.

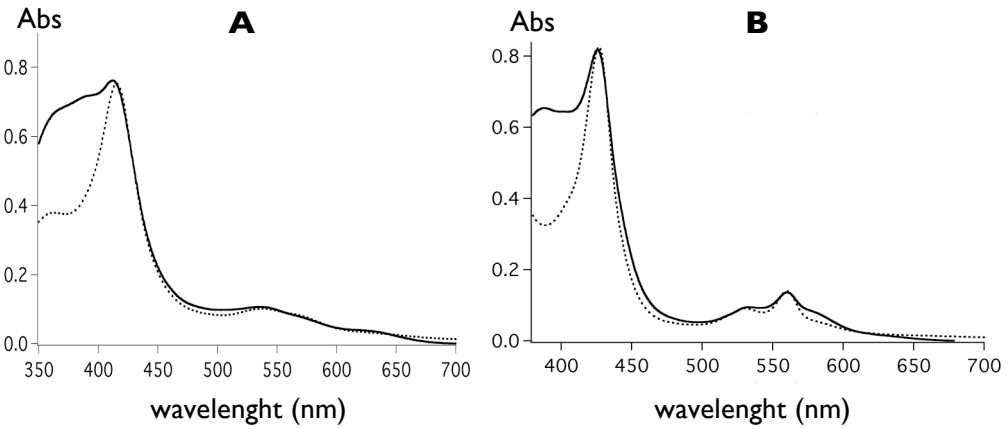

Figure 4.14. The spectrum of the ferric (A) and the ferrous (B) holo-DNR H187A mutant (black line) compared with the spectrum of the ferrous holo-DNR (dotted line)

The results obtained with the DNRH187A mutant suggested that the His 187 is involved in the heme iron coordination. Since the affinity of heme/protein complexes is known to be mainly controlled by the rate of heme dissociation from the holo-protein (Hargrowe *et al.,* 1996) we calculated the heme dissociation rate from the holo-DNRH187A mutant which is much faster (2± 0.4 × 10^{-3} s^{-1}) than that obtained with the holo-DNR wt (Giardina *et al.*, 2008).

At the same time, all the DNR mutants were assayed by the *in vivo* β-gal reporter system, in order to understand if the lack of a histidine of DNR putatively involved in the coordination of the heme jeopardizes the protein/DNA interaction. The DNR mutants have been expressed constitutively in the TOP10 *E. coli* strain together with the pRS-NOR plasmid and the β-galactosidase activity was measured under anaerobiosis (white bar) and under anaerobiosis in the presence of 10 μM NO (violet bars).

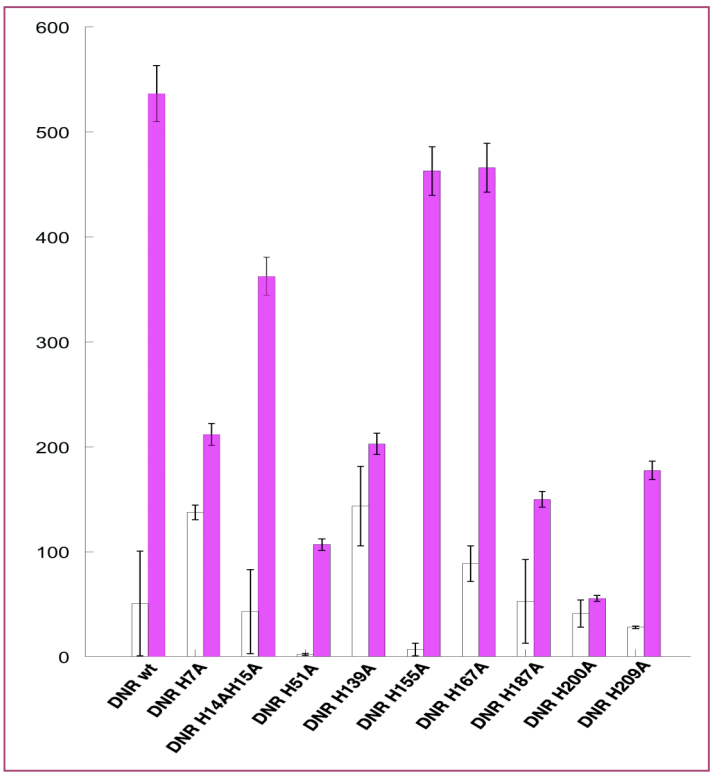

Figure 4.15 The DNR mutants have been expressed constitutively in the TOP10 *E. coli* strain together with the pRS-NOR plasmid and the β-galactosidase activity was measured under anaerobiosis (white bars) and under anaerobiosis in the presence of NO (violet bars).

The results show a high variability in the activity of the different mutants suggesting that a simple correlation between activity and interaction of the histidines with the heme cofactor is difficult to find (see discussion section). However, the DNRH187A mutant, which has particular features *in vitro*, such as a significant spectral change compared to the wild type (Fig. 4.14), also shows low activity *in vivo* (Fig 4.15) (manuscript in preparation). To demonstrate that the lack of activation by some DNR mutants in the *E. coli* strain was not due to the lack of expression of the mutated proteins, we have verified by Western blot that the proteins were correctly produced in the bacterium (data not shown).

DISCUSSION

Denitrifiers can use nitrate instead of oxygen as the final electron acceptor in the respiratory chain, by reducing it to dinitrogen (Zumft, 1997). The complete denitrification pathway involves four enzymes: the nitrate reductase (NAR), the nitrite reductase (NIR), the nitric oxide reductase (NOR) and the nitrous oxide reductase (NOS), operating sequentially to reduce nitrate to dinitrogen gas via nitrite (NO2-), nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). The expression and the activity of the NIR and NOR enzymes are tightly controlled because it is mandatory for the bacteria to keep the concentration of intracellular NO below cytotoxic levels, to limit nitrosative stress.

In *Pseudomonas aeruginosa* the expression of genes of the denitrification pathway is controlled by the NO-responsive regulator DNR (Dissimilative Nitrate respiration Regulator) belonging to the CRP-FNR superfamily of transcription factors. In denitrifiers the NO dependence of the transcriptional activity of promoters regulated by transcription factors of the DNR and NnrR subgroups of the CRP-FNR superfamily has suggested that they may act as NO sensors *in vivo* (Zumft, 2002).

Recent findings indicate that NO is also an important signaling molecule *in vivo*, being able, at concentration far below cytotoxic levels, to induce dispersal of *P. aeruginosa* biofilms (Barraud *et al*., 2006); these results strongly suggest that denitrification, regulation of the NO-homeostasis and pathogenesis are strictly related in denitrifying bacteria.

The understanding of the biochemical mechanisms used to respond to specific redox signals (such as NO) is a crucial point in order to dissect the *in vivo* function of a sensor protein. The molecular mechanism underlying the activity of DNR is still elusive, mainly because a reliable system to probe the NO-dependent DNA binding of this regulator was missing to date.

Recently it was shown, by our group, that the DNR protein binds heme *in vitro* forming stable complexes and that holo-DNR reacts with NO or CO obtaining derivatives typical of heme-proteins (Giardina et al., 2008). Moreover, to deeply understand the role of the heme molecule in the NO-dependent DNR activity, EMSA assays were carried out and a partial binding of the holo-

DNR to the target *nirS* promoter was obtained (Giardina *et al.,* 2008). Due to the difficulty to characterize the protein activity *in vitro*, we have decided to characterize it *in vivo.*

5.1 E. coli-based reporter system: DNR specifically requires N-oxides to activate the nor promoter.

The effect of N-oxides on the expression of the *nir, nor* and *nos* genes by DNR has been previously demonstrated, *in vivo*, in *P. aeruginosa* (Arai et al., 1999, 2003). The first aim of our work was to obtain a NO-dependent DNR transcriptional activity in a heterologous model organism such as *E. coli*, unable to carry out denitrification, to develop a reliable system useful to characterize the DNR protein and its site-specific mutants. A ß-gal assay was performed under anaerobic conditions in the presence of N-oxides, using the putative DNR target promoter *nor* from *P. aeruginosa* (Fig. 4.2). The choice of this promoter is supported by previous evidence showing that in *P. aeruginosa*, in the presence of N-oxides, *nor* is more strongly activated by DNR than *nir* (Arai *et al*., 1999). In our system DNR is able to transactivate the *P. aeruginosa nor* promoter in *E. coli* under anaerobic conditions and in the presence of NO (Fig.4.2), suggesting that the transcriptional machine of this bacterium can be efficiently used with a *P. aeruginosa* promoter such as the *nor* promoter (Castiglione *et al.,* 2009). A similar response to NO *in vivo* has been reported for the DNR homologue NNR in *Paracoccus denitrificans* (Van Spanning *et al.,* 1999) and, for the same regulator, also in *E. coli* using the FNR-dependent *E. coli melR* promoter (Hutchings *et al*., 2000).

The activation observed in our experiments in the presence of 2 mM nitrite (Fig. 4.2) can be explained since it is known that, in *E. coli*, nitrite is a source of NO due to the side activity of nitrite reductase, which reduces nitrite to ammonia (Corker & Poole, 2003). Nitrite is not directly involved in the activation of DNR since very low activity was observed in air in the presence of 2 mM nitrite, a condition under which NO is not produced (Darwin et al., 1993). The inactivation of DNR under aerobic conditions is in agreement with

its biological function to regulate the denitrification pathway in *P. aeruginosa,* which occurs under anaerobiosis.

As shown in fig. 4.1, the *nor* promoter fragment used in this studies includes 150 bp upstream of the ATG of the *norCB* gene; this fragment contains the canonical recognition motif for the transcriptional activator protein FNR, named FNR-box, centred -64,5 bases upstream of the ATG translation initiation codon. Interestingly, no activation of the *nor* promoter was observed in the TOP10 strain containing only the pRS-NOR plasmid under anaerobic conditions (Fig. 4.2), suggesting that the endogenous *E. coli* FNR protein is unable to recognize this promoter. This result was unexpected because we thought that the FNR *E. coli* protein, which recognizes the *melR* promoter (Barnard *et al.,* 2003) (fig 4.4a) under anaerobic conditions, could activate also the *nor* promoter due to the high degree of conservation of the FNR-box target sequence among the two promoters (Fig 4.4a). For this reason we verified if the TOP10 strain used in our study was fenotipically FNR+; our results confirm the presence of a functional FNR protein. Analyzing the region located between the palindromic TTGAT motif of the consensus FNR-box in the *nor* promoter we noticed a high GC content (Fig. 4.4a). Recently it has been demonstrated that the FNR protein favours an AT-rich motif against a GC-rich motif in this region (Scott et al., 2003); this may explain the observed lack of activation. We cannot exclude however that other features of the *nor* promoter may preclude the productive interaction between FNR and the *E. coli* transcriptional machinery. On the other hand, although the *E. coli* FNR protein seems unable to activate the *nor* promoter, the DNR protein is able to recognize in *P. aeruginosa* an FNR-dependent *E. coli* promoter (Hasegawa et al., 1998) making the *scenario* more complicated. In the future we plan to investigate this point in more detail, using a *nor/melR* chimeric promoter: the region of the *nor* fragment located between the palindromic TTGAT motif will be changed to that of the FNR target promoter *melR*.

5.2 DNR binds specifically the nor promoter

First of all, we have been able to demonstrate the specificity of the DNR/*nor* promoter interaction in the *E. coli* background (Castiglione *et al.,* 2009). We carried out the ß-gal assay with mutants harbouring mutations both in the HTH domain and in the FNR-box of the *nor* promoter. We have analyzed the N152stop mutant, which, as shown in the crystal structure (figure 1.8), lacks the last 75 amino acids and hence the entire DNA binding domain (Giardina et al., 2008). The inability of the N152stop mutant to transactivate the *nor* promoter under anaerobiosis in the presence of NO (Fig. 4.5a) suggests that the NO-dependent activation of the *nor* promoter requires the direct binding of DNR to the target DNA. To more deeply investigate the DNR/DNA interaction, we have also characterized the activity of a site-specific mutant in which Glu 193 is substituted with an Asp. This residue is highly conserved in the CRP/FNR superfamily (Green *et al*., 2001) and was shown to be involved in the direct interaction of the FNR protein with the FNR-box sequence on the target promoter (Spiro *et al.,* 1990). The inability of this mutant of DNR to activate the *nor* promoter under anaerobiosis and in the presence of NO is in agreement with the predicted involvement of this glutamate in binding the FNR-box.

Finally, for the first time we performed a detailed characterization of the *nor* promoter demonstrating that the NO-dependent DNR/DNA interaction was also blocked if the *nor* promoter carries mutations in the FNR-box consensus sequence: either an inversion of two bases in the FNR-box (TT**AG**T-N_4-TTGAT) or a single base change (A_4 to G) (TTG**G**T-N_4-TTGAT). The sensitivity of the FNR-box to the A_4 to G mutation in the consensus sequence was shown also for the *narGHJI* promoter, an *E. coli* FNR target (Walker *et al.,* 1991). Therefore, for the first time, we demonstrated that a single nucleotide substitution in the FNR-box is sufficient to completely disrupt the activity of the DNR protein on the *nor* promoter.

5.3 DNR requires heme to bind the nor promoter

The mechanism whereby DNR can sense the signal molecule is not yet understood; it is certain, however that DNR, which lacks conserved cysteines, is unable to form a Fe – S center that can interact with the signal molecule as reported for the O_2-sensor FNR (Khoroshilova *et al*., 1995, 1997; Green *et al*., 1996; Jordan *et al*., 1997).

As previously mentioned, spectroscopical evidence demonstrates the formation of a stable DNR/heme complex, suggesting that the heme is the cofactor involved in the NO-sensing of the protein. Moreover, the only evidence in the literature in which a member of the DNR class of regulators is able to bind *in vitro* the target DNA was obtained by an EMSA assay using the *P. aeruginosa* DNR protein bound to heme (Giardina *et al*., 2008). In the present ph.D. thesis, it was demonstrated that the heme is required for DNR activity *in vivo,* by measuring the NO-dependent activation of the *nor* promoter in a heme-deficient *E.coli* strain (Castiglione *et al.,* 2009), which lacks a key intermediate in the biosynthesis of heme (Harris *et al.,* 1993) (Fig.4.7). This result confirms previous evidence obtained *in vitro* and supports the idea that a heme-based mechanism is the most likely to explain the NO-dependent regulation of the function of DNR. A similar approach was successfully employed to show that heme biosynthesis is essential for the sensing activity of NNR from *Paracoccus denitrificans* (Lee *et al.,* 2006).

Another heme-based mechanism within the proteins belonging to the CRP-FNR superfamily has been thoroughly characterized for *Rhodospirillum rubrum* CooA (Shelver *et al.,* 1997); in this case the CO molecule binds to the heme iron and triggers a conformational change, thus regulating the transcriptional activity (Lanzillotta *et al.,* 2000).

Given that heme is essential for DNR activation we tested if DNR is able to discriminate between different gases during its activity. Therefore, using the *E. coli* system to compare the activity obtained under anaerobiosis in the presence of NO to that obtained in the presence of CO we demonstrated that DNR responses differently to the two molecules. These findings are in agreement with previous results obtained *in vitro*, in which the reduced form of the DNR-heme complex was shown to bind *in vitro* both NO and CO

(Giardina *et al.*, 2008). The adduct of NO with the heme-DNR complex is pentacoordinate, whereas the CO bound species is hexacoordinate. We can conclude that NO is the specific and unique signal required for DNR function; this selective activation in response to a particular ligand is shared with other heme-based gas sensors (e.g., CooA and H-NOX domains) (Andrew *et al.*, 2001; Boon *et al.*, 2005; Gilles-Gonzalez & Gonzalez, 2005).

5.4 Structural features of the DNR protein

To bind DNA, the CRP-FNR transcription factors must be in an active or ON conformation; ligand binding, in fact, triggers the conformational changes from an OFF conformation (unable to bind DNA) to an ON conformation (able to bind DNA). Once the regulator is activated (ON conformation) the two recognition helices of the HTH motif are in the correct position to bind the cognate DNA sequence by interaction with the major groove as shown in Figures 1.5 and 5.1a for CRP from *E. coli* whose structure has been solved in complex with DNA. On the contrary, when these regulators are in the inactive form (OFF conformation), the DNA binding domain of each monomer can change its orientation dramatically by rotating around the connection with the dimerization helix (called *hinge*). Recently, the structure of the wtDNR protein was solved (Giardina *et al.,* 2009); the comparison between DNR and CRP reveals that wtDNR has crystallized in an OFF conformation, in which the recognition helices of the HTH motif are oriented downward looking at the sensing domain (5.1 a and b), a topology that would not be effective in binding DNA.

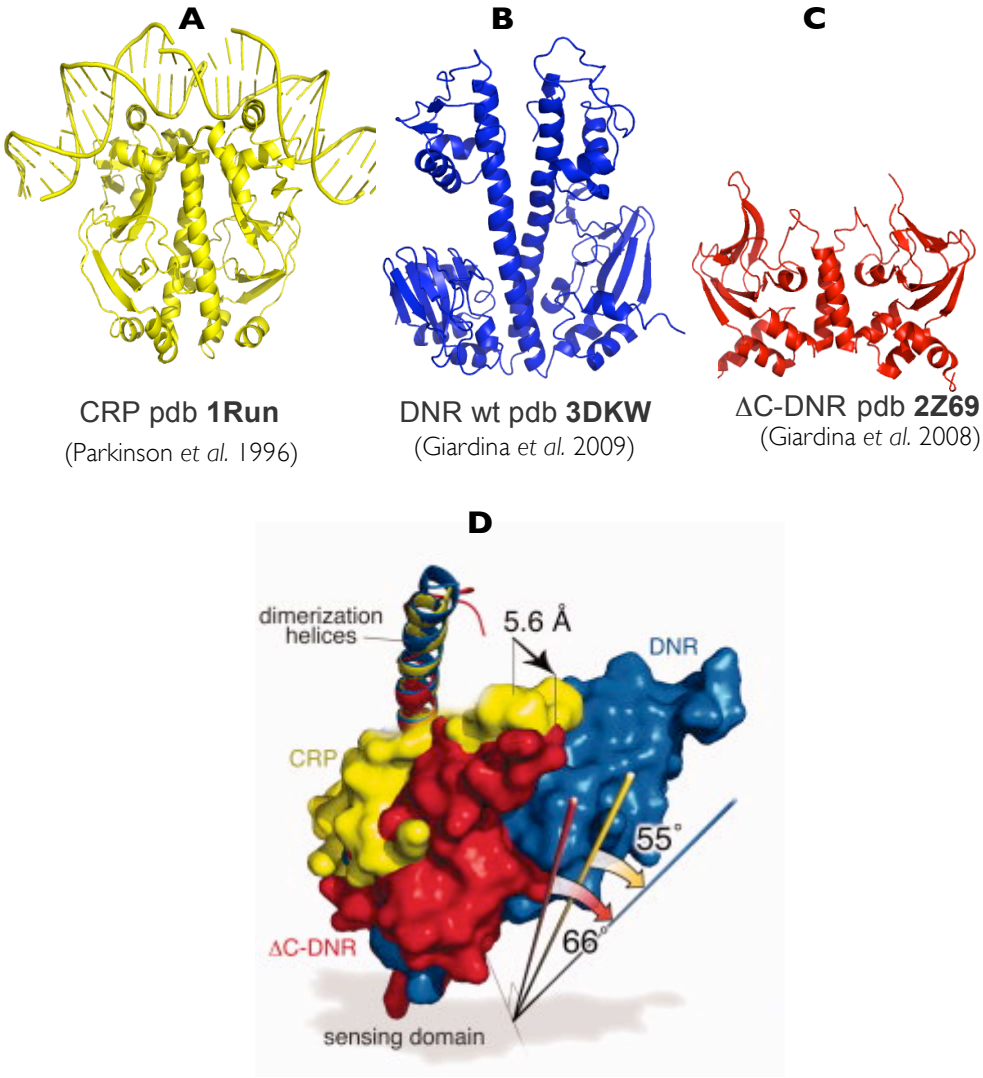

Figure 5.1 comparison between the CRP, wtDNR and ΔC-DNR structures: A) CRP structure with the DNA bound (PDBid: 1run, Parkinson *et al.*, 1996) B) wtDNR structure (PDBid: 3DKW, Giardina *et al.*, 2008) C) ΔC-DNR structure (PDBid: 2Z69, Giardina *et al.*, 2009). D) Superposition of the CRP (yellow), ΔC-DNR (red) and DNR (blue) sensing domains (surface rapresentation) showing the angles of rotation around an axis perpendicular to the direction of the dimerization helix. The DNA binding domains have been removed for clarity. Comparing the ΔC-DNR and DNR structures, it is evident that the sensing domain of wtDNR is rotated with respect to the same domain in ΔC-DNR.

Moreover, comparison of the wtDNR with CooA (OFF conformation) reveals that wtDNR shows indeed a different position of the sensing domain, suggesting that the wtDNR structure in a different OFF position, thus named *fully*OFF.

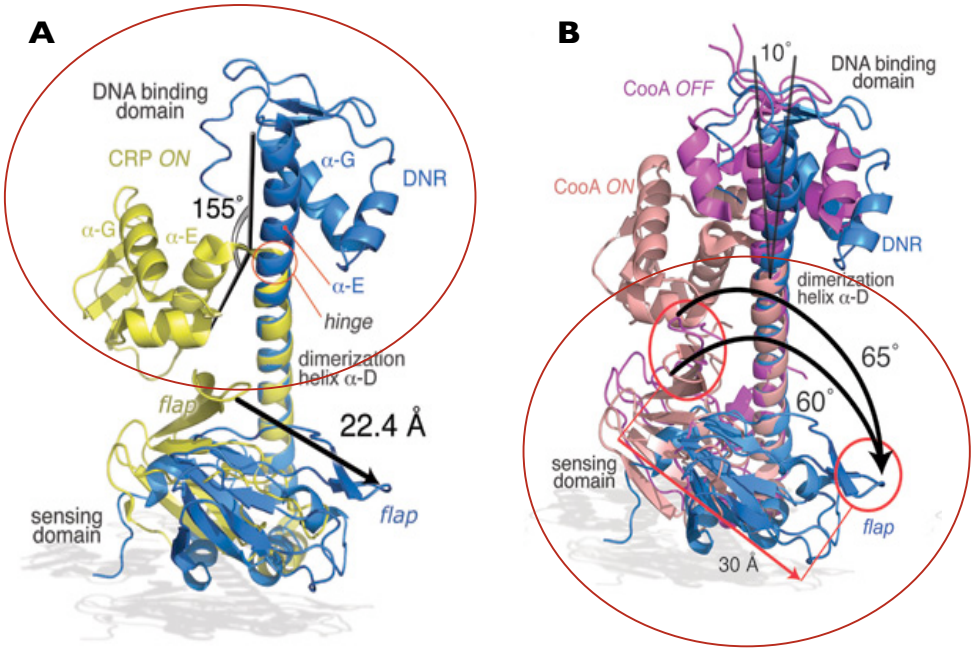

Figure 5.2 (A) Superposition of the dimerization helices of wtDNR (in blue) and CRP (in yellow; pdb id:1run) monomers showing the different positions of the DNA binding domains and sensing domains in the active ON conformation (CRP) and in the inactive fully OFF conformation (wtDNR). (B) Superposition of the dimerization helices of wtDNR (in blue) and CooA OFF and ON conformations (in magenta and salmon, respectively, pdb id: 1ft9 and 2hkx) showing different positions of the DNA binding domains and sensing domains.

Looking carefully at the two 3D structures of the DNR protein, ΔC-DNR and wtDNR (Fig. 5.1 b and c) we clearly observe different features not due to changes in the overall fold but due to the peculiar orientation of the sensing domain with respect to the dimerization helix, which is dramatically different in the two proteins (Fig. 5.1 d). After superposition of the dimerization helix of the two structures, it is evident that the sensing domain of wtDNR is rotated with respect to the same domain in ΔC-DNR, the latter being more similar to

the active form of CRP suggesting that this DNR mutant could be in a ON conformation. For this reason we have created "in silico" a chimera by fusing ΔC-DNR with an HTH domain modelled on the structure of CRP in complex with the DNA (Fig. 5.3).

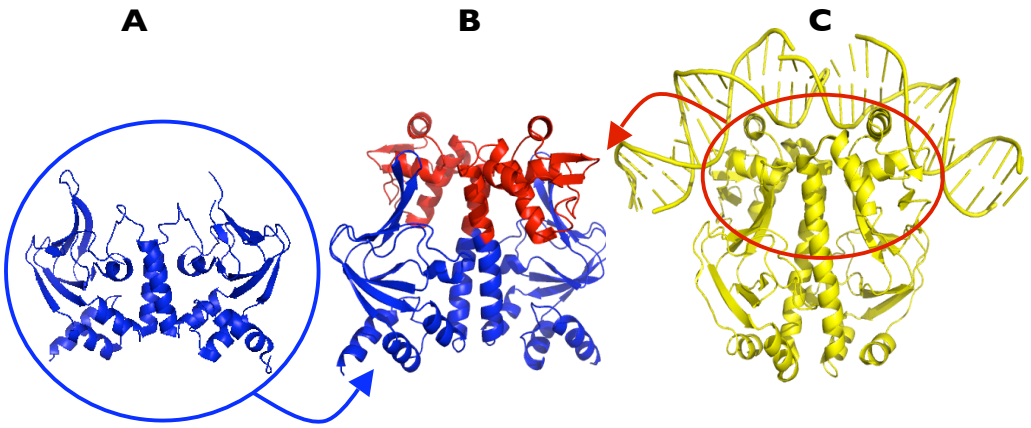

Figure 5.3 Chimera construction: A) crystal structure of ΔC-DNR (PDBid: 2Z69) B) chimera of a hypothetical ON conformation of DNR obtained by fusing the structure of ΔC-DNR (blue) with an HTH domain (red) modelled on the structure of CPR in complex with the DNA (PDBid: 1run) (C).

The model obtained represents a hypothetical ON conformation of DNR. This structure is quite different to the structure of the OFF wtDNR, suggesting that the large conformational change described for other transcription factors belonging to the CRP-FNR superfamily is also taking place in DNR. Moreover, this conformational change allows the formation of a large hydrophobic pocket between the sensing domain and the dimerization helix in which the heme could be held.

The DNR protein has been crystallized in the apo form but not yet in the holo form. For this reason, to deeply investigate on the Fe-heme environment of the holo-DNR, we have characterized the protein in solution by the XANES technique in collaboration with Prof. Arcovito (Cattolica of Sacro Cuore University of Rome) and Prof. Della Longa (University of L'Aquila). The results obtained confirmed that, in the absence of further available models for axial coordination (for example from tyrosine), the heme iron in formally ferric

DNR is assigned to the low-spin hexacoordinated species and that it is coordinated by two histidines; moreover they confirm the 5-coordination of the ferrous DNR-NO derivative.
Intriguingly, the results show an equilibrium in solution between two species, an hexa- and a tetra-coordinate one due to a partial destabilization of the axial ligation in ferrous DNR, thus suggesting that the heme cofactor is not steadily bound to the protein as in "canonical" hemo-proteins, but presents high degree of freedom inside the protein.

5.5 *DNR activation mechanism: current hypothesis*

As described above, DNR activation requires a large conformational change to bind the DNA: the heme cofactor may have a crucial role in protein structure rearrangement during its activation. As suggested by the XANES experiments, the heme cofactor is not stably bound to the protein but an equilibrium exists between the associated and the dissociated form; this hypothesis is also supported by the fact that the DNR protein always crystallized in a apo form. These findings demonstrated that the holo-DNR behaviour is different with respect to other canonical hemo-protein.
Our *in vivo* results also show that the protein requires NO binding to fulfil its biological role. Based on these results we suggest a model of DNR activation involving 3 different conformational states whose interconversion requires a dramatic reorganization of the protein structure (fig. 5.4).
Our hypothesis is that wtDNR activation may be driven initially by heme association, that promotes a first conformational rearrangement from the fully OFF towards a pseudo ON conformation (represented by ΔC-DNR), followed by NO binding to the heme with final conversion to the biologicaly active state of the protein.

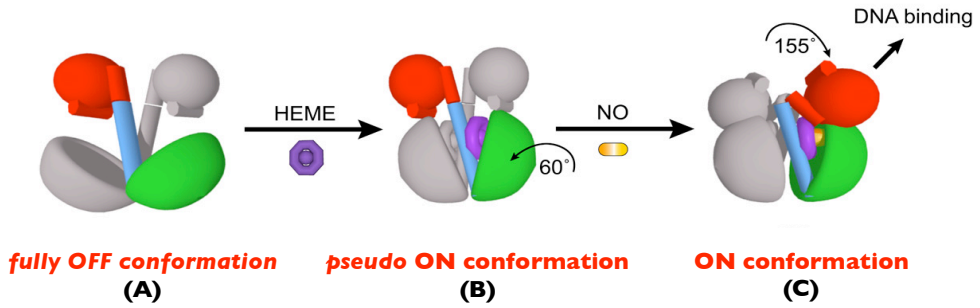

Figure 5.4 Model of activation of the DNR protein: wtDNR activation may be driven initially by heme association, that promotes a first conformational rearrangement from the fully OFF (A) corresponding to the wtDNR crystal structure, toward a pseudoON (B) corresponding to the sensing domain represented by the ΔC-DNR crystal structure conformation, followed by NO binding to the heme with final conversion to the biologically active state of the protein (C).

Our data are in agreement with the proposal that heme association to DNR is a prerequisite for NO binding and effective transcriptional regulation.

5.6 DNR as a heme binding protein

The hypothesis of DNR activation illustrated above has prompted us to further investigate both *in vitro* and *in vivo* mechanism of heme binding by protein engineering. Since the XANES experiments have shown that two histidines are involved in the coordination of the heme iron in the heme/DNR complex, we investigated which histidines of the protein were involved in binding the heme iron. We have mutated all the histidines of DNR to alanines obtaining 8 single mutants and one double mutant. These mutants were studied *in vitro* to evaluate effect of mutation on the heme environment and *in vivo* to understand if the substitution of a histidine putatively involved in the coordination of the heme jeopardizes the protein activity. In Figure 5.5 the wtDNR OFF structure and the wtDNR hypothetical ON model are shown with the histidines marked with different colours.

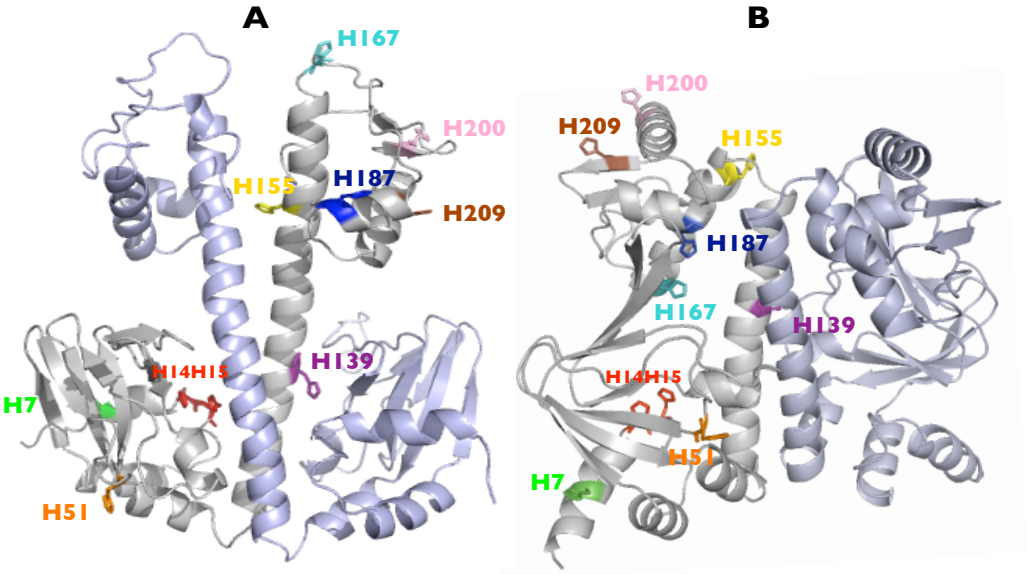

Figure 5.5 crystal structure of wtDNR A) and model of the wtDNR hypothetical ON structure B). The histidines of the protein are marked with different colours: H7, light green; H14 and H15, red; H51, orange; H139, magenta; H155, yellow; H167, sky blue; H187, blue; H200 pink; H209, brown.

Contrary to the expectation, all mutant holo-proteins displayed similar absorption spectra to the wt suggesting that the hexa-coordination of the heme iron is mantained; only the H187A shows some significant change in the spectrum indicating an involvement of this residue in the heme/DNR binding, although, also in this mutant, the heme iron appears partially to be still hexacoordinate. These results strongly suggest again that the holo-DNR behaviour is different with respect other canonical hemo-protein thus supporting the novel mechanism of heme/protein complex formation described above. All these findings could place DNR within the recent group of heme-responsive proteins which have been described by other groups (Igarashi *et al*, 2008; Kitanishi *et al*., 2008; Yang *et al.,* 2005) in the last years. In all these cases, site-directed mutagenesis of the heme axial ligand of the heme-binding proteins does not always result in the expected spectral changes. For Igarashi *et al*. (2008) in the heme-regulated eukaryotic initiation factor 2 (eIF2) kinase (HRI) a redox- dependent ligand switching from Cys to a

another residue upon reduction of Fe(III) to Fe(II) heme occurs; this ligand exchange is a characteristic feature of these heme-responsive proteins. A similar result was obtained by Yang *et al*., (2005) on the iron response regulator (Irr) protein from *Bradyrhizobium japonicum*. This protein has a characteristic motif consisting of three consecutive His residues, His-117/His-118/His-119, which may be potential axial ligand candidates for the heme iron complex and none of single or double mutants displayed heme absorption spectral changes; only the triple His mutant lost heme affinity. In fact, in these proteins a residue located in the proximity of the native axial ligand may switch to coordinate to the heme iron, even in mutant proteins, thus hampering unequivocal identification of the heme axial ligand. This may be caused by protein flexibility of the heme-responsive protein, which is critical for heme binding. The heme binding thus regulates catalysis, protein-protein interactions or transcriptional activity by mediating structural changes induced by heme association/dissociation. This mechanism is not unlikely, thinking that heme *per se* also plays a regulatory role in various important physiological functions in organisms. As an example, Bach1 controls the transcription of ß-globin and heme oxygenase 1 genes in response to the heme concentration (Ochiai *et al*., 2008). These ligand switching phenomena induced by mutation of the heme axial ligand are not generally observed for globin proteins where the heme is effectively coordinated by specific residues, whose mutation dramatically alter the spectroscopical properties (Liong *et al*., 2001).

In parallel, all the mutants characterized *in vitro* were also characterized *in vivo* (Fig. 4.15). An high variability in the activity of different mutants was observed suggesting that is difficult to find a simple correlation between activity and interaction of the histidines with the heme cofactor. To simplify the analysis of the other mutants we can classify them into different categories, each involving a different feature of DNR.

Heme/DNR complex stability:

The H187A mutant is the only residue which has particular features *in vitro*, such as significant spectral changes compared to the wt (see above); this mutant also shows low activity *in vivo*. This effect is, in our opinion, due to the destabilization of the heme-protein complex. In agreement with this interpretation, recent experiments showed that the presence of heme significantly increase the stability of wt DNR to GuHCl-induced protein unfolding; this stabilization is lost in the H187A mutant. In addition, the heme dissociation rate of H187A (measured at 20 °C in the presence of excess sperm whale apomyoglobin (apo-Mb)) turn out to be faster than in wt DNR ones. These findings suggest a destabilization of the heme/protein complex in the holo-mutant H187A.

It is intriguingly to notice that only in the hypothetical ON conformation of wtDNR (Fig. 5.6) the H187 residue points towards the sensing domain, in particular to a large pocket in which the heme cofactor may be held.

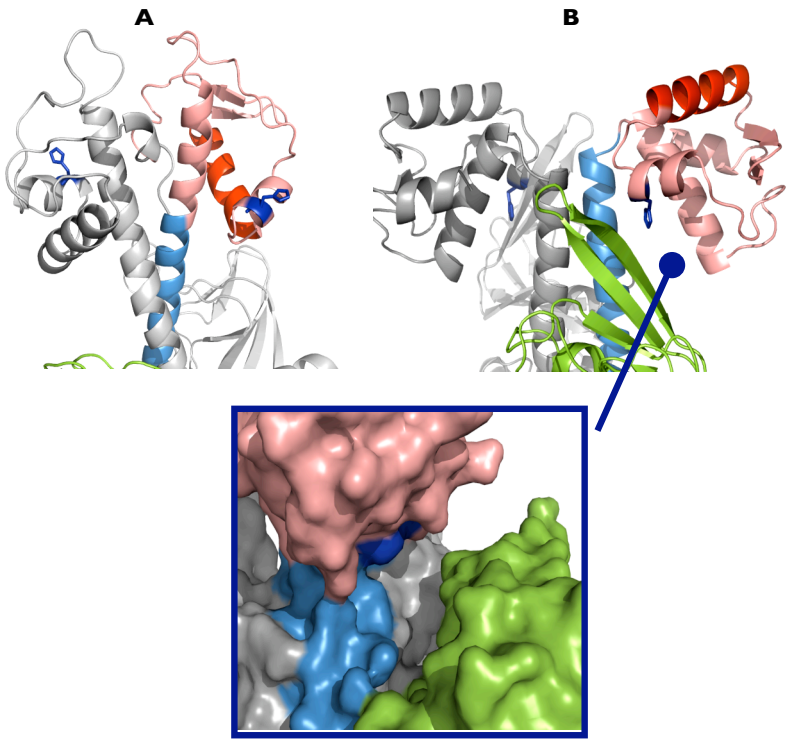

Figure 5.6 detail of the structure of the wtDNR protein in the OFF conformation (A) and of the model in the hypothetical ON conformation (B). In the panel a particular of the pocket. H187 is marked in blues.

All these evidences suggest a crucial role of this histidine in binding of the heme-protein to DNR.

In light of what discussed above the involvement of a histidine in the heme coordination doesn't necessary correspond to a negative activation of the corresponding mutant *in vivo*. The double mutant H14AH15A and the H167A mutant present a wt-like spectra *in vitro* and activity *in vivo*; however, we cannot rule out completely the possibility that these histidines may be still involved in the coordination of the heme. These residues are located in the hydrophobic pocket of the wt DNR ON model (fig. 5.5 B) thus suggesting that an interaction with the heme cofactor may occur.

The H139A mutant, which have wild-type like features *in vitro,* present low activity *in vivo.* The 139 residue is located in the dimerization helix, facing the sensing domain: due to the high plasticity of the protein this aminoacid may be involved in the coordination of the heme cofactor.

Stability of the DNR conformations:

Looking at the activity of the H155A mutant in the presence of the NO molecule (pink bar, fig. 4.15) in comparison to that under anaerobic conditions (white bar, fig. 4.15) we can observe that the basal activity in anaerobiosis without NO is surprisingly low. Histidine 155 (fig. 5.5, yellow histidine) in the wt OFF structure is located in the hinge of the protein, a region connecting the dimerization helix and the HTH domain which undergoes a large change upon activation.

Mutation of His 155 to Ala may introduce an extra hydrophobic contact which stiffens the hinge region thus favouring the *fully*OFF conformation. Therefore binding of the heme cofactor under anaerobiosis is less likely to occur in the H155A mutant (Fig. 4.15, white bar). In the presence of NO, which may drive the equilibrium towards the active conformation, the H155 residue faces the solvent and thus does not play any role; for this reason the mutant is fully active in the presence of NO.

DNR/DNA interaction:

The His 200 is positioned in one of the helix involved in the interaction with the DNA, therefore the complete inability of the H200A mutant to bind the *nor* promoter (fig. 4.15) could be simply due to a loss of a positive charge in the area of binding. The His 209 is located near this recognition helix: in the *in vivo* assay the H209A mutant is not totally inactive as the H200A, but presents a low activity. Being H209 not directly placed in the DNR/DNA interface its the mutation probably destabilizes the interaction to a lower extent.

DNR/RNA Polymerase interaction

The low activity of the H7A and the H51A mutants (pink bars fig. 4.15, green and orange histidines in the structure in fig. 5.5) could be explained given that the two residues could be involved in the DNR/RNA Polymerase interaction. In the structural alignment between DNR and CRP, histidine 7 is located in the same position of an histidine of the CRP protein, which contribute to the formation of the Activating Region 2, involved in the protein/Polymerase interaction (Li *et al.,* 1998) and located at the N-terminal domain of the protein. The His 51 is also located in this area, filling in an external and lateral position, useful for the interaction of the Polymerase. These two histidines present a favourable position for this interaction both in the OFF structure and the in hypothetical ON model.

5.7 Future perspectives

All the available functional and structural evidence lead us to place the DNR protein in the novel heme-sensor group of proteins; moreover, the hypothesis that a mechanism with 3 different conformational states exists, is also supported by the *in vivo* and *in vitro* evidence reported above.

In this mechanism it seems that step by step the protein loses degrees of freedom and plasticity because it is increasingly constrained in a rigid and active structure with the heme and the NO stably bound.

An encouraging result has been recently obtained by Dr. Giorgio Giardina who has been able to crystalle DNR under anaerobic condition in the presence of NO: for the first time clusters of small neadle-shaped red crystals (fig. 5.7), which indicate the presence of heme bound to the protein, were obtained. The crystallization conditions will be optimized, but compared to the unsuccessful aerobic crystallization trials on the holo-protein, this result strongly suggests that anaerobiosis and NO drive the protein complex towards a more rigid conformation, more prone to crystallize.

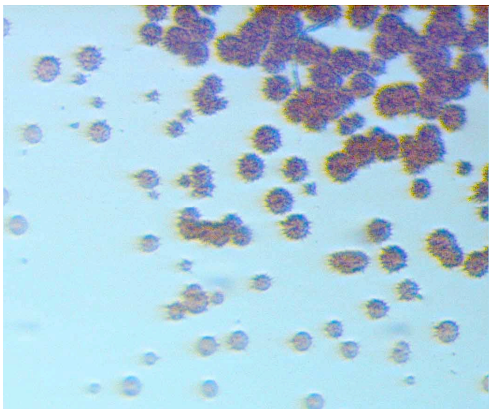

Figure 5.7 clusters of small needle-shaped red crystals wt DNR. These crystals has been obtained by Dr. Giorgio Giardina by crystallizing the DNR protein under anaerobic condition in the presence of NO; the red colour indicates the presence of the heme in complex with the protein.

The crystallographic results indicate that NO-dependent DNR activity is under allosteric control, and the heme is obviously involved in the structural modulation. This hypothesis is in agreement with very recent results obtained on CRP (Popovych *et al.*, 2009), whereby the cAMP molecule elicits an allosteric transition that switches *E. coli* CRP from the OFF to the ON state; this transition involves not only the structural reorganization but also the folding of a segment of the protein which acquires the ON conformation.
An investigation to probe the significance of this allosteric transition in the mechanism of sensing *in vitro* and *in vivo* is challenging and possibly prone to direct experiments.

REFERENCES

Andrew, C. R., Green, E. L., Lawson, D. M. & Eady, R. R. (2001). Resonance Raman studies of cytochrome c' support the binding of NO and CO to opposite sides of the heme: implications for ligand discrimination in heme-based sensors. *Biochemistry* **40**, 4115-4122.

Aono, S. (2003). Biochemical and biophysical properties of the CO-sensing transcriptional activator CooA. *Acc Chem Res* **36**, 825-831.

Antonini, E. & Brunori, M. (1971). Hemoglobin and myoglobin in their reactions with ligands. In (Neuberger, A. & Tatum, E. L., eds), *North-Holland Publishing Company*, Amsterdam, The Netherlands

Arai, H., Hayashi, M., Kuroi, A., Ishii, M. & Igarashi, Y. (2005). Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. *J Bacteriol* **187**, 3960-3968.

Arai, H., Igarashi, Y. & Kodama, T. (1995). Expression of the nir and nor genes for denitrification of Pseudomonas aeruginosa requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett* **371**, 73-76.

Arai, H., Kodama, T. & Igarashi, Y. (1997). Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in Pseudomonas aeruginosa. *Mol Microbiol* **25**, 1141-1148.

Arai, H., Kodama, T. & Igarashi, Y. (1999). Effect of nitrogen oxides on expression of the nir and nor genes for denitrification in Pseudomonas aeruginosa. *FEMS Microbiol Lett* **170**, 19-24.

Arai, H., Mizutani, M. & Igarashi, Y. (2003). Transcriptional regulation of the nos genes for nitrous oxide reductase in Pseudomonas aeruginosa. *Microbiology* **149**, 29-36.

Barnard, A. M., Green, J. & Busby, S. J. (2003). Transcription regulation by tandem-bound FNR at Escherichia coli promoters. *J Bacteriol* **185**, 5993-6004.

Barraud, N., Hassett, D. J., Hwang, S. H., Rice, S. A., Kjelleberg, S. & Webb, J. S. (2006). Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. *J Bacteriol* **188**, 7344-7353.

Barraud, N., Schleheck, D., Klebensberger, J., Webb, J. S., Hassett, D. J., Rice, S. A. & Kjelleberg, S. (2009). Nitric oxide signaling in Pseudomonas aeruginosa biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J Bacteriol* **191**, 7333-7342.

Bastian, N. R. & Hibbs, J. B., Jr. (1994). Assembly and regulation of NADPH oxidase and nitric oxide synthase. *Curr Opin Immunol* **6**, 131-139.

Boon, E. M. & Marletta, M. A. (2005). Ligand discrimination in soluble guanylate cyclase and the H-NOX family of heme sensor proteins. *Curr Opin Chem Biol* **9**, 441-446.

Botsford, J. L. & Harman, J. G. (1992). Cyclic AMP in prokaryotes. *Microbiol Rev* **56**, 100-122.

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

Buchanan, P. J., Ernst, R. K., Elborn, J. S. & Schock, B. (2009). Role of CFTR, Pseudomonas aeruginosa and Toll-like receptors in cystic fibrosis lung inflammation. *Biochem Soc Trans* **37**, 863-867.

Busby, S. & Ebright, R. H. (1999). Transcription activation by catabolite activator protein (CAP). *J Mol Biol* **293**, 199-213.

Camilli, A. & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science* **311**, 1113-1116.

Castiglione, N., Rinaldo, S., Giardina, G. & Cutruzzola, F. (2009). The transcription factor DNR from Pseudomonas aeruginosa specifically requires nitric oxide and haem for the activation of a target promoter in Escherichia coli. *Microbiology*.

Cooper, M., Tavankar, G. R. & Williams, H. D. (2003). Regulation of expression of the cyanide-insensitive terminal oxidase in Pseudomonas aeruginosa. *Microbiology* **149**, 1275-1284.

Corker, H. & Poole, R. K. (2003). Nitric oxide formation by Escherichia coli. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. *J Biol Chem* **278**, 31584-31592.

Crack, J. C., Le Brun, N. E., Thomson, A. J., Green, J. & Jervis, A. J. (2008). Reactions of nitric oxide and oxygen with the regulator of fumarate and nitrate reduction, a global transcriptional regulator, during anaerobic growth of Escherichia coli. *Methods Enzymol* **437**, 191-209.

Drago, L. (2009). [Bacteria and biofilm in respiratory tract infections]. *Infez Med* **17 Suppl 2**, 3-9.

Driscoll, J. A., Brody, S. L. & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. *Drugs* **67**, 351-368.

Eiglmeier, K., Honore, N., Iuchi, S., Lin, E. C. & Cole, S. T. (1989). Molecular genetic analysis of FNR-dependent promoters. *Mol Microbiol* **3**, 869-878.

Eiting, M., Hageluken, G., Schubert, W. D. & Heinz, D. W. (2005). The mutation G145S in PrfA, a key virulence regulator of Listeria monocytogenes, increases DNA-binding affinity by stabilizing the HTH motif. *Mol Microbiol* **56**, 433-446.

Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., Poole, R. K., Penn, C. W. & Park, S. F. (2005). NssR, a member of the Crp-Fnr superfamily from Campylobacter jejuni, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Mol Microbiol* **57**, 735-750.

Evans PR. (1997). *Scala*. Joint CCP4 and ESF-EACBM. newsletter on protein. Crystallography 33 22-24.

Feelisch, M., Stamler, J.S. (1996) *Methods in nitric oxide research.* John Wiley & Sons, Chichester,UK.

Fux, C. A., Costerton, J. W., Stewart, P. S. & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends Microbiol* **13**, 34-40.

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

Giardina, G., Rinaldo, S., Castiglione, N., Caruso, M. & Cutruzzola, F. (2009). A dramatic conformational rearrangement is necessary for the activation of DNR from Pseudomonas aeruginosa. Crystal structure of wild-type DNR. *Proteins*.

Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M. & Cutruzzola, F. (2008). NO sensing in Pseudomonas aeruginosa: structure of the transcriptional regulator DNR. *J Mol Biol* **378**, 1002-1015.

Gilles-Gonzalez, M. A. & Gonzalez, G. (2005). Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *J Inorg Biochem* **99**, 1-22.

Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J. & Guest, J. R. (1996). Reconstitution of the [4Fe-4S] cluster in FNR and demonstration of the aerobic-anaerobic transcription switch in vitro. *Biochem J* **316 (Pt 3)**, 887-892.

Green, J., Scott, C. & Guest, J. R. (2001). Functional versatility in the CRP-FNR superfamily of transcription factors: FNR and FLP. *Adv Microb Physiol* **44**, 1-34.

Guest, J.R., Green, J., Irvine, A.S. and Spiro, S. (1996). The FNR modulon and FNR-regulated gene expression. In: Regulation of Gene Expression in *Escherichia coli* (Lin, E.C.C. and Lynch, A.S., Eds.), pp. 317-342. *Chapman and Hall*, New York.

Gusarov, I. & Nudler, E. (2005). NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci U S A* **102**, 13855-13860.

Gusarov, I., Shatalin, K., Starodubtseva, M. & Nudler, E. (2009). Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science* **325**, 1380-1384.

Hasegawa, N., Arai, H. & Igarashi, Y. (1998). Activation of a consensus FNR-dependent promoter by DNR of Pseudomonas aeruginosa in response to nitrite. *FEMS Microbiol Lett* **166**, 213-217.

Hassett, D. J., Cuppoletti, J., Trapnell, B. & other authors (2002). Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* **54**, 1425-1443.

Hassett, D. J., Sutton, M. D., Schurr, M. J., Herr, A. B., Caldwell, C. C. & Matu, J. O. (2009). Pseudomonas aeruginosa hypoxic or anaerobic biofilm infections within cystic fibrosis airways. *Trends Microbiol* **17**, 130-138.

Hentzer, M. & Givskov, M. (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J Clin Invest* **112**, 1300-1307.

Hoboth, C., Hoffmann, R., Eichner, A., Henke, C., Schmoldt, S., Imhof, A., Heesemann, J. & Hogardt, M. (2009). Dynamics of adaptive microevolution of hypermutable Pseudomonas aeruginosa during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis* **200**, 118-130.

Hoeren, F. U., Berks, B. C., Ferguson, S. J. & McCarthy, J. E. (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* **218**, 49-57.

Hu, R. G., Wang, H., Xia, Z. & Varshavsky, A. (2008). The N-end rule pathway is a sensor of heme. *Proc Natl Acad Sci U S A* **105**, 76-81.

Huie, R. E. & Padmaja, S. (1993). The reaction of no with superoxide. *Free Radic Res Commun* **18**, 195-199.

Hutchings, M. I., Shearer, N., Wastell, S., van Spanning, R. J. & Spiro, S. (2000). Heterologous NNR-mediated nitric oxide signaling in Escherichia coli. *J Bacteriol* **182**, 6434-6439.

Igarashi, J., Kitanishi, K., Martinkova, M., Murase, M., Iizuka, A., and Shimizu, T. (2008) The role of thiolate-heme proteins, other than the P450 cytochromes, in regulation of heme -sensor proteins. *Acta Chim. Slo*V. **55**, 67–74.

Jordan, P. A., Thomson, A. J., Ralph, E. T., Guest, J. R. & Green, J. (1997). FNR is a direct oxygen sensor having a biphasic response curve. *FEBS Lett* **416**, 349-352.

Joyce, M. G., Levy, C., Gabor, K. & other authors (2006). CprK crystal structures reveal mechanism for transcriptional control of halorespiration. *J Biol Chem* **281**, 28318-28325.

Khoroshilova, N., Beinert, H. & Kiley, P. J. (1995). Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding. *Proc Natl Acad Sci U S A* **92**, 2499-2503.

Khoroshilova, N., Popescu, C., Munck, E., Beinert, H. & Kiley, P. J. (1997). Iron-sulfur cluster disassembly in the FNR protein of Escherichia coli by O2: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. *Proc Natl Acad Sci U S A* **94**, 6087-6092.

Kiley, P. J. & Beinert, H. (1998). Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol Rev* **22**, 341-352.

Kitanishi, K., Igarashi, J., Hayasaka, K., Hikage, N., Saiful, I., Yamauchi, S., Uchida, T., Ishimori, K. & Shimizu, T. (2008). Heme-binding characteristics of the isolated PAS-A domain of mouse Per2, a transcriptional regulatory factor associated with circadian rhythms. *Biochemistry* **47**, 6157-6168.

Korner, H., Sofia, H. J. & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev* **27**, 559-592.

Lanzilotta, W. N., Schuller, D. J., Thorsteinsson, M. V., Kerby, R. L., Roberts, G. P. & Poulos, T. L. (2000). Structure of the CO sensing transcription activator CooA. *Nat Struct Biol* **7**, 876-880.

Lee, Y. Y., Shearer, N. & Spiro, S. (2006). Transcription factor NNR from Paracoccus denitrificans is a sensor of both nitric oxide and oxygen: isolation of nnr* alleles encoding effector-independent proteins and evidence for a haem-based sensing mechanism. *Microbiology* **152**, 1461-1470.

Lesile, A.G.W. (1992). Recent changes to the MOSFLM package for processing film and image plate data. Joint CCP4 + ESF-EACBM newsletter on protein. *Crystallography*. n°**26**.

Liong, E. C., Dou, Y., Scott, E. E., Olson, J. S. & Phillips, G. N., Jr. (2001). Waterproofing the heme pocket. Role of proximal amino acid side chains in preventing hemin loss from myoglobin. *J Biol Chem* **276**, 9093-9100.

McKay, D. B. & Steitz, T. A. (1981). Structure of catabolite gene activator protein at 2.9 A resolution suggests binding to left-handed B-DNA. *Nature* **290**, 744-749.

Moreau-Marquis, S., Stanton, B. A. & O'Toole, G. A. (2008). Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulm Pharmacol Ther* **21**, 595-599.

Nathan, C. F. & Hibbs, J. B., Jr. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* **3**, 65-70.

Ochiai, S., Mizuno, T., Deie, M., Igarashi, K., Hamada, Y. & Ochi, M. (2008). Oxidative stress reaction in the meniscus of Bach 1 deficient mice: potential prevention of meniscal degeneration. *J Orthop Res* **26**, 894-898.

Parkinson, G., Gunasekera, A., Vojtechovsky, J., Zhang, X., Kunkel, T. A., Berman, H. & Ebright, R. H. (1996). Aromatic hydrogen bond in sequence-specific protein DNA recognition. *Nat Struct Biol* **3**, 837-841.

Poole, R. K. & Hughes, M. N. (2000). New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol Microbiol* **36**, 775-783.

Popovych, N., Tzeng, S. R., Tonelli, M., Ebright, R. H. & Kalodimos, C. G. (2009). Structural basis for cAMP-mediated allosteric control of the catabolite activator protein. *Proc Natl Acad Sci U S A* **106**, 6927-6932.

Price, M. S., Chao, L. Y. & Marletta, M. A. (2007). Shewanella oneidensis MR-1 H-NOX regulation of a histidine kinase by nitric oxide. *Biochemistry* **46**, 13677-13683.

Richardson, A. R., Soliven, K. C., Castor, M. E., Barnes, P. D., Libby, S. J. & Fang, F. C. (2009). The Base Excision Repair system of Salmonella enterica serovar typhimurium counteracts DNA damage by host nitric oxide. *PLoS Pathog* **5**, e1000451.

Rinaldo, S., Giardina, G., Brunori, M. & Cutruzzola, F. (2006). N-oxide sensing and denitrification: the DNR transcription factors. *Biochem Soc Trans* **34**, 185-187.

Romeo, T. (2006). When the party is over: a signal for dispersal of Pseudomonas aeruginosa biofilms. *J Bacteriol* **188**, 7325-7327.

Sarti, P., Fiori, P. L., Forte, E., Rappelli, P., Teixeira, M., Mastronicola,

D., Sanciu, G., Giuffre, A. & Brunori, M. (2004). Trichomonas vaginalis degrades nitric oxide and expresses a flavorubredoxin-like protein: a new pathogenic mechanism? *Cell Mol Life Sci* **61**, 618-623.

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M. & Jahn, D. (2007). The anaerobic regulatory network required for Pseudomonas aeruginosa nitrate respiration. *J Bacteriol* **189**, 4310-4314.

Schultz, S. C., Shields, G. C. & Steitz, T. A. (1991). Crystal structure of a CAP-DNA complex: the DNA is bent by 90 degrees. *Science* **253**, 1001-1007.

Scott, C., Partridge, J. D., Stephenson, J. R. & Green, J. (2003). DNA target sequence and FNR-dependent gene expression. *FEBS Lett* **541**, 97-101.

Spiro, S. (1994). The FNR family of transcriptional regulators. *Antonie Van Leeuwenhoek* **66**, 23-36.

Spiro, S. (2007). Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev* **31**, 193-211.

Spiro, S., Gaston, K. L., Bell, A. I., Roberts, R. E., Busby, S. J. & Guest, J. R. (1990). Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. *Mol Microbiol* **4**, 1831-1838.

Stone, J. R., Sands, R. H., Dunham, W. R. & Marletta, M. A. (1995). Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cyclase. *Biochem Biophys Res Commun* **207**, 572-577.

Unden G. (1998). Transcriptional regulation and energetics of alternative respiratory pathways in facultatively anaerobic bacteria, *Biochim. Biophys. Acta*, **1365**, 220-224.

Van Alst, N. E., Picardo, K. F., Iglewski, B. H. & Haidaris, C. G. (2007). Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in Pseudomonas aeruginosa. *Infect Immun* **75**, 3780-3790.

Van Spanning, R. J., Houben, E., Reijnders, W. N., Spiro, S., Westerhoff, H. V. & Saunders, N. (1999). Nitric oxide is a signal for NNR-mediated transcription activation in Paracoccus denitrificans. *J Bacteriol* **181**, 4129-4132.

Vollack, K. U. & Zumft, W. G. (2001). Nitric oxide signaling and transcriptional control of denitrification genes in Pseudomonas stutzeri. *J Bacteriol* **183**, 2516-2526.

Walker, M. S. & DeMoss, J. A. (1991). Promoter sequence requirements for Fnr-dependent activation of transcription of the narGHJI operon. *Mol Microbiol* **5**, 353-360.

Williams, P. & Camara, M. (2009). Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* **12**, 182-191.

Wink, D. A. & Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med* **25**, 434-456.

Yang, J., Ishimori, K. & O'Brian, M. R. (2005). Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein. *J Biol Chem* **280**, 7671-7676.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M. & other authors (2002). Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593-603.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533-616.

ATTACHMENTS

Cutruzzola', F., Rinaldo, S., Castiglione, N., Giardina, G., Pecht, I., and Brunori, M., (2009) Nitrite reduction: an ubiquitous function from a pre-aerobic past. Bioessays. Aug; 31(8):885-91.

Castiglione N, Rinaldo S, Giardina G, Cutruzzolà F. (2009) The transcription factor DNR from Pseudomonas aeruginosa specifically requires nitric oxide and haem for the activation of a target promoter in Escherichia coli. Microbiology. 2009 May 28. [Epub ahead of print]

Giardina, G., Rinaldo, S., Castiglione, N., Caruso M., Cutruzzola', F. (2009) A dramatic conformational rearrangement is necessary for the activation of DNR from Pseudomonas aeruginosa. Crystal structure of wild type DNR. Proteins. Oct; 77(1):174-80.

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New insights into the activity of *Pseudomonas aeruginosa* cd_1 nitrite reductase

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Abstract

The cytochrome cd_1 nitrite reductases are enzymes that catalyse the reduction of nitrite to nitric oxide (NO) in the bacterial energy conversion denitrification process. These enzymes contain two different redox centres: one covalently bound *c*-haem, which is reduced by external donors, and one peculiar d_1-haem, where catalysis occurs. In the present paper, we summarize the current understanding of the reaction of nitrite reduction in the light of the most recent results on the enzyme from *Pseudomonas aeruginosa* and discuss the differences between enzymes from different organisms. We have evidence that release of NO from the ferrous d_1-haem occurs rapidly enough to be fully compatible with the turnover, in contrast with previous hypotheses, and that the substrate nitrite is able to displace NO from the d_1-haem iron. These results shed light on the mechanistic details of the activity of cd_1 nitrite reductases and on the biological role of the d_1-haem, whose presence in this class of enzymes has to date been unexplained.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is able to colonize low-oxygen environments such as the airways of cystic fibrosis patients, where it survives using the anaerobic respiratory pathway of denitrification [1]. In the denitrification process, nitrate is reduced to dinitrogen by four different enzymes (namely nitrate, nitrite, nitric oxide and nitrous oxide reductases) [2], according to the following pathway:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

This pathway is expressed under low oxygen tension in the presence of N-oxides such as nitrate and nitrite [2]. Intracellular NO concentration is strictly controlled by the co-ordinated activity of the enzymes nitrite and nitric oxide reductases, which catalyse NO production and removal respectively [1,2].

The NiR (nitrite reductase) from *Ps. aeruginosa* (*Pa*-cd_1NiR) is a homodimer containing in each monomer one covalently bound *c*-haem, which is reduced by external donors, and one d_1-haem, where catalysis occurs [3]. The d_1-haem, a cofactor unique to the denitrifiers containing the cd_1NiR, is synthesized starting from δ-aminolevulinic acid via uroporphyrinogen III. The d_1-haem, which was unequivocally identified as a 3,8-dioxo-17-acrylate-porphyrindione (Figure 1), has also been confirmed by inspection of the crystal structures of cd_1NiRs [4,5]. The presence of the electronegative oxo groups shifts the redox potential (E_0') of the iron to more positive values, relative to protoporphyrin IX.

EPR, NMR and MCD (magnetic circular dichroism) measurements [6–8] showed that the d_1-haem in *Pa*-cd_1NiR is a low-spin hexa-co-ordinate species in the ferric state and a high-spin pentaco-ordinate species in the ferrous state. The so-called proximal ligand is provided by His^{192}. In the low-spin ferric form, the sixth ligand is a hydroxide ion in the oxidized *Pa*-cd_1NiR structure. However, in the oxidized *Paracoccus pantotrophus* cd_1NiR (*Pp*-cd_1NiR), the sixth ligand is provided by the phenolate of Tyr^{25}, a residue belonging to the N-terminal segment and thus to the *c*-domain; this state is catalytically inert (resting) and needs to be activated by reduction [9,10]. A low-spin ferric d_1-haem-associated α-band in the 636–644 nm region, seen in all NiRs, may be consistent with a common histidine–OH or histidine–tyrosine ligation; however, whether the latter form is on or off the catalytic pathway remains unclear. Two conserved histidine residues (His^{327} and His^{369}) are present in the distal pocket of the d_1-haem in *Pa*-cd_1NiR and are both involved in catalysis (see below) [11].

Reduction of nitrite: substrate binding and product release

In vitro, *Pa*-cd_1NiR catalyses both the reduction of nitrite to NO and the reduction of dioxygen to water. Reduced d_1-haem binds the oxidants (NO_2^-, O_2) as well as other haem ligands, such as NO, carbon monoxide (CO) and cyanide (CN^-). Nitrite reduction is, however, the physiologically relevant activity of the enzyme; reduced *Pa*-cd_1NiR binds nitrite with high affinity ($K_m = 6\ \mu M$) [11] and produces NO with a turnover number of $6\ s^{-1}$ (at pH 7.0) [12]. The activity

Key words: cytochrome cd_1, haem d_1, nitric oxide, nitrite reductase, *Pseudomonas aeruginosa*.
Abbreviations used: NiR, nitrite reductase; *Pa*-cd_1NiR, *Pseudomonas aeruginosa* cd_1 NiR; *Pp*-cd_1NiR, *Paracoccus pantotrophus* cd_1NiR.
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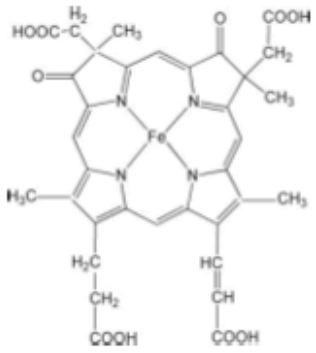
Figure 1 | Chemical structure of d_1-haem



is pH-dependent with an optimum at pH 6.0–6.6, depending on the electron donor used [12,13].

The mechanistic details of nitrite reduction are still a subject of much debate. In the present paper, we summarize the current understanding of the reaction in *Pa*-cd_1NiR in the light of the most recent results and discuss the differences between enzymes from different sources. Finally, the features of the reaction with nitrite will be compared with those with oxygen, with special attention to the role of the d_1-haem.

In the catalytic cycle, the substrate (i.e. nitrite) binds to the fully reduced enzyme ($c^{2+}d_1^{2+}$) and is then dehydrated to yield NO and oxidized d_1-haem (Figure 2). Reduction of the d_1-haem occurs (by intramolecular electron transfer) from the *c*-haem which in turn is reduced by external electron donors. In contrast with *b*-type haem-containing proteins, ferrous cd_1NiR binds with high-affinity anions such as the substrate nitrite ($K_m = 6\ \mu M$) [11], or the inhibitor cyanide ($K_d = 9.5\ \mu M$) [14]. This peculiar and physiologically relevant feature depends on the presence of the unique cofactor d_1-haem and of two conserved histidine residues (see above) on the distal side of the d_1-haem pocket [5]; in fact the affinity for anions (cyanide) is significantly decreased in the H369A mutant ($K_d = 123\ \mu M$) [14]. The affinity for nitrite of the oxidized d_1-haem in *Pa*-cd_1NiR is low [15]; recent studies carried out on the *Pp*-cd_1NiR [16,17] show binding of nitrite to the ferric enzyme, but this process occurs at a rate which is much lower than the turnover number [9], and thus its physiological relevance is questionable. The high affinity for nitrite of reduced *Pa*-cd_1NiR has important implications for the catalytic mechanism and is discussed further below.

As mentioned above, nitrite binding is followed by dehydration and formation of the product NO bound to the oxidized d_1-haem. The release of NO from the ferric d_1-haem might be assisted by the displacement of NO by the hydroxide ion, which is the physiological ligand of the oxidized d_1-haem (Figure 2, pathway 1). NO

Figure 2 | Proposed catalytic scheme for the reaction of nitrite with *Pa*-cd_1NiR

The fully reduced enzyme ($c^{2+}d_1^{2+}$) reacts with nitrite, producing NO in the active site. In pathway 1, the release of NO occurs from the oxidized d_1-haem, in order to avoid the formation of the $c^{2+}d_1^{2+}$-NO species, as it was previously proposed [21]. According to recent results on the reactivity of *Pa*-cd_1NiR [12,23], pathway 2 is also possible. Under high reductive pressure, release of NO can occur from the fully reduced NO-bound species ($c^{2+}d_1^{2+}$-NO) by displacement with an incoming nitrite molecule. The scheme implicitly assumes that functional interactions between the two monomers can be neglected, which may not necessarily be the case.

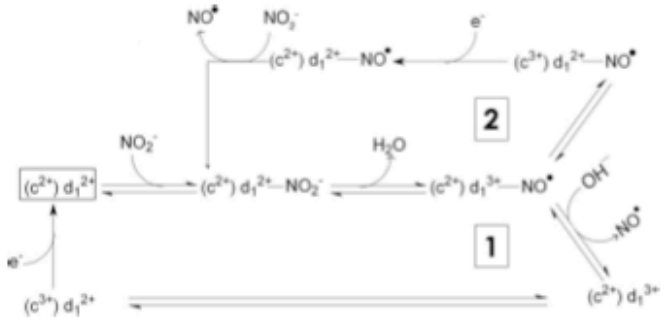


Table 1 | Dissociation and association rate constants for NO binding to reduced *Pa*-cd$_1$NIR and other haemoproteins

Protein	pH	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	Reference(s)
Wild-type *Pa*-cd$_1$NIR	7.0	3.9×10^8	$k_1 = 27.5^*$; $k_2 = 3.8^*$	[12]
			$k = 70^\dagger$	[23]
H369A *Pa*-cd$_1$NIR	7.0	1.9×10^8	$k_1 = 16.8^*$; $k_2 = 2.3^*$	[12]
Haemoglobin (human)	7.0	2.6×10^7	1×10^{-3}, 1×10^{-5}	[26,27]
Cytochrome *c* oxidase	7.4	$(0.4–1.0) \times 10^8$	4×10^{-3}	[28]

*The NO dissociation rate constant from the fully reduced wild-type and H369A mutant *Pa*-cd$_1$NIR has been measured using human deoxyhaemoglobin as an NO scavenger.
†The NO dissociation rate constant from the fully reduced *Pa*-cd$_1$NIR wild-type has also been determined by using potassium cyanide as a ligand of the ferrous d_1-haem in a displacement experiment.

production from *Pa*-cd$_1$NIR can be demonstrated under steady-state conditions; the nitrite reductase activity is pH-dependent with a turnover number of 11 s^{-1} at pH 6.2, which is significantly decreased at pH 8.0 (2 s^{-1}) [12]. Direct measurements of NO release from the ferric d_1-haem are not available, even when the reaction with nitrite is measured in the absence of excess electrons [18]. However, a recent piece of work on *Pp*-cd$_1$NIR shows that NO release is facilitated by the presence of a scavenger of electrons such as the oxidized copper protein pseudoazurin, suggesting that both substrates (nitrite and the electron donor) are necessary for the enzyme to turnover and that the oxidized *Pp*-cd$_1$NIR can release NO [19]. Also in the *Pp*-cd$_1$NIR, NO release critically depends on pH [20].

In the catalytic cycle, if the enzyme is reduced before NO is released, the $c^{2+}d_1^{2+}$–NO adduct is formed. For a long time, this species was assumed to be a 'dead-end' enzyme [21], since it is known that NO is a strong ligand of ferrous haemoproteins [22]. However, we have shown recently that reduced *Pa*-cd$_1$NIR dissociates NO very rapidly (up to 70 s^{-1}) [12,23] (Table 1) and thus should not be considered as a truly inhibited derivative; in fact, pre-incubation of ferrous *Pa*-cd$_1$NIR with NO does not inhibit the enzyme, and nitrite reduction can still be measured [12]. The exceptionally rapid release of NO must be a specific feature of the d_1-haem, since reduced *b*-type haem-containing proteins do form very stable adducts with NO (Table 1).

The pH-dependence of the turnover rate and of other kinetic data implies that a protonable residue is involved in controlling the activity of the enzyme. More than one step in the catalytic cycle requires protons or protonated amino acid side chains: (i) binding of the substrate is enhanced by the presence of a positive charge located near to the d_1-haem; and (ii) protonation of nitrite is necessary to achieve the dehydration step crucial in the catalysis. The conserved His^{327} and His^{369} residues in *Pa*-cd$_1$NIR are the most likely candidates to be involved in the pH-dependence. The catalytic efficiency towards nitrite is severely affected by the histidine-to-alanine mutation at positions 327 and 369 and both histidine mutants display a 100-fold decrease in the turnover rate [11]. At pH 8.0 and in the presence of excess nitrite, these mutants are also quickly trapped in the $c^{2+}d_1^{2+}$–NO derivative ($t_{1/2} = 50$ s). For the H369A mutant, we have shown that this is not due to an increased affinity for NO (Table 1) [12]. The decreased affinity for nitrite in the H369A mutant is in part responsible for the observed accumulation of the $c^{2+}d_1^{2+}$–NO derivative in this protein, as demonstrated by the following experiment. If the $c^{2+}d_1^{2+}$–NO adduct of *Pa*-cd$_1$NIR is rapidly mixed with a nitrite solution [23], replacement of NO by nitrite is observed in the wild-type enzyme within the first 50 ms ($k_{obs} \sim 70$ s^{-1}) and a second kinetic process (nitrite reduction) then follows at a rate compatible with the turnover number (4 s^{-1} at pH 7.0) [12]. This experiment confirms that the $c^{2+}d_1^{2+}$–NO derivative of *Pa*-cd$_1$NIR is competent to react with nitrite and cannot be considered a truly inhibited state. During catalysis, nitrite may displace the product (i.e. NO) bound to the ferrous enzyme which can thereby enter a new catalytic cycle (Figure 2, pathway 2). If the same experiment is carried out with the H369A mutant, no nitrite–NO replacement is observed and the $c^{2+}d_1^{2+}$–NO derivative accumulates [23]. In summary, the behaviour of the H369A mutant is likely to be due to its diminished capability to stabilize nitrite and, more generally, anions.

As mentioned above, *in vitro* *Pa*-cd$_1$NIR is also competent in the reduction of O_2 to water. The pH-dependence of the oxidase activity is very similar to that of the nitrite reduction activity (S. Rinaldo and F. Cutruzzolà, unpublished work), suggesting that a protonable residue is also involved in the control of O_2 reduction. A comparative kinetic study of this reaction was carried out in the wild-type *Pa*-cd$_1$NIR and in three mutants of the amino acid residues close to the d_1-haem on the distal side (Y10F, H369A and H327A/H369A) [24]. The results clearly indicate that His^{369} is the key residue in the control of reactivity, since its replacement by alanine, shown previously to affect the reduction of nitrite [24], also impairs the reaction with O_2, affecting both the properties and lifetime of the intermediate species, in particular that of a peroxy-intermediate on-pathway to the final product of the reaction, i.e. water.

Our findings allow us to extend our previous conclusion that the conserved distal histidine residues are essential for the binding to reduced d_1-haem of different anions, whether a substrate such as nitrite, a ligand such as cyanide or

an intermediate in the O_2 reduction cycle. Moreover, we proposed that His^{369} may exert a protective role towards degradation of the d_1-haems, by preventing formation and adverse effects of O_2 radical species (never significantly present in wild-type cd_1NiR). This finding has physiological implications for NiR stability in the cell, given that denitrification can occur also under low O_2 tensions and that strict anaerobiosis is not required for the biosynthesis and activity of cd_1NiR in *Ps. aeruginosa* and other denitrifiers.

Concluding remarks

These results shed light on the mechanistic details of the activity of cd_1NiRs and on the biological role of the peculiar d_1-haem, whose presence in this class of enzymes was unexplained to date.

Reduced *Ps. aeruginosa* cd_1NiR displays a significantly lowered affinity for NO, at least three orders of magnitude less then *b*-type haem-containing proteins. The chemical structure of this cofactor may explain both the high dissociation rate of the NO from the reduced iron of the d_1-haem and the high affinity for anions such as the substrate. At least one of the two conserved histidine residues, i.e. His^{369}, is also involved in the stabilization of the substrate.

The $c^{3+}d_1^{2+}$–NO adduct, which was historically considered to be a 'dead end' state [21], seems to be a genuine intermediate in the catalytic cycle and not an inhibited enzyme; this viewpoint implies that release of NO from the ferrous d_1-haem occurs rapidly enough to be fully compatible with the turnover, in contrast with the previous hypothesis in which NO release occurs only from the ferric d_1-haem. The capability of the reduced enzyme to dissociate NO rapidly allows the possibility of reconsidering not only the current model of the catalytic cycle of cd_1NiRs, but also, more generally, the reactivity of haem with NO.

Tetrapyrroles are essential molecules in living organisms and perform a multitude of functions in all kingdoms. Their synthesis is achieved in cells via a complex biosynthetic machinery which is unlikely to be maintained, if unnecessary. We propose that ancient haems, such as the d_1-haem of cd_1-NiR or the sirohaem of bacterial and plant nitrite and sulfite reductases, are molecular fossils which have survived the evolutionary pressure because their role is strategic to the survival of the organism where they are found today [25]. The peculiar NO-releasing propensity of the d_1-haem of *Ps. aeruginosa* NiR, shown recently by our group [12], is, in our opinion, an example of this strategy: the hypothesis is that the d_1-haem structure might be a prerequisite for the high rate of NO dissociation from the ferrous form, a property which is crucial to enzymatic activity and cannot be achieved with a more common *b*-type haem.

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References

1 Yoon, S.S., Hennigan, R.F., Hilliard, G.M., Ochsner, U.A., Parvatiyar, K., Kamani, M.C., Allen, H.L., DeKievit, T.R., Gardner, P.R., Schwab, U. et al. (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev. Cell **3**, 593–603

2 Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61**, 533–616

3 Rinaldo, S. and Cutruzzolà, F. (2007) Nitrite reductases in denitrification. In Biology of the Nitrogen Cycle (Bothe, H., Ferguson, S. and Newton, W.E., eds), pp. 37–55, Elsevier, Amsterdam

4 Fülöp, V., Moir, J.W., 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

5 Nurizzo, D., Silvestrini, M.C., Mathieu, M., Cutruzzolà, F., Bourgeois, D., Fülöp, V., Hajdu, J., Brunori, M., Tegoni, M. and Cambillau, C. (1997) N-terminal arm exchange is observed in the 2.15 Å crystal structure of oxidized nitrite reductase from *Pseudomonas aeruginosa*. Structure **5**, 1157–1171

6 Muhoberac, B.B. and Wharton, D.C. (1983) Electron paramagnetic resonance study of the interaction of some anionic ligands with oxidised *Pseudomonas* cytochrome *c* oxidase. J. Biol. Chem. **258**, 3019–3027

7 Timkovich, R. and Cork, M.S. (1983) Magnetic susceptibility measurements on *Pseudomonas* cytochrome cd_1. Biochem. Biophys. Acta **742**, 162–168

8 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

9 Richter, C.D., Allen, J.W., Higham, C.W., Koppenhofer, A., Zajicek, R.S., Watmough, N.J. and Ferguson, S.J. (2002) Cytochrome cd_1, reductive activation and kinetic analysis of a multifunctional respiratory enzyme. J. Biol. Chem. **277**, 3093–3100

10 Gordon, E.H., Sjögren, T., Löfqvist, M., Richter, C.D., Allen, J.W., Higham, C.W., Hajdu, J., Fülöp, V. and Ferguson, S.J. (2003) Structure and kinetic properties of *Paracoccus pantotrophus* cytochrome cd_1 nitrite reductase with the d_1 heme active site ligand tyrosine 25 replaced by serine. J. Biol. Chem. **278**, 11773–11781

11 Cutruzzolà, F., Brown, K., Wilson, E.K., Bellelli, A., Arese, M., Tegoni, M., Cambillau, C. and Brunori, M. (2001) The nitrite reductase from *Pseudomonas aeruginosa*: essential role of two active-site histidines in the catalytic and structural properties. Proc. Natl. Acad. Sci. U.S.A. **98**, 2232–2237

12 Rinaldo, S., Arcovito, A., Brunori, M. and Cutruzzolà, F. (2007) Fast dissociation of nitric oxide from ferrous *Pseudomonas aeruginosa* cd_1 nitrite reductase: a novel outlook on the catalytic mechanism. J. Biol. Chem. **282**, 14761–14767

13 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

14 Sun, W., Arese, M., Brunori, M., Nurizzo, D., Brown, K., Cambillau, C., Tegoni, M. and Cutruzzolà, F. (2002) Cyanide binding to cd_1 nitrite reductase from *Pseudomonas aeruginosa*: role of the active-site His^{369} in ligand stabilization. Biochem. Biophys. Res. Commun. **291**, 1–7

15 Timkovich, R. and Cork, M.S. (1982) Nitrogen-15 nuclear magnetic resonance investigation of nitrite reductase-substrate interaction. Biochemistry **21**, 3794–3797

16 van Wonderen, J.H., Knight, C., Oganesyan, V.S., George, S.J., Zumft, W.G. and Cheesman, M.R. (2007) Activation of the cytochrome cd_1 nitrite reductase from *Paracoccus pantotrophus*: reaction of oxidized enzyme with substrate drives a ligand switch at heme c. J. Biol. Chem. **282**, 28207–28215

17 Oganesyan, V.S., Cheesman, M.R. and Thomson, A.J. (2007) Magnetic circular dichroism evidence for a weakly coupled heme-radical pair at the active site of cytochrome cd_1, a nitrite reductase. Inorg. Chem. **46**, 10950–10952

18 George, S.J., Allen, J.W., Ferguson, S.J. and Thorneley, R.N. (2000) Time-resolved infrared spectroscopy reveals a stable ferric heme-NO intermediate in the reaction of *Paracoccus pantotrophus* cytochrome cd_1 nitrite reductase with nitrite. J. Biol. Chem. **275**, 33231–33237

19 Sam, K.A., Fairhurst, S.A., Thorneley, R.N., Allen, J.W. and Ferguson, S.J. (2008) Pseudoazurin dramatically enhances the reaction profile of nitrite reduction by *Paracoccus pantotrophus* cytochrome cd_1 and facilitates release of product nitric oxide. J. Biol. Chem. **283**, 12555–12563

20 Sam, K.A., Tollanc, J.D., Fairhurst, S.A., Higham, C.W., Lowe, D.J., Thomeley, R.N., Allen, J.W. and Ferguson, S.J. (2008) Unexpected dependence on pH of NO release from *Paracoccus pantotrophus* cytochrome cd_1. Biochem. Biophys. Res. Commun. **371**, 719–723
21 Averill, B.A. (1996) Dissimilatory nitrite and nitric oxide reductases. Chem. Rev. **96**, 2951–2964
22 Khaitonov, V.G., Sharma, V.S., Magde, D. and Koesling, D. (1997) Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins, including soluble guanylate cyclase. Biochemistry **36**, 6814–6818
23 Rinaldo, S., Brunori, M. and Cutruzzolà, F. (2007) Nitrite controls the release of nitric oxide in *Pseudomonas aeruginosa cd*$_1$ nitrite reductase. Biochem. Biophys. Res. Commun. **363**, 662–666
24 Centola, F., Rinaldo, S., Brunori, M. and Cutruzzolà, F. (2006) Critical role of His^{369} in the reactivity of *Pseudomonas aeruginosa* cytochrome *cd*$_1$-nitrite reductase with oxygen. FEBS J. **273**, 4495–4503
25 Rinaldo, S., Brunori, M. and Cutruzzolà, F. (2008) Ancient hemes for ancient catalysts. Plant Signaling Behav. **3**, 135–136
26 Azizi, F., Kielbasa, J.E., Aceyiga, A.M., Maree, R.D., Frazier, M., Yakubu, M., Shields, H., King, S.B. and Kim-Shapiro, D.B. (2005) Rates of nitric oxide dissociation from hemoglobin. Free. Radical Biol. Med. **39**, 145–151
27 Moore, E.G. and Gibson, Q.H. (1976) Cooperativity in the dissociation of nitric oxide from hemoglobin. J. Biol. Chem. **251**, 2788–2794
28 Sarti, P., Giuffrè, A., Forte, E., Mastronicola, D., Barone, M.C. and Brunori, M. (2000) Nitric oxide and cytochrome *c* oxidase: mechanisms of inhibition and NO degradation. Biochem. Biophys. Res. Commun. **274**, 183–187

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PROTEINS
STRUCTURE · FUNCTION · BIOINFORMATICS

A dramatic conformational rearrangement is necessary for the activation of DNR from *Pseudomonas aeruginosa*. Crystal structure of wild-type DNR

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ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* can grow in low oxygen, because it is capable of anaerobic respiration using nitrate as a terminal electron acceptor (denitrification). An intermediate of the denitrification pathway is nitric oxide, a compound that may become cytotoxic at high concentration. The intracellular levels of nitric oxide are tightly controlled by regulating the expression of the enzymes responsible for its synthesis and degradation (nitrite and nitric oxide reductases). In this article, we present the crystallographic structure of the wild-type dissimilative nitrate respiration regulator (DNR), a master regulator controlling expression of the denitrification machinery and a putative target for new therapeutic strategies. Comparison with other structures among the CRP-FNR class of regulators reveals that DNR has crystallized in a conformation that has never been observed before. In particular, the sensing domain of DNR has undergone a rotation of more than 50° with respect to the other structures. This suggests that DNR may undergo an unexpected and very large conformational rearrangement on activation.

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Key words: nitric oxide; nitrite reductase; transcription factor; denitrification; hemeprotein; heme sensor; NO sensing.

INTRODUCTION

Pseudomonas aeruginosa is an extensively investigated opportunistic pathogen that is able to colonize different environments and is responsible for a wide number of nosocomial infections[1]; among these, the most severe is certainly chronic lung infection of cystic fibrosis patients. Pseudomonal infections are life threatening.

P. aeruginosa is a facultative aerobe, and thereby is able to stably colonize the thick mucous layer produced by the host's inflammatory response. Cells lying near the edge of the mucous layer rapidly deplete oxygen (O_2) creating a gradient where O_2 rapidly drop to low levels. Under these O_2-depleted (microaerobic) conditions *P. aeruginosa* grows in thick biofilms probably using both microaerobic respiration and the denitrifying redox chain to supply energy by the stepwise reduction of nitrate (NO_3^-) to nitrogen (N_2).[2–4]

It is accepted that the ability to denitrify is also a component of pseudomonal resistance to nitric oxide (NO) produced by the host–defence system, and that biofilm formation is responsible for increased drug resistance.[5] Given that according to recent work, NO and nitrite (NO_2^-) are also crucial players in the regulation of both denitrification[6] and biofilm formation,[7,8] it becomes mandatory to unveil the role of these molecules during the various stages of infection to propose new therapeutic strategies.

In *P. aeruginosa* the expression of the denitrification machinery is controlled by ANR (anaerobic regulator of arginine deaminase and nitrate reduction), a global O_2 sensor that at low $p(O_2)$, activates different anaerobic pathways and induces the expression of the key transcription factor DNR (dissimilative nitrate respiration regulator).[9] DNR is required for the N-oxides-dependent transcriptional activation of genes involved in the denitrification pathway.[10]

To date, a description at molecular level of the mechanism that controls DNR activation is still missing. Both ANR and DNR belong to the CRP-FNR superfamily of transcription factors.[11] These regulators, structurally related to CRP (cyclic AMP receptor protein)[12] and FNR (fumarate and nitrate reduction regulator)[13] from *Escherichia coli*, are homodimers, each monomer con-

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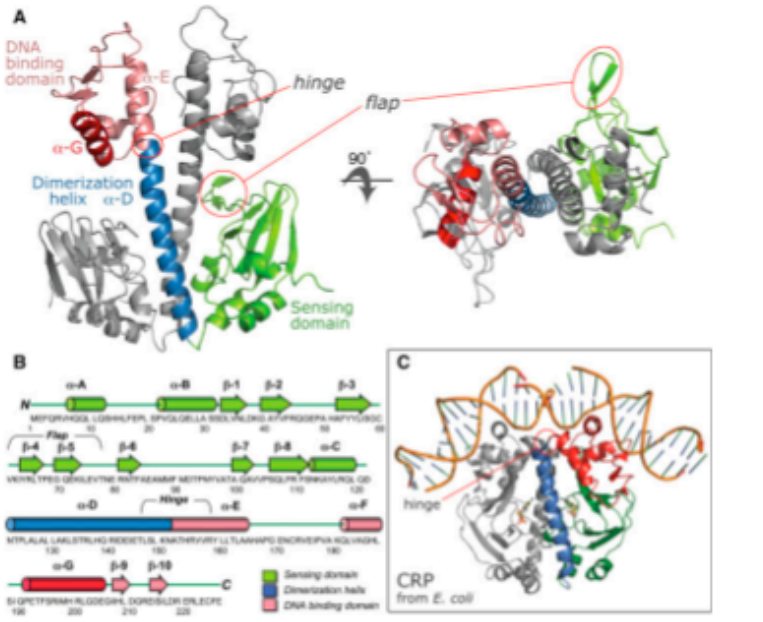

Figure 1

Structure of wtDNR from *P. aeruginosa*. (**A**) 3D structure of wtDNR dimer, side and top views. The monomer domains are highlighted with different colors: *green* the sensing domain; *blue* the dimerization helix; *salmon* the DNA binding domain (the recognition helix α-G is shown in *red*). The structural features *hinge* and *flap* are highlighted by red circles. The same color code is used throughout the figure. (**B**) Sequence and corresponding secondary structure of wtDNR. (**C**) 3D structure of CRP from *E. coli* in complex with DNA[27] (pdb id:1run).

sisting of three distinct domains: (i) a sensing domain (N-terminal); (ii) a dimerization α-helix; and (iii) a DNA binding domain (C-terminal), as shown in Figure 1(C) for CRP. The CRP-FNR regulators control many enzymatic pathways and respond to a large number of signals such as cAMP, anoxia, the redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate, or temperature.[11] Ligand binding triggers the conformational changes from an *OFF* conformation (unable to bind DNA) to an *ON* conformation (able to bind DNA). While ANR is an O_2 sensor containing an iron-sulfur cluster,[14] it is yet unknown how DNR responds to NO or other N-oxides.

We have recently solved the 3D structure of DNR-N152stop (ΔCDNR) a C-terminal deletion mutant of DNR lacking the DNA binding domain and have shown that this truncated protein (152 a.a.) as well as the wild-type (wt)DNR (227 a.a.), are able to bind protoheme in vitro with a 1:1 stoichiometry.[15]

In this article, we present the 3D crystallographic structure of the wtDNR. A structural comparison with both ΔCDNR and CRP suggests that, among the CRP-FNR type of regulators, DNR activation may be controlled by a novel activation mechanism.

METHODS

Protein cloning, expression, and purification

Cloning and heterologous expression of the *dnr* gene is described in Ref. 15 except that in the present article[5], cells were grown at 20°C instead of 25°C. wtDNR was purified according to the same protocol used for the mutant DNR-N152stop described in the same article.

Crystallization and data collection

After screening a wide rage of conditions with a crystallization robot (PHOENIX; Art Robbins Instruments), crystals of native DNR were obtained by sitting drop vapor diffusion method mixing 1 μL of protein solution (5 mg/mL in 150 m*M* NaCl, 20 m*M* TRIS pH 7.2 buffer) with 1 μL of the 500 μL reservoir solution—20% (w/v) PEG3350, 0.2*M* ammonium tartrate. Crystal grew in 2 to 3 days as very thin plates. Before freezing in liquid nitrogen crystals were soaked into a solution of mother liquor and 25% (v/v) ethylene glycol.

Data were collected at 100 K at European Synchrotron Radiation Facility (ESRF) (ID23-1), Grenoble (France). The best crystal diffracted weakly to 3.6 Å resolution and belongs to space group *C*2. Data collection strategy was calculated with BEST.[16] Data were integrated using MOSFLM[17] and, due to radiation damage, only the first 220 frames (154°) were scaled with SCALA.[18] The asymmetric unit (AU) consists of 10 monomers (five noncrystallographic dimers). See Table I for complete data collection statistics.

Table I
Data Collection and Structure Refinement Statistics for wtDNR

Data collection			
Beamline	ESRF ID23-1		
Space group	C2		
Cell dimensions			
a, *b*, *c* (Å)	245.28	121.47	82.55
β (°)	97.53		
Resolution range (Å)[a]	100.0 – 3.6 (3.79–3.60)		
Wavelength (Å)	0. 972		
R_{merge} (%)[a]	17.0 (48.6)		
$I/\sigma I$[a]	6.7 (1.9)		
Completeness (%)[a]	96.9 (97.7)		
Unique reflections[a]	27033 (3855)		
Redundancy[a]	2.7 (2.7)		
Matthews coef. (Å³/Da)	2.4		
Solvent content (%)	47.6		
Monomers/AU	10		
Refinement statistics			
Resolution range (Å)	100.0–3.6		
R_{work} (%)	32.6		
R_{free} (%)[b]	37.4		
N. residues	2145		
Ramachandran analysis (%)			
Core	85.1		
Allowed	11.5		
Generously allowed	1.4		
Disallowed	2.0		
Average B-factor (Å²)	52.5		
RMS deviations[c]			
Bond lengths (Å)	0.001		
Bond angles (°)	0.25		
RCSB Protein Data Bank entry	3dkw		

[a]Values in parentheses are for highest-resolution shell.
[b]The R_{free} was calculated with 5% of the data omitted from structure refinement.
[c]A matrix weighting term of 10^{-4} was used during refinement in Refmac5 to tighten geometry.

Structure determination and refinement

Phases were obtained by molecular replacement method with PHASER[19] in the CCP4 suite.[20] Only the β-barrel core of the N-terminal domain of DNR-N152stop (ΔCDNR) (pdb id: 2z69) was used as a search model. The final MR solution consisted of six of the 10 monomers (five dimers) in the AU. The missing molecules and the dimerization helices were placed manually where electron density from the $2F_o-F_c$ map (contoured at 1.5σ) together with positive electron density from the F_o-F_c map (contoured at 3.5σ) became visible as the model improved. Finally, positive electron density belonging to the helices of the helix-turn-helix (HTH) motif became visible and it was possible to fit a model of the C-terminal DNA-binding domain obtained with MODELLER,[21] thus obtaining the final 10 molecules in the AU.

Building and refinement was iteratively carried out using COOT[22] and REFMAC5.[23] All data from 100.0 to 3.6 Å resolution were included in the refinement. During refinement the geometric parameters were restrained and structure idealization steps were alternated with model building. To avoid over-refinement and model bias a polyalanine model was iteratively produced from the working model and side chains were added only in the positive F_o-F_c electron density map obtained from the polyalanine model. Despite the presence of five dimers, no NCS averaging could be used because of the differences among the monomers, and each of the 10 monomers had to be inspected carefully after every refinement round. Refinement process was stopped when no substantial improvement in maps quality could be observed. Only conventional rotamers were used both for visible and nonvisible side chains. Nonvisible atoms were included in the final model with zero occupancy. The final model consists of 2145 residues. The geometrical quality of the model was assessed using PROCHECK.[24,25] Final statistics are reported in Table I. The coordinates and the structure factors of DNR have been deposited in the RCSB Protein Data Bank with accession code 3dkw.

Figures

Figures in this article were made using PyMol™ (DeLano, W.L. The PyMOL Molecular Graphics System, 2002: http://www.pymol.org).

Movie

The movie in supplementary materials was made using eMovie.[26] The movie is a morph from wtDNR crystal structure to a chimera model in which the first 140 N-terminal residues belong to the crystal structure of ΔCDNR while the last 87 residues (the DNA binding domain) are modeled on CRP structure (pdb id: 1 run).

The movie shows qualitatively the entity of the hypothetical conformational change needed for DNR activation.

RESULTS AND DISCUSSION

Crystal structure of wtDNR

The structure of wtDNR was solved by molecular replacement at 3.6 Å resolution. The protein crystallized as a homodimer in the *apo* form. The 3D structure is shown in Figure 1(A) together with the secondary structure organization [Fig. 1(B)]. As expected, the monomer fold of DNR is similar to that of the other transcription factors of the CRP-FNR superfamily. In particular (i) the sensing domain (N-terminal; residues 1–123) is formed by a β-barrel core (β-sheets 1–8) and three α-helices (α-helix A–C); (ii) α-helix D (residues 124–152) is the dimerization helix; and (iii) the DNA binding domain (C-terminal; residues 153–227) is constituted by three α-helices (α-helix E to G) and two β-sheets (β-9 and 10), where α–G is the recognition helix of the HTH motif responsible for DNA binding.

Comparison of wtDNR with ΔCDNR

Comparing the structure of wtDNR with the C-terminal deletion mutant of the regulator, ΔCDNR,[15] a striking difference immediately appears. After superposition of the dimerization helices of the two structures, it is evident that the sensing domain of wtDNR is rotated by 66° with respect to the same domain in ΔCDNR [Fig. 2(A)]. It is important to note that in the known structures of CRP-FNR regulators, the scaffold of the sensing domain is structurally conserved, although the sequence identity is often lesser than 25%. Thus, for this class of regulators, we can consider the entire sensing domain as a rigid body (as a first approximation). In particular, in the case of wtDNR and ΔCDNR which share the same sequence, the two sensing domains are similar and superpose with a Root Mean Square Deviation (RMSD) of 0.75 Å (Cα). Clearly, the difference between the two structures is not due to differences in the overall fold but due to the peculiar orientation of the sensing domains with respect to the dimerization helix D, which is dramatically different for the two proteins.

Comparison of wtDNR with CRP: *ON* and *OFF* conformations

To bind DNA, the CRP-FNR transcription factors must be in an active or *ON* conformation, as shown in Figure 1(C) for CRP from *E. coli* which, after activation by two molecules of cAMP, binds DNA.[27] Once the regulator is activated (*ON* conformation) the two recognition helices of the HTH motif are in the correct position to bind the cognate DNA sequence by interaction with the major groove [Fig. 1(C)]. CRP is the first structural homologue of DNR whose structure has been solved in complex with DNA; thus, it is considered the paradigm for the *ON* conformation. On the contrary, when this class of regulators is inactive or in the *OFF* conformation, the DNA binding domain of each monomer can change its orientation dramatically by rotating around the dimerization helix.[28–30] As a consequence, the region connecting the two domains is called the *hinge* [connection between α-helix D and α-helix E; Fig. 1(A,B)].[31]

The comparison between DNR and CRP reveals that wtDNR has crystallized in an *OFF* conformation. Interestingly, both DNA binding domains of the wtDNR dimer are rotated by 155° with respect to the *ON* conformation of CRP [Fig. 2(B)], with α-helix E being now an extension of α-helix D. This yields a single long helix composed by α-helix D and α-helix E on the same axis [Fig. 1(A,B)]. In this *fully OFF* conformation of wtDNR, the recognition helices of the HTH motif are oriented downward looking at the sensing domain [Fig. 1(A)], a topology that would not be effective in binding DNA.

The *fully OFF* conformation of the DNA binding domain has been already observed for two DNR homologues CprK from *Desulfitobacterium hafniense*[30] and CooA from *Rhodospirillum rubrum*[28] (see below for a detailed comparison). The novel and unexpected feature in the wtDNR structure is the position of the sensing domain. In particular, the *fully OFF* wtDNR conformation results in a rotation of the two monomers that *twist* around the two long helices [dimerization helix-D plus α-helix E, see top view Fig. 1(A)]. This twist affects the overall position of the sensing domain that, in wtDNR, is different not only from ΔCDNR but, as we will see, from all the other known structures. By comparison with CRP, the sensing domain of wtDNR is rotated by 55°, as shown in Figure 2(A). Superposition of the sensing domain of the two structures (Cα) results in an RMSD of 2.08 Å (sequence identity of 23.6%); thus once again we can consider the β-barrel core (β-sheets 1–8) of these structures as a rigid body. We will now focus on the position of the *flap* in the sensing domain [β-hairpin composed by β-sheets 4 and 5; Fig. 1(A,B)]. The *flap* is an important structural element of the CRP-FNR class of regulators, that interacts with the DNA binding domain in the *ON* conformation.[32] In wtDNR the *flap* is more than 20 Å away from its position in a hypothetical *ON* conformation as derived from the CRP structure [Fig. 2(B)].

Comparison of wtDNR with CooA and CprK: position of the sensing domain

CooA[28,33] is a CRP-FNR regulator activated by carbon monoxide (CO) binding to the heme cofactor. The position of the DNA binding domain in the *OFF* conformation[28] of the protein is similar to the *fully OFF* wtDNR, except for a 10° angle between α-helix E and

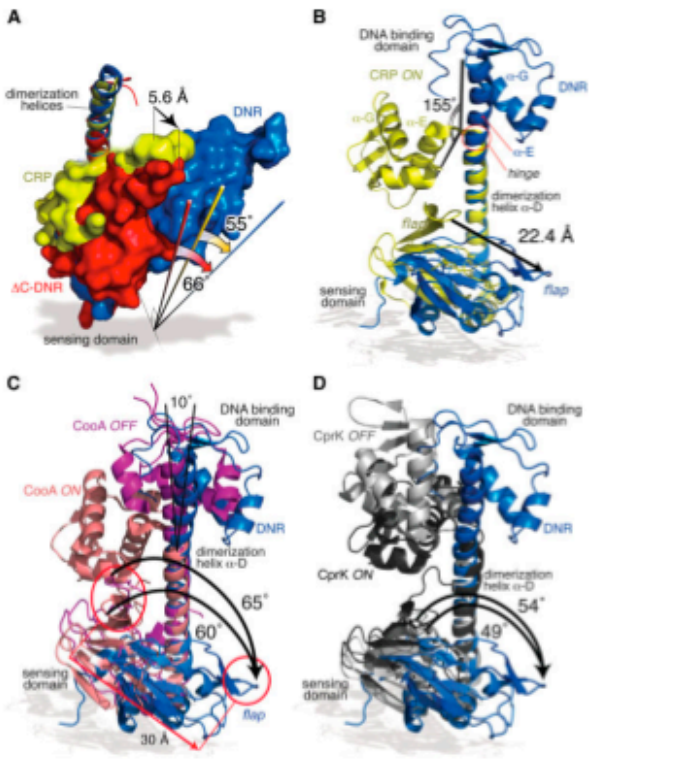

Figure 2

wtDNR peculiar *fully OFF* conformation. (**A**) Different positions of the sensing domains (surface representation) of wtDNR (*blue*), ΔC-DNR[15] (*red*; pdb id 2z69), and CRP[27] (*yellow*; pdb id:1run) showing the angles of rotation around an axis perpendicular to the direction of the superposed dimerization helices. The DNA binding domains have been removed for clarity. (**B**) Superposition of the dimerization helices of wtDNR (in *blue*) and CRP[27] (in *yellow*; pdb id:1run) monomers showing the different positions of the DNA binding domains and sensing domains in the active *ON* conformation (CRP) and in the inactive *fully OFF* conformation (wtDNR). (**C**) Superposition of the dimerization helices of wtDNR (in *blue*) and CooA *OFF*[28] and *ON*[33] conformations (in *magenta* and *salmon*, respectively, pdb id: 1fl9 and 2hkc) showing different positions of the DNA binding domains and sensing domains. (**D**) Superposition of the dimerization helices of wtDNR (in *blue*) and CprK *OFF*[30] and *ON*[30] conformations (in *light* and *dark grey*, respectively, pdb id: 3e6d and 3e6c) showing the different positions of the DNA binding domains and sensing domains.

the corresponding helix of CooA [Fig. 2(C)]. On the contrary, the sensing domain results rotated, with respect to the same domain in both CooA *OFF*[28] and *ON* (CO:protein complex)[33] structures by 65° and 60°, respectively. The distance between the *flap* regions in wtDNR and in both CooA structures is more than 30 Å as shown in Figure 2(C).

CprK is a DNR homologue activated by *o*-chlorophenolacetic acid (OCPA) binding to the reduced protein. A recent article reports the structures of the protein in both

the *OFF* conformation and *ON* conformation (protein:ligand:DNA complex).[30] Again the major difference with wtDNR structure is the position of the sensing domain, which is almost invariant in both CprK inactive and active conformations, while results rotated, with respect to wtDNR, by 49° and 54°, respectively [Fig. 2(D)].

The *fully OFF* structure of wtDNR is therefore peculiar with respect to the other *OFF* structures known for this class of regulators, which all display a different orientation of the DNA binding domain around the *hinge* zone, while the position of the sensing domain is invariant.[28–30] We can hence regard the wtDNR *fully OFF* structure as a new and atypical quaternary structure among the CRP-FNR superfamily of transcription factors, since none of the other DNR structural homologues displays such a peculiar domain organization.

Hypothesis on DNR activation mechanism

Since both CooA (protein:ligand complex)[33] and CprK (protein:ligand:DNA complex)[30] display an active conformation which is similar to CRP (superposition of Cα: RMSD 2.28 Å and 3.09 Å, respectively), we can expect the active form of DNR to share the same topology. Thus the *fully OFF* conformation reported here suggests that to be able to bind DNA, wtDNR is likely to undergo a huge conformational change involving (i) a rotation of almost 160° of the C-terminal DNA binding domain around the *hinge*, and (ii) a concomitant rotation of more than 60° of the whole sensing domain around the junction between this domain and the dimerization helix α-D (see movie in supplementary materials).

Within this scenario, it is challenging to unveil the mechanism that drives such a dramatic conformational change, apparently controlling DNR activation.

All the characterized CRP-FNR regulators are activated by binding of small molecules to the sensing domain, which may contain cofactors such as iron–sulfur clusters or heme. Although the activation mechanism is still poorly understood, it is accepted that ligand binding to the sensing domain induces a conformational change, which is transferred through the dimerization helix and the *flap* to the DNA binding domain.[31,34] In this activation mechanism, the overall position of the sensing domain is not supposed to change during the switch from the *OFF* to the *ON* conformation. Given the *fully OFF* crystal structure of wtDNR reported in this study, the activation mechanism for DNR seems to be different, and probably more complex, since the ligand association event should trigger a much larger conformational rearrangement.

We have recently shown that both wtDNR and ΔCDNR bind in vitro one Fe(III)protoporphyrin-IX complex (hemin) per monomer with high affinity.[15] However, despite extensive attempts, we could not obtain crystals of the heme–protein complex. Considering the spectroscopic and kinetic properties of the wtDNR–heme complex,[15] we are keen to think that DNR could be one member of a growing group of so-called heme-responsive/sensing proteins, identified in recent years, which are noncanonical heme binding proteins that respond to heme association/dissociation.[35,36] In fact the ΔCDNR structure, which in this scenario should mimic the *ON* conformation of the sensing domain, displays a hydrophobic cleft that could be the putative heme binding site; this cleft is absent in the wtDNR *fully OFF* structure due to the remarkable rotation of 66° of the sensing domain [Fig. 2(A)]. Since from the structural analysis wtDNR seems to be an extremely flexible protein (flexibility that may account for the extreme difficulty to obtain well diffracting crystals), we think that *apo*DNR should be able to undergo a ligand-linked large conformational rearrangement necessary to switch to the active conformation.

In summary our hypothesis, which needs further experimental support, is that wtDNR activation may be driven initially by heme association, that promotes a first conformational rearrangement from the *fully OFF* toward a *pseudoON* conformation (represented by ΔCDNR), followed by NO binding to the heme with final conversion to the biologically active state of the protein.

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REFERENCES

1. Hakki M, Limaye AP, Kim HW, Kirby KA, Corey L, Boeckh M. Invasive *Pseudomonas aeruginosa* infections: high rate of recurrence and mortality after hematopoietic cell transplantation. Bone Marrow Transplant 2007;39:687–693.
2. Hassett DJ, Sutton MD, Schurr MJ, Herr AB, Caldwell CC, Matu JO. *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. Trends Microbiol 2009;17:130–138
3. Alvarez-Ortega C, Harwood CS. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol Microbiol 2007;65:153–165.
4. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, Rowe JJ, Iglewski BH, McDermott TR, Mason RP, Wozniak DJ, Hancock RE, Parsek MR, Noah TL, Boucher RC, Hassett DJ. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 2002;3:593–603.
5. Hassett DJ, Cuppoletti J, Trapnell B, Lymar SV, Rowe JJ, Yoon SS, Hilliard GM, Parvatiyar K, Kamani MC, Wozniak DJ, Hwang SH, McDermott TR, Ochsner UA. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv Drug Deliv Rev 2002;54:1425–1443.
6. Spiro S. Regulators of bacterial responses to nitric oxide. FEMS Microbiol Rev 2007;31:193–211.
7. Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol 2006;188:7344–7353.

8. Van Alst NE, Picardo KF, Iglewski BH, Haidaris CG. Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in *Pseudomonas aeruginosa*. Infect Immun 2007;75:3780–3790.
9. Arai H, Kodama T, Igarashi Y. Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. Mol Microbiol 1997;25:1141–1148.
10. Arai H, Igarashi Y, Kodama T. Expression of the nir and nor genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. FEBS Lett 1995;371:73–76.
11. Korner H, Sofia HJ, Zumft WG. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. FEMS Microbiol Rev 2003;27:559–592.
12. Schultz SC, Shields GC, Steitz TA. Crystal structure of a CAP-DNA complex: the DNA is bent by 90 degrees. Science 1991;253:1001–1007.
13. Spiro S. The FNR family of transcriptional regulators. Antonie Van Leeuwenhoek 1994;66:23–36.
14. Yoon SS, Karabulut AC, Lipscomb JD, Hennigan RF, Lymar SV, Groce SL, Herr AB, Howell ML, Kiley PJ, Schurr MJ, Gaston B, Choi KH, Schweizer HP, Hassett DJ. Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. EMBO J 2007;26:3662–3672.
15. Giardina G, Rinaldo S, Johnson KA, Di Matteo A, Brunori M, Cutruzzola F. NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. J Mol Biol 2008;378:1002–1015.
16. Popov AN, Bourenkov GP. Choice of data-collection parameters based on statistic modelling. Acta Crystallogr D Biol Crystallogr 2003;59:1145–1153.
17. Leslie AGW. Recent changes to the MOSFLM package for processing film and image plate data. Joint CCP4 + ESF-EACBM newsletter on protein. Crystallography 1992;n.26.
18. Evans PR. Scala. Joint CCP4 and ESF-EACBM newsletter on protein. Crystallography 1997;3322–24.
19. McCoy AJ, Grosse-Kunstleve RW, Storoni LC, Read RJ. Likelihood-enhanced fast translation functions. Acta Crystallogr D Biol Crystallogr 2005;61:458–464.
20. CCP4 (Collaborative Computational Project N4. The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 1994;50:760–763.
21. Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A. Comparative protein structure modeling using Modeller. Curr Protoc Bioinformatics 2006; Ch 5, Unit 5.6.
22. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 2004;60(Pt 12 Pt 1): 2126–2132.
23. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D Biol Crystallogr 1997;53(Pt 3):240–255.
24. Morris AL, MacArthur MW, Hutchinson EG, Thornton JM. Stereochemical quality of protein structure coordinates. Proteins 1992;12:345–364.
25. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 1993;26:283–291.
26. Hodis E, Schreiber G, Rother K, Sussman JL. eMovie: a storyboard-based tool for making molecular movies. Trends Biochem Sci 2007;32:199–204.
27. Parkinson G, Gunasekera A, Vojtechovsky J, Zhang X, Kunkel TA, Berman H, Ebright RH. Aromatic hydrogen bond in sequence-specific protein DNA recognition. Nat Struct Biol 1996; 3:837–841.
28. Lanzilotta WN, Schuller DJ, Thorsteinsson MV, Kerby RL, Roberts GP, Poulos TL. Structure of the CO sensing transcription activator CooA. Nat Struct Biol 2000;7:876–880.
29. Eiting M, Hageluken G, Schubert WD, Heinz DW. The mutation G148S in PrfA, a key virulence regulator of Listeria monocytogenes, increases DNA-binding affinity by stabilizing the HTH motif. Mol Microbiol 2005;56:433–446.
30. Levy C, Pike K, Heyes DJ, Joyce MG, Gabor K, Smidt H, van der Oost J, Leys D. Molecular basis of halorespiration control by CprK, a CRP-FNR type transcriptional regulator. Mol Microbiol 2008; 70:151–167.
31. Kim J, Adhya S, Garges S. Allosteric changes in the cAMP receptor protein of Escherichia coli: hinge reorientation. Proc Natl Acad Sci USA 1992;89:9700–9704.
32. Yu S, Lee JC. Role of residue 138 in the interdomain hinge region in transmitting allosteric signals for DNA binding in *Escherichia coli* cAMP receptor protein. Biochemistry 2004;43:6662–6669.
33. Borjigin M, Li H, Lanz ND, Kerby RL, Roberts GP, Poulos TL. Structure-based hypothesis on the activation of the CO-sensing transcription factor CooA. Acta Crystallogr D Biol Crystallogr 2007;63(Pt 3):282–287.
34. Harman JG. Allosteric regulation of the cAMP receptor protein. Biochim Biophys Acta 2001;1547:1–17.
35. Kitanishi K, Igarashi J, Hayasaka K, Hikage N, Saiful I, Yamauchi S, Uchida T, Ishimori K, Shimizu T. Heme-binding characteristics of the isolated PAS-A domain of mouse Per2, a transcriptional regulatory factor associated with circadian rhythms. Biochemistry 2008;47:6157–6168.
36. Igarashi J, Murase M, Iizuka A, Pichierri F, Martinkova M, Shimizu T. Elucidation of the heme binding site of heme-regulated eukaryotic initiation factor 2alpha kinase and the role of the regulatory motif in heme sensing by spectroscopic and catalytic studies of mutant proteins. J Biol Chem 2008;283:18782–18791.

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The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*

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Pseudomonas aeruginosa is a well-known pathogen in chronic respiratory diseases such as cystic fibrosis. Infectivity of *P. aeruginosa* is related to the ability to grow under oxygen-limited conditions using the anaerobic metabolism of denitrification, in which nitrate is reduced to dinitrogen via nitric oxide (NO). Denitrification is activated by a cascade of redox-sensitive transcription factors, among which is the DNR regulator, sensitive to nitrogen oxides. To gain further insight into the mechanism of NO-sensing by DNR, we have developed an *Escherichia coli*-based reporter system to investigate different aspects of DNR activity. In *E. coli* DNR responds to NO, as shown by its ability to transactivate the *P. aeruginosa norCB* promoter. The direct binding of DNR to the target DNA is required, since mutations in the helix–turn–helix domain of DNR and specific nucleotide substitutions in the consensus sequence of the *norCB* promoter abolish the transcriptional activity. Using an *E. coli* strain deficient in haem biosynthesis, we have also confirmed that haem is required *in vivo* for the NO-dependent DNR activity, in agreement with the property of DNR to bind haem *in vitro*. Finally, we have shown, we believe for the first time, that DNR is able to discriminate *in vivo* between different diatomic signal molecules, NO and CO, both ligands of the reduced haem iron *in vitro*, suggesting that DNR responds specifically to NO.

INTRODUCTION

Pseudomonas aeruginosa is one of the most important opportunistic pathogens; it is a Gram-negative bacterium which colonizes the inflamed lungs of cystic fibrosis patients, causing persistent infections (Yoon *et al.*, 2006). *P. aeruginosa* is able to grow in the absence of oxygen, through anaerobic metabolism, which is important for infectivity and for the formation of biofilm (Hassett *et al.*, 2002; Barraud *et al.*, 2006), a surface-associated antibiotic-resistant microbial community (Singh *et al.*, 2000). The molecular race between host and pathogen thus includes strategies that are centred on the ability of *P. aeruginosa* to survive under oxygen-limited conditions; cells lying near the edge of the mucous layer rapidly deplete oxygen (O_2), creating a gradient where O_2 drops to very low levels. Under these microaerobic conditions *P. aeruginosa* grows in thick biofilms probably employing both microaerobic respiration and the denitrifying redox chain (Yoon *et al.*, 2002; Alvarez-Ortega & Harwood, 2007; Platt *et al.*, 2008). The complete denitrification pathway involves four enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase (NOR) and nitrous oxide reductase, operating sequentially to reduce nitrate to dinitrogen gas via nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). The expression and the activity of the NIR and NOR enzymes are tightly controlled because it is mandatory for the bacteria to keep the concentration of intracellular NO below cytotoxic levels, to limit nitrosative stress.

In *P. aeruginosa* the denitrification pathway is regulated by redox signalling, through a cascade of transcription factors; in particular, the global oxygen-sensing regulator ANR (anaerobic regulation of arginine deaminase and nitrate reduction) (Galimand *et al.*, 1991), a homologue of the *Escherichia coli* oxygen sensor FNR protein, activates, under anaerobic conditions, the gene coding for the transcription factor DNR (dissimilatory nitrate respiration regulator), which, in the presence of N-oxides, promotes the expression of the *nir*, the *nor* and the *nos* genes (Arai *et al.*, 1995, 1997, 1999, 2003).

Abbreviations: ALA, 5-aminolaevulinic acid; CRP, cAMP receptor protein; DNR, dissimilatory nitrate respiration regulator; FNR, fumarate and nitrate reductase regulator; HTH, helix–turn–helix; SNP, sodium nitroprusside; β-gal, β-galactosidase.

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The DNR transcription factor belongs to the CRP/FNR (cAMP receptor protein/fumarate and nitrate reductase regulator) superfamily of regulators (Zumft, 2002; Körner *et al.*, 2003). The members of this superfamily are usually homodimers, each monomer being formed by three domains (McKay & Steitz, 1981): (i) an N-terminal sensing domain (also referred to as the effector domain) with the typical fold of the cAMP-binding domain of CRP; (ii) a long dimerization α-helix recruited to form the dimer interface; and (iii) a C-terminal DNA-binding domain that contains a helix–turn–helix (HTH) motif.

N-oxide sensors belong to different subgroups of the CRP/FNR superfamily, namely the DNR, NnrR and FNR subgroups (Rinaldo *et al.*, 2006), which recognize the same consensus sequence in their target promoters, the FNR box (TTGATN$_4$ATCAA) (Eiglmeier *et al.*, 1989; Hoeren *et al.*, 1993; Hasegawa *et al.*, 1998); these proteins contain a highly conserved amino acid sequence motif Glu–SerArg (E–SR), directly involved in the interaction with the FNR box (Green *et al.*, 2001). Another transcription factor belonging to the E subgroup of the CRP/FNR superfamily, i.e. NssR from *Campylobacter jejuni*, has been recently described to act, perhaps indirectly, as a NO sensor (Elvers *et al.*, 2005).

Previous studies, carried out in *P. aeruginosa* PAO1 with the *nirS*, the *norC* and the *nosZ* promoters fused to the *lacZ* reporter gene, showed that the DNR transcription factor responds *in vivo* to N-oxides (Arai *et al.*, 1999, 2003). A similar response to NO *in vivo* has been reported for the DNR homologue NNR in *Paracoccus denitrificans* (Van Spanning *et al.*, 1999) and, for the same regulator, also in *E. coli* using the FNR-dependent *E. coli melR* promoter (Hutchings *et al.*, 2000).

Understanding of the biochemical mechanisms used to respond to specific redox signals (such as NO) is crucial in order to dissect the *in vivo* function of a sensor protein. The molecular mechanism underlying the activity of DNR is still elusive, mainly because a reliable system to probe the NO-dependent DNA binding of this regulator has not been available. Therefore to investigate in detail the NO-dependent activity of *P. aeruginosa* DNR, we have studied the DNR-mediated transcriptional activity in *E. coli*, a species which is unable to carry out denitrification. A β-galactosidase (β-gal) assay was performed under anaerobic conditions in the presence of N-oxides, using the putative DNR target promoter *norCB* (hereinafter *nor*) from *P. aeruginosa*. The choice of this promoter is supported by previous evidence showing that in *P. aeruginosa*, in the presence of N-oxides, *nor* is more strongly activated by DNR than *nirS* (Arai *et al.*, 1999). In the first part of this paper we report the successful development of this system, and the characterization of the NO-dependent activity of DNR in *E. coli*.

To gain further insight into the mechanism of NO-sensing by DNR or other homologous transcription factors, in the second part of the paper we describe how the system developed in *E. coli* was successfully used to investigate different aspects of the DNR activity. We have demonstrated the specificity of the DNR–DNA interaction, analysing both the transcriptional activity of DNR mutants and the effect of mutations in the FNR box of the *nor* promoter. Since spectroscopic and structural evidence and *in vitro* functional assays suggested the involvement of haem in DNR activity (Giardina *et al.*, 2008), we have also confirmed the importance of this cofactor in the mechanism of DNR-mediated activation of the *nor* promoter in *E. coli*.

Finally, in order to test whether DNR responds specifically to NO, we investigated its activity in the presence of carbon monoxide (CO).

METHODS

Plasmid construction and mutagenesis. The *dnr* gene was cloned into the pACYc184 vector (Biolabs) under the constitutive *tet* promoter; an extra *Eco*RV restriction site and the ribosome-binding site were also added, yielding the plasmid pACY-DNR. The *nor–lacZ* reporter system was obtained by cloning the *nor* promoter from the *P. aeruginosa* PAO1 genome first into the *Eco*RI and *Bam*HI sites of the pUC18 vector, yielding the plasmid pUC-NOR and subsequently into the *Eco*RI and *Bam*HI sites of the pRS415 vector, yielding the plasmid pRS-NOR. The *nor* promoter fragment used in this work includes 150 bp upstream of the ATG of the *norCB* gene (Fig. 1). Site-directed mutagenesis was done using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions, with pACY-DNR or pUC-NOR as a template. Two mutants were obtained in the HTH region of DNR: N152stop, previously described (Giardina *et al.*, 2008), which lacks the entire HTH domain, and E193D, which has a substitution (Glu to Asp) in position 193. The mutations introduced in the FNR box of the *nor* promoter were as follows: a double mutation in which the GA bases in positions 3 and 4 have been substituted with AG (TT**AG**TN$_4$ATCAA, named *nor*1), and a single mutation in which the A base in position 4 has been changed to a G (TTGGTN$_4$ATCAA, named *nor*2). The *nor*1 and *nor*2 fragments were then inserted into the *Eco*RI and *Bam*HI sites of the pRS415 vector, yielding, respectively, plasmids pRS-NOR1 and pRS-NOR2.

***In vivo* β-galactosidase assay.** Plasmids pACY-DNR and pRS-NOR were transformed into *E. coli* TOP10 (Invitrogen) [F$^-$ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara–leu*)*7697 galE15 galK16 rpsL*(Str^R) *endA1* λ$^-$] or *E. coli* C600 [F$^-$ *tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1* λ$^-$ (*hemA*::kan)] (Δ*hemA*) (a kind gift of Cécile Wandersman, Institut Pasteur, Paris, France). The TOP10 strain is phenotipically FNR$^+$, as tested in a control experiment with an FNR-dependent repoter fusion (pRW50 carrying the *FF-pnirB* fragment) (Wing *et al.*, 1995) (data not shown). The rich medium for bacterial growth was Luria–Bertani (LB) broth [tryptone (10 g l^{-1}), yeast extract (5 g l^{-1}), NaCl (10 g l^{-1})], supplemented with 0.4 % (w/v) glucose (LG broth) and 0.4 mM ALA (5-aminolaevulinic acid hydrochloride), as indicated.

DNR activity was tested by the β-gal assay at least in duplicate according to the method of Miller (1972) on at least two independently grown cultures. The aerobic cultures were grown in 50 ml medium in 250 ml flasks at 37 °C to OD_{600} 0.4 and were shaken at 200 r.p.m. Anaerobic growth was carried out by transferring 10 ml of the aerobic samples from the flasks to filled tubes sealed with a rubber cap. After 30 min of growth to remove the residual oxygen, selected samples were induced with either 10 μM

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1    GCCGCTGCTGATGGCCCTGGCGGTGGGCGCTGCGCTGTACCTGGCGCGGATGAATAACTG  60
                    FNR-box
61   AGTAAATCGTTCGGGAATCTTGATTGCCATCAAGCGGGTTCGCCGCCGCCGCTTCCTAGA  120
                                    norCB
121  ATCGCCGCACCACCCATTCCAGGAGGCCGCATGTCCGAGACCTTTACCAAAGGC-------  174
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Fig. 1. Sequence of the *P. aeruginosa nor* promoter fragment used in this study (150 bp). The putative binding site for the DNR protein is boxed and the bases of the consensus FNR box are highlighted in bold; the initial part of the coding sequence of the *norCB* gene, not included in the PCR fragment, is shown in grey and the translational start point is underlined.

aqueous NO from a saturated solution (2 mM) or 2 mM nitrite using a gas-tight syringe or, if indicated, with 5 % (v/v) CO gas; the cultures were then incubated without shaking for 1 h before β-gal was assayed. For the N152stop mutant, the growth temperature was switched from 37 °C to 20 °C after 1 h of growth. For the Δ*hewA* strain, the rich medium was supplemented with ALA when indicated.

For Western blot analysis, cultures were grown under the same conditions as used for the β-galactosidase assays; cells harvested and disrupted by boiling in the loading buffer (1.50 mM Tris/HCl pH 6.8, 300 mM DTT, 6 % SDS, 0.3 % bromophenol blue, 30 %, v/v, glycerol) were separated by SDS-PAGE and Western blot analysis was carried out. The DNR protein was detected using a polyclonal rabbit antibody (Davids Biotechnologies) and a goat anti-rabbit IgG (Sigma) alkaline phosphatase-conjugated secondary antibody.

RESULTS AND DISCUSSION

In vivo activation of DNR by N-oxides in the heterologous *E. coli* system

The effect of N-oxides on the expression of the *nir*, *nor* and *nos* genes activated by DNR has been previously demonstrated in *P. aeruginosa* (Arai *et al.*, 1999, 2003). The primary aim of our work was to characterize the NO-dependent DNR transcriptional activity in a heterologous model organism such as *E. coli*, which is unable to carry out denitrification. Plasmid pRS-NOR, carrying the *P. aeruginosa nor* promoter (Fig. 1) was transformed into *E. coli* TOP10, with or without plasmid pACY-DNR, harbouring the *dnr* gene under the control of a constitutive *tet* promoter. A β-gal assay according to the method of Miller (1972) was carried out under aerobic and anaerobic conditions in the presence of different N-oxides.

As shown in Fig. 2, DNR was able to transactivate the *nor* promoter in *E. coli* under anaerobic conditions and in the presence of 10 μM NO. The maximal activation of the *nor* promoter was observed at 10 μM NO, testing a range of NO concentrations from 5 μM to 50 μM; the increase of the NO concentration from 10 μM to 50 μM had no influence on the cell growth but slightly decreased the DNR activation (data not shown). An activation of the *nor* promoter under anaerobic conditions comparable to that obtained with NO gas was observed using 100 μM sodium nitroprusside (SNP), a nitrosylating agent and a source of NO^+ (data not shown).

The activation observed in the presence of 2 mM nitrite (Fig. 2) can be explained since it is known that, in *E. coli*, nitrite is a source of NO, due to the activity of nitrite reductase, which reduces nitrite to ammonia (Corker & Poole, 2003). Nitrite is not directly involved in the activation of DNR since no activity was observed in air in the presence of 2 mM nitrite, a condition in which NO is not produced (Darwin *et al.*, 1993).

As shown in Fig. 2, DNR is unable to activate the *nor* promoter under aerobic conditions. We also tested the

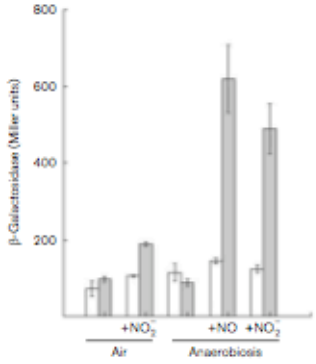

Fig. 2. Activation of the *P. aeruginosa nor* promoter requires DNR and N-oxides. The DNR protein was expressed constitutively in *E. coli* TOP10 containing plasmid pRS-NOR, in which the *nor* promoter is fused to *lacZ* (grey bars). As a control, the β-galactosidase activity was assayed under the same experimental conditions in *E. coli* TOP10 containing only the pRS-NOR vector (white bars). Cultures were grown aerobically and anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μM NO or 2 mM sodium nitrite. β-Galactosidase activity was measured 1 h after induction in duplicate in three independently grown cultures; mean values are shown, together with experimental error bars (SD).

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activity of the protein in air in the presence of SNP; the use of SNP as a source of NO^+ under aerobic conditions has been previously reported for the NorR protein (Hutchings *et al.*, 2002). No DNR activity was observed in air in the presence of SNP (data not shown), in agreement with the physiological role of DNR, which, in *P. aeruginosa*, is involved in the regulation of denitrification, an alternative respiratory metabolism activated under oxygen-limiting conditions (Zumft, 1997).

Our results show that DNR is able to activate its target promoter in *E. coli*, suggesting that the transcriptional machinery of this bacterium can be efficiently used with a *P. aeruginosa* promoter such as the *nor* promoter. A similar NO-dependent transactivation in *E. coli* was obtained with the NNR protein from *Paracoccus denitrificans*; in the latter case, however, an FNR-dependent *E. coli* promoter was used (Hutchings *et al.*, 2000).

The *nor* promoter fragment used in this study includes 150 bp upstream of the ATG of the *norCB* gene; this fragment contains the canonical recognition motif for the transcriptional activator protein FNR, named the NR box, centred −64.5 bases upstream of the ATG (Fig. 1). Interestingly, no activation of the *nor* promoter was observed in the TOP10 strain containing only plasmid pRS-NOR under anaerobic conditions (Fig. 2), suggesting that the endogenous FNR protein is unable to recognize this promoter. A preliminary *in silico* analysis of the *P. aeruginosa nor* promoter (identifying a good candidate for a −10 sequence centred around base 119.5) and its comparison with the *norC* promoter of *Pseudomonas stutzeri* (in which the transcriptional start site has been mapped: Zumft *et al.*, 1994), suggests that the FNR box could be located as in class II promoters; this hypothesis will require to be further confirmed experimentally. Interestingly, however, the region located between the palindromic TTGAT motif of the consensus FNR box in the *nor* promoter has a high GC content (Fig. 1), possibly explaining the lack of activation, given that it is known that FNR favours an AT-rich motif against a GC-rich motif in this region (Scott *et al.*, 2003). We cannot exclude, however, that other features of the *nor* promoter may preclude productive interaction between FNR and the *E. coli* transcriptional machinery. Interestingly, although the *E. coli* FNR protein seems unable to activate the *nor* promoter (this work), the DNR protein is able to recognize in *P. aeruginosa* an FNR-dependent *E. coli* promoter (Hasegawa *et al.*, 1998), making the scenario more complicated.

DNR binds specifically the *nor* promoter

To demonstrate the specificity of the DNR–*nor* promoter interaction in the *E. coli* system described above, we performed the β-gal assay with different mutants which have mutations in the HTH DNA recognition motif of the DNR protein or in the FNR box of the *nor* promoter.

The inability of the N152stop mutant, which lacks the last 75 amino acids and hence the entire DNA-binding domain of DNR (Giardina *et al.*, 2008), to transactivate the *nor* promoter under anaerobiosis in the presence of NO (Fig. 3a) suggests that the NO-dependent activation of the *nor* promoter requires the direct binding of DNR to the target DNA. To further analyse the DNR–DNA interaction, we focused our attention on the Glu-193 residue of the protein, a position highly conserved in the CRP/FNR superfamily (Green *et al.*, 2001); this glutamate residue was shown to be involved in the direct interaction of the FNR protein with the FNR box sequence on the target promoter (Spiro *et al.*, 1990). In agreement with the predicted involvement of this glutamate in binding the FNR box, the Glu 193 to Asp (E193D) variant of the DNR protein was unable to activate the *nor* promoter (Fig. 3a) under anaerobiosis in the presence of NO. This result confirms the specificity of the interaction of DNR with the *nor* promoter. The lack of activation by the two DNR mutants in the *E. coli* strain is not due to the lack of expression of the mutated proteins, which are correctly produced in the bacterium as verified by Western blotting (data not shown).

We also probed the DNR–DNA interaction by producing specific base substitutions in the FNR box of the *nor* promoter. Two mutants were produced: the first one carries an inversion of two bases in the FNR box (TTAGT-N_4-TTGAT) (*nor*1) and the second one carries a single base change (A_4 to G) (TTGGT-N_4-TTGAT) (*nor*2). The corresponding plasmids (see Methods) were transformed into an *E. coli* strain harbouring the plasmid pACYDNR and the β-gal activity under anaerobic conditions with or without NO gas was measured. As shown in Fig. 3(b), DNR was unable to activate both mutant promoters under anaerobiosis in the presence of NO. These results confirm again the specificity of the interaction between DNR and the target DNA in the *E. coli* system. Moreover we have demonstrated, to our knowledge for the first time, that a single nucleotide substitution in the FNR box is sufficient to completely disrupt the activity of the DNR protein on the *nor* promoter. The sensitivity of the FNR box to the A_4 to G mutation in the consensus sequence was shown also for the *narGHJI* promoter, an *E. coli* FNR target (Walker & DeMoss, 1992).

DNR activity requires haem biosynthesis

The mechanism whereby DNR can sense the signal molecule is not yet understood; it is certain, however, that DNR, which lacks conserved cysteines, is unable to form an Fe–S centre that can interact with the signal molecule as reported for the O_2 sensor FNR (Khoroshilova *et al.*, 1995, 1997; Green *et al.*, 1996; Jordan *et al.*, 1997). Moreover, spectroscopic evidence supports the hypothesis that haem is involved in NO sensing; in fact the DNR–haem complex is stable and can bind NO in the ferrous state to yield a pentacoordinate derivative (Giardina *et al.*, 2008). In

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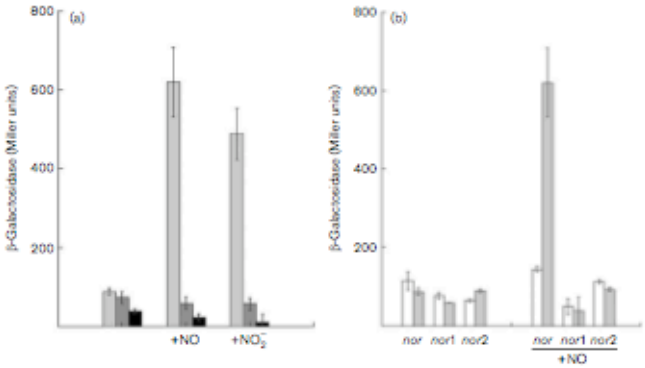

Fig. 3. Activation of the *nor* promoter requires the direct binding of DNR. (a) The N152stop protein, lacking the DNA-binding domain (dark grey bars) or the E193D protein (black bars) was expressed constitutively in *E. coli* TOP10 together with plasmid pRS-NOR and the β-galactosidase activity was measured. For comparison, the β-galactosidase activity of the wild-type protein is also shown (grey bars). (b) Plasmid pRS415 containing the wild-type *nor* promoter or the mutant promoters *nor*1 (FNR box: TTAGTN$_4$ATCAA) or *nor*2 (FNR box: TTG(G)TN$_4$ATCAA) was expressed constitutively in *E. coli* TOP10 together with pACY-DNR (grey bars). As a control, the β-galactosidase activity was measured under the same experimental conditions in *E. coli* TOP10 containing only the plasmids with the wild-type and mutant promoters (white bars). Cultures were grown anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μM NO. β-Galactosidase activity was measured 1 h after induction in duplicate in three independently grown cultures; mean values are shown, together with experimental error bars (SD).

addition, the only evidence in the literature in which a member of the DNR class of regulators binds the target DNA *in vitro* was obtained by an electophoretic mobility shift assay using the *P. aeruginosa* DNR protein bound to haem (Giardina *et al.*, 2008). The hypothesis of a haem-based sensing is reasonable also in the light of a similar haem-based mechanism well known for the *Rhodospirillum rubrum* CooA protein (Shelver *et al.*, 1997); in this case the CO molecule binds to the haem iron and triggers a conformational change, thus regulating the transcriptional activity (Lanzilotta *et al.*, 2000). These findings suggest that a haem-based mechanism is the most likely to explain the NO-dependent regulation of the function of DNR.

To demonstrate that the haem is required for DNR activity *in vivo*, we measured the NO-dependent activation of the *nor* promoter in a haem-deficient (Δ*hemA*) *E. coli* strain, which lacks the HemA protein involved in the production of ALA, a key intermediate in the biosynthesis of haem (Harris *et al.*, 1993). A similar approach was successfully employed to show that haem biosynthesis is essential for the sensing activity of NNR from *Paracoccus denitrificans* (Lee *et al.*, 2006).

Plasmid pRS-NOR was transformed into the *E. coli* Δ*hemA* strain with or without plasmid pACY-DNR, and cells were grown in the presence or in the absence of 0.4 mM ALA, under anaerobiosis with or without NO. To evaluate the basal activity of the *nor* promoter, we also measured the β-gal activity in the *E. coli* Δ*hemA* strain containing only plasmid pRS-NOR.

The results (Fig. 4) show that only in the presence of exogenous ALA is a NO-dependent DNR activity comparable to that seen in the TOP10 strain observed; this demonstrates that in *E. coli* haem is required to support the activation of the *nor* promoter by DNR *in vivo*, confirming previous evidence obtained *in vitro*.

DNR discriminates between different diatomic gases

Given that haem is essential for DNR activation we tested whether DNR is able to discriminate between different gases. We have previously shown that the reduced form of the DNR–haem complex is able to bind both NO and CO *in vitro* (Giardina *et al.*, 2008). In particular, the adduct of NO with the haem–DNR complex is pentacoordinate, whereas the CO-bound species is hexacoordinate. Therefore, we used the *E. coli* system developed here to carry out a β-gal assay under anaerobic conditions in the

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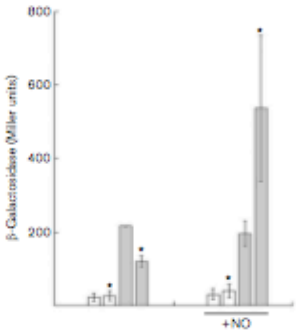

Fig. 4. Haem dependence of DNR-mediated β-galactosidase activity. The DNR protein was expressed constitutively in the Δ*hemA E. coli* strain together with plasmid pRS-NOR (grey bars). As a control, the β-galactosidase activity was assayed under the same experimental conditions in the Δ*hemA E. coli* strain containing only the pRS-NOR vector (white bars). Cultures were grown anaerobically in LG medium to exponential phase, and induced, as indicated, by the addition of 10 μM NO; samples marked with asterisk on the graph refer to cultures grown in the presence of 0.4 mM ALA. β-Galactosidase activity (Miller, 1972) was measured 1 h after induction (grey bars) in duplicate in three independently grown cultures; mean values (Miller units) are shown, together with experimental error bars (SD).

presence of CO. The very low (25.5 ± 3 Miller units) β-gal activity, measured under these conditions shows that DNR is inactive in the presence of CO, suggesting that the protein responds differently to the two diatomic signal molecules, i.e. NO and CO. These results are in agreement with our previous *in vitro* evidence that the haem coordination state of the NO and CO complexes in DNR is different (Giardina *et al.*, 2008). In conclusion, NO is the specific and unique signal required for DNR function; this selective activation in response to a particular ligand is shared with other haem-based gas sensors (e.g. CooA and H-NOX domains) (Andrew *et al.*, 2001; Boon & Marletta, 2005; Gilles-Gonzalez & Gonzalez, 2005).

Conclusions

We have shown in this work that the *P. aeruginosa* DNR protein is active in a heterologous system (*E. coli*) and can use the transcriptional apparatus of this bacterium to bind specifically to the *P. aeruginosa nor* promoter in order to regulate its activation. Moreover, we show that haem is the cofactor mediating the NO-dependent DNR regulation in *E. coli*; this finding reinforces the *in vitro* results previously obtained by our group. We have demonstrated, we believe for the first time, that DNR is able to discriminate between different diatomic signal molecules as NO and CO.

We can therefore conclude that the heterologous system described has shed new light on the features of the *P. aeruginosa* DNR protein and, in the future, will allow us to assay the activity of site-directed mutants targeting residues putatively involved in haem binding and in NO-sensing.

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REFERENCES

Alvarez-Ortega, C. & Harwood, C. S. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol Microbiol* **65**, 153–165.

Andrew, C. R., Green, E. L., Lawson, D. M. & Eady, R. R. (2001). Resonance Raman studies of cytochrome *c*′ support the binding of NO and CO to opposite sides of the heme: implications for ligand discrimination in heme-based sensors. *Biochemistry* **40**, 4115–4122.

Arai, H., Igarashi, Y. & Kodama, T. (1995). Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett* **371**, 73–76.

Arai, H., Kodama, T. & Igarashi, Y. (1997). Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Mol Microbiol* **25**, 1141–1148.

Arai, H., Kodama, T. & Igarashi, Y. (1999). Effect of nitrogen oxides on expression of the *nir* and *nor* genes for denitrification in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **170**, 19–24.

Arai, H., Mizutani, M. & Igarashi, Y. (2003). Transcriptional regulation of the *nos* genes for nitrous oxide reductase in *Pseudomonas aeruginosa*. *Microbiology* **149**, 29–36.

Barraud, N., Hassett, D. J., Hwang, S. H., Rice, S. A., Kjelleberg, S. & Webb, J. S. (2006). Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 7344–7353.

Boon, E. M. & Marletta, M. A. (2005). Ligand discrimination in soluble guanylate cyclase and the H-NOX family of heme sensor proteins. *Curr Opin Chem Biol* **9**, 441–446.

Corker, H. & Poole, R. K. (2003). Nitric oxide formation by *Escherichia coli*. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. *J Biol Chem* **278**, 31584–31592.

Darwin, A., Hussain, H., Griffiths, L., Grove, J., Sambongi, Y., Busby, S. & 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.

Eiglmeier, K., Honoré, N., Iuchi, S., Lin, E. C. & Cole, S. T. (1989). Molecular genetic analysis of FNR-dependent promoters. *Mol Microbiol* **3**, 869–878.

Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., Poole, R. K., Penn, C. W. & Park, S. F. (2005). NssR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*,

«paper no. mic028027 charlesworth ref: mic028027»

regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Mol Microbiol* **57**, 755–750.

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

Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M. & Cutruzzolà, F. (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol* **378**, 1002–1015.

Gilles-Gonzalez, M. A. & Gonzalez, G. (2005). Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *J Inorg Biochem* **99**, 1–22.

Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J. & Guest, J. R. (1996). Reconstitution of the [4Fe–4S] cluster in FNR and demonstration of the aerobic–anaerobic transcription switch in vitro. *Biochem J* **316**, 887–892.

Green, J., Scott, C. & Guest, J. R. (2001). Functional versatility in the CRP-FNR superfamily of transcription factors: FNR and FLP. *Adv Microb Physiol* **44**, 1–34.

Harris, W. F., Burkhalter, R. S., Wen, L. & Timkovich, R. (1993). Enhancement of bacterial porphyrin biosynthesis by exogenous aminolevulinic acid and isomer specificity of the products. *Bioorg Chem* **21**, 209–220.

Hasegawa, N., Arai, H. & Igarashi, Y. (1998). Activation of a consensus FNR-dependent promoter by DNR of *Pseudomonas aeruginosa* in response to nitrite. *FEMS Microbiol Lett* **166**, 213–217.

Hassett, D. J., Cuppoletti, J., Trapnell, B., Lymar, S. V., Rowe, J. J., Yoon, S. S., Hilliard, G. M., Parvatiyar, K., Kamani, M. C. & other authors (2002). Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* **54**, 1425–1443.

Hoeren, F. U., Berks, B. C., Ferguson, S. J. & McCarthy, J. E. (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* **218**, 49–57.

Hutchings, M. I., Shearer, N., Wastell, S., van Spanning, R. J. & Spiro, S. (2000). Heterologous NNR-mediated nitric oxide signaling in *Escherichia coli*. *J Bacteriol* **182**, 6434–6439.

Hutchings, M. I., Mandhana, N. & Spiro, S. (2002). The NorR protein of *Escherichia coli* activates expression of the flavoerubredoxin gene *norV* in response to reactive nitrogen species. *J Bacteriol* **184**, 4640–4643.

Jordan, P. A., Thomson, A. J., Ralph, E. T., Guest, J. R. & Green, J. (1997). FNR is a direct oxygen sensor having a biphasic response curve. *FEBS Lett* **416**, 349–352.

Khoroshilova, N., Beinert, H. & Kiley, P. (1995). Association of a polynuclear iron–sulfur center with a mutant FNR protein enhances DNA binding. *Proc Natl Acad Sci U S A* **92**, 2499–2503.

Khoroshilova, N., Popescu, C., Münck, E., Beinert, H. & Kiley, P. (1997). Iron–sulfur cluster disassembly in the FNR protein of *Escherichia coli* by O_2: [4Fe–4S] to [2Fe–2S] conversion with loss of biological activity. *Proc Natl Acad Sci U S A* **94**, 6087–6092.

Körner, H., Sofia, H. J. & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev* **27**, 559–592.

Lanzilotta, W. N., Schuller, D. J., Thorsteinsson, M. V., Kerby, R. L., Roberts, G. P. & Poulos, T. L. (2000). Structure of the CO sensing transcription activator CooA. *Nat Struct Biol* **7**, 876–880.

Lee, Y. Y., Shearer, N. & Spiro, S. (2006). Transcription factor NNR from *Paracoccus denitrificans* is a sensor of both nitric oxide and oxygen: isolation of *nnr** alleles encoding effector-independent proteins and evidence for a haem-based sensing mechanism. *Microbiology* **152**, 1461–1470.

McKay, D. B. & Steitz, T. A. (1981). Structure of catabolite gene activator protein at 2.9 Å resolution suggests binding to left-handed B-DNA. *Nature* **290**, 744–749.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Platt, M. D., Schurr, M. J., Sauer, K., Vazquez, G., Kukavica-Ibrulj, I., Potvin, E., Levesque, R. C., Fedynak, A., Brinkman, F. S. & other authors (2008). Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. *J Bacteriol* **190**, 2739–2758.

Rinaldo, S., Giardina, G., Brunori, M. & Cutruzzola, F. (2006). N-oxide sensing and denitrification: the DNR transcription factors. *Biochem Soc Trans* **34**, 185–187.

Scott, C., Partridge, J. D., Stephenson, J. R. & Green, J. (2003). DNA target sequence and FNR-dependent gene expression. *FEBS Lett* **541**, 97–101.

Shelver, D., Kerby, R. L., He, Y. & Roberts, G. P. (1997). CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. *Proc Natl Acad Sci U S A* **94**, 11216–11220.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**, 762–764.

Spiro, S., Gaston, K. L., Bell, A. I., Roberts, R. E., Busby, S. J. & Guest, J. R. (1990). Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. *Mol Microbiol* **4**, 1831–1838.

Van Spanning, R. J., Houben, E., Reijnders, W. N., Spiro, S., Westerhoff, H. V. & Saunders, N. (1999). Nitric oxide is a signal for NNR-mediated transcription activation in *Paracoccus denitrificans*. *J Bacteriol* **181**, 4129–4132.

Walker, M. S. & DeMoss, J. A. (1992). Role of alternative promoter elements in transcription from the *nar* promoter of *Escherichia coli*. *J Bacteriol* **174**, 1119–1123.

Wing, H. J., Williams, S. M. & Busby, S. J. (1995). Spacing requirements for transcription activation by *Escherichia coli* FNR protein. *J Bacteriol* **177**, 6704–6710.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. & other authors (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593–603.

Yoon, S. S., Coakley, R., Lau, G. W., Lymar, S. V., Gaston, B., Karabulut, A. C., Hennigan, R. F., Hwang, S. H., Buettner, G. & other authors (2006). Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions. *J Clin Invest* **116**, 436–446.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533–616.

Zumft, W. G. (2002). Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. *J Mol Microbiol Biotechnol* **4**, 277–286.

Zumft, W. G., Braun, C. & Cuypers, H. (1994). Nitric oxide reductase from *Pseudomonas stutzeri*. Primary structure and gene organization of a novel bacterial cytochrome *bc* complex. *Eur J Biochem* **219**, 481–490.

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

Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M. & Cutruzzolà, F. (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J Mol Biol* **378**, 1002–1015.

Gilles-Gonzalez, M. A. & Gonzalez, G. (2005). Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *J Inorg Biochem* **99**, 1–22.

Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J. & Guest, J. R. (1996). Reconstitution of the [4Fe–4S] cluster in FNR and demonstration of the aerobic–anaerobic transcription switch in vitro. *Biochem J* **316**, 887–892.

Green, J., Scott, C. & Guest, J. R. (2001). Functional versatility in the CRP-FNR superfamily of transcription factors: FNR and FLP. *Adv Microb Physiol* **44**, 1–34.

Harris, W. F., Burkhalter, R. S., Wen, L. & Timkovich, R. (1993). Enhancement of bacterial porphyrin biosynthesis by exogenous aminolevulinic acid and isomer specificity of the products. *Bioorg Chem* **21**, 209–220.

Hasegawa, N., Arai, H. & Igarashi, Y. (1998). Activation of a consensus FNR-dependent promoter by DNR of *Pseudomonas aeruginosa* in response to nitrite. *FEMS Microbiol Lett* **166**, 213–217.

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Hutchings, M. I., Mandhana, N. & Spiro, S. (2002). The NorR protein of *Escherichia coli* activates expression of the flavoerubredoxin gene *norV* in response to reactive nitrogen species. *J Bacteriol* **184**, 4640–4643.

Jordan, P. A., Thomson, A. J., Ralph, E. T., Guest, J. R. & Green, J. (1997). FNR is a direct oxygen sensor having a biphasic response curve. *FEBS Lett* **416**, 349–352.

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Körner, H., Sofia, H. J. & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev* **27**, 559–592.

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Lee, Y. Y., Shearer, N. & Spiro, S. (2006). Transcription factor NNR from *Paracoccus denitrificans* is a sensor of both nitric oxide and oxygen: isolation of *nnr** alleles encoding effector-independent proteins and evidence for a haem-based sensing mechanism. *Microbiology* **152**, 1461–1470.

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Scott, C., Partridge, J. D., Stephenson, J. R. & Green, J. (2003). DNA target sequence and FNR-dependent gene expression. *FEBS Lett* **541**, 97–101.

Shelver, D., Kerby, R. L., He, Y. & Roberts, G. P. (1997). CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. *Proc Natl Acad Sci U S A* **94**, 11216–11220.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**, 762–764.

Spiro, S., Gaston, K. L., Bell, A. I., Roberts, R. E., Busby, S. J. & Guest, J. R. (1990). Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. *Mol Microbiol* **4**, 1831–1838.

Van Spanning, R. J., Houben, E., Reijnders, W. N., Spiro, S., Westerhoff, H. V. & Saunders, N. (1999). Nitric oxide is a signal for NNR-mediated transcription activation in *Paracoccus denitrificans*. *J Bacteriol* **181**, 4129–4132.

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Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533–616.

Zumft, W. G. (2002). Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. *J Mol Microbiol Biotechnol* **4**, 277–286.

Zumft, W. G., Braun, C. & Cuypers, H. (1994). Nitric oxide reductase from *Pseudomonas stutzeri*. Primary structure and gene organization of a novel bacterial cytochrome *bc* complex. *Eur J Biochem* **219**, 481–490.

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Nitrite reduction: a ubiquitous function from a pre-aerobic past

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In eukaryotes, small amounts of nitrite confer cytoprotection against ischemia/reperfusion-related tissue damage *in vivo*, possibly *via* reduction to nitric oxide (NO) and inhibition of mitochondrial function. Several hemeproteins are involved in this protective mechanism, starting with deoxyhemoglobin, which is capable of reducing nitrite. In facultative aerobic bacteria, such as *Pseudomonas aeruginosa*, nitrite is reduced to NO by specialized heme-containing enzymes called cd_1 nitrite reductases. The details of their catalytic mechanism are summarized below, together with a hypothesis on the biological role of the unusual d_1-heme, which, in the reduced state, shows unique properties (very high affinity for nitrite and exceptionally fast dissociation of NO). Our results support the idea that the nitrite-based reactions of contemporary eukaryotes are a vestige of earlier bacterial biochemical pathways. The evidence that nitrite reductase activities of enzymes with different cellular roles and biochemical features still exist today highlights the importance of nitrite in cellular homeostasis.

Keywords: hemeprotein; nitrite; nitric oxide; nitrite reductase; *Pseudomonas aeruginosa*

Introduction

Nitric oxide (NO) is a crucial chemical messenger that, at low concentration, controls a plethora of important cellular processes in both eukaryotes and prokaryotes. In multicellular organisms, complex biological events ranging from *trans*-membrane signaling and neuronal communication to inhibition of platelet aggregation, vasodilation, and smooth muscle relaxation are directly or indirectly controlled by NO. However, when concentration rises above μM, NO becomes harmful and causes deleterious effects, such as tissue inflammation, chronic infection, malignant transformations, and degenerative diseases. On the other hand, high concentrations of NO are produced by macrophages to fight invading prokaryotic pathogens and parasites.[1,2]

NO reacts preferentially with hemes and labile 4Fe–4S clusters and thereby inhibits mitochondrial respiratory complexes and hence aerobic respiration.[3–6] Moreover, NO can modify protein residues through nitration (NO_2^- group modification) or S-nitrosation (NO^+ binding to thiols), or react with transition metals to form nitrosyl derivatives.[7] The reaction of solvated NO with superoxide ($O_2^{\cdot-}$) yields peroxynitrite ($OONO^-$), a strong oxidant of biological molecules.[8]

The synthesis of NO in eukaryotic cells proceeds *via* O_2-dependent oxidation of L-arginine, catalyzed by NO synthases (NOS);[9,10] its steady-state production is tightly regulated. In prokaryotes, NO synthesis under aerobic conditions is catalyzed by bNOS, the bacterial counterpart of NOS; here NO is not involved in cell signaling, but rather in the biosynthetic nitration of tryptophanyl moieties (*Bacillus subtilis* NOS)[11] and in cytoprotection against oxidative stress.[12,13] Since NOS catalyzes an O_2-dependent reaction, the onset of hypoxic conditions may hamper the production of NO from L-arginine. Hypoxia is a pathological condition in which the body as a whole, or a specific organ, is deprived of adequate O_2 supply. Nevertheless, it is known that in eukaryotes NO-dependent biological processes may also occur under hypoxic stress conditions. For example, NO production is associated with cytoprotection against ischemia/reperfusion (IR) injury[14] by inhibiting not only complex IV but also complex I of the respiratory chain.

Until quite recently, the source of NO produced under hypoxic conditions in eukaryotes was unknown, whereas NO synthesis under anaerobic conditions in bacteria is known to start from nitrite and has been studied in considerable detail. The discovery that in eukaryotes nitrite can be a source of NO under hypoxic conditions has given novel relevance to this compound, which was previously considered to be physiologically irrelevant.

This paper summarizes and compares the mechanisms of nitrite-derived NO production in eukaryotes and in bacteria,

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; Hb, hemoglobin; Mb, myoglobin; cd_1NIR, cd_1 nitrite reductase; Pa-cd_1NIR, cd_1 nitrite reductase from *Pseudomonas aeruginosa*; RBC, red blood cell.

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with special attention to the role of heme-containing proteins. Nitrite reductase (NIR) activity is carried out by enzymes and proteins with intrinsically different cellular roles and biochemical features, spread across the two kingdoms; the wide distribution of this activity highlights the importance of nitrite in cellular homeostasis. Since long before the advent of O_2, nitrates and nitrites were successfully employed as electron acceptors in bacterial respiration, we conclude that the nitrite-based reactions of contemporary mammalian cells may be considered as "molecular fossils" of earlier bacterial biochemical processes prevailing in the pre-aerobic world.

Nitrite-derived NO production in eukaryotes

The most recent actor in hypoxic NO signaling in eukaryotes is nitrite, a compound which was previously considered to be physiologically irrelevant[15] and a simple end product of endogenous NO metabolism. Plasma concentrations of nitrite are in the high nM range, whereas tissue levels can reach up to 10 μM.[16,17] Several hemeproteins, iron–sulfur cluster-containing proteins, and molybdenum-based reductases, present in subcellular compartments, have recently been shown to be able to reduce nitrite to NO and seem to act in a concerted manner. Possibly, other enzymes catalyzing nitrite reduction will be identified in the years to come.

Several examples of NIR activities have been found in blood, tissues, and mitochondria. All of them can be ascribed to proteins that, under aerobic conditions, play an O_2-dependent biological role, but reveal NIR activity under hypoxic conditions. In blood, the reaction of nitrite with deoxyhemoglobin (deoxy-Hb) has been proposed to represent a source of NO bioactivity, according to the following reaction[18,19]:

$$NO_2^- + Hb^{2+} \rightarrow NO + Hb^{3+} + OH^- \quad (1)$$

Kinetic studies demonstrated that the Hb-dependent NIR activity is allosterically controlled by the quaternary structure of the protein.[19] *In vivo* experiments have shown that NO can be produced from nitrite by deoxygenated red blood cells (RBC) or by deoxy-Hb.[20–22] The main conundrum lies in the expectation that the NO produced via reaction [1] is likely to be trapped by the large excess of deoxy-Hb in the RBC, yielding a stable ferrous nitrosyl complex characterized by a very slow dissociation rate constant (k_{off} between 10^{-3} and 10^{-5} s^{-1}).[23,24] In order to achieve a productive physiological effect, NO must escape from the RBC after formation; how NO trapping by deoxy-Hb is avoided and how NO is released is still an unclear matter. Possible chemical "tricks" bypassing this obstacle include oxidative denitrosylation carried out by nitrite[25,26] or the reaction of the ferric Hb-nitrite complex with NO to form N_2O_3,[27] which may diffuse out of the RBC, later forming NO or acting by nitrosylating a thiol. Finally, heme Fe(II)NO complexes of Hb can be oxidatively converted to S-nitrosothiol (CysβSNO),[28] which maintains NO bioactivity.

In the red muscle and in the heart, the NIR activity during hypoxic stress has been assigned to myoglobin (Mb).[29] Recent findings[30] in an Mb-knockout mouse model provide unequivocal evidence that deoxy-Mb reduces nitrite thereby forming NO that regulates mitochondrial respiration and cardiac contractility during IR (Fig. 1). In non-muscle tissues, NO may be generated from nitrite *via* other pathways. Recent experiments[21,31] suggest that a major part of the nitrite-derived NO production occurs in tissue and not in blood, challenging the physiological relevance of Hb as a NIR.

As stated above, a major concern on the crucial role of Hb and Mb in the nitrite-dependent NO formation in hypoxia is the well-known voracious capacity of deoxy/oxy-Hb and Mb to scavenge free NO, together with the high concentration of the two proteins in the cell (RBC and myocyte, respectively); this problem must be addressed with high priority. In other tissues, nitrite-derived NO production is due to the activity of both xanthine oxidase and aldehyde oxidase, both belonging to the family of molybdenum-containing proteins.[32] These enzymes were shown to produce NO from nitrite (100 μM) under anaerobic conditions using a reducing substrate like NADH (500 μM); under these experimental conditions, which may possibly reproduce a pathological state in the tissues, NO production in the presence of ferrous Hb has not been observed.[31]

It is well known that mitochondria exposed to hypoxia and reperfusion with O_2 generate abundant and potentially damaging reactive oxygen species (ROS). The respiratory chain is directly involved in the induction of some hypoxic nuclear genes (hypoxic signaling). As recently proposed,[33] mitochondrially produced NO functions in a signaling pathway to the nucleus by reacting with the superoxide produced by hypoxic mitochondria, yielding peroxynitrite ($ONOO^-$), which may directly or indirectly modify specific proteins and activate

Figure 1. Proposed role of myoglobin as a NIR in the red muscle.[30] The NO produced by the NIR activity of deoxymyoglobin inhibits the respiratory chain. When the complex I is inhibited, the production of ROS is reduced, exerting a cytoprotective effect.

hypoxic signaling. Under stress conditions, enough NO seems to be produced from nitrite either directly within the mitochondria[30–35] or in the cytoplasm, as it occurs in myocytes where deoxy-Mb acts as a NIR.[30,36] Therefore, inhibition of the respiratory electron transport chain by NO (and particularly complex I and IV) may be envisaged as a mechanism limiting ROS-induced cell damage under O_2 shortage and reperfusion. The correlation between NIR activity and oxidative phosphorylation capacity in different organs suggests that nitrite serves a cell regulatory role (*e.g.*, the modulation of intermediary metabolism),[22] beyond its capacity to elicit hypoxic vasodilation.[37,38]

Although the present paper is focused mainly on animal cells, it is worth mentioning that NO synthesis from nitrite could play a strategic role also in plants, where no obvious NOS homologue has yet been identified.[39] Several reports have shown that NO synthesis can be catalyzed by plant nitrate reductase,[39] a molybdenum-containing enzyme associated with nitrogen assimilation; moreover, also plant Hb can produce NO under hypoxic conditions.[40]

Nitrite-derived NO production in facultative aerobic bacteria

As detailed above, in eukaryotes the conversion of nitrite to NO seems part of a conserved regulatory mechanism that is able to match O_2 homeostasis to intermediary metabolism. The concept of respiratory adaptation in response to environmental changes, which in Eukarya is coming to light over the last 5 years, is a well-known feature of bacteria that are able to re-program their metabolism to cope with O_2 availability. A striking example can be found in facultative aerobes such as denitrifiers (including human pathogens like *Pseudomonas aeruginosa* and *Neisseria meningitidis*), which were shown to adapt to the variable supply of O_2 and nitrate/nitrite by regulating expression and activity of the various reductases involved in respiratory metabolism.

The human pathogen *P. aeruginosa* is responsible for severe nosocomial infections, in particular in chronic respiratory diseases such as cystic fibrosis. *P. aeruginosa* is capable of anaerobic growth by respiration using nitrate or nitrite as terminal electron acceptors[41] (Fig. 2). NO_3^- and NO_2^- are present in the respiratory airways of infected individuals and NO_2^- levels as high as 600 μM[42] have been estimated, a concentration that is permissive for anaerobic growth of *P. aeruginosa in vitro* and *in vivo*. During anaerobic growth, *P. aeruginosa* controls the levels of NO by orchestrating its synthesis and degradation,[43] given that overproduction may cause a metabolic suicide of the pathogen.[44] Moreover, literature data suggest that exposure to 15 mM NO_2^- at pH 6.5 kills a mucoid variant of *P. aeruginosa* which is abundant in the airways of cystic fibrosis patients,[45]

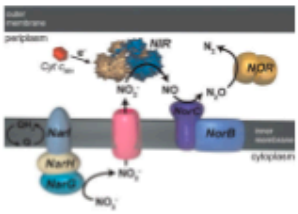

Figure 2. The denitrification respiratory chain of *P. aeruginosa*. The transformation of nitrate into molecular nitrogen is achieved by stepwise reduction carried out by four reductases, namely nitrate reductase (NarGHI), cd_1 NIR, NO reductase (NorCB), and nitrous oxide reductase (N_2OR). In the case of NIR, which is located in the bacterial periplasm and accepts electrons from cytochrome c_{551} (Cyt c_{551}), the three-dimensional structure is depicted (PDB code: 1NIR).

suggesting that NO itself may be involved in the antimicrobial effect. A proteomic and microarray study carried out on *P. aeruginosa*[46] clearly indicates that treatment with NO_2^- leads to activation of the denitrification genes and of several other genes involved in intermediate metabolism.

In the denitrification pathway, NO is produced from nitrite by the enzyme NIR. Two distinct classes of nitrite reductases are found in bacteria, both yielding NO as the main product. The two types of nitrite reductase (NIR) contain either copper (CuNIR) or heme (cd_1NIR) as cofactor, the heme-containing enzymes occurring more frequently.

In the present paper, we focus on the mechanism of nitrite reduction by cd_1NIR from *P. aeruginosa* (Pa-cd_1NIR).[47,48] We discuss the essential catalytic features of this heme-containing enzyme, giving special attention to the mechanism involved in avoiding product inhibition, *i.e.*, trapping of the active-site reduced heme by the product NO, an issue which is still controversial in eukaryotic hemeproteins acting as NIRs, and thus relevant in the context of the present discussion. A detailed description of the mechanism of nitrite reduction by CuNIR is presented elsewhere.[47,49]

Pa-cd_1NIR is a homodimer containing one c-heme and one d_1-heme group in each subunit (Fig. 3A). The active site contains the specialized d_1-heme, to which the substrate NO_2^- is bound and reduced.[41] This cofactor (Fig. 3B) is unique to the cd_1NIRs[41,50] and is synthesized *via* a specialized pathway present only in denitrifiers (strongly induced in *P. aeruginosa* upon nitrite treatment). Electrons are transferred from cytochrome c_{551} to the c-heme moiety of the enzyme,[51] and thereby internally to the active site Fe (III) d_1-heme.

Nitrite reduction to NO is the physiologically relevant activity of cd_1NIR[52,53]; indeed the expression of cd_1NIR is

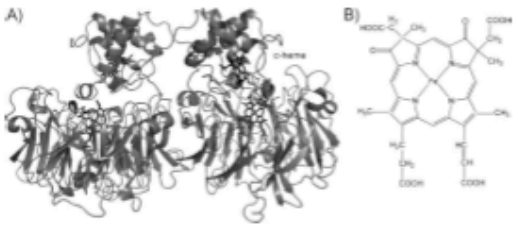

Figure 3. (**A**) Three-dimensional structure of the homodimeric Pa-cd$_1$NIR as obtained by crystallography. Each monomer is composed of an alfa-helical domain containing the c-heme (top) and a beta-propeller domain containing the d$_1$-heme (bottom). (**B**) Chemical structure of the d$_1$-heme.

induced by low O_2 tensions and presence of nitrogen oxides.[41] NO is produced efficiently by Pa-cd$_1$NiR (turnover number = 6 s^{-1} at pH7.0)[54] and the activity is pH-dependent with an optimum between pH 5.8 and 6.5.[52,54] In the catalytic cycle, nitrite binds to the fully reduced enzyme ($c^{2+}d_1^{2+}$) and is then reduced and dehydrated to yield NO. The reduced d$_1$-heme was shown to bind nitrite with high affinity (K_d = 6 μM),[55] contrary to b-type heme-containing proteins. This peculiar and physiologically relevant feature does not only depend on the unique chemical structure of the d$_1$-heme but also on the presence of two conserved histidines (His327 and His369) in the active-site pocket.[56] Upon mutagenesis of the latter histidine residues into Ala, the affinity for nitrite drops and the turnover rate is reduced 100-fold.[55] The high affinity for nitrite of reduced Pa-cd$_1$NiR has important implications for the catalytic mechanism as discussed below.

Similarly to other ferrous hemeproteins,[24] the formation of the complex between NO and the reduced d$_1$-heme might be detrimental. Indeed, if electrons are transferred from the c-heme to the d$_1$-heme before NO dissociation, the d_1^{2+}NO complex may yield a "dead end" species,[57] given that NO binds tightly to ferrous hemeproteins.[23,24] However, several features of Pa-cd$_1$NiR act in concert to limit this potentially catastrophic event.

First of all, the internal electron transfer rate is tightly controlled by an allosteric mechanism,[58,59] which is proposed to be mediated by a large conformational change which includes the dissociation of the ferric d$_1$-heme OH^- ligand upon reduction.[58,60] Notably, when the hydroxide ligand is destabilized by mutagenesis, a significant increase in the intramolecular c-to-d$_1$ electron transfer rate is observed.[59]

Most importantly, we have recently shown that the rate constant of NO dissociation from reduced Pa-cd$_1$NIR is fast (up to 70 s^{-1}),[54] which is several orders of magnitude greater than that of heme b-containing proteins. Consequently, the affinity of Pa-cd$_1$NIR for NO is lowered and the ferrous enzyme is not firmly inhibited by NO.[54,61] Noteworthy, nitrite reduction can still be monitored after pre-incubation of reduced Pa-cd$_1$NIR with NO.[54]

We have also shown (by rapid kinetics) that nitrite can displace the NO bound to the ferrous enzyme,[61] allowing the enzyme to enter a new catalytic cycle. Therefore, the high affinity of the active-site ferrous d$_1$-heme for nitrite is a crucial aspect of the catalytic mechanism which contributes to NO dissociation. In agreement with this observation, if the affinity of Pa-cd$_1$NiR for nitrite is decreased (as in the His369Ala mutant), the $c^{2+}d^{2+}$NO derivative accumulates.[55,61] The observation that NO and nitrite can compete for binding may suggest that the formation of N_2O_3 could in principle occur, *e.g.*, in a reaction similar to that proposed for Hb.[27] This event is, however, highly unlikely, mainly because during catalysis the d$_1$-heme is maintained in the reduced state by internal electron transfer from the c-heme. Moreover, even if some ferric d$_1$-heme is formed, this oxidation state has a low affinity for nitrite,[62] a feature which likely limits further reaction with free NO to produce N_2O_3.

In summary, the allosteric control of the internal electron transfer rate, the high affinity for nitrite and the exceptionally fast NO dissociation rate ensure that, when nitrite is available, nitrite reductase is active and can fulfil its physiological role in spite of the steady-state production of NO.

Conclusions

In eukaryotes, small amounts of nitrite have been demonstrated to exert potent cytoprotective effects against IR-related tissue damage *in vivo*, possibly via modulation of mitochondrial function. These findings may pave new roads for the development of therapeutics.[63,64] The physiological

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effect of nitrite is due to its conversion to NO by a variety of proteins/enzymes acting as NIRs. Several hemeproteins, including Hb and Mb, have been assigned a role in this protective mechanism. Some concern still remains since the NO (produced from nitrite) must escape entrapment by the ferrous hemeproteins in order to reach its biological targets. This difficult problem has been somewhat neglected based on the fact that very low levels of NO are necessary to fulfill its role in living cells[65]; thus even if the majority of nitrite-derived NO was captured by Hb or Mb, enough NO would still be available to diffuse freely outside the cell.

In facultative aerobic bacteria, such as *P. aeruginosa*, nitrite is used as an electron acceptor and is converted to NO by the specialized class of heme-containing enzymes called cd_1NIRs.[41] The mechanistic details of the activity of cd_1NIRs are being unraveled, highlighting the essential role of the peculiar d_1-heme, whose presence in these enzymes was to date a mystery. As mentioned above, the high affinity of reduced d_1-heme for nitrite[55] and the exceptionally fast NO dissociation[56] are trademarks of the d_1-heme, and are tuned by two conserved histidines present in the active site of Pa-cd_1NIR. We have recently proposed[66] that ancient hemes, such as the d_1-heme of cd_1-NIR or the siroheme of bacterial and plant nitrite and sulfite reductases, are molecular fossils which have survived evolutionary pressure because their role is strategic to the organism where they are found today. The peculiar NO dissociation propensity of the reduced d_1-heme of *P. aeruginosa* cd_1NIR is a component of this strategy, which could not be achieved by the more common b-type heme.

Finally, the evidence that NIR activities catalyzed by enzymes with different cellular roles and biochemical features still exist today highlights the importance of nitrite in cellular homeostasis. We support the hypothesis, already put forward by other authors,[15,63,64,67] that the nitrite-based reactions of contemporary mammalian cells are a vestige of earlier biochemical pathways of prokaryotes. In eukaryotes the same signaling cascade is activated by both the NO derived from nitrite (as in ancient prokaryotes) and that produced by NOS. This may suggest that an ancestral NO-dependent signaling cascade was already present when the only source of NO was nitrite.

The early availability of NO and its oxidation products (nitrate and nitrite) in the primordial atmosphere has been demonstrated experimentally.[68] Indeed it is accepted that nitrates and nitrites were available long before the advent of O_2 and were employed as electron acceptors by a set of "ancient" energy-converting enzymes closely related to those found in contemporary denitrification. The original hypothesis by Saraste and coworkers,[69] who proposed that oxidases have evolved from NO-reductases, has gained acceptance with time as experiments yielded more relevant data.[70–72] The reduction of nitrite to NO under hypoxic conditions, documented in eukaryotes only recently, may therefore be considered a vestigial function originating from the denitrifying metabolism and having been operative long before the advent of aerobic respiration.

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REFERENCES

1. **Bastian, N. R. and Hibbs, J. B.,** Assembly and regulation of NADPH oxidase and nitric oxide synthase. *Curr Opin Immunol* 1994. **6**: 131–139.
2. **Nathan, C. F. and Hibbs, J. B.,** Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* 1991. **3**: 65–70.
3. **Carr, G. J. and Ferguson, S. J.,** Nitric oxide formed by nitrite reductase of *Paracoccus denitrificans* is sufficiently stable to inhibit cytochrome oxidase activity and is reduced by its reductase under aerobic conditions. *Biochim Biophys Acta* 1990. **1017**: 57–62.
4. **Brown, G. C. and Cooper, C. E.,** Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* 1994. **356**: 295–298.
5. **Cleeter, M. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H.,** Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett* 1994. **345**: 50–54.
6. **Clementi, E., Brown, G. C., Feelisch, M. and Moncada, S.,** Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci U S A* 1998. **95**: 7631–7636.
7. **Bonner, F. T. and Stedman, G.,** The chemistry of nitric oxide and redox related species. In: Feelisch, M. and Stamler, J. S. editors. Methods in nitric oxide research. Chichester, John Wiley and sons Ltd., 1996. p. 3–18.
8. **Huie, R. E. and Padmaja, S.,** The reaction of NO with superoxide. *Free Radic Res Commun* 1993. **18**: 195–199.
9. **Palacios, M., Knowles, R. G., Palmer, R. M. and Moncada, S.,** Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. *Biochem Biophys Res Commun* 1989. **165**: 802–809.
10. **Alderton, W. K., Cooper, C. E. and Knowles, R. G.,** Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001. **357**: 593–615.
11. **Kers, J. A., Wach, M. J., Krasnoff, S. B., Widom, J., Cameron, K. D., *et al.*** Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. *Nature* 2004. **429**: 79–82.
12. **Gusarov, I. and Nudler, E.,** NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci U S A* 2005. **102**: 13855–13860.
13. **Gusarov, I., Starodubtseva, M., Wang, Z. Q., McQuade, L., Lippard, S. J., *et al.*** Bacterial nitric-oxide synthases operate without a dedicated redox partner *J Biol Chem.* 2008. **283**: 13140–13147.
14. **Dezfulian, C., Raat, N., Shiva, S. and Gladwin, M. T.,** Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc Res* 2007. **75**: 327–338.
15. **Bryan, N. S.,** Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med* 2006. **41**: 691–701.
16. **Grau, M., Hendgen-Cotta, U. B., Brouzos, P., Drexhage, C., Rassaf, T., *et al.*** Recent methodological advances in the analysis of nitrite in the

human circulation: nitrite as a biochemical parameter of the L-arginine/NO pathway. *J Chromatogr, B Analyt Technol Biomed Life Sci* 2007; **851**: 106–123.
17. **Shiva, S. and Gladwin, M. T.,** Shining a light on tissue NO stores: near infrared release of NO from nitrite and nitrosylated hemes. *J Mol Cell Cardiol* 2009; **46**: 1–3.
18. **Huang, Z., Shiva, S., Kim-Shapiro, D. B., Patel, R. P., Ringwood, L. A., *et al.*** Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J Clin Invest* 2005; **115**: 2099–2107.
19. **Gladwin, M. T. and Kim-Shapiro, D. B.,** The functional nitrite reductase activity of the heme-globins. *Blood* 2008; **112**: 2636–2647.
20. **Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., *et al.*** Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 2003; **9**: 1498–1505.
21. **Webb, A. J., Milsom, A. B., Rathod, K. S., Chu, W. L., Qureshi, S., *et al.*** Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. *Circ Res* 2008; **103**: 957–964.
22. **Feelisch, M., Fernandez, B. O., Bryan, N. S., Garcia-Saura, M. F., Bauer, S., *et al.*** Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. *J Biol Chem* 2008; **283**: 33927–33934.
23. **Moore, E. G. and Gibson, Q. H.,** Cooperativity in the dissociation of nitric oxide from hemoglobin. *J Biol Chem* 1976; **251**: 2788–2794.
24. **Kharitonov, V. G., Sharma, V. S., Magde, D. and Koesling, D.,** Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins, including soluble guanylate cyclase. *Biochemistry* 1997; **36**: 6814–6818.
25. **Grubina, R., Huang, Z., Shiva, S., Joshi, M. S., Azarov, I., *et al.*** Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. *J Biol Chem* 2007; **282**: 12916–12927.
26. **Gladwin, M. T., Grubina, R. and Doyle, M. P.,** The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxidative denitrosylation, and nitrite reductase/anhydrase. *Acc Chem Res* 2009; **42**: 157–167.
27. **Basu, S., Grubina, R., Huang, J., Conradie, J., Huang, Z., *et al.*** Catalytic generation of N2O3 by the concerted nitrite reductase and anhydrase activity of hemoglobin. *Nat Chem Biol* 2007; **3**: 785–794.
28. **Angelo, M., Singel, D. J. and Stamler, J. S.,** An S-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate. *Proc Natl Acad Sci U S A* 2006; **103**: 8366–8371.
29. **Shiva, S., Huang, Z., Grubina, R., Sun, J., Ringwood, L. A., *et al.*** Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res* 2007; **100**: 654–661.
30. **Hendgen-Cotta, U. B., Merx, M. W., Shiva, S., Schmitz, J., Becher, S., *et al.*** Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2008; **105**: 10256–10261.
31. **Li, H., Cui, H., Kundu, T. K., Alzawahra, W. and Zweier, J. L.,** Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem* 2008; **283**: 17855–17863.
32. **Mendel, R. R. and Bittner, F.,** Cell biology of molybdenum. *Biochim Biophys Acta* 2006; **1763**: 621–635.
33. **Castello, P. R., David, P. S., McClure, T., Crook, Z. and Poyton, R. O.,** Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab* 2006; **3**: 277–287.
34. **Castello, P. R., Woo, D. K., Ball, K., Wojcik, J., Liu, L., *et al.*** Oxygen-regulated isoforms of cytochrome c oxidase have differential effects on its nitric oxide production and on hypoxic signaling. *Proc Natl Acad Sci U S A* 2008; **105**: 8203–8208.
35. **Basu, S., Azarova, N. A., Font, M. D., King, S. B., Hogg, N., *et al.*** Nitrite reductase activity of cytochrome C. *J Biol Chem* 2008; **283**: 32590–32597.
36. **Cossins, A. and Berenbrink, M.,** Myoglobin's new clothes. *Nature* 2008; **454**: 416–417.
37. **Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G.,** Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987; **84**: 9265–9269.
38. **Palmer, R. M., Rees, D. D., Ashton, D. S. and Moncada, S.,** L-arginine is the physiological precursor for the formation of nitric oxide in endothelium dependent relaxation. *Biochem Biophys Res Commun* 1988; **153**: 1251–1256.
39. **Wilson, I. D., Neill, S. J. and Hancock, J. T.,** Nitric oxide synthesis and signalling in plants. *Plant Cell Environ* 2008; **31**: 622–631.
40. **Dordas, C., Hasinoff, B. B., Rivoal, J. and Hill, R. D.,** Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. *Planta* 2004; **219**: 66–72.
41. **Zumft, W. G.,** Cell biology and molecular basis of denitrification. *Microbiol. Mol Biol Rev* 1997; **61**: 533–616.
42. **Jones, K. L., Hegab, A. H., Hillman, B. C., Simpson, K. L., Jinkins, P. A., *et al.*** Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. *Pediatr Pulmonol* 2000; **30**: 76–85.
43. **Hassett, D. J., Cuppoletti, J., Trapnell, B., Lymar, S. V., Rowe, J. J., *et al.*** Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* 2002.
44. **Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., *et al.*** *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* 2002; **3**: 593–603.
45. **Yoon, S. S., Coakley, R., Lau, G. W., Lymar, S. V., Gaston, B., *et al.*** Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions. *Clin Invest* 2006; **116**: 436–446.
46. **Platt, M. D., Schurr, M. J., Sauer, K., Vazquez, G., Kukavica-Ibrulj, I., *et al.*** Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. *J Bacteriol* 2008; **190**: 2759–2758.
47. **Rinaldo, S. and Cutruzzolà, F.,** Nitrite reductases in denitrification. In: Bothe, H., Ferguson, S. and Newton, W. E. editors. Biology of the Nitrogen Cycle. Amsterdam, Elsevier, 2007. pp. 37–55.
48. **Rinaldo, S., Arcovito, A., Giardina, G., Castiglione, N., Brunori, M., *et al.*** New insights into the activity of Pseudomonas aeruginosa cd1 nitrite reductase. *Biochem Soc Trans* 2008; **36**: 1155–1159.
49. **Adman, E. T. and Murphy, M. E. P.,** Copper nitrite reductase. In: Messerschmidt, A., Huber, R., Poulos, T. and Wieghardt, K. editors. Handbook of metalloproteins. New York, Wiley, 2001. p. 1381–1389.
50. **Allen, J. W., Barker, P. D., Daltrop, O., Stevens, J. M., Tomlinson, E. J., *et al.*** Why isn't 'standard' heme good enough for c-type and d1-type cytochromes? *Dalton Trans* 2005; **7**: 3410–3418.
51. **Vijgenboom, E., Busch, J. E. and Canters, G. W.,** *In vivo* studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that azu expression is under control of rpoS and ANR. *Microbiology* 1997; **143**: 2853–2863.
52. **Yamanaka, T., Ota, A. and Okunuki, K.,** A nitrite reducing system reconstructed with purified cytochrome components of *Pseudomonas aeruginosa*. *Biochim Biophys Acta* 1961; **53**: 294–308.
53. **Richter, C. D., Allen, J. W., Higham, C. W., Koppenhofer, A., Zajicek, R. S., *et al.*** Cytochrome cd1, reductive activation and kinetic analysis of a multifunctional respiratory enzyme. *J Biol Chem* 2002; **277**: 3093–3100.
54. **Rinaldo, S., Arcovito, A., Brunori, M. and Cutruzzolà, F.,** Fast dissociation of nitric oxide from ferrous *Pseudomonas aeruginosa* cd1 nitrite reductase. A novel outlook on the catalytic mechanism. *J Biol Chem* 2007; **282**: 14761–14767.
55. **Cutruzzolà, F., Brown, K., Wilson, E. K., Bellelli, A., Arese, M., *et al.*** The nitrite reductase from *Pseudomonas aeruginosa*: essential role of two active-site histidines in the catalytic and structural properties. *Proc Natl Acad Sci U S A* 2001; **98**: 2232–2237.
56. **Nurizzo, D., Silvestrini, M. C., Mathieu, M., Cutruzzolà, F., Bourgeois, D., *et al.*** N-terminal arm exchange is observed in the 2.15 A crystal structure of oxidized nitrite reductase from *Pseudomonas aeruginosa*. *Structure* 1997; **5**: 1157–1171.
57. **Averill, B. A.,** Dissimilatory Nitrite and Nitric Oxide Reductases. *Chem Rev* 1996; **96**: 2951–2964.

58. **Farver, O., Kroneck, P. M., Zumft, W. G. and Pecht, I.,** Allosteric control of internal electron transfer in cytochrome cd1 nitrite reductase. *Proc Natl Acad Sci U S A* 2003. **100**: 7622–7625.
59. **Farver, O., Brunori, M., Cutruzzolà, F., Rinaldo, S., Wherland, S.,** *et al.* Intramolecular electron-transfer in *Pseudomonas aeruginosa* cd1 nitrite reductase – thermodynamics and kinetics. *Biophys J* 2008. **96**: 2849–2856.
60. **Nurizzo, D., Cutruzzolà, F., Arese, M., Bourgeois, D., Brunori, M.,** *et al.* Conformational changes occurring upon reduction and NO binding in nitrite reductase from *Pseudomonas aeruginosa*. *Biochemistry* 1998. **37**: 13987–13996.
61. **Rinaldo, S., Brunori, M. and Cutruzzolà, F.,** Nitrite controls the release of nitric oxide in *Pseudomonas aeruginosa* cd1 nitrite reductase. *Biochem Biophys Res Commun* 2007. **353**: 662–666.
62. **Timkovich, R. and Cork, M. S.,** Nitrogen-15 nuclear magnetic resonance investigation of nitrite reductase-substrate interaction. *Biochemistry* 1982. **21**: 3794–3797.
63. **Lundberg, J. O., Weitzberg, E. and Gladwin, M. T.,** The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discovery* 2008. **7**: 156–167.
64. **Butler, A. R. and Feelisch, M.,** Therapeutic uses of inorganic nitrite and nitrate: from the past to the future. *Circulation* 2008. **117**: 2151–2159.
65. **Ignarro, L. J.,** Nitric oxide: a unique endogenous signaling molecule in vascular biology. *Biosci Rep* 1999. **19**: 51–71.
66. **Rinaldo, S., Brunori, M. and Cutruzzolà, F.,** Ancient hemes for ancient catalysts. *Plant Signaling Behav* 2008. **3**: 135–136.
67. **Gladwin, M. T., Schechter, A. N., Kim-Shapiro, D. B., Patel, R. P., Hogg, N.,** *et al.* The emerging biology of the nitrite anion. *Nat Chem Biol* 2005. **1**: 308–314.
68. **Navarro-González, R., McKay, C. P. and Mvondo, D. N.,** A possible nitrogen crisis for Archaean life due to reduced nitrogen fixation by lightning. *Nature* 2001. **412**: 61–64.
69. **Hendriks, J., Gohlke, U. and Saraste, M.,** From NO to OO: nitric oxide and dioxygen in bacterial respiration. *J Bioenerg Biomembr* 1997. **30**: 15–24.
70. **Zumft, W. G. and Körner, H.,** Enzyme diversity and mosaic gene organization in denitrification. *Antonie Van Leeuwenhoek* 1998. **71**: 43–58.
71. **Giuffrè, A., Stubauer, G., Sarti, P., Brunori, M., Zumft, W. G.,** *et al.* The heme-copper oxidases of *Thermus thermophilus* catalyze the reduction of nitric oxide: evolutionary implications. *Proc Natl Acad Sci U S A* 1999. **96**: 14718–14723.
72. **Ducluzeau, A. L., van Lis, R., Duval, S., Schoepp-Cothenet, B., Russell, M. J.,** *et al.* Was nitric oxide the first deep electron sink? *Trends Biochem Sci* 2009. **34**: 9–15.