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

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The nitrite reductase and nitric oxide reductase regulator (NNR) from *Paracoccus denitrificans* activates transcription in response to nitric oxide (NO). The mechanism of NO sensing has not been elucidated for NNR, or for any of its orthologues from the FNR/CRP family of transcriptional regulators. Using regulated expression of the *nnr* gene in *Escherichia coli*, evidence has now been obtained to indicate that activation of NNR by NO does not require *de novo* synthesis of the NNR polypeptide. In anaerobic cultures, NNR is inactivated slowly following removal of the source of NO. In contrast, exposure of anaerobically grown cultures to oxygen causes rapid inactivation of NNR, suggesting that the protein is inactivated directly by oxygen. By random and site-directed mutagenesis, two variants of NNR were isolated (with substitutions of arginine at position 80) that show high levels of activity in anaerobic cultures in the absence of NO. These proteins remain substantially inactive in aerobic cultures, suggesting that the substitutions uncouple the NO- and oxygen-signalling mechanisms, thus providing further evidence that NNR senses both molecules. Structural modelling suggested that Arg-80 is close to the C-helix that forms the monomer–monomer interface in other members of the FNR/CRP family and plays an important role in transducing the activating signal between the regulatory and DNA binding domains. Assays of NNR activity in a haem-deficient mutant of *E. coli* provided preliminary evidence to indicate that NNR activity is haem dependent.

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INTRODUCTION

Bacteria capable of denitrification synthesize nitric oxide (NO) from nitrite under anaerobic growth conditions using either a cytochrome *cd₁* or a copper-containing nitrite reductase. The NO is subsequently reduced to nitrous oxide by a membrane-associated NO reductase (Watmough *et al.*, 1999; Zumft, 1997). A recurring theme amongst denitrifying organisms is the regulation of expression of the genes encoding the nitrite and NO reductases by an NO-responsive transcription factor, either an FNR/CRP family member or a sigma-54-dependent enhancer binding protein (Zumft, 2002). In *Paracoccus denitrificans*, the FNR/CRP family member NNR (nitrite reductase and nitric oxide reductase regulator) activates transcription of the *nir* and *nor* operons,

which encode the respiratory nitrite and NO reductases, respectively (Van Spanning *et al.*, 1995, 1997). A number of lines of evidence indicate that NNR is responsive *in vivo* to NO and can be activated by physiological (e.g. nitrate and nitrite) and non-physiological (e.g. nitroprusside) sources of NO and NO⁺ (Hutchings *et al.*, 2000; Van Spanning *et al.*, 1999). Thus, the enzymes that synthesize and consume NO in the denitrification pathway are co-ordinately activated by NO, and this is thought to be a strategy to prevent the accumulation of toxic concentrations of NO (Baker *et al.*, 1998; Zumft, 2002). Proteins related to NNR that are similarly NO-responsive have been characterized: NnrR from *Rhodobacter sphaeroides* (Kwiatkowski & Shapleigh, 1996); DnrD from *Pseudomonas stutzeri* (Vollack *et al.*, 1999); and DNR from *Pseudomonas aeruginosa* (Hasegawa *et al.*, 1998). NnrR from *Bradyrhizobium japonicum* may also be a sensor of NO (Mesa *et al.*, 2003). However, these NO-responsive regulators do not fall into a single well-defined clade within the wider FNR/CRP family (Korner *et al.*, 2003; Mesa *et al.*, 2003; Vollack *et al.*, 1999), and

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Abbreviations: ALA, 5-aminolaevulinic acid; AR, activating region; EMB, eosin methylene blue; GSNO, S-nitrosoglutathione; NNR, nitrite reductase and nitric oxide reductase regulator; SNP, sodium nitroprusside.

alignments of the sequences of these proteins reveal very few conserved residues that might point towards a mechanism of signal recognition. The two groupings of NNR-like proteins correspond to the proteins that activate expression of genes encoding a cytochrome *cd₁* or a copper-containing nitrite reductase (Mesa *et al.*, 2003). It is not known whether these phylogenetic groupings reflect mechanistic differences, and evidence for the biochemical basis of activation of NNR and related proteins by NO has remained elusive. Site-directed mutagenesis of the genes encoding NNR and NnrR (from *R. sphaeroides*) has implicated several residues in their function, but in no case has a mechanistic explanation been forthcoming (Hutchings *et al.*, 2000; Laratta & Shapleigh, 2003).

We have previously shown that NNR is active and signal-responsive in *Escherichia coli* (Hutchings *et al.*, 2000), an organism that does not have a denitrification pathway but that can reduce nitrate to ammonia during anaerobic growth. In this heterologous system, NNR activity can be activated by nitroprusside, a convenient source of NO⁺, or by nitrate and nitrite, which very likely act as sources of NO produced as a by-product of respiratory metabolism (Hutchings *et al.*, 2000). Others have also shown that NO is produced during nitrate and nitrite respiration in *E. coli*, and it has recently been suggested that the respiratory nitrite reductase Nrf is the major source (Corker & Poole, 2003; Ji & Hollocher, 1988; Van Doorslaer *et al.*, 2003). Activation of NNR in the heterologous background is most easily explained by a model in which NO, or a related molecule, interacts directly with the protein, since the participation of other proteins in the signalling mechanism would require those proteins to be conserved in *E. coli*. NNR activity cannot be detected in aerobic cultures of *E. coli* (Hutchings *et al.*, 2000), which makes good physiological sense, given that enzymes of the denitrification pathway are only required and expressed in anaerobically growing cultures (Baker *et al.*, 1998). The absence of NNR activity in an aerobic culture suggests either that the protein is directly inactivated by oxygen (or some other signal of aerobic metabolism), or that the molecule that activates NNR is absent or unstable in aerobically growing cells. In this paper, we show that NNR is rapidly inactivated following a shift to aerobic growth conditions, in a way that is consistent with direct inactivation of the protein. Further, we report the isolation of mutant *nnr* alleles that encode NNR proteins that have significant activity in anaerobic cultures in the absence of NO, but remain inactive in aerobic cultures. The properties of these proteins also demonstrate that NNR activity is inhibited *in vivo* by a signal of aerobic metabolism, and therefore that NNR is a dual sensor of both oxygen and NO. A haem-based mechanism would provide a means for NNR to sense both NO and oxygen, and we provide preliminary evidence to suggest that NNR activity requires haem.

METHODS

Bacterial strains and plasmids, media and growth conditions. *E. coli* strain JRG1728 [Δ lacX74 *galU galK rpsL* Δ (*ara-leu*)

Δ (*tyrR-fnr-rac-trg*)] was used as the host for assays of NNR activity (Hutchings *et al.*, 2000). For experiments using a single-copy NNR reporter fusion, JRG1728 was lysogenized with a lambda phage (a gift from David Lee, University of Birmingham, UK) carrying a consensus FNR binding site (FF), located 41.5 bp upstream of the transcription start site of the *melR* promoter fused to *lacZ* (Li *et al.*, 1998). Strain DH10B [*mcrA* Δ (*mrr hsdRMS mcrBC*) ϕ 80d*lacZ* Δ M15 Δ lacX74 *deoR recA endA araD* Δ (*ara leu*) *galU galK rpsL nupG*] was used for routine DNA manipulations, and XL1-Red [*endA gyrA thi hsdR supE relA lac mutD mutS mutT::Tn10*] from Stratagene was used for random mutagenesis. H500 (*hemA::kan*) was a gift from K. Ito (University of Kyoto, Japan) and C. Wandersman (University of Paris, France). The *hemA::kan* mutation was transferred to JRG1728 by transduction with bacteriophage P1. The plasmids used were pRW2A/FF (from Steve Busby), containing the multiple-copy FF-*melR* reporter fusion; pBAD24 (Guzman *et al.*, 1995) for controlled expression of *nnr*; and pNNR, a pUC18 derivative which expresses *nnr* from the *lac* promoter (Hutchings *et al.*, 2000). The rich medium for bacterial growth was L broth (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 5 g NaCl l⁻¹), supplemented with 0.5% (w/v) glucose for anaerobic growth. The minimal medium was either M9 (Miller, 1992) or a mineral salts medium (Spencer & Guest, 1973), supplemented with glucose, maltose and arabinose, as indicated. Eosin methylene blue (EMB) lactose medium (Miller, 1992) was used to screen for *nnr* alleles with altered phenotypes. Anaerobic cultures were grown in filled standing bottles, conditions which for *E. coli* are physiologically equivalent to strict anoxia (Constantinidou *et al.*, 2006). NO was added to cultures from a saturated solution (2 mM) using a gas-tight syringe.

DNA manipulations. For cloning into pBAD24, *nnr* and its mutant alleles were amplified from pNNR (or the corresponding derivative) by PCR, with a 5' primer that introduced a *KpnI* site immediately downstream of the ATG codon of *nnr*. The genes were cloned into the *KpnI* and *XbaI* sites of pBAD24 (Guzman *et al.*, 1995). The *KpnI* site is immediately downstream of an ATG codon in the vector, so this procedure introduced valine and proline codons immediately after the initiating codon of *nnr*. Since the genes are active *in vivo*, there is no reason to believe that this change influences NNR activity.

Mutagenesis of the *nnr* gene. The cloned *nnr* gene on pNNR was transformed into *E. coli* XL1-Red. Transformants were pooled, inoculated into L broth and grown overnight. The culture was inoculated into fresh L broth and grown overnight again and this procedure was repeated for a third culture. Then plasmid DNA was prepared, and the insert containing *nnr* was purified and subcloned into pUC18. The resulting plasmid DNA was transformed into JRG1728 containing the chromosomal FF-*melR*-*lacZ* fusion and colonies were screened for gain-of-function phenotypes on EMB plates incubated aerobically. Loss-of-function *nnr* alleles were isolated on EMB plates supplemented with 50 mM nitrate. Site-directed mutagenesis of pNNR was by a PCR-based method, as previously described by Hutchings *et al.* (2000).

Analytical procedures. β -Galactosidase was assayed according to the method of Miller (1992). Western blotting with a polyclonal anti-NNR antiserum was carried out as previously described by Hutchings *et al.* (2000).

RESULTS

Activation of NNR *in vivo* by sources of NO

It has been demonstrated previously that NNR activity can be activated in *P. denitrificans* by nitroprusside, a nitrosylating agent and source of NO⁺, and circumstantial evidence has also been presented to suggest that NO itself activates

NNR (Van Spanning *et al.*, 1999). In an *E. coli* reporter system, NNR could likewise be activated by nitroprusside, and by nitrate and nitrite, which very likely act as sources of traces of NO made as a by-product of respiratory metabolism (Corker & Poole, 2003; Hutchings *et al.*, 2000). We were interested to establish the range of compounds that might activate NNR *in vivo*, so have tested aqueous NO, along with S-nitrosoglutathione (GSNO), which is a potential source of NO as well as being a major sink for NO *in vivo*. Apparent NNR activity was assayed in a strain expressing *nnr* from a multi-copy plasmid and with an NNR-regulated promoter (FF-*melR*) fused to *lacZ* on a lambda prophage. In this system, β -galactosidase activity provides an indirect measure of transcription activation by NNR. Cultures were grown anaerobically in defined media to exponential phase and then exposed to inducer for 2 h before assaying β -galactosidase. Under these conditions, 200 μ M nitrite was an efficient inducer of NNR activity (909 units of β -galactosidase, compared to <20 units in control cultures not exposed to any inducer). High levels of NNR activity were also seen when 100 μ M nitroprusside (2440 units), 50 mM nitrate (427 units), 50 μ M GSNO (2857 units), or 1 μ M aqueous NO (911 units) were used as inducers.

NNR activation does not require *de novo* protein synthesis

We were interested to explore whether NNR synthesized under non-activating conditions can be activated by subsequent exposure to NO, in other words, whether activation requires *de novo* synthesis of NNR polypeptide. We cloned the *nnr* gene into pBAD24, which allows activation of *nnr* expression by addition of arabinose to growth media, and repression by addition of glucose (Guzman *et al.*, 1995). When the *nnr* clone in pBAD24 was introduced into the reporter strain, we found that expression of β -galactosidase became dependent on arabinose, in an anaerobic culture supplemented with an inducer of NNR (Fig. 1a). Western blots confirmed that NNR abundance in the cell was regulated by arabinose in these experiments, and was below the detection limit in cultures amended with glucose (data not shown). We then grew the reporter strain in an aerobic culture containing arabinose, conditions which allow the synthesis of NNR in an inactive form. In mid-exponential phase, cultures were washed thoroughly, then resuspended in medium containing glucose (to repress further *nnr* expression) and nitrate as an inducer of NNR activity. Following the switch in growth conditions, NNR was rapidly activated, as indicated by the immediate synthesis of β -galactosidase (Fig. 1b). The activity of the *lacZ* reporter levelled off after approximately one doubling of the culture, presumably because active NNR was diluted out of the cells as a consequence of growth. Accordingly, in the presence of arabinose (to allow continued *nnr* expression), β -galactosidase accumulated at the same rate following the shift in growth conditions, but did not show the same levelling off (Fig. 1b). The results of this experiment clearly demonstrate that

Fig. 1. Regulated expression of NNR from the *araBAD* promoter. Results shown are typical of several similar experiments. (a) Strain JRG1728 lysogenized with a lambda phage containing an NNR-regulated *lacZ* fusion was transformed with a pBAD24-derived plasmid expressing *nnr* from the *araBAD* promoter. Cultures were grown anaerobically in L broth supplemented with 50 mM nitrate and the indicated concentration of arabinose for assays of β -galactosidase. The inset shows the same data on expanded scales. (b) A culture of the same strain was grown aerobically in L broth supplemented with 2% arabinose. At 120 min, the culture was washed and divided into two. One culture (filled squares) was grown under anaerobic conditions in the presence of nitrate and 0.2% glucose, and the second culture (open squares) was grown in the same way but with 0.2% arabinose instead of glucose. MU, Miller units.

inactive NNR can be activated, with no requirement for new protein synthesis.

NNR is inactivated slowly by removal of the activating signal

We designed an experiment to test whether removal of the activating signal under anaerobic conditions leads to the inactivation of NNR. A culture was grown anaerobically in the presence of arabinose to drive NNR synthesis, and nitrate to activate the protein. In exponential phase, cells

were washed, resuspended in medium lacking arabinose and supplemented with glucose (to prevent further NNR synthesis), and grown under anaerobic conditions in the absence of nitrate. Following the transition, there was an ~1 h lag before any loss of NNR activity was detectable and thereafter, NNR activity declined at a rate close to that predicted, assuming inactivation of the protein and loss of β -galactosidase activity by dilution in the growing culture (Fig. 2a). Thus, removal of the activating signal (in this case nitrate) did not lead to instantaneous and rapid inactivation of NNR. The loss of NNR activity in the later part of the experiment presumably reflected turnover of the activating signal, since inclusion of nitrate in the medium for this experiment allowed NNR activity to continue to increase throughout the time period (not shown).

NNR is inactivated rapidly by a shift to aerobic growth conditions

We next performed a similar experiment, in which an anaerobic culture expressing active NNR was shifted to aerobic conditions, in the presence of glucose to prevent further NNR synthesis. Following the shift, β -galactosidase activity declined rapidly at a rate close to that predicted, assuming immediate inactivation of NNR and dilution of β -galactosidase as a consequence of cell growth (Fig. 2b). Thus, it seems clear that NNR was inactivated following a shift to aerobic growth conditions, which can be explained either by direct inactivation of NNR by oxygen, or by rapid removal of the activating signal in the presence of oxygen. The results of the aerobic-to-anaerobic shift experiment also imply that the inactivation of NNR is fully reversible.

Comparing the results of the two experiments, it is clear that NNR is inactivated rapidly following a transition to aerobic conditions, but not under anaerobic conditions in the absence of nitrate. *E. coli* is capable of metabolizing NO under both aerobic and anaerobic conditions, using its flavohaemoglobin and flavorubredoxin, respectively (Gardner & Gardner, 2002). *E. coli* cells grown anaerobically and exposed to NO consume NO at similar rates in the presence and absence of oxygen (Gardner & Gardner, 2002). Thus, the difference in the apparent rates of inactivation of NNR under aerobic and anaerobic conditions is unlikely to reflect a difference in the rates of NO turnover.

Isolation of *nnr* alleles encoding gain-of-function proteins

The only residue in the signalling domain that is completely conserved amongst NNR and its presumed orthologues is Phe-82. We have previously shown this residue to be essential for normal NNR activity (Hutchings *et al.*, 2000). Our attention has therefore focussed on this region of the NNR protein and we have begun to substitute residues close to Phe-82 in the primary structure. The first substitution made was of Arg-80 by alanine, with surprising consequences. The R80A variant of NNR showed significantly increased activity in aerobic cultures and greatly increased

Fig. 2. Inactivation of NNR under anaerobic and aerobic conditions. Results shown are typical of several similar experiments. Cultures of the reporter strain expressing *nnr* from the *araBAD* promoter were grown anaerobically in L broth supplemented with 0.2% arabinose and 50 mM nitrate. (a) At 150 min the culture was washed thoroughly and grown under anaerobic conditions in the presence of 0.2% glucose, to prevent further NNR synthesis. β -Galactosidase activity was assayed periodically (filled circles). Open circles show the predicted β -galactosidase activity, calculated on the assumption that NNR was completely inactivated at the time of the shift, and that β -galactosidase activity was lost by dilution in the growing culture. (b) At 180 min, the culture was washed and grown under aerobic conditions. Symbols are as for (a). MU, Miller units.

activity in anaerobic cultures in the absence of sources of NO (Table 1). Indeed, this protein showed a high level of activity in unamended minimal medium in anaerobic cultures, conditions under which the wild-type protein was almost completely inactive (Table 1). Thus, the R80A variant of NNR had become independent of the activating signal

Table 1. β -Galactosidase activity directed by NNR* proteins

Wild-type and mutant *nnr* alleles were expressed from the *lac* promoter (in pUC18) or the *araBAD* promoter (in pBAD24) in a strain containing a chromosomal fusion of an NNR-regulated promoter to *lacZ*. Cultures were grown aerobically and anaerobically in minimal medium to exponential phase, and were induced by the addition of 20 μ M SNP, and β -galactosidase activity was measured 2 h after induction. Where added, nitrate was present throughout growth. Cultures containing derivatives of pBAD24 were grown in minimal medium supplemented with maltose as the carbon and energy source (to prevent catabolite repression) and 0.2% (w/v) arabinose to activate the *araBAD* promoter. β -Galactosidase activity was measured (Miller, 1992) in duplicate in three independently grown cultures; mean values (Miller units) $\pm$ SD are shown.

NNR	Vector	Aerobic growth		Anaerobic growth		
		No addition	SNP	No addition	SNP	Nitrate
Wild-type	pUC18	51 $\pm$ 1	27 $\pm$ 1	28 $\pm$ 1	1748 $\pm$ 17	1026 $\pm$ 3
R80A	pUC18	434 $\pm$ 28	517 $\pm$ 10	942 $\pm$ 8	1657 $\pm$ 14	964 $\pm$ 63
R80H	pUC18	348 $\pm$ 1	413 $\pm$ 1	710 $\pm$ 7	1499 $\pm$ 37	1004 $\pm$ 18
Wild-type	pBAD24	16 $\pm$ 2	31 $\pm$ 1	49 $\pm$ 10	337 $\pm$ 34	65 $\pm$ 20
R80A	pBAD24	17 $\pm$ 1	84 $\pm$ 5	342 $\pm$ 4	523 $\pm$ 126	1511 $\pm$ 124
R80H	pBAD24	18 $\pm$ 1	66 $\pm$ 4	771 $\pm$ 187	568 $\pm$ 67	1619 $\pm$ 155

(NO) and, by analogy with other FNR/CRP family members (see below), we designate this phenotype NNR*. In aerobic cultures, NNR R80A (when expressed from the *lac* promoter in pUC18) had an activity >20-fold higher than the basal level of activity seen with the wild-type protein, but nevertheless significantly lower than the activity seen in anaerobic cultures (Table 1).

We sought additional gain-of-function and NNR* proteins, in an effort to shed further light on the NNR mechanism. The *nnr* gene was subjected to random mutagenesis by propagation of pNNR in a mutator strain of *E. coli*. The mutagenized DNA was introduced into the reporter strain, and transformants with increased β -galactosidase activity were sought by screening on EMB and X-Gal indicator media. We were able to isolate loss-of-function mutants (some of which are described below) from this screening at a frequency of $\sim$ 0.4%, confirming the effectiveness of the mutagenesis. From a total of $\sim$ 4000 colonies, one was isolated with a slightly more intense Lac⁺ phenotype on an aerobically incubated EMB indicator plate, and a fully Lac⁺ phenotype on an anaerobically incubated X-Gal indicator plate (with a subsequent incubation in the presence of oxygen to allow development of the blue pigment). Sequence analysis of this clone indicated that it too had a mutation in the codon corresponding to Arg-80, which was substituted with histidine in the encoded protein. The R80H protein had a qualitatively similar NNR* phenotype to that of NNR R80A (Table 1). Like NNR R80A, the R80H protein was essentially independent of NO for activation in anaerobic cultures, and had significant activity in aerobic cultures. Western blotting (not shown) confirmed that NNR* proteins were present in the cell at a similar abundance to that of the wild-type protein when expressed from the same vector. Thus the phenotype cannot be explained by (and is not consistent with) a non-specific stabilization of the protein.

Effect of NNR abundance on activity

Partially active variants of transcription factors like NNR can show artefactually high activities in *in vivo* assays dependent on reporter fusions, especially if the protein is overexpressed (Kerby *et al.*, 2003). The reason is, presumably, that an overexpressed protein with partial activity (i.e. where the equilibrium is shifted towards the inactive species) may nevertheless fully occupy a single-copy promoter, and therefore drive high levels of transcription. This effect has been observed for other FNR/CRP family members, including FNR itself and CooA (Kerby *et al.*, 2003; Moore & Kiley, 2001). Similarly, the significantly increased activity of NNR* proteins (under aerobic conditions, and anaerobic conditions in the absence of NO) may reflect only a small increase in specific activity. The ideal solution is to assay DNA binding *in vitro*, but we have not so far been able to identify suitable conditions for detecting activity in purified NNR. Another approach is to measure activity *in vivo*, under conditions where the transcription factor is expressed at a low level and so is limiting for promoter activity (Kerby *et al.*, 2003; Moore & Kiley, 2001). We therefore utilized regulated expression in pBAD24 to further characterize the activity of the R80A and R80H variants of NNR. NNR activity was measured in cultures grown in minimal medium containing maltose as the carbon and energy source (to avoid catabolite repression of the *araBAD* promoter) and supplemented with 0.2% arabinose. At this concentration of arabinose, NNR activity was submaximal (Fig. 1a), presumably because the protein abundance in the cell was limiting for promoter occupancy. Under these assay conditions, wild-type NNR showed a smaller response to nitroprusside and no response to nitrate (Table 1), presumably reflecting the lower abundance of the protein in the cell and the fact that nitrate respiration generates very low concentrations of NO. The NNR* proteins still had significant activity under anaerobic

conditions in the absence of NO, but had very low activity in aerobic cultures (Table 1). This result implies that the NO-independent activity of the star proteins did not arise from a small increase in specific activity that was magnified by overexpression from the *lac* promoter. When the star proteins were expressed at levels limiting for gene expression, they had the same activity as the wild-type protein in aerobic cultures, but sevenfold (R80A) and 16-fold (R80H) higher activity (compared to the wild-type protein) in cultures grown anaerobically in the absence of an activating signal. Thus, the mutation has a specific effect on the NO signalling mechanism, rather than a general effect on NNR activity that would be manifested under both aerobic and anaerobic conditions (for example by increasing the strength of the interaction with RNA polymerase).

Structural model of NNR

To gain further insight into the possible role of Arg-80, we modelled the structure of NNR, using the known structures of CRP (Fig. 3) and CooA (not shown) as the templates. In both cases, Arg-80 was predicted to be in the signalling domain of NNR, lying on the surface of a cleft between this domain and the long C-helix that forms the interface between monomers (Fig. 3). Comparison of the two models (active and inactive conformations) suggests that there may be conformational changes in both the C-helix and the region of the protein surrounding Arg-80. His-134 in the C-helix is the residue closest to Arg-80 in both models. Interestingly, in alignments of all of the known NO-sensing orthologues of NNR, arginine is uniquely found at position 80 (NNR numbering) in NNR, and His-134 is substituted by arginine in every other protein (Fig. 3). Thus, in NNR, Arg-80 and His-134 are predicted to be physically close in the tertiary structure, and these residues co-vary in NNR compared to its relatives. This is suggestive of a mechanistic relationship between Arg-80 and His-134. We tested the importance of His-134 by substituting it with arginine and found that the altered protein was inactive (data not shown). Thus, the substitution of histidine for arginine at this position of the C-helix of NNR orthologues may be mechanistically significant.

Isolation of *nnr* alleles encoding loss-of-function proteins

From the 4000 colonies generated by the random mutagenesis procedure, 17 appeared to express inactive forms of NNR. Excluding frameshifts, truncations and duplications, three unique mutations were identified amongst the 17, encoding NNR proteins with Y175C, A83V and G57S substitutions. Of these, NNR G57S was undetectable in cell extracts by Western blotting, suggesting that the substitution had destabilized the protein. The isolation of A83V as an inactive mutant is further evidence of the importance of residues in NNR close to the conserved Phe-82.

The fact that NNR is active in *E. coli* implies a productive interaction with the heterologous *E. coli* RNA polymerase.

Fig. 3. (a) Structural model of NNR made by 3D-Jigsaw (Bates *et al.*, 2001) using the structure of CRP (Passner *et al.*, 2000; PDB ID number 1G6N) as the template. The carbon backbone only is shown, except for the side chains of Arg-80 and His-134, which are highlighted. The long alpha helix (C-helix) forms the major interface between CRP monomers in the dimeric structure. (b) Alignment of sequences flanking Arg-80 and His-134 of NNR (shown in bold) with the equivalent regions of the known NO sensors DnrD (*Ps. stutzeri*), NnrR (*R. sphaeroides*) and DNR (*Ps. aeruginosa*), and CRP and CooA. The two alignments were extracted from a single alignment of a larger number of members of the FNR/CRP family, made using T-Coffee (Notredame *et al.*, 2000).

That interaction is presumably equivalent to the well-characterized interactions between the *E. coli* FNR and CRP proteins and RNA polymerase, which rely on surface-exposed activating regions (ARs) on the transcriptional activators (Busby & Ebright, 1999). We have previously speculated that activation by NNR at Class II promoters (where the activator binding site is centred 41.5 bp upstream of the transcription start site) requires an AR equivalent to the AR3 of CRP and FNR (Busby & Ebright, 1999; Hutchings *et al.*, 2000; Lamberg *et al.*, 2002). AR3 in CRP is a surface loop and its activity requires Glu-58 (Busby & Ebright, 1999); the conserved Glu-87 in FNR also has a role in AR3 activity (Lamberg *et al.*, 2002). To test whether NNR activity in *E. coli* required a functional AR3, we substituted the corresponding glutamate (at position 70) with

alanine and found that the altered protein was inactive in our *in vivo* assay (data not shown). The phenotype is consistent with a role for Glu-70 of NNR as an AR3 residue.

Haem-dependence of NNR activity

The pattern of activity of NNR is superficially similar to the haem-based sensor CooA, which is activated by carbon monoxide (CO) and is inactive in aerobic cultures (Thorsteinsson *et al.*, 2001). A haem-based mechanism would provide a plausible explanation for the observation that NNR can be activated by NO and apparently inactivated by oxygen. To test this idea, we transduced a *hemA* mutation into our reporter strain and assayed NNR activity in cultures grown in the absence and presence of exogenous 5-aminolaevulinic acid (ALA), which restores haem biosynthesis to *hemA* strains. In the absence of ALA, NNR was significantly less responsive to nitrite, nitroprusside and aqueous NO, but high levels of activity were restored by addition of ALA to the growth medium (Table 2). Cultures continued to grow in the 2 h following addition of the inducing compound, indicating that loss of NNR activity was not a simple consequence of sensitivity of the *hemA* mutant to the inducing compound. Loss of the response to nitrite was to be expected in the *hemA* mutant, given that the generation of NO from nitrite probably requires the activity of haem-containing enzymes. However, the loss of the response to NO and nitroprusside can only be explained in terms of a direct or indirect requirement of NNR for haem. NNR surprisingly showed high activity in cultures of the *hemA* mutant grown without ALA and in the presence of nitrate (Table 2). These cultures were capable of reducing nitrate to nitrite (not shown), which is a haem-dependent

pathway. There is evidence for a *hemA*-independent mechanism for haem biosynthesis in *E. coli* and *Salmonella typhimurium* (Elliott & Roth, 1989; Jahn *et al.*, 1991), and we speculate that anaerobic growth on nitrate may stimulate the activity of this second pathway.

DISCUSSION

Regulatory proteins that respond to NO are commonly found in denitrifying bacteria, which make NO as an intermediate of denitrification during anaerobic growth (Zumft, 2002). Here, we have shown that NNR can be activated in anaerobic cultures with micromolar concentrations of aqueous NO, metabolic and chemical sources of nitric oxide (nitrate, nitrite and GSNO), and the nitrosylating agent sodium nitroprusside (SNP). This spectrum of compounds that can activate NNR *in vivo* may provide some clues as to the activation mechanism. NO *per se* is not a nitrosylating agent and so cannot, for example, nitrosylate thiols unless an oxidizing agent (oxygen or a transition metal ion) is present (Poole & Hughes, 2000). NO can however react with haem and non-haem iron centres, and with Fe-S clusters. GSNO is transported into the cell through a peptide permease, whereupon it can modify intracellular targets, either by rather slow homolytic decomposition to NO or by the heterolytic transfer of NO⁺ (De Groote *et al.*, 1995). GSNO reacts with haemoglobin to form nitrosyl haemoglobin, in which NO is bound to the iron of the ferrous haem (Spencer *et al.*, 2000). Nitroprusside does not directly release NO (except through photolytic decomposition), but is an effective nitrosylating agent (Poole & Hughes, 2000), and is a potent activator of soluble guanylate cyclase, a haem-based NO sensor (Martin *et al.*, 2003). The fact that NO activates NNR in the absence of oxygen argues against thiol nitrosylation as the activating mechanism, and the sole cysteine of NNR is partially dispensable for activity (Hutchings *et al.*, 2000). The dispensability of the cysteine residue also argues against modification of an Fe-S cluster as the activation mechanism, and we have shown that NNR retains activity in an *icsS* mutant that has a defect in Fe-S cluster assembly (N. Shearer & S. Spiro, unpublished observations). All of the compounds that activate NNR are consistent with a haem-based mechanism for NO sensing. The partial loss of NNR activity in a *hemA* mutant is further evidence for a haem-based sensing mechanism. Biochemical efforts to demonstrate that purified NNR contains or can bind haem have so far proved unsuccessful, so we cannot exclude the possibility that the requirement for haem is indirect. In other words, there may be a haem-containing sensor protein (analogous to the eukaryotic soluble guanylate cyclase) that generates a secondary signal that activates NNR, although this model cannot easily be reconciled with the phenotypes of NNR* proteins. It has been reported that the NO-sensing DnrD protein of *Ps. stutzeri* purifies with a bound haem, but a specific function cannot be attributed to the haem (Vollack & Zumft, 2001). Cultures of *P. denitrificans* which have been depleted for haem (by treatment with the ALA analogue laevulinic acid) synthesize the apo-form

Table 2. Haem dependence of NNR-regulated β -galactosidase activity

A *hemA* mutant of the NNR reporter strain was grown in minimal medium supplemented as indicated with ALA (50 $\mu\text{g ml}^{-1}$). Cultures were grown anaerobically (except where indicated otherwise) to mid-exponential phase, induced by the indicated addition (except in the case of nitrate, which was present throughout growth) and then assayed for β -galactosidase activity 2 h later. Aerobic control cultures grown in the absence of ALA were supplemented with catalase (1 $\mu\text{g ml}^{-1}$) which is required for growth of the *hemA* mutant under these conditions. β -Galactosidase activity was measured (Miller, 1992) in duplicate in three independently grown cultures; mean values (Miller units) $\pm$ SD are shown.

Addition	-ALA	+ALA
None (aerobic)	47 $\pm$ 1	6 $\pm$ 1
None (anaerobic)	233 $\pm$ 32	126 $\pm$ 1
1 μM NO	115 $\pm$ 8	1346 $\pm$ 10
50 mM nitrate	583 $\pm$ 25	910 $\pm$ 5
2 mM nitrite	229 $\pm$ 14	1397 $\pm$ 63
20 μM SNP	209 $\pm$ 4	1550 $\pm$ 10

of the periplasmic cytochrome *cd₁* nitrite reductase (Page & Ferguson, 1990). Thus, the NNR-activated *nirS* gene can continue to be transcribed in cells that are depleted for haem. One possible explanation is that the imposed haem limitation (which allows growth to continue for at least one generation) does not deplete haem sufficiently to abolish activity of the cytoplasmic NNR protein, but does prevent export of haem to the periplasm for assembly of the nitrite reductase. Also, expression of the *P. denitrificans* nitrite reductase is not fully dependent on NNR (Van Spanning *et al.*, 1999). In *Ps. stutzeri*, the NO-responsiveness of the NNR orthologue DnrD is not abolished by a mutation that prevents biosynthesis of haem *d₁* (Vollack & Zumft, 2001), implying that haem *d₁* is not required for DnrD activity. This is consistent with the fact that NNR is active in *E. coli*, an organism that does not make haem *d₁*.

We have sought signal-independent variants of NNR (NNR*) in the hope that characterization of such proteins might shed further light on the regulatory mechanism. NNR* proteins with substitutions of Arg-80 were isolated; these proteins appear to have a perturbed signal-recognition mechanism, such that they are independent of the activating signal (NO). As far as we can tell, the two proteins substituted at position 80 (with alanine and histidine) have similar properties, despite the very different physicochemical characteristics of the two amino acids. The NNR* proteins remain sensitive to aerobic growth conditions. NNR is inactive during anaerobic growth in the absence of NO and during aerobic growth. If lack of activity under aerobic growth conditions reflects the rapid metabolism of the activating signal (most likely NO), then the two inactive states of NNR would be equivalent, in that they both result simply from the absence of NO. If this were the case, gain-of-function mutations should have similar effects under aerobic and anaerobic (in the absence of NO) conditions. This is not the case, since the star mutations selectively enhance the activity of NNR under anaerobic conditions (Table 1). Thus, it is likely that NNR is directly inactivated by O₂ or perhaps by an indirect signal of aerobic metabolism. Rapid inactivation of NNR is consistent with the observation that nitrite reductase mRNA abundance decays to the basal level within 1 h of switching to aerobic growth in cultures of *P. denitrificans* (Baumann *et al.*, 1996).

FNR* proteins have been isolated and have proved crucial in establishing the mechanism of oxygen sensing. FNR is inactivated by oxygen and FNR* proteins, with increased activity in the presence of oxygen, have mutations that stabilize an oxygen-sensitive Fe-S cluster (Bates *et al.*, 2000) or increase the tendency of the protein to dimerize in the presence of oxygen (Lazazzera *et al.*, 1993; Moore & Kiley, 2001). CoxA from *Rhodospirillum rubrum* is another FNR/CRP family member which contains a haem and is activated by CO binding to the haem. Like NNR, CoxA is inactive in anaerobic cultures in the absence of its signal (CO) and is inactive in aerobic cultures (Thorsteinsson *et al.*, 2001). CO-independent CoxA variants (CooA*) have been

isolated, some of which have substitutions in the C-helix of the protein that forms the major monomer-monomer interface (Kerby *et al.*, 2003). Introduction of a novel haem ligand in the C-helix also generates a CooA* phenotype (Youn *et al.*, 2002). For both CoxA and CRP there are also signal-independent variants that have substitutions in a hinge region connecting the effector-binding and DNA-binding domains; these mutations presumably lock the protein in an active conformation (Aono *et al.*, 1997). In an effort to understand the basis for the star phenotypes of the NNR R80A and R80H proteins, we have modelled the structure of NNR by homology-based methods, using the structures of CoxA in the inactive conformation (Lanzilotta *et al.*, 2000) and CRP in the active conformation (Passner *et al.*, 2000) as templates. In the NNR model, Arg-80 is predicted to be in the effector-binding domain, with its side chain projecting towards the C-helix at the monomer-monomer interface, where it approaches to within ~5.5 Å of a histidine residue at position 134 (Fig. 3). Interestingly, Arg-80 is not conserved amongst orthologues of NNR; indeed, NNR appears to be unique in the wider FNR/CRP family in having an arginine at this position. His-134 is also not conserved amongst NNR orthologues, and occurs rarely at this position in FNR/CRP family members. Thus, given the phenotypes of the R80A and R80H proteins, we are drawn to the conclusion that NNR orthologues that sense NO may not have a common mechanism for so doing. The equivalent position to Arg-80 in CRP is occupied by an aspartate residue, which is salt-bridged to Arg-123 in the C-helix (at the position equivalent to His-134 of NNR). Arg-123 of CRP is one of the cAMP ligands, and substitutions at this position have a severe effect on CRP activity (Moore *et al.*, 1992). Several residues in the C-helix of CoxA are close to the haem, and the L116K substitution in the C-helix is thought to generate a novel axial ligand to the haem (Youn *et al.*, 2002). Leu-116 of CoxA is three residues away from His-134 of NNR in a sequence alignment and is displaced by three positions from His-134 in helical wheel projections (of the known structure of CoxA and the modelled structure of NNR). Thus, taken together, the evidence from related systems is consistent with the idea that Arg-80 and/or His-134 approach closely to a site in NNR in which signals (NO and O₂) are sensed, or that substitutions at Arg-80 perturb the structural rearrangements around the C-helix that are presumably required for activation (Kerby *et al.*, 2003; Moore & Kiley, 2001).

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