

A non-haem iron centre in the transcription factor NorR senses nitric oxide

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Nitric oxide (NO), synthesized in eukaryotes by the NO synthases, has multiple roles in signalling pathways and in protection against pathogens^{1,2}. Pathogenic microorganisms have apparently evolved defence mechanisms that counteract the effects of NO and related reactive nitrogen species. Regulatory proteins that sense NO mediate the primary response to NO and nitrosative stress^{3–9}. The only regulatory protein in enteric bacteria known to serve exclusively as an NO-responsive transcription factor is the enhancer binding protein NorR (refs 9–11). In *Escherichia coli*, NorR activates the transcription of the *norVW* genes encoding a flavorubredoxin (FIRd) and an associated flavoprotein, respectively, which together have NADH-dependent NO reductase activity^{10,12–14}. The NO-responsive activity of NorR raises important questions concerning the mechanism of NO sensing. Here we show that the regulatory domain of NorR contains a mononuclear non-haem iron centre, which reversibly binds NO. Binding of NO stimulates the ATPase activity of NorR, enabling the activation of transcription by RNA polymerase. The mechanism of NorR reveals an unprecedented biological role for a non-haem mononitrosyl-iron complex in NO sensing.

To gain insight into the nature of the active form of NorR, we sought evidence for the presence of an NO adduct in *E. coli* whole cells using electron paramagnetic resonance (EPR) spectroscopy. Whole cells exposed to NO under anaerobic conditions exhibited an EPR signal in the $g = 2$ region, which probably arises from complexes formed between NO and cellular and/or medium components (Fig. 1b, spectra 2 and 4). Cells expressing NorR and exposed to NO exhibited a new EPR signal in the $g = 4$ region, with two apparent g values at $g = 4.19$ and $g = 3.82$ (Fig. 1b, spectrum 4), which was absent from similarly treated cells either not expressing NorR (Fig. 1b, spectrum 2), or expressing NorR but not exposed to NO (Fig. 1b, spectrum 3). Thus, the formation of the $g = 4$ signal requires both the expression of NorR and treatment with NO. Similar EPR spectra have been observed after reaction of non-haem iron enzymes with NO (refs 15–19), suggesting that NorR possesses a non-haem iron centre.

Transcriptional activation by sigma 54 (σ^{54})-dependent activators is usually controlled by sensory modules¹¹, and in the case of NorR the amino-terminal GAF domain (named for cyclic GMP-specific and stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA) is the probable site of signal sensing (Fig. 1a). Deletion of the GAF domain (NorR Δ GAF) results in constitutive signal-independent transcriptional activation by NorR *in vivo* (Fig. 1e), implying that the function of the GAF domain is to sense the signal and inhibit the ATPase activity of the central AAA⁺ domain (Fig. 1a) when NorR is in its inactive state^{3,10}. Cells expressing only the GAF domain of NorR (GAF_{NorR}) and exposed to NO exhibited an EPR signal in the $g = 4$ region, identical to the signal observed with full-length NorR (Fig. 1c, spectrum 4). In contrast, cells expressing the GAF-truncated and constitutively active form of NorR, NorR Δ GAF,

did not exhibit the $g = 4$ EPR signal (Fig. 1d, spectrum 1), demonstrating that this signal does not arise from other proteins as a consequence of NorR-dependent activation of gene expression, and that the paramagnetic centre is located within the GAF domain of NorR.

NorR and GAF_{NorR} purified from overexpressing cells under aerobic conditions were both devoid of iron. However, when purified to homogeneity under anaerobic conditions (Supplementary Fig. 1a), NorR contained 0.3 Fe atoms per monomer. To increase the yield, we attempted the reconstitution of these preparations with ferrous iron in the absence of oxygen. After reconstitution and purification to remove free iron, GAF_{NorR} (Supplementary Fig. 1b) and full-length

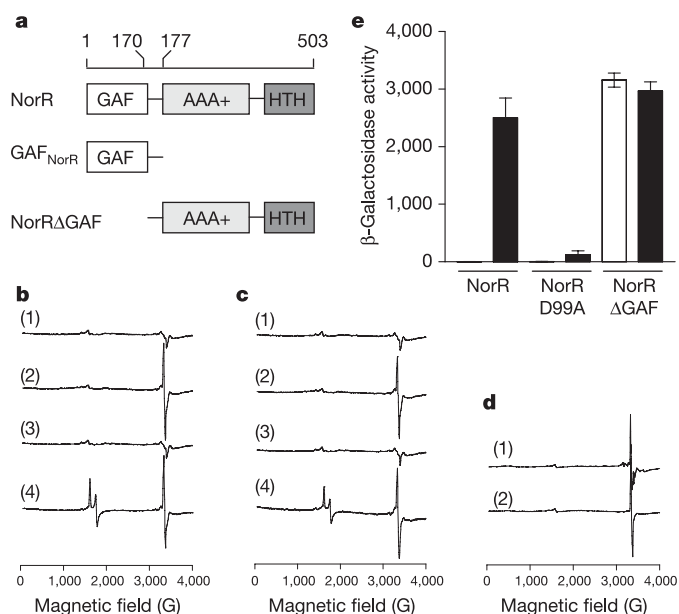


Figure 1 | Whole-cell EPR spectra and *in vivo* transcriptional activation by NorR. **a**, Schematic representation of the GAF, AAA⁺ and helix-turn-helix (HTH) domains of NorR and its derivatives, showing the relative amino acid positions of each domain. **b**, **c**, EPR spectra recorded from *E. coli* BL21(DE3) cells expressing NorR (**b**) and GAF_{NorR} (**c**). In both instances, spectra were obtained from cultures treated as follows: spectrum 1, no treatment; spectrum 2, treatment with NO; spectrum 3, induced for protein expression; spectrum 4, induced for protein expression and then treated with NO. **d**, EPR spectra recorded from cultures expressing NorR Δ GAF (spectrum 1) and NorR D99A (spectrum 2), induced for protein expression and then treated with NO. **e**, Activation of *norV-lacZ* expression *in vivo* by plasmids carrying wild-type NorR, NorR D99A or NorR Δ GAF. Cultures were grown either in the absence (open bars) or presence (filled bars) of potassium nitrite as described in the Supplementary Methods. Error bars show standard deviation between repeat experiments.

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NorR contained 0.7 and 0.9 Fe atoms per monomer, respectively, suggesting that NorR coordinates a single mononuclear iron atom per monomer.

The iron centre in NorR seems to be in the reduced state because, in the absence of NO, EPR signals indicative of a ferric species were not detected either in whole cells (spectra 3 in Fig. 1b, c) or with purified reconstituted proteins (Supplementary Fig. 2). When exposed to NO, NorR-Fe(II) and GAF_{NorR}-Fe(II), unlike the apo-proteins, exhibited strong EPR signals characterized by the *g* values 4.19, 3.82 and 2.00, and *E/D* ≈ 0.03 (Fig. 2, spectra 1 and 2). The same EPR signal was obtained either with NO gas, or when using the pre-decomposed NO donor MAHMA NONOate (see Methods). The EPR signals are identical to those recorded from whole cells, indicating that the nitrosyl species observed *in vivo* can be entirely attributed to NO-modification of the Fe²⁺ centre in NorR. The set of *g* values is characteristic of five- and six-coordinated non-haem mononitrosyl {Fe(NO)}⁷ (*S* = 3/2) complexes^{15–19}, where the super-script refers to the number of valence electrons shared by the iron and NO, according to the Enemark and Feltham formalism²⁰. Furthermore, the identity of the EPR spectra obtained with full-length NorR and GAF_{NorR} (Fig. 2) indicates that all of the protein ligands to the nitrosyl-iron species are located in the GAF domain, and that the other domains of NorR do not influence the electronic properties of the mononitrosyl-iron centre. Titration of the nitrosyl-iron species by EPR revealed that 95% of the iron in both NorR and GAF_{NorR} is involved in the binding of NO. No significant differences were detected in the molecular mass of NorR (analysed by mass spectrometry under denaturing conditions) before and after treatment with NO (55,102 ± 2 and 55,103 ± 2 Da, respectively), demonstrating that NO does not form any additional covalent adduct with NorR (Supplementary Fig. 3).

To determine whether the iron centre in NorR is required for its activity, we substituted a candidate ligand to the iron, the strictly conserved aspartate residue at position 99 in the GAF domain of NorR, for alanine (NorR D99A). This mutation prevented the formation of the *g* = 4 EPR signal, both *in vivo* (Fig. 1d, spectrum 2) and *in vitro* (Fig. 2, spectrum 3). In contrast to wild-type NorR, the anaerobically purified NorR D99A mutant protein did not contain iron. Moreover, unlike wild-type NorR, the NorR D99A mutant was unable to activate expression from a *norVW-lacZ* reporter construct

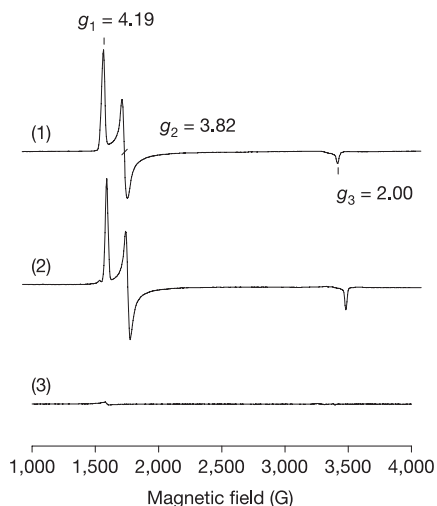


Figure 2 | EPR spectra of NO-treated purified proteins. Spectrum 1, purified NorR-Fe(II); spectrum 2, GAF_{NorR}-Fe(II); and, spectrum 3, NorR D99A. Protein concentrations were 40 μM for NorR-Fe(II), 90 μM for GAF_{NorR}-Fe(II) and 100 μM for NorR D99A. Identical spectra were obtained when proteins were treated with two equivalents of NO from a predecomposed MAHMA NONOate solution, or with NO gas.

in vivo (Fig. 1e), demonstrating that the loss of Fe²⁺ binding correlates with the absence of NorR activity.

The central catalytic AAA⁺ domain of enhancer binding proteins is required to couple nucleotide hydrolysis to the formation of open promoter complexes by σ⁵⁴-RNA polymerase²¹. Because the transcriptional activation mediated by this domain of NorR is apparently inhibited by the GAF domain in the absence of the signal, we anticipated that the coordination of NO to the Fe(II) centre might activate NorR. As a positive control for activity assays, we used the truncated form of NorR (NorRΔGAF), which lacks the GAF domain and gives rise to signal-independent transcriptional activation *in vivo* (Fig. 1a, e)^{3,10}. Isomerization of the σ⁵⁴-RNA polymerase holoenzyme was detected as a heparin-resistant super-shifted nucleoprotein complex on non-denaturing gels (Fig. 3a, lanes 8 and 9). Formation of the super-shifted species required not only NorRΔGAF, σ⁵⁴-RNA polymerase, integration host factor (IHF) and ATP, but also an initiating nucleotide, with CTP being more effective than UTP (Fig. 3a). NorR apoprotein prepared under aerobic conditions

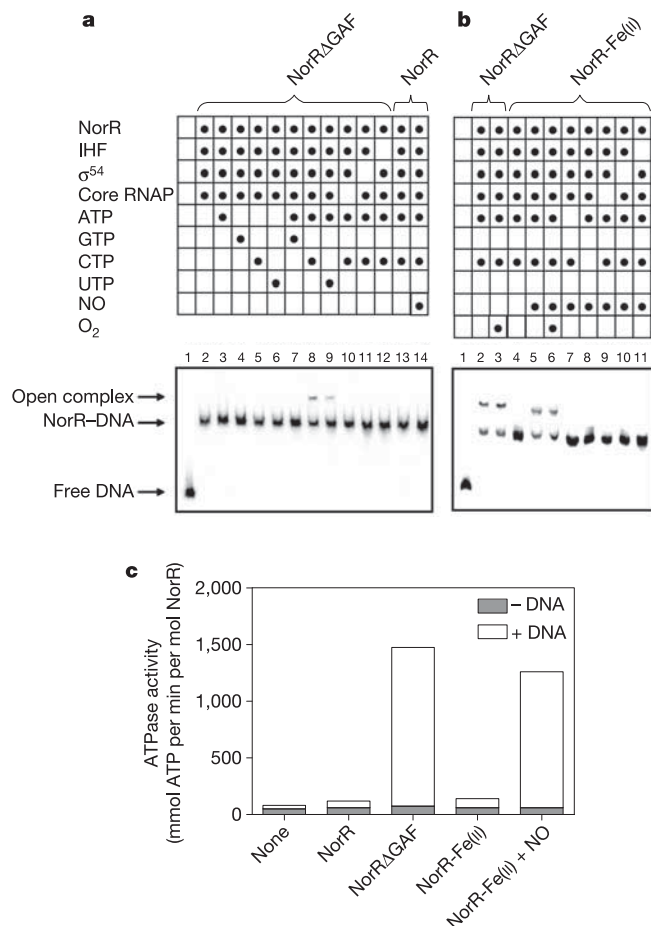


Figure 3 | Formation of open promoter complexes and the ATPase activity of NorR. **a**, Open promoter complex assays with NorRΔGAF (lanes 2–12) and NorR apoprotein (lanes 13 and 14). Reactions were performed under anaerobic conditions with the components indicated by the bullet points above each lane, in addition to DNA and buffer. RNAP, RNA polymerase. **b**, Open promoter complex formation by NorRΔGAF (lanes 2 and 3) and NorR-Fe(II) (lanes 4–11). Reactions were performed under anaerobic conditions except in lanes 3 and 6, which were incubated in air. **c**, ATPase activities of NorR derivatives. Rates of ATP hydrolysis were monitored at 340 nm for 20 min at 37 °C (filled bars). Then, 5 nM of the *norR-norVW* DNA fragment was added and the rates were monitored for an additional 20 min at 37 °C (open bars). ATPase activities are expressed as specific activity relative to protein concentration and the bars represent the sum of the values obtained without and with DNA.

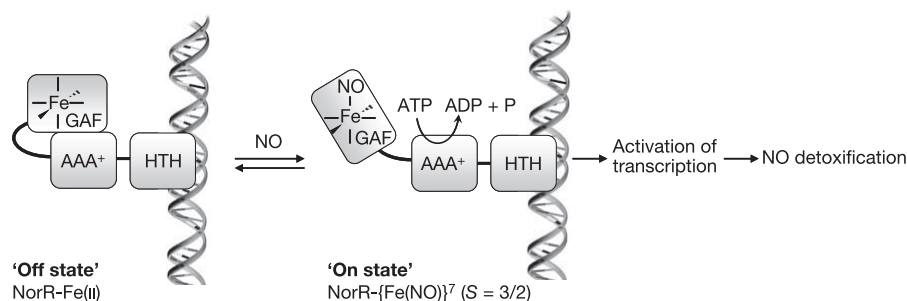


Figure 4 | Schematic of the proposed mechanism for transcriptional activation by NorR. NorR is represented in the 'off state' as a NorR-Fe(II)-DNA complex, and is activated by NO to an 'on state' with ATPase activity. NorR is shown with a tripartite domain architecture comprising the carboxy-terminal HTH DNA binding domain, the central catalytic AAA⁺ domain and the N-terminal GAF domain containing a five- or six-coordinate

mononuclear non-haem ferrous iron ion (here shown as a six-coordinated iron centre for clarity). The binding of NO leads to a {Fe(NO)}⁷ (*S* = 3/2) mononitrosyl complex where the interaction between the GAF domain and the AAA⁺ domain is released, allowing ATP hydrolysis that is further coupled to transcriptional activation. For clarity, NorR is represented as a monomer.

(and thus devoid of iron) was unable to drive the formation of open promoter complexes either in the presence or absence of NO (Fig. 3a, lanes 13 and 14). In contrast, a super-shifted species with mobility similar to that of the open complex formed in the presence of NorRΔGAF was seen in reactions containing NorR-Fe(II) treated with NO (Fig. 3b, lanes 2 and 3 versus lanes 5 and 6). These data demonstrate that both the iron centre and NO are required for open complex formation and that no other cofactors are involved in NorR-dependent activation. Open complexes were formed in reactions performed in air (Fig. 3b, lane 6), and EPR spectra of NO-treated ferrous NorR were not modified after exposure to air. Therefore, NO-activated NorR-Fe(II) is apparently not sensitive to oxygen, which is consistent with the presence of NorR activity in aerobic cultures^{9,14}.

The requirement for ATP in the open complex assays probably reflects the need for nucleotide hydrolysis by the AAA⁺ domain of NorR in remodelling the σ^{54} -RNA polymerase-promoter complex to activate transcription²². We therefore assayed the ATPase activity of NorR to obtain further evidence that the protein is activated by the formation of a nitrosyl-iron complex. All forms of NorR, including the constitutively active NorRΔGAF, NorR-Fe(II) and NO-treated NorR-Fe(II), had similar basal levels of activity. However, the ATPase activities of NorRΔGAF and NO-treated NorR-Fe(II) markedly increased in the presence of DNA containing NorR binding sites (Fig. 3c). Stimulation of ATPase activity on binding to specific enhancers has also been demonstrated for some other σ^{54} -dependent activators²³. The enhancer dependency of the ATPase activity potentially increases the specificity of NO signalling by NorR towards the regulation of specific target promoters. Clearly, the DNA-dependent ATPase activity of full-length NorR requires activation of the iron-containing species by NO, which may account for the signal-dependent isomerization of σ^{54} -RNA polymerase that we observe in the open complex assays.

Using an NO electrode, we obtained a dissociation constant, $K_d = 50 \pm 10$ nM at pH 8.5 and 30 °C (Supplementary Fig. 4), which indicates that NorR is sufficiently sensitive to respond to the micromolar levels of NO released by macrophages during inflammation²⁴, and the nanomolar levels made endogenously by nitrite reduction (Supplementary Methods). Determination of approximate rate constants for NO binding, with myoglobin as a competitor ($k_{\text{off}} > 1 \text{ s}^{-1}$ and $k_{\text{on}} > 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), suggests that NorR is a rapid and efficient NO sensor.

We have identified a mononuclear non-haem iron centre in the GAF domain of NorR, which can be modified by a single NO molecule to generate a mononitrosyl {Fe(NO)}⁷ (*S* = 3/2) species. We propose that formation of this species triggers a conformational change in the GAF domain that relieves intramolecular repression of the AAA⁺ domain (Fig. 4), allowing enhancer-stimulated ATPase activity, open complex formation and transcription initiation by

σ^{54} -RNA polymerase. This mechanism enables direct transduction of the NO signal by NorR to the activation of NO detoxification processes. Diverse signal transduction pathways involving GAF domains have been described²⁵, but NorR provides the first example of a GAF domain coordinating a transition metal to sense and transduce a signal. EPR spectroscopy showed that the nitrosyl complex in the GAF domain of NorR belongs to the {Fe(NO)}⁷ (*S* = 3/2) family. To our knowledge, activation of NorR is the first example of a biological process involving a {Fe(NO)}⁷ (*S* = 3/2) complex. Previously reported NO signalling mechanisms involve either dinitrosyl-iron {Fe(NO)₂}⁹ complexes^{5,6,8,26}, or (as in the case of soluble guanylate cyclase) a haem-containing mononitrosyl complex of the {Fe(NO)}⁷ (*S* = 1/2) type²⁷. In contrast to most dinitrosyl-iron complexes²⁶, NO binding is reversible in mononitrosyl systems. Mononitrosyl low-spin (*S* = 1/2) haem systems and mononitrosyl high-spin (*S* = 3/2) non-haem systems are clearly distinct owing to their electronic and spatial structures^{18,19,28,29}, which may influence NO binding properties, ligand selectivity and mechanisms of NO signal transduction. The system we describe here has no precedent in NO signalling pathways and may provide a paradigm for NO coordination in NO receptors containing non-haem iron.

METHODS

Bacterial strains and plasmids. The pNorR2 plasmid containing the *norR* gene of *E. coli* in the pET21a vector has been described previously¹². Two derivatives of pNorR2 containing genes that express deleted versions of NorR were constructed. One is a deletion of the N-terminal domain from amino acids 1 to 170, and expresses the protein designated NorRΔGAF. The second expresses the NorR GAF domain only (designated GAF_{NorR}), from residues 1 to 177. For open complex and ATPase activity assays, the *norR-norVW* intergenic region was amplified and cloned into the *Sma*I site of pUC19 to yield the pNPTprom construct. Restriction digestion of pNPTprom with *Eco*RI and *Bam*HI generates a 362 base pair (bp) fragment spanning the entire *norR-norVW* intergenic region and includes the first 86 bp and 62 bp of the *norR* and *norV* coding sequences, respectively.

Nitric oxide treatment. Two different sources of NO were used to prepare NO solutions: the NO donor, MAHMA NONOate ((Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl)amino]]diazene-1-ium-1,2-diolate), and NO gas. Stock solutions of MAHMA NONOate were prepared in 10 mM NaOH. NO solutions were prepared by decomposition of a solution of MAHMA NONOate in buffer I (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 5% glycerol) at 30 °C for 20 min ($t_{1/2} = 3$ min, pH 7.5, 30 °C) under anaerobic conditions. NO gas was purified by bubbling through 1 M KOH. Saturated solutions (2 mM) were prepared by bubbling pure NO gas into buffer I in anaerobic conditions.

Electron paramagnetic resonance experiments. For whole-cell experiments, freshly transformed bacteria were grown anaerobically at 30 °C in 25 ml of Luria-Bertani medium containing 1% (w/v) glucose and 100 $\mu\text{g ml}^{-1}$ carbenicillin. Expression of NorR and GAF_{NorR} was induced at an absorbance ($A_{600 \text{ nm}}$) of 0.6 with 50 μM isopropyl- β -D-thiogalactoside. After 3 h, 50 μl of 100 mM MAHMA NONOate was added and incubation continued for 15 min ($t_{1/2} = 3$ min,

pH 7.5, 30 °C). The cells were collected at 1,800g for 10 min, and the pellets were resuspended in 0.5 ml of 100 mM Tris-HCl (pH 7.5), 25 mM NaCl, 10% (v/v) glycerol. Cell suspensions were transferred to an EPR tube and immediately frozen. For EPR spectroscopy of purified proteins, samples were prepared by reaction of NorR-Fe(II) and GAF_{NorR}-Fe(II) with two equivalents of NO from predecomposed MAHMA NONOate or NO gas in anaerobic conditions. To observe the $g = 2$ contribution, the solution was incubated for a further 2 min with the cap open, to allow free NO to equilibrate with the gas phase, and then the solution was frozen. After this incubation, free NO was not observed in solution. X-band EPR spectra were recorded on a Bruker ER 200 D-SRC spectrometer under the following conditions: frequency, 9.477 GHz (for whole cells) or 9.440 GHz (for pure protein); power, 2 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz; temperature 8 K. Spin concentrations were measured by integration of the EPR absorption spectra. The resulting areas were compared to the signal from aqueous Cu(H₂O)₆ (1 mM) recorded with identical instrument settings. EPR simulations were obtained with WinEPR Simfonia software (Bruker).

Protein purification. All proteins were purified to homogeneity (Supplementary Methods and Supplementary Fig. 1). Protein concentrations were determined by quantitative amino acid analysis (Alta Biosciences). According to these quantitative analyses, we have determined the absorption coefficients, at a wavelength of 280 nm, to be 42,000 M⁻¹ cm⁻¹, 6,500 M⁻¹ cm⁻¹ and 35,000 M⁻¹ cm⁻¹ for NorR, GAF_{NorR} and NorRΔGAF, respectively. Total iron concentrations were determined by inductively coupled plasma mass spectrometry (Harwell Scientific).

Open complex assays. Open complex formation was assayed as described previously³⁰ under anaerobic conditions using a modified buffer system to avoid iron chelation (Supplementary Methods).

ATPase activity assays. ATPase activity was measured using an assay in which the production of ADP is coupled to the oxidation of NADH by lactate dehydrogenase and pyruvate kinase. The oxidation of NADH was monitored by A_{340 nm} at 37 °C for 20 min. All reaction mixtures contained ATP (30 mM), phosphoenolpyruvate (1 mM), NADH (0.3 mM), pyruvate kinase (7 U), lactate dehydrogenase (23 U) in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2 mM MgCl₂ and 300 nM of either NorR, NorRΔGAF or NorR-Fe(II). Where indicated, NorR-Fe(II) was pretreated with 20 μM NO. The DNA template added was the same *norR-norVW* promoter fragment (5 nM) as used for the open complex assays and is described in the Supplementary Methods.

Other protocols. Detailed procedures used for site-directed mutagenesis and assays of NorR activity *in vivo*, mass spectrometry, determination of the NO dissociation constant and kinetic parameters, open complex assays, protein purifications and details of chemical and biochemicals used are provided in the Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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