NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp

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Nitric oxide (NO) is a signalling and defence molecule of major importance in biology. The flavohaemoglobin Hmp of *Escherichia coli* is involved in protective responses to NO. Because *hmp* gene transcription is repressed by the O_2-responsive regulator FNR, we investigated whether FNR also senses NO. The $[4Fe–4S]^{2+}$ cluster of FNR is oxygen labile and controls protein dimerization and site-specific DNA binding. NO reacts anaerobically with the Fe–S cluster of purified FNR, generating spectral changes consistent with formation of a dinitrosyl-iron–cysteine complex. NO-inactivated FNR can be reconstituted, suggesting physiological relevance. FNR binds at an FNR box within the *hmp* promoter (P_{hmp}). FNR samples inactivated by either O_2 or NO bind specifically to P_{hmp}, but with lower affinity. Dose-dependent up-regulation of P_{hmp} *in vivo* by NO concentrations of pathophysiological relevance is abolished by *fnr* mutation, and NO also modulates expression from model FNR-regulated promoters. Thus, FNR can respond to not only O_2, but also NO, with major implications for global gene regulation in bacteria. We propose an NO-mediated mechanism of *hmp* regulation by which *E.coli* responds to NO challenge.
Keywords: flavohaemoglobin/FNR/gene regulation/iron–sulfur clusters/nitric oxide sensing

Introduction

Nitric oxide (NO) is a signalling and defence molecule of major importance, but the biochemical basis of resistance to NO and reactive nitrogen species (RNS) is poorly understood. NO and RNS pose distinct biochemical challenges by reacting under appropriate conditions with several key biomolecules including metalloproteins, thiol groups of proteins and low molecular weight thiols such as glutathione and homocysteine (Hcy) (Poole and Hughes, 2000). These cellular targets may themselves serve as signal transducers, sensing the presence of NO and RNS, resulting in changes in gene expression and consequent synthesis of protective enzymes. Regulation of transcriptional activation in some cases integrates the responses to free radicals of both oxidative and nitrosative stresses. Thus, in *Escherichia coli*, the *soxRS* and *oxyR* regulons, known to respond to superoxide anion and hydrogen peroxide, respectively, are also induced by NO (Nunoshiba *et al*., 1993; Hausladen *et al*., 1996; Ding and Demple, 2000). These global antioxidant regulators afford protection against NO or nitrosothiols (Nunoshiba *et al*., 1993; Hausladen *et al*., 1996), but the resistance mechanisms are not entirely known, despite their importance in allowing bacteria to combat, for example, macrophage microbicidal activity (De Groote *et al*., 1997).

The most fully understood mechanism for detoxification of NO involves the bacterial flavohaemoglobin (Hmp). Hmp synthesis is activated primarily by NO or RNS, and the physiological function of Hmp in protecting *E.coli* from NO is established (Poole *et al*., 1996; Membrillo-Hernández *et al*., 1999; Poole and Hughes, 2000). Aerobically, Hmp detoxifies NO by acting as an NO oxygenase (Gardner *et al*., 1998a; Hausladen *et al*., 1998) or denitrosylase (Hausladen *et al*., 2001) and affords inducible protection of aconitase activity (Gardner *et al*., 1998b) and respiration (Stevanin *et al*., 2000). Anaerobically, Hmp serves as an NO reductase, generating nitrous oxide (Hausladen *et al*., 1998; Kim *et al*., 1999).

Consistent with the role of Hmp in NO detoxification, the flavohaemoglobin-encoding gene of *E.coli*, *hmp*, is up-regulated by NO and RNS; this appears not to involve SoxRS (Poole *et al*., 1996). We have reported (Membrillo-Hernández *et al*., 1998) a mechanism of *hmp* gene regulation that involves interaction between *S*-nitrosothiols and Hcy. Intracellular Hcy is an important co-regulator of several genes involved in methionine biosynthesis, via its effects on MetR, a LysR family DNA-binding protein. One gene activated by MetR with Hcy as cofactor is *glyA*, which, in *E.coli*, is adjacent to *hmp* and divergently transcribed from it. Elevated Hcy levels, achieved either by exogenous Hcy or in certain *met* mutants, decrease *hmp* expression. Since Hcy has been shown to be nitrosated by *S*-nitrosoglutathione (GSNO) (Membrillo-Hernández *et al*., 1998), such nitrosating agents can deplete Hcy pool sizes, and are postulated to enhance MetR binding at a site proximal to *hmp*, and up-regulate *hmp* transcription (Membrillo-Hernández *et al*., 1998). It is important to recognize that this mechanism does not explain *hmp* regulation by NO itself. First, NO induces *hmp* expression anoxically, under which conditions NO will not nitrosate Hcy. Secondly, although the *S*-nitrosoHcy generated on reaction with GSNO breaks down to release NO, which could itself be the inducer, the reaction of Hcy with sodium nitroprusside (SNP) (which also induces *hmp*) forms a more stable species from which
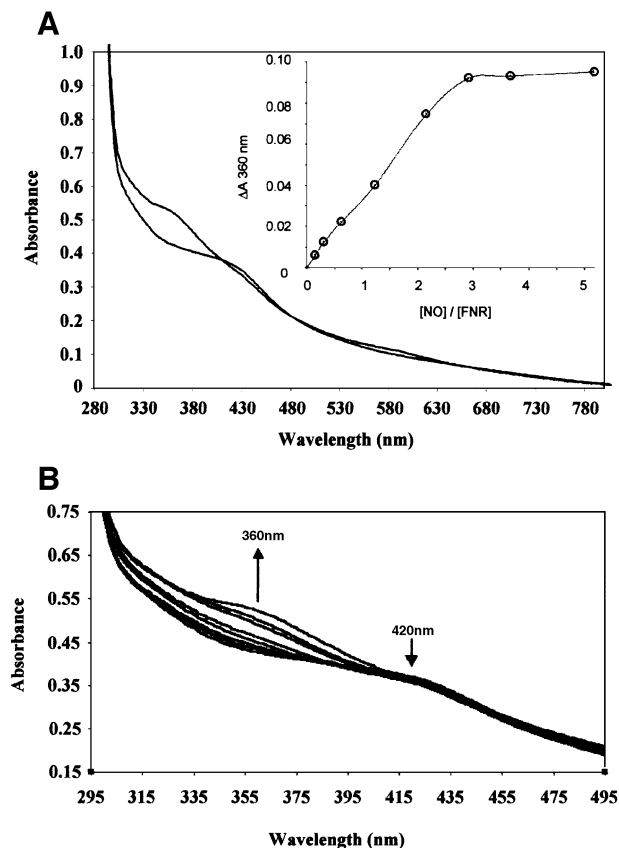
A

B

Fig. 1. Effect of NO on FNR. (**A**) Optical spectra of [4Fe–4S]$^{2+}$ FNR (27 μM, monomer) before and after treatment with aliquots of 4.17 mM proline NONOate to give a final concentration of 140 μM NO (72 μM NONOate). Inset: a plot of ΔA_{360} versus the ratio of [NO]/[FNR]. When no further change was apparent, a sample was withdrawn for EPR analysis. (**B**) Optical spectra of the same FNR suspension acquired after sequential additions of proline NONOate. The lowest spectrum is FNR in the absence of NO and the uppermost one is at a [NO]/[FNR] ratio of 5.2. The [NO]/[FNR] ratio increases from 0, 0.2, 0.3, 0.6, 1.2, 2.2, 2.9, 3.7 to 5.2, giving rise to dose-dependent absorbance increases at 360 nm.

NO is not released. Thus other mechanisms for *hmp* regulation by NO, particularly anoxically, must be present.

Anaerobically, the global regulator FNR (Kiley and Beinert, 1999; Green *et al.*, 2001) is also involved in the regulation of *hmp* since an *fnr* mutation enhances *hmp-lacZ* expression (Poole *et al.*, 1996). Inspection of the *hmp* promoter reveals a DNA-binding site for FNR in a repressing position, but how FNR contributes to NO-mediated regulation has remained obscure. Up-regulation of *hmp* in *Salmonella* is achieved by a third mechanism involving the iron uptake regulator Fur (Crawford and Goldberg, 1998).

Here, we report an anaerobic regulatory mechanism of *hmp* induction directly mediated by NO, involving nitrosylation of the [4Fe–4S]$^{2+}$ clusters in the FNR protein. NO sensing by FNR has far-reaching implications for a full understanding of its global regulatory functions.

Results

FNR is an NO sensor

The starting point for this work was the observation that the oxygen-responsive global transcription factor FNR
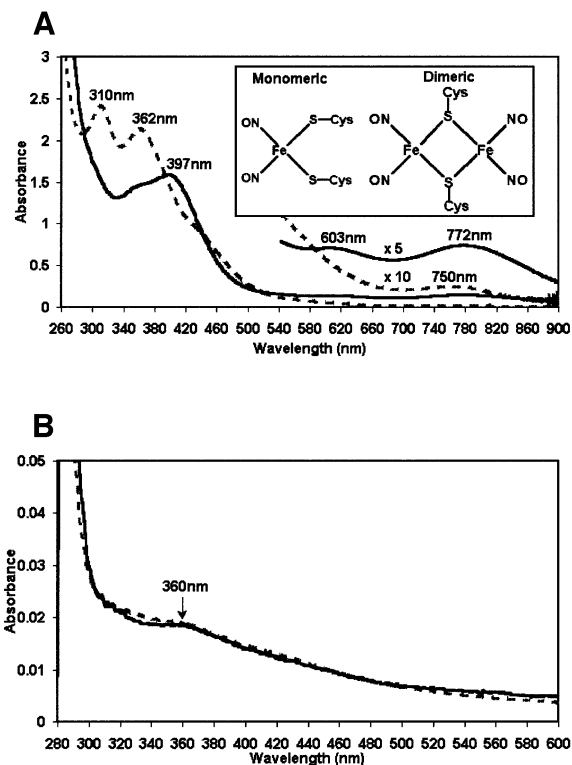
A

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Fig. 2. Optical spectra of DNIC complexes. (**A**) Model complexes. The monomeric species (445 μM; solid line) displays characteristic absorption maxima at 397 nm (ε ~3580/M/cm), 603 nm (ε ~299/M/cm) and 772 nm (ε ~312/M/cm) (Costanzo *et al.*, 2001). The dimeric species (250 μM; dashed line) displays characteristic absorption maxima at 310 nm (ε ~9650/M/cm), 362 nm (ε ~8529/M/cm) and 750 nm (ε ~100 M/cm). Inset: structures proposed for monomeric and dimeric DNIC species (Costanzo *et al.*, 2001). (**B**) Comparison between experimental (dashed line) and simulated (black line) spectra of DNICs. The simulated spectrum consists of contributions from 20 and 80% of the monomeric and dimeric DNIC species, respectively, plus apoFNR.

represses expression of the NO-detoxifying protein Hmp during anaerobic growth (Poole *et al.*, 1996). FNR senses oxygen via the assembly–disassembly of [4Fe–4S]$^{2+}$ clusters (Green *et al.*, 1996; Khoroshilova *et al.*, 1997; Kiley and Beinert, 1999). Iron–sulfur clusters of other proteins have been shown to react with NO, and thus the clusters of FNR might also constitute a mechanism to account for NO responsiveness of *hmp* expression. To address this possibility, the effects of NO on the [4Fe–4S]$^{2+}$ clusters of FNR were investigated *in vitro*.

The anoxic, reconstituted FNR protein (holo-FNR) showed the characteristic broad absorbance at ~420 nm (Khoroshilova *et al.*, 1995; Green *et al.*, 1996; Lazzazera *et al.*, 1996; Jordan *et al.*, 1997) and was devoid of electron parmagnetic resonance (EPR) signals. On addition of the fast NO-releasing compound, proline NONOate, the broad absorbance at 420 nm was replaced progressively by a prominent shoulder at 360 nm and a decrease in absorbance at 420 nm with an isosbestic point near 480 nm (Figure 1A). A plot of ΔA_{360} versus NO concentration shows that the reaction is complete at a [NO]/[FNR] ratio of <3.0 (Figure 1A inset and B).

Dinitrosyl-iron–cysteine (DNIC) complexes have relatively intense optical absorption bonds in the near UV. Figure 2A displays the absorption spectra of yellow and
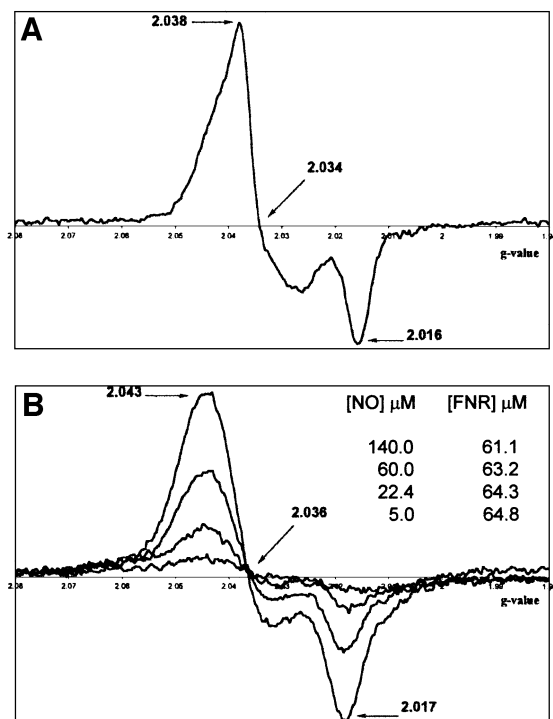
Fig. 3. EPR spectra of [4Fe–4S]$^{2+}$ FNR. (**A**) The FNR sample used in Figure 1, after treatment with 140 μM NO (72 μM proline NONOate; 27 μM FNR monomer). (**B**) Increasing magnitudes of EPR signals elicited by addition of aliquots of NO solution (NO and FNR concentrations were as indicated). The temperature was 77 K. Microwave power and frequency were in (A) 2.007 mW and 9.653 GHz, and in (B) 2.000 mW and 9.669 GHz, respectively. Modulation amplitude, frequency and receiver gain were in (A) 5 gauss (0.5 mT), 100 kHz and 5.02×10^5 and in (B) 10 gauss (1 mT), 100 kHz and 1.00×10^5, respectively.

green DNICs having dimeric and monomeric structures (inset), respectively. The extinction coefficients for the two species are as follows: dimeric clusters $\varepsilon_{310} = 9650$/M/cm, $\varepsilon_{362} = 8529$/M/cm and $\varepsilon_{750} = 100$/M/cm; monomeric form $\varepsilon_{362} \sim 3580$/M/cm, $\varepsilon_{603} = 300$/M/cm and $\varepsilon_{772} = 312$/M/cm. The dimeric DNIC is EPR silent, whereas the monomeric form has one unpaired electron, S = 1/2, with an axial g-tensor, g = 2.038, 2.024 and 2.06. The EPR spectrum of a sample taken from the titration of FNR with proline NONOate at a [NO]/[FNR] ratio of ~5 shows just such an axial EPR spectrum (Figure 3A). This signal is strongly reminiscent of the DNIC of cysteine (Kennedy *et al.*, 1997; Vanin *et al.*, 1998). Integration of the g = 2.038 signal gave only 20% of an electron spin per [4Fe–4S]$^{2+}$ cluster. A minor species appearing at g = 2.15 may arise from a thiyl radical (data not shown; Kennedy *et al.*, 1997). The EPR spectra measured at low temperature (not shown) revealed a trace amount of a [3Fe–4S]$^{1+}$ cluster at g = 2.01.

In a parallel set of experiments, sequential additions of NO solution or air-saturated buffer (~230 μM O$_2$) to reconstituted FNR also caused absorbance decreases at 420 nm. Some difficulty was experienced with slow protein precipitation causing baseline variations but, during this titration with NO, samples were withdrawn for EPR spectroscopy. The main species detected at 70 K

again was typical for a monomeric DNIC (Figure 3B). For example, the EPR spectra of NO-treated FNR are very similar to the EPR spectra of the d^7 form of DNIC-aconitase (Kennedy *et al.*, 1997). At 10 K, varying intensities of the signal for the [3Fe–4S]$^{1+}$ cluster (g = 2.02) were also seen, as previously observed when [4Fe–4S]$^{2+}$ FNR is briefly exposed to oxygen (Green *et al.*, 1996).

The experimental EPR results (Figure 3) and optical spectra (Figure 1) suggest a mixture of the yellow and green forms of DNIC. We therefore simulated the optical spectrum by combining spectra of the monomeric and dimeric forms in the ratio 20:80. The result is shown in Figure 2B. The match to the experimental spectrum (Figure 2B, dashed line) is good, although the presence of some light-scattering precipitate causes background absorption sloping towards the UV.

An FNR sample exposed to NO solution and lacking the 420 nm peak was flushed with N$_2$ gas for 60 min, then used in a standard procedure previously shown to be effective for reconstitution of the Fe–S cluster (Jordan *et al.*, 1997). Importantly, after overnight incubation, the protein showed the same optical features (420 nm maximum) as an NO-untreated, but reconstituted, sample (data not shown).

These data strongly suggest that the [4Fe–4S]$^{2+}$ cluster of FNR can react with NO to form a mixture of monomeric and dimeric DNICs, and that the NO-treated protein remains native and can be reconstituted. Thus the conversion of the native [4Fe–4S]$^{2+}$ cluster to DNICs could provide the molecular basis of a direct NO-responsive switch.

Interaction of FNR with wild-type and mutant P$_{hmp}$

A putative FNR box, TTGAG----ATCAA, with a strong resemblance to the consensus FNR-binding sequence is centred at position +5.5 in the P$_{hmp}$ region. To determine whether the putative FNR box is actually recognized by FNR, mutations were made in which the central G (underlined above) was mutated to A, and the corresponding C (also underlined), in the second half of the box, was changed to T. The interaction of FNR with its target sequence was then analysed by gel retardation. At holo-FNR concentrations as low as 0.4 and 0.5 μM, 9 and 20% of the wild-type P$_{hmp}$, respectively, is retarded (Figure 4A). No retardation could be detected when the FNR box was mutated and incubated with holo-FNR (Figure 4D). Interestingly, wild-type P$_{hmp}$ was also retarded (11 and 26%) by dioxygen- and NO-treated FNR lacking the 420 nm peak, but this occurred only with higher protein concentrations (i.e. >>2 μM) (Figure 4B and C). The retardation profile suggests that holo-FNR, as well as O$_2$- and NO-treated FNR, may bind P$_{hmp}$ in a similar, namely dimeric, form, but with different affinities. The protein–DNA dissociation constants (K_d) for the three FNR forms were determined by gel retardation and repressor–operator interaction analyses. Both titration methods showed similar results. O$_2$- and NO-treated FNR had similar affinities for P$_{hmp}$ with apparent dissociation constants of $K_d = 2.5$–3 μM. The affinity for holo-FNR, $K_d = 1$ μM, is greater than that of either O$_2$- or NO-treated FNR.
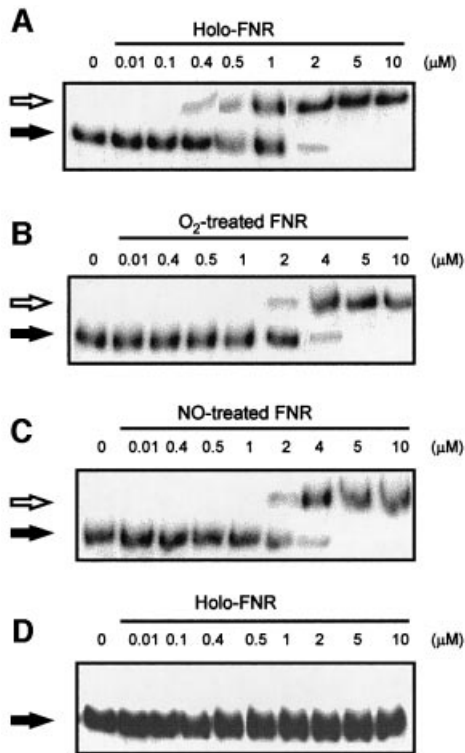
Fig. 4. DNA binding by holo-FNR, O_2- and NO-treated FNR proteins. Gel retardation assays were performed by incubating the ^{32}P-labelled P_{hmp} DNA fragment (wild-type) with increasing amounts of (**A**) holo-FNR, (**B**) O_2-treated FNR and (**C**) NO-treated FNR protein. The ^{32}P-labelled P_{hmp} DNA fragment (mutated) was incubated with increasing amounts of (**D**) holo-FNR. Arrows indicate the FNR–target DNA (black) and the FNR–P_{hmp} complex (white). Protein concentrations (monomer) are shown.

DNase I protection by holo-FNR, O_2-treated and NO-treated FNR

DNase I footprinting confirmed the different binding affinities of holo-FNR, O_2- and NO-treated FNR obtained from titration experiments. A 26 bp region overlapping the FNR box and located between –6 and +20 in P_{hmp} was protected by the addition of 0.2 µM holo-FNR (Figure 5A). Increasing protein concentrations also revealed hypersensitive sites. Once the FNR concentration reached 2–12 µM, an AT-rich hexamer centred at –15.5 appears to be protected from cleavage (Figure 5A and C). The same footprinting pattern was observed with both O_2-treated FNR (data not shown) and NO-treated FNR (Figure 5B). Consistent with gel retardation results, 0.2 µM O_2- or NO-treated FNR failed to protect the FNR box and protection was seen from 2 µM protein (Figure 5B). Again, the AT-rich hexamer and the hypersensitive sites were revealed by adding further protein. However, in the case of NO-treated FNR (Figure 5B), the hypersensitive +1G and +2A sites were substituted by a –1T site. Also, extensive non-specific DNA protection could be seen upstream of the hypersensitive sites found immediately 5′ to the AT-rich hexamer when higher concentrations of NO-treated FNR were used, indicating that this form of the protein not only has a lower affinity for DNA but also binds with reduced specificity (Figure 5B). *Escherichia coli* RNA polymerase
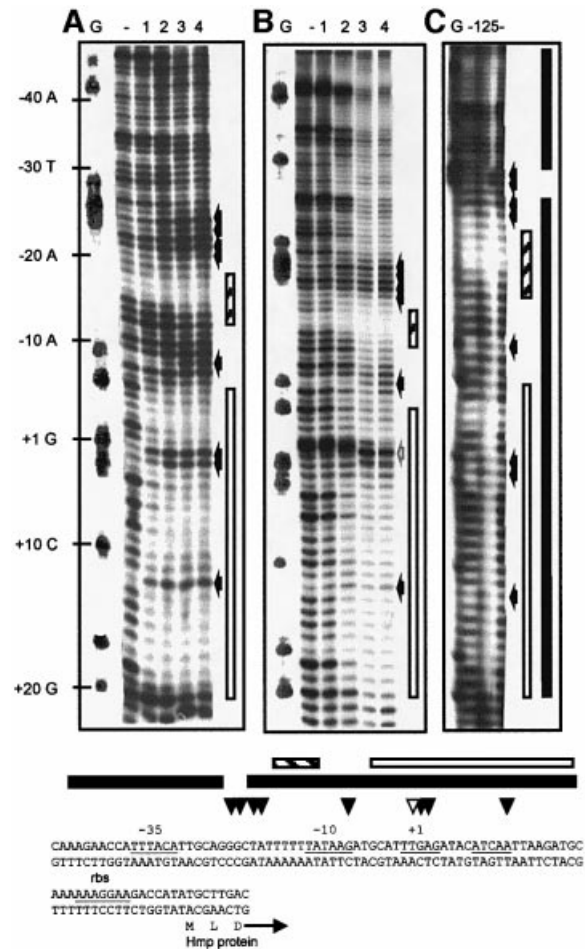

Fig. 5. DNase I protection by holo-FNR, NO-treated FNR and RNAP bound to P_{hmp}. The ^{32}P-labelled P_{hmp} DNA fragment was digested with DNase I in the presence of various concentrations of (**A** and **C**) holo-FNR, (**B**) NO-treated FNR and (C) RNAP. Template strand patterns shown were obtained with no FNR (lanes -), 0.2 µM (lanes 1), 2 µM (lanes 2), 12 µM (lanes 3) and 20 µM (lanes 4) FNR protein (as monomer), and with 69 nM RNAP (lane 5). Lanes G provide a calibration for the GC base pairs in the P_{hmp} region. Hypersensitive sites are marked with black or white arrowheads. Hollow boxes show the region protected by dimeric FNR binding. Striped boxes show the position of a DNase I-protected AT-rich hexamer. The filled boxes indicate the region bound by RNAP σ^{70} subunit. The –10 and –35 hexamers, the ribosome-binding site (rbs), the transcription start site (+1) and the FNR box are indicated on the P_{hmp} sequence shown below.

(RNAP) was used in DNase I footprinting assays to determine whether the σ^{70}-holoenzyme recognizes P_{hmp}. By itself, RNAP bound to a 64 bp DNA region (–44 to +20) containing the –10 and –35 hexamers, as well as the FNR box (Figure 5C).

In vivo modulation of FNR activity by NO

Regulation of *hmp* transcription by NO and RNS appears complex, and at least one regulatory mechanism involving MetR is known (Membrillo-Hernández *et al.*, 1998; Poole and Hughes, 2000). In order to assess the modulation of FNR activity by NO *in vivo*, we used an *E.coli* strain carrying a single copy of a P_{hmp}-*lacZ* fusion (RKP2178). Previously, a bolus addition of a solution of NO gas was used to demonstrate up-regulation of *hmp* but, in view of the reactivity of NO and the demonstration that *E.coli* cells
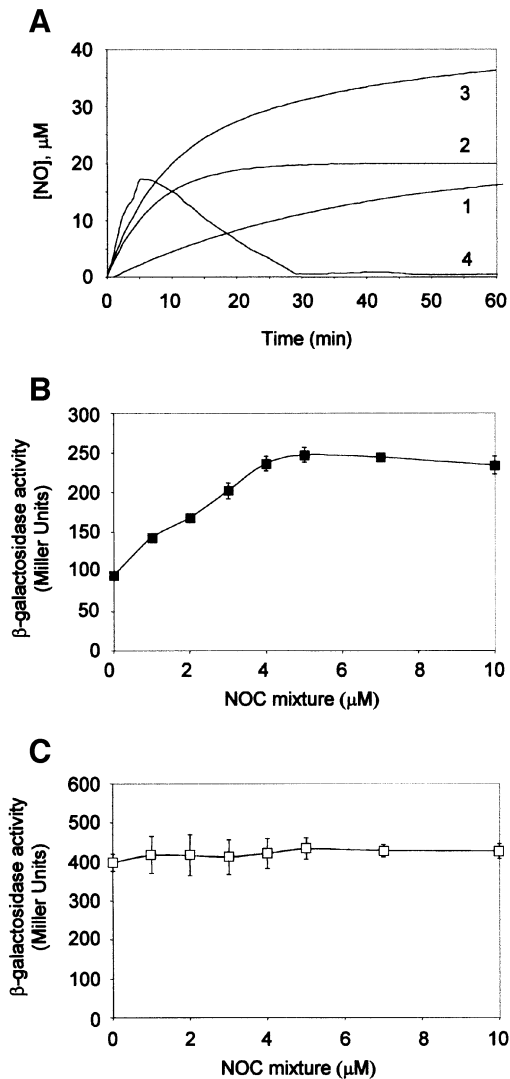
Fig. 6. NO derepresses *hmp* expression *in vivo* by reacting with FNR. (**A**) Release of NO by the NOC mixture over 1 h. Traces 1 and 2 are the theoretical NO release patterns at 37°C (in PBS pH 7.4) by NOC-7 (half-life 5 min) and NOC-5 (half-life 25 min), respectively. Trace 3 is the total NO release by the NOC mixture (addition of traces 1 and 2). Trace 4 is the measured NO release by the NOC mixture under the same conditions of NO challenge in anoxic LB-containing cells (OD_{600} of ~0.2) at 37°C. Anaerobic cultures of (**B**) RKP2178 [Φ(*hmp-lacZ*)1] and (**C**) RKP2185 (RKP2178 but *fnr-271*::Tn*10*) were challenged with different amounts of NOC mixture. The β-galactosidase activity is the mean of three independent repetitions of the experiment; bars show standard deviations.

Fig. 7. NO modulates gene expression *in vivo* by reacting with FNR. Anaerobic cultures of *E.coli* strains carrying (**A**) the FNR-inducible promoter [FF(−71.5)::*lacZ*] or (**B**) the FNR-repressible promoter (FF*galΔ4::lacZ*) were treated with different amounts of NO. β-galactosidase activity is the mean of three independent experiments.

consume NO, even anaerobically (Kim *et al.*, 1999), we have used both NO gas solutions (see later, Figure 7) and NO provided by its release from NOC-5 and NOC-7. Figure 6A (traces 1–3) shows the predicted progress of NO release from both NOC-5 and NOC-7 (10 μM of each, final concentration). The release from NOC-5 (half-life of 25 min at pH 7.0, 37°C) is shown as trace 1, and from the donor with a much shorter half life, NOC-7 (5 min at pH 7.0, 37°C) as trace 2. Trace 3 is the sum of the calculated individual NO release profiles. The actual NO concentration in solution (trace 4) was monitored continuously during anaerobic growth with an NO electrode; during the first 10 min of culture, the measured and
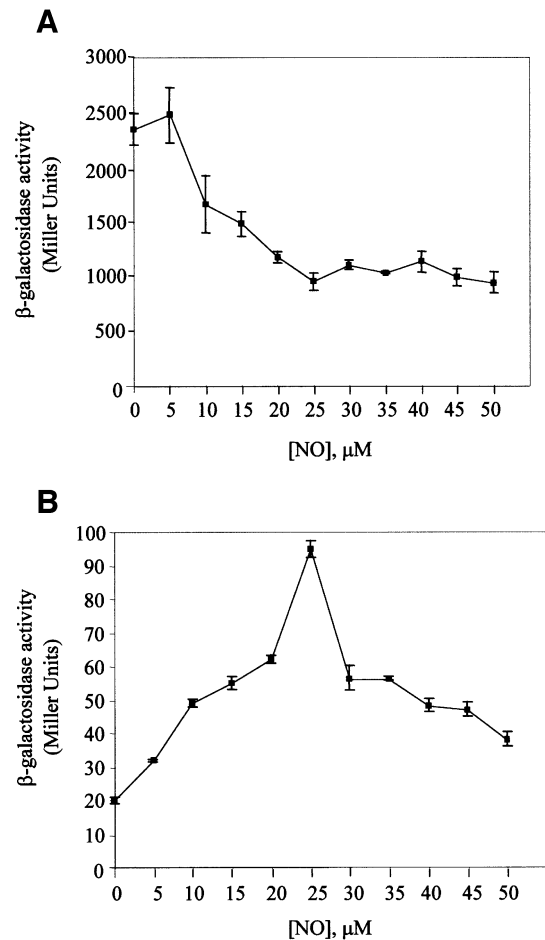
theoretical NO levels were in good agreement but thereafter NO concentration declined. Nevertheless, significant NO in the range of concentrations previously shown to up-regulate a P_{hmp}-*lacZ* fusion (Poole *et al.*, 1996) were sustained in the experiment.

To determine whether these concentrations of NOC compounds or NO generated were cytotoxic, the anaerobic growth of cultures of strains RKP2178 [Φ(*hmp-lacZ*)] or RKP2185 [Φ(*hmp-lacZ*) *fnr*] (see below) was measured. NOC-5 and NOC-7 were added (5 or 10 μM of each) to exponentially growing cultures that had been pre-grown in sealed tubes to a Klett reading of 20–25. In the first 30 min after NOC addition, growth rates declined slightly but then resumed the rates characteristic of control cultures (data not shown). In an additional test of toxicity, measurements of viable cell numbers were made during a similar anaerobic experiment. Wild-type *hmp*^{+} cells were resistant to 500 μM of each NOC compound, whereas *hmp* mutant cells (strain RKP4600) were sensitive to 50 μM of each NOC compound (results not shown). We have also shown that *E.coli* can be grown at NOC concentrations as high as 5 mM (M.Binet, J.Laver, H.Cruz-Ramos, R.K.Poole, unpublished data). Thus, NOC compounds in cultures

sustain NO in solution over 30 min at concentrations that up-regulate *hmp* yet are not toxic for growth.

β-galactosidase activities were assayed following the addition of different concentrations of an NOC mixture. Increasing concentrations of NOC compounds elicited an increase in Φ(*hmp-lacZ*) activity in a wild-type background (Figure 6B) but, in an *fnr* strain, NOC addition was without effect on the derepressed level of Φ(*hmp-lacZ*) activity (Figure 6C). These results are consistent with NO reacting with FNR *in vivo* with consequent derepression of *hmp* transcription, but additional regulatory mechanisms are suggested by the fact that the derepressed level in the wild-type strain (Figure 6B) is lower than in the *fnr* mutant (Figure 6C).

In order to assess (i) the modulation of FNR activity by NO *in vivo* in a simpler system, and (ii) the implications for regulation of other genes in the FNR regulon, we used the pRW50[FF(−71.5)::*lacZ*] and pRW50(FF*gal*Δ4::*lacZ*) fusion plasmids (Williams *et al.*, 1998) providing relatively simple semi-synthetic promoters, at which induction or repression is totally dependent on anaerobic FNR binding. These constructs were used to transform Δ*lac* and Δ*lac* Δ*fnr* strains. The β-galactosidase activities of the anaerobically grown strains were assayed following the addition of different volumes of NO solution. It was anticipated that any disruption by NO of FNR might reduce its inducing activity on FF(−71.5)::*lacZ* and derepress the FF*gal*Δ4::*lacZ* promoter fusion. The titration *in vivo* of the FNR-inducible promoter [FF(−71.5)] with NO showed a progressive decrease in β-galactosidase activity, reaching only 40% of the NO-untreated level at 25 μM NO (Figure 7A). The titration curve of the FNR-repressible promoter (FF*gal*Δ4) showed a bell-like shape (Figure 7B). Promoter derepression was observed from 5 to 10 μM NO (1.6- to 2.5-fold increase, respectively) and the highest reporter activity (~5-fold increase) was reached with 25 μM NO. These data showed that NO is effective as a signal molecule in a relatively narrow concentration range. NO concentrations above 25 μM appear to destabilize *lacZ* transcripts.

As expected, the FF(−71.5) promoter (strain RKP2761) was inactive in the absence of *fnr* (93 ± 13 Miller units of β-galactosidase when 10, 20, 30 or 50 μM NO was added; data not shown). Again, as expected, the FF*gal*Δ4 promoter (strain RKP2762) was active in the *fnr* background regardless of the presence of NO (210 ± 30 Miller units of β-galactosidase when 10, 20, 30 or 50 μM NO was added). In control experiments, β-galactosidase activities were measured on strains [RKP2672 (Δ*lac*) and RKP2760 (Δ*lac* Δ*fnr*)] carrying the pRW50 plasmid but without the promoter-*lacZ* fusion; in all cases, the activities were low (<2 Miller units of β-galactosidase) and showed no pattern of variation during anaerobic growth in the presence of NO.

Discussion

Here we present direct evidence of FNR binding to the P$_{hmp}$ *in vitro* by gel retardation, repressor–operator interaction and footprinting studies. The FNR box centred at position +5.5 was bound specifically by holo-FNR. RNAP occupied a DNA region overlapping the FNR box. Thus, this regulatory element is located appropriately for repression of transcription initiation by promoter occlusion. Point mutations of the central GC pairs within the FNR box were sufficient to abolish protein–DNA interaction, demonstrating the presence of a single FNR-binding site in P$_{hmp}$. Other FNR-repressed promoters, such as *ndh*, *narX* and *fnr*, contain more than one FNR box (Guest *et al.*, 1996), and complex regulatory mechanisms involving FNR have been described (Meng *et al.*, 1997; Marshall *et al.*, 2001). To our knowledge, P$_{hmp}$ is the first described naturally occurring promoter where repression by the *E.coli* FNR protein appears relatively simple, i.e. involving a single FNR box in the repressing position.

The DNA-binding activity of FNR was affected by either O$_2$ or NO treatment, resulting in a lower P$_{hmp}$ affinity and specificity. Nevertheless, some residual DNA-binding ability was observed after treatment of the FNR protein with either O$_2$ or NO. Such residual DNA-binding activity has been reported previously for O$_2$-treated FNR (Lazzazera *et al.*, 1996). However, in other work with different DNA targets, treatment of FNR with O$_2$ (Jordan *et al.*, 1997) and either O$_2$ or NO (Wu *et al.*, 2000) abolished DNA binding. Thus, it is likely that the DNA target influences the ability of O$_2$- or NO-treated FNR to bind. The footprinting data for NO-treated FNR indicate similar, but not identical, DNA distortions occurring upon binding of O$_2$-treated FNR. DNase I-hypersensitive sites arise from protein-induced bending that creates kinks in the DNA structure. The DNase I hypersensitivities and the protected AT-rich hexamer found immediately 5′ to the FNR box remained, whereas the +1 FNR half-site appeared less susceptible to the endonuclease when NO-treated FNR was bound. Whether this difference in DNA binding relates to a different FNR structure due to nitrosylation requires further studies since the structure of the FNR protein has not yet been determined.

NO has been demonstrated to react with the Fe–S cluster of several proteins, including NADH dehydrogenase, succinate dehydrogenase, aconitase and SoxR. The present work demonstrates that a [4Fe–4S]$^{2+}$ cluster can react with NO to form at least two known products, a dimeric and a monomeric DNIC. This is the first time that the dimeric, yellow, EPR-silent, form of a DNIC has been proposed to occur as a protein-bound cluster. The evidence comes from the optical spectrum taken together with the low spin integration of the EPR-active, green monomeric species. The detailed chemistry of the reaction of NO with a susceptible [4Fe–4S]$^{2+}$ cluster was not clearly established by the study with aconitase (Kennedy *et al.*, 1997). The aconitase studies also failed to obtain more than ~70% of the EPR-active, S = 1/2 DNIC species. The optical spectra suggest that in this case also a mixture of DNIC forms may be generated.

The results showing up to three NO per Fe–S cluster to complete the overall reaction in FNR suggest a multistep process. The inorganic chemistry of an iron–cysteine–NO mixture shows that the equilibrium

$$yellow \leftrightarrow green$$
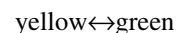
is driven to the right by increasing the cysteine to iron ratio. In a protein, this ratio will be raised if iron is lost. Hence, iron released from [4Fe–4S]$^{2+}$ by NO will shift the equilibrium between the two forms to the right. A possible reaction scheme for FNR, therefore, is that NO converts
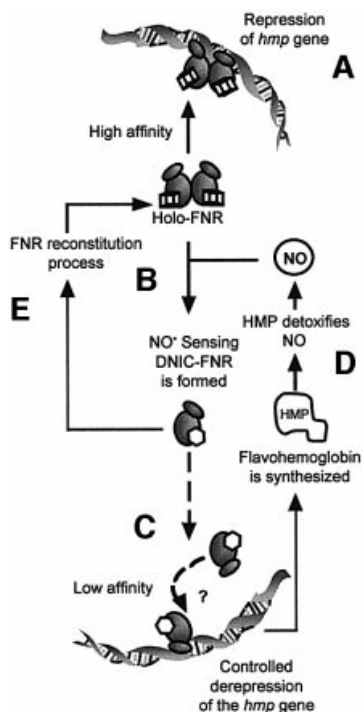
Fig. 8. NO-mediated mechanism of anaerobic up-regulation of the *E.coli hmp* gene. FNR forms are represented by ovals. Cubes and hexagons represent the $[4Fe–4S]^{2+}$ and nitrosylated clusters, respectively. DNA (P_{hmp}), NO and Hmp molecules are also shown. (**A**) Holo-FNR–DNA binding and repression of *hmp* under anaerobic conditions. (**B**) FNR senses NO by reaction with $[4Fe–4S]^{2+}$ clusters to generate DNIC–FNR. This post-transcriptional modification is likely to promote monomerization of FNR. (**C**) NO-treated FNR (and apo-FNR) can eventually bind P_{hmp} with a lower affinity to avoid sudden and complete derepression of the *hmp* gene. (**D**) The NO signal transduction through FNR allows derepression of *hmp* transcription. Synthesized Hmp protein detoxifies NO to produce N_2O or NO_3^- in the absence or presence of O_2, respectively (Poole and Hughes, 2000). (**E**) When the NO concentration is reduced, putative Fe–S cluster-repairing mechanisms can reconstitute the NO sensor, FNR.

the $[4Fe–4S]^{2+}$ cluster to the $[2Fe–2S]^{2+}$ form, as is the case with O_2, followed by further reaction to generate a dimeric then a monomeric DNIC. A reaction between a $[2Fe–2S]$ cluster and NO was proposed in the case of the reaction with SoxR (Ding and Demple, 2000). In either scheme, the $[4Fe–4S]^{2+}$ cluster would undergo a ligational and, hence, substantial conformational change.

We previously demonstrated that NO acts as an effector in FNR regulation of *Azotobacter vinelandii* gene expression (Cruz-Ramos *et al.*, 2000; Wu *et al.*, 2000). FnrP from *Paracoccus denitrificans* also appears sensitive to SNP, as judged by effects *in vivo* (Hutchings *et al.*, 2002). However, NO sensing by *E.coli* FNR has special physiological significance since FNR regulates expression of Hmp, which protects against NO and nitrosative stress. The biological significance of this protein is emphasized by our recent finding (Stevanin *et al.*, 2002) that Hmp protects *Salmonella* from NO-related killing by human macrophages, and similar findings have been obtained with *E.coli* (T.M.Stevanin, R.C.Read and R.K.Poole, unpublished data). That the sensing of micromolar NO concentrations is physiologically relevant is suggested by the results of Krieglstein *et al.* (2001) who report the

production of NO metabolites (NO_x) by polymorphonuclear leukocytes to be in the range 12–37 μM. Similarly, total NO metabolites (measured as nitrite) produced by RAW 264.7 macrophages co-cultured with arginase-deficient *Helicobacter pylori* are in the approximate range 15–22 μM (Gobert *et al.*, 2001). Here, both adding a solution of NO gas and by maintenance of NO in cultures using NOC compounds, we demonstrate that FNR can sense pathophysiological levels of NO (5–10 μM) *in vivo*. If intracellular NO concentrations are similar, the FNR concentration in cells (estimated to be 1 μM; Unden and Duchêne, 1987) is appropriate for NO sensing. The present work strongly suggests an NO-mediated mechanism of anaerobic up-regulation of the *E.coli hmp* gene by nitrosylation of the FNR $[4Fe–4S]^{2+}$ clusters (Figure 8).

The results suggest that other FNR-regulated genes should be affected by NO, but we are unaware of any cases described thus far in which gene regulation in bacteria is so regulated. The products of other FNR-repressed genes, such as *acnA* (aconitase), *sucAB* (α-ketoglutarate dehydrogenase), *sucCD* (succinyl CoA synthetase), *fumA* (fumarase A) and *icd* (isocitrate dehydrogenase) might, for example, represent a response to NO challenge by maintaining flux through the tricarboxylic acid cycle. Other *E.coli* genes directly repressed by FNR include *fpg/mutM* (Lee *et al.*, 1998), *sodA* (Hassan and Sun, 1992) and *sodC* (Gort *et al.*, 1999). These genes respond to oxidative stress and the products have roles in defence responses. In addition, superoxide dismutases SodA (MnSOD) and SodC (CuZnSOD) in the cytoplasm and periplasm, respectively, might contribute to NO stress tolerance by removing superoxide that can react with NO to form the toxic species, peroxynitrite. The *fpg/mutM* gene product, oxidized guanine glycosylase, repairs oxidative DNA damage (Chung *et al.*, 1991), removing 8-hydroxyguanine and formamidopyrimidine residues in DNA, although deamination of DNA normally is attributed to NO (Wink *et al.*, 1991).

Evidence that NO signal transduction is achieved by other Fe–S proteins is also presented in eukaryotic systems by the iron regulatory protein IRP1, which has a dual function. As an aconitase, IRP1 possesses a $[4Fe–4S]$ cluster and catalyses the isomerization of citrate and isocitrate. As a cluster-depleted protein, it binds specific RNA structures called iron-responsive elements (IREs) found in several mRNAs, like those encoding the ferritin subunits and transferrin receptor (Hentze and Kühn, 1996; Richardson and Ponka, 1997). The enzymatic/regulatory IRP1 activity is sensitive to endogenous chemical signals provided by nitrosative stress (Pantopoulos and Hentze, 1995; Bouton *et al.*, 1997; Oliveira *et al.*, 1999; Wardrop *et al.*, 2000). Exposure of IRP1 to NO or peroxynitrite flux elicited an increase in IRE-binding activity to the detriment of aconitase activity. SNAP-generated NO increased IRP1 regulatory activity, while SNP-generated NO^+ decreased this activity as a function of concentration (Wardrop *et al.*, 2000). At present, the mechanism(s) of these effects on IRP1 remains unclear.

In summary, we have shown that direct modification of the FNR $[4Fe–4S]^{2+}$ clusters to form protein-bound dinitrosyl-iron–thiol complexes serve as a functional switch in NO-responsive control of gene expression. Specifically, NO inactivation of FNR results in up-

regulation of synthesis of Hmp, a protein with key roles in NO resistance. This study reveals FNR to be a major NO-responsive regulator in bacteria, although other NO sensors surely remain to be discovered.

Materials and methods

Bacterial strains and medium
Escherichia coli VJS676 [Δ(*argF-lacZ*)*U169*; wild-type] and derivatives RKP2178 [Φ(*hmp-lacZ*)1], RKP2185 (RKP2178 *fnr-271*::Tn*10*) and RKP4600 (same as RKP4545, i.e. *hmp*::Tn*5*) have been described previously (Poole *et al.*, 1996; Membrillo-Hernández *et al.*, 1999). The FNR-inducible promoter, FF(–71.5)::*lacZ*, and FNR-repressible promoter, FF*gal*Δ4::*lacZ*, were cloned into pRW50 (Williams *et al.*, 1998). Cells were grown in rich medium (LB), initial pH 7.0. Kanamycin, tetracycline and rifampicin were used at final concentrations of 100, 35 and 50 µg/ml, respectively.

Treatment with NO and nitric oxide releasers
To release NO slowly in cultures, an anoxic equimolar mixture of NOC (Calbiochem) compounds was used: NOC-5 (3-[2-hydroxy-1-(1-methyl-ethyl)-2-nitrosohydrazino]-1-propanamine) and NOC-7 (3-[2-hydroxy-1-methyl-2-nitrosohydrazino]-*N*-methyl-1-propanamine) freshly prepared within an anaerobic glove box (Mk3, Don Whitley Scientific Ltd) in buffer (25 mM Tris pH 10.0, 100 mM NaCl) and kept at 4°C. The amount of NO released by the NOC mixture in an LB culture (OD_{600} of ~0.2) at 37°C was measured at least three times by using simultaneously (Mills *et al.*, 2001) O_2 (Rank Bros.) and NO electrodes (World Precision Instruments ISO-NO Mark II) over a 1 h period. The NOC mixture was added by injection into the stirred perspex chamber on depletion of all measurable oxygen by the cells (~5 min). Calibration of the amount of NO was carried out in the same conditions by injection of various volumes of a freshly prepared anoxic solution of NO (~1.9 mM in H_2O at room temperature; Poole *et al.*, 1996). Theoretical NO release (two NO per NOC) was calculated using half-lives of NOCs in phosphate-buffered saline (PBS; pH 7.4) at 37°C for NOC-7 and NOC–5 of 5 and 25 min, respectively (Calbiochem). Anaerobic cultures of strains RKP2178 and RKP2185 were grown to an OD_{600} of ~0.2 as described previously (Poole *et al.*, 1996) in screw-cap glass tubes filled with medium to the brim at 37°C in a water bath. The NOC mixture was added within the glove box to final concentrations in the range of 1–10 µM, and the anaerobic incubation continued for 1 h. Since NOCs are polyamines that can have regulatory effects on gene expression, the control culture (zero NO) was treated with 10 µM of a NOC mixture that was left at room temperature until no signal from the NO electrode was detectable. After treatment with NOC, rifampicin (50 µg/ml) was added to the cultures to stop gene transcription caused by exposure to oxygen. Bacteria were pelleted, washed with PBS and stored at –20°C before β-galactosidase assay.

For studies of strains carrying the pRW50[FF(–71.5)::*lacZ*] and pRW50(FF*gal*Δ4::*lacZ*) fusion plasmids, anaerobic cultures were diluted 20-fold into 10 ml of anoxic LB medium containing tetracycline (35 µg/ml) in 15 ml centrifuge tubes. After 4 h incubation in the anaerobic box (~25°C), NO was added to final concentrations up to 50 µM, the incubation was continued for 2 h and chloramphenicol (34 µg/ml) was added to the cultures to stop protein synthesis and avoid oxygen interference. Bacteria were pelleted, washed with 5 ml of PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 pH 7.4) and stored at –20°C before β-galactosidase assay.

Toxicity of NO solutions
To determine the toxicity of NO, cultures of strains VJS676 (wild-type) and RKP4600 (*hmp*) were grown aerobically at 37°C to an OD_{600} of ~0.2. The cultures were divided, one-half being left for 30 min in the glove box at 37°C with slow shaking (50 r.p.m.) in order to obtain anaerobic conditions, and the other half left growing aerobically at 37°C with shaking (100 r.p.m.). Then, cultures were diluted to the same OD_{600} (~0.2) with either aerobic or anaerobic LB medium. Cultures were challenged in triplicate with 3, 5, 10, 20, 50, 100 and 500 µM NOC mixture. After 1 h further incubation, the cultures were serially diluted in medium and plated in triplicate on nutrient agar plates containing antibiotic, when appropriate. Plates were incubated overnight at 30°C and colonies were counted. Results were expressed as a percentage of the viable counts in cultures not exposed to the NOC mixture. Anaerobic growth curves were obtained by inoculating 80 µl of an overnight culture into sealed tubes filled to the brim with 8 ml of LB medium. Cultures were

grown at 37°C on a slowly shaking platform for 90 min before NOC-5 and NOC-7 were added from stock solutions at high pH. A control tube lacked NOC compounds.

Purification, reconstitution and O_2 treatment of FNR
FNR protein was overproduced, purified and reconstituted as described previously (Jordan *et al.*, 1997). FNR samples in TCB (50 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$) were transferred from the anaerobic box in sealed cuvettes to a spectrophotometer (Beckman DU®650). Air-saturated TCB (~230 µM O_2 at 23°C) was added to the sample by injection in the glove box.

Preparation of the NO-treated protein
Two methods were used. In the first, reconstituted FNR protein was treated in the glove box with aliquots of a fresh anoxic solution of NO. Progress of the reaction was monitored as above. In the second, reactions with NO were performed using the fast release agent, proline NONOate (Alexis Chemicals), and the FNR protein was resuspended in nitrate-containing working buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 100 mM NaCl, 100 mM $NaNO_3$ pH 7.5). Proline NONOate was stored at ~1°C in buffer (same as the NOC mixture). Release of NO occurs with a half-time of ~13 s when titrated in working buffer at ~25°C. The amount of NO released per NONOate was measured by capturing the NO with the haem protein Fe(II)-Mb, and estimated to be 1.9 ± 0.1. FNR was held in a sealed cuvette within the glove box and titrated by injection with aliquots of a 4.17 mM stock solution of NONOate. After the addition of each aliquot, the cuvette was placed immediately into a spectrophotometer (Hitachi U3200) scanning at 120 nm/min. At the end point of the titration, an aliquot of NO-treated FNR was withdrawn and put into an EPR tube within the glove box.

Preparation of DNIC complexes
To determine the optical characteristics of DNIC complexes, an anaerobic solution of 500 µM $(NH_4)_2Fe(SO_4)_2$, 1 mM L-cysteine, 25 mM HEPES, 100 mM NaCl pH 7.5 was injected with an aliquot of proline NONOate to give a final concentration of 692 µM (equivalent to 1.38 mM NO). The resulting solution yielded the yellow or dimeric species. Conversion of the dimeric species into the green or monomeric species was achieved by increasing the L-cysteine concentration to 23.5 mM. Optical spectra were obtained using a Hitachi U3200 spectrophotometer and sealed anaerobic cuvettes.

EPR spectroscopy
EPR spectra were recorded on an X-band Bruker EMX spectrometer (9.64 GHz) equipped with an ESR-900 helium-flow cryostat and a TE-102 microwave cavity (Oxford Instruments). Field intensity was monitored using a microwave counter model 2440 (Marconi Instruments). Spin intensities of paramagnetic samples were estimated from integrations of EPR absorption spectra using 1 mM Cu(II)/10 mM EDTA as the standard solution.

Construction of mutant P_{hmp} and labelling
In order to change the FNR box in P_{hmp}, primers RP86 (5′-TAT-AAGATGCATTTA̱AGATAC*ATTAAT*TAAGATGCAAAAAAAGG-3′, incorporating the underlined mutations, G→A and C→T, and the italicized *Vsp*I restriction site) and RP87 (complementary to RP86) were used in an asymmetric temperature cycling reaction with *Pwo* polymerase and plasmid pRKP1613 as the template. Construct pRKP1613 is the pGEM-7zf(+) cloning vector (Promega) carrying the 628 bp *Bam*HI–*Sac*I fragment from pPL341 (Vasudevan *et al.*, 1991). The parental (methylated) plasmid DNA was then removed by treatment with *Dpn*I, and the remaining DNA was amplified in *E.coli*. The resulting plasmid pRKP1614 was confirmed by *Vsp*I restriction and sequencing. Purified 335 bp *Sac*I–*Fok*I DNA fragments from pRKP1613 and pRKP1614 (P_{hmp}) were labelled with [α-^{32}P]dCTP and Klenow polymerase (Promega). For FNR–DNA interaction studies, purified labelled fragments were left at least overnight in the anaerobic box.

FNR–DNA interactions
Anaerobic gel retardation analyses were as in Wu *et al.* (2000), except that the ^{32}P-labelled DNA fragment used was P_{hmp} (0.732 pmol in 10 µl). Reconstituted FNR protein was tested for DNA-binding activity after titration with air-saturated TCB or with an NO solution. After drying, gels were applied to a Bio-Rad phosphorimager model GS-363 and analyzed by Molecular Image® System (Bio-Rad) with the local background redefined as zero.

Repressor–operator interaction analyses were performed essentially as in Riggs *et al.* (1970). Dilutions of FNR protein were mixed with ^{32}P-labelled P_{hmp} in anoxic TCB supplemented with bovine serum albumin (50 μg/ml) and salmon sperm DNA (10 μg/ml) in a final volume of 100 μl. Reactions were incubated for 10 min, before 2 ml of TCB was added to the tubes. After mixing, two reaction samples (1 ml) were filtered through cellulose nitrate filters (25 mm diameter, 0.45 μm pore size; Schleicher and Schuell) previously soaked for 30 min in anoxic TCB. Filters were washed once with 0.5 ml of TCB to eliminate free P_{hmp} then subjected to scintillation counting. The fraction of FNR–P_{hmp} complex was plotted versus the log of FNR concentration, according to the equation $K_d = $ [FNR][DNA]/[FNR·DNA], where [FNR]>>[DNA]. The apparent K_d for the individual assays is the concentration of FNR (as monomer) giving half-maximal binding; the mean of two sets of experiments was taken.

DNase I footprinting reactions of P_{hmp} were carried out anaerobically using anoxic solutions of the Core Footprinting System (Promega) and following the manufacturer's instructions. Reactions with FNR protein and RNAP (USB) were loaded onto an 8% polyacrylamide sequencing gel. The *Sac*I–*Fok*I fragment from pRKP1613 was also cleaved by dimethyl sulfate/piperidine hydrolysis for use as a DNA sequence ladder.

Acknowledgements

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