Published in final edited form as:

Biochemistry. 2008 December 9; 47(49): 13084–13092. doi:10.1021/bi801342x.

Transcription factor NsrR from *Bacillus subtilis* senses nitric oxide with a 4Fe-4S cluster

Erik T. Yukl, Mohamed A. Elbaz, Michiko M. Nakano, and Pierre Moënne-Loccoz*

Department of Science and Engineering, School of Medicine, Oregon Health & Science University, 20,000 NW Walker Road, Beaverton, Oregon 97006-8921

Abstract

In *Bacillus subtilis*, NsrR is required for the upregulation of ResDE-dependent genes in the presence of nitric oxide (NO). NsrR was shown to bind to the promoters of these genes and inhibit their transcription *in vitro*. NO relieves this inhibition by an unknown mechanism. Here we use spectroscopic techniques (UV-vis, resonance Raman, and EPR) to show that anaerobically isolated NsrR from *B. subtilis* contains a [4Fe-4S]²⁺ cluster which reacts with NO to form dinitrosyl iron complexes. This method of NO sensing is analogous to that of the FNR protein of *Escherichia coli*. The Fe-S cluster of NsrR is also reactive toward other exogenous ligands such as cyanide, dithiothreitol, and O₂. These results, together with the presence of only three cysteine residues in NsrR, suggest that the 4Fe-4S cluster contains a non-cysteinyllabile ligand to one of the iron atoms, leading to high reactivity. Size exclusion chromatography and crosslinking experiments show that NsrR adopts a dimeric structure in its [4Fe-4S]²⁺ holo form as well as in the apo form. These findings provide a first steppingstone to investigate the mechanism of NO sensing in NsrR.

The ability to adapt to anaerobic conditions is vital for a great diversity of microorganisms. It is particularly true of pathogenic organisms which may encounter oxygen limitation within their hosts. Such bacteria also commonly face other stresses such as nitric oxide (NO) produced by phagocytic cells as part of the immune response of the host (1). Thus, an understanding of how bacteria sense and respond to these conditions is of considerable importance.

An example of the bacterial response to oxygen limitation comes from *Bacillus subtilis* which, in the absence of oxygen, can grow by nitrate respiration (2). The ResDE two-component regulatory system is required for the induction of genes involved in nitrate respiration (3). These genes include *fnr*, (the gene encoding anaerobic transcription factor) (4), *nasDEF* (nitrite reductase genes) (5), and *hmp* (flavohemoglobin gene) (6). However, anaerobic conditions alone are not sufficient for the full induction of the ResDE-controlled genes, in particular *hmp* and *nasDEF*, and the presence of NO is required to attain the full induction of these genes (7). The effect of NO is abrogated in an *nsrR* mutant strain, indicating a role for NsrR in NO-mediated control of the ResDE regulon (8).

NsrR belongs to the Rrf2 family of transcription regulators and is widely found in various bacteria (9). Sequence analyses predict that NsrR contains a helix-turn-helix which likely binds to the promoter region of its target genes (9). *In vivo* studies of *nsrR* mutants and the effect of

*To whom correspondence should be addressed. Tel: 503-748-167, Fax: 503-7481464, Email: ploccoz@ebs.ogi.edu. Corresponding author: Pierre Moënne-Loccoz, Oregon Health & Science University, 20,000 NW Walker Road, Beaverton, Oregon 97006. Tel: 503-748-1673; Fax: 503-748-1464; Email: ploccoz@ebs.ogi.edu.

Supporting Information available: A table listing the strains and plasmids used in this study, a comparison of the RR and EPR spectra of the NH-NsrR and CH-NsrR, and of the room temperature and low temperature RR spectrum of the [4Fe-4S]²⁺ cluster of NH-NsrR. This material is available free of charge via Internet at <http://pubs.acs.org>.

NO on NsrR-controlled genes have shown that NsrR is an NO-responsive transcription repressor in *Escherichia coli* (10), *B. subtilis* (8), *Salmonella enterica* serovar Typhimurium (11), *Neisseria gonorrhoeae* (12) and *Neisseria meningitidis* (13). In addition, NsrR was shown to repress ResDE-dependent *in vitro* transcription of *hmp* and *nasD* (8). The critical role of NsrR in modulating Hmp levels in both aerobic and anaerobic conditions and its impact on oxidative and nitrosative stress in macrophages was recently exemplified in *Salmonella enterica* serovar Typhimurium (14). However, any sort of structural information on NsrR remains sparse.

NsrR bears significant homology (30% identity, 48% similarity) to IscR, a 2Fe-2S cluster-containing transcriptional regulator of Fe-S cluster synthesis genes in *E. coli* (15). Notably, among the residues conserved between these proteins are three cysteine residues likely to coordinate the iron sulfur cluster in IscR, although the number of residues separating the first Cys from the CX₅C motif varies in IscR and NsrR orthologs. A transient brownish color in aerobically purified NsrR suggested that NsrR also contains an Fe-S cluster sensitive to O₂-mediated decomposition (8).

Other transcriptional regulators bearing Fe-S clusters whose activities are modulated by NO have been reported. NO binds to the [2Fe-2S]⁺ cluster of SoxR to form a dinitrosyl iron complex (DNIC) that activates transcription of *soxS* (16). Similarly, FNR contains a [4Fe-4S]²⁺ that reacts with NO. However, in this case, NO-binding inhibits the transcriptional regulation activity of FNR (17). Finally, NO has been shown to react with the 4Fe-4S cluster of aconitase to convert this enzyme into an iron regulatory protein (IRP-1) that controls mRNA translation and stability by interacting with iron responsive elements (IREs) (18-20). Thus, there are precedents for the utilization of Fe-S clusters as NO sensors to regulate the activity of Fe-S proteins involved in diverse cellular physiology.

In this report, we use UV-vis, resonance Raman (RR), and EPR spectroscopies to demonstrate that anaerobically purified NsrR harbors a [4Fe-4S]²⁺ cluster. Size exclusion chromatography and chemical crosslinking experiments were used to determine that NsrR is dimeric in both holo and apo forms. Exposure of this cluster to O₂ leads to its destruction via a [3Fe-4S]⁺ intermediate. The [4Fe-4S]²⁺ cluster of NsrR also reacts with NO to form at least two distinct forms of dinitrosyl-iron complexes. Other exogenous ligands such as dithiothreitol (DTT) and cyanide (CN⁻) are also shown to bind to the Fe-S cluster. We conclude that the 4Fe-4S cluster of NsrR contains at least one labile ligand, either a hydroxide/aqua ligand or a non-Cys endogenous residue, e.g., an aspartate residue as in the 4Fe-4S cluster of *P. furiosus* ferredoxin (21-23).

Materials & Methods

Purification of N-terminal and C-terminal His₆-tagged NsrR proteins (NH-NsrR & CH-NsrR)

The various strains and plasmids used in this study are listed in Table S1 (see Supporting Information). NH-NsrR expression plasmid, pMMN648, was previously constructed (8). To produce CH-NsrR, plasmid pMMN740 was constructed as follows. The *nsrR* gene was amplified by PCR from pMMN638 (8) using the primers oMN05-296 (5'-GGCGCGGGCATATGAAGTTAACCAATTATAC-3') and oMN06-304 (5'-CGCTCTCGAGTTCCTTCATTTTAAAGC-3'). The resulting PCR product was digested with *Nde*I and *Xho*I and cloned into pET-23a(+) (Novagen) digested with the same enzymes. The construct was verified by DNA sequencing analysis. *E. coli* BL21 (DE3)/pLysS carrying pMMN648 or pMMN740 was grown at 37°C in 1 to 2 L of M9-glucose medium (24) supplemented with 40 μM ferric ammonium citrate, 50 μg/ml of ampicillin and 5 μg/ml of chloramphenicol. At an OD₆₀₀ of 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After incubating at 37°C for 3 h, cells were collected by

centrifugation at $5,000 \times g$, resuspended in the culture medium, and transferred into a 1-L sealed bottle. The cell suspension was sparged with argon and kept overnight at 4°C. Cells were broken by passing through a French press placed in a plastic anaerobic glove bag (Glas-Col, LLC) continuously flushed with argon. The cleared lysate was recovered by centrifugation in a sealed tube at $15,000 \times g$ for 20 min. Subsequent purification steps were performed in an anaerobic chamber containing less than 1 ppm O₂ (Omni-Lab System; Vacuum Atmospheres Co.). All buffers and solutions were purged with argon and kept in the anaerobic chamber before use.

Cleared lysate was mixed with 15 ml of Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN or Sigma) in buffer A (50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 30 mM imidazole). After 1 h incubation, the column was washed with 10 volumes of buffer A followed by 2 volumes of buffer A containing 1.0 M KCl, 2 volumes buffer A containing 60 mM imidazole, and 2 volumes buffer A containing 100 mM imidazole. His₆-NsrR was eluted with buffer A containing 300 mM imidazole. Fractions containing His₆-NsrR were pooled and exchanged into an imidazole-free buffer containing 50 mM Tris-HCl and 100 mM KCl at pH 8.5 using HiTrap™ Desalting columns (GE Healthcare). The C-terminal His-tag protein was eluted from the Ni-NTA column in an identical fashion but 5 mM dithiothreitol (DTT) was added to the exchange buffer to prevent protein precipitation likely due to the poor Fe-S incorporation observed in this construct.

Complementation of *nsrR* with His₆-NsrR

To examine whether CH-NsrR complements the *nsrR* null mutation in *B. subtilis*, two plasmids pMMN749 and pMMN750 were generated. pMMN749 was constructed by cloning the erythromycin-resistance gene isolated from pDG646 (25) into pMMN740 digested with *HincII* and *XbaI* and pMMN750 was constructed by cloning the tetracycline-resistance gene from pDG1515 (25) into pMMN740 digested with *HincII*. pMMN749 and pMMN750 were used to transform a *B. subtilis* strain JH642 (*nsrR*⁺) to generate ORB7270 and ORB7285, respectively. The transformants were obtained by a single crossover recombination at the *nsrR* locus, resulting in transcription of *ch-nsrR* from the native *nsrR* promoter (Figure S1). ORB7270 and ORB7285 were transduced with SPβ phage carrying a transcriptional *nasD-lacZ* fusion (5) to generate ORB7284 and ORB7287, respectively.

Complementation experiments were carried out by determining expression of *nasD-lacZ* in ORB7284 and ORB7287 together with LAB2854 (wild type carrying *nasD-lacZ*) (5) and ORB6188 (*nsrR* mutant carrying *nasD-lacZ*) (8). Cells were grown under anaerobic conditions in 2xYT (26) supplemented with 1% glucose and 0.2% KNO₃ or 2xYT supplemented with 0.5% glucose and 0.5% pyruvate with appropriate antibiotics. Cells were collected at 1-h intervals and β-galactosidase activity was measured as previously described (26) and was shown in Miller units (24).

Western blot analysis

LAB2854 (wild type), ORB6188 (*nsrR*), ORB7284, and ORB7287 (both carrying *ch-nsrR*) were grown anaerobically in 2xYT supplemented with 0.5% glucose and 0.5% pyruvate and cells were harvested at T₀ (the end of exponential growth). Cell lysate was prepared as described previously (27), and 15 μg of total protein was applied to SDS-polyacrylamide (15%) gel. After electrophoresis, the proteins were electroblotted to nitrocellulose membrane filters. The detection of CH-NsrR was carried out using anti-NsrR antibody raised in rabbit, followed by anti-rabbit secondary antibody conjugated alkaline phosphatase as described (8).

Iron and protein determinations

The iron content of NsrR was determined spectrophotometrically with the ferrene assay (28). Specifically, 30 μ L samples of NsrR at two different concentrations were incubated at room temperature with 3 μ L 38% HCl for 10 min. The sample was centrifuged at $15,000 \times g$ for 15 min to remove denatured protein before addition of 50 μ L 3 M sodium acetate and 5 μ L of 1 M ascorbic acid pH 5.5. Finally, 5 μ L of 3.0 mM 3-(2-Pyridyl)-5,6-bis(5sulfo-2furyl)-1,2,4-triazine disodium salt hydrate (ferrene, Aldrich) was added. Absorbance was measured at 593 nm ($\epsilon_{593} = 35.5 \text{ mM}^{-1}\text{cm}^{-1}$) and compared to two blanks lacking either NsrR or ferrene. As expected the same procedure applied to apo-NsrR did not reveal any significant iron. Protein concentrations were determined by Bradford assay (29) using bovine serum albumin (Sigma-Aldrich) as a standard. Total amino acid analyses of the holo- and apo-proteins (AAA Service Laboratory Inc, Damascus, Oregon) suggest that the Bradford assay overestimates NsrR concentration by $\sim 25\%$. Because NsrR contains no tryptophan, calculated 280 nm molar extinction coefficient on the basis of the amino acid primary sequence is unreliable. Instead, concentrations of NsrR monomers are calculated using 280 nm molar extinction coefficients deduced from the total amino acid analyses of holo-NsrR ($\epsilon_{280} = 38 \text{ mM}^{-1} \text{ cm}^{-1}$) and apo NsrR ($\epsilon_{280} = 24.3 \text{ mM}^{-1} \text{ cm}^{-1}$).

Size exclusion chromatography

Size exclusion chromatography was performed using a BioSil® SEC 250 pre-packed size exclusion column (Bio-Rad) with a Varian HPLC system. Protein sample (60 to 100 μ M) was loaded and eluted at 1.0 mL/min with argon-purged buffer containing 100 mM potassium phosphate pH 7.5, 150 mM KCl, and 2.5 mM DTT. Elution profiles were monitored by absorbance at 280 nm. The column was calibrated using a gel filtration standard containing thyroglobulin, γ -globulin, ovalbumin, myoglobin, and vitamin B₁₂ (Bio-Rad).

Chemical crosslinking

Holo-NsrR was exchanged into the appropriate buffer using HiTrap™ Desalting columns (GE Healthcare). Apo-NsrR was acquired by extensive aerobic dialysis (~ 24 h) of holo protein against 50 mM Tris-HCl, 100 mM KCl, 5 mM DTT, and 10 mM EDTA pH 8.5 at room temperature, followed by dialysis into 50 mM potassium phosphate buffer pH 7.5, 100 mM KCl, and 5 mM DTT. Holo and apo-NsrR at 30 to 80 μ M were anaerobically incubated with 1.0 mM dimethyl suberimidate dihydrochloride (Fluka) and 100 mM KCl in 50 mM potassium phosphate buffer pH 7.5. After 1 h incubation at room temperature, the reaction was quenched with the addition of a 4-fold volume of pure acetic acid (Sigma-Aldrich) and each sample was diluted to ~ 10 μ M for analysis by SDS-PAGE (12% gel).

Spectroscopic characterization of NsrR

All sample preparation steps were performed in the anaerobic chamber. When necessary, samples for spectroscopic analysis were concentrated using filtering devices (Microcon 10 kD cutoff, Biomax, Millipore). Reduced protein was generated by the addition of excess sodium dithionite in the presence or absence of DTT. Oxidized NsrR was generated either by the addition of excess potassium ferricyanide followed by its removal using desalting spin columns (Zeba™ 0.5 mL; Pierce) or by the addition of DTT to 4.0 mM followed by exposure to O₂ gas. Addition of NO to the headspace of an NsrR sample was performed with a gastight Hamilton syringe to reach a partial pressure of 0.5 atm. After ~ 5 min incubation, the samples were exposed to the inert atmosphere of the glove box to release the excess NO, and transferred to EPR tubes (Wilmad Lab Glass) sealed with rubber septa. Alternatively, NsrR samples were incubated with varying concentration of diethylamine NONOate (Sigma-Aldrich) for 1 h at room temperature or diluted 2.5 fold with NO-saturated buffer in a sealed tube and incubated

5 min. Cyanide complexes were generated by 15-min incubation of reduced NsrR with 20 mM NaCN.

All UV-vis spectra were obtained using a Cary 50 spectrophotometer (Varian). EPR spectra were obtained on a Bruker E500 X-band EPR spectrometer equipped with a superX microwave bridge and a dual mode cavity with a helium flow cryostat (ESR900, Oxford Instruments, Inc.) for measurements at 5 - 10 K and a super HiQ cavity resonator (ESR4122, Bruker) for measurements at 100 K. The experimental conditions, i.e., temperature, microwave power, and modulation amplitude, were varied to optimize the detection of all potential EPR active species and ensure nonsaturating conditions. Quantitation of the EPR signals was performed with $\text{Cu}^{\text{II}}(\text{EDTA})$ standards.

Room temperature and low-temperature (110 K) RR spectra were obtained using a 90° and backscattering geometry, respectively. All spectra were collected on a custom McPherson 2061/207 spectrograph (set at 0.67 m with variable gratings) equipped with a Princeton Instruments liquid- N_2 -cooled CCD detector (LN-1100PB). Excitation at 488 nm was provided by an Innova I90C-3 argon ion laser and Rayleigh scattering was attenuated using a long-pass filter (RazorEdge®, Semrock). Frequencies were calibrated relative to indene and CCl_4 (room temperature) or aspirin (110 K) and are accurate within $\pm 1 \text{ cm}^{-1}$.

Results

CH-NsrR complements an *nsrR* mutant in *B. subtilis*

This study uses both N-terminal and C-terminal His₆-tagged proteins called NH-NsrR and CH-NsrR, respectively. In order to determine whether NH-NsrR is functional in *B. subtilis*, attempts were made to express *nh-nsrR* from the *nsrR* native promoter in a *B. subtilis* *nsrR* mutant strain, but the N-terminally tagged protein is not produced in *B. subtilis*. Since CH-NsrR and NH-NsrR purified from *E. coli* produce equivalent spectroscopic signatures (see below), we constructed the strains that express *ch-nsrR* from the *nsrR* promoter (see *Material & Methods*) and determined whether CH-NsrR is expressed in *B. subtilis* at a level similar to the native NsrR protein. Strains ORB7284 and ORB7287 do not produce the native NsrR protein, and instead produce the tagged NsrR with a molecular weight corresponding to CH-NsrR (Figure 1). Furthermore, the level of CH-NsrR produced in the *B. subtilis* strains is similar to that of NsrR in the wild-type strain (LAB2854). These results demonstrate that ORB7284 and ORB7287 are suitable for examining whether CH-NsrR regulates ResDE-controlled genes in the same manner as does native NsrR.

The native NsrR represses *nasD* transcription in anaerobic cultures in the absence of nitrate, while *nasD* expression is derepressed when nitrate is supplied in the medium (Figure 2A). As shown previously (8), the effect of nitrate is due to NO generated during nitrate respiration. The *nsrR* null mutation alleviates the repression of *nasD* in the absence of nitrate (Figure 2B). In the two strains producing CH-NsrR (Figure 2C & 2D), *nasD* expression is comparable to that in the wild-type background, indicating that CH-NsrR represses *nasD* transcription and the repressor activity is sensitive to NO. These results justify the use of CH-NsrR in this spectroscopic study for understanding the Fe-S chemistry that modulates NsrR activity in response to NO.

Holo-NsrR is a dimeric protein containing a $[\text{4Fe-4S}]^{2+}$ cluster

Anaerobically purified NsrR is greenish-brown in color and displays a broad visible absorption feature around 412 nm (Figure 3) characteristic of proteins containing $[\text{4Fe-4S}]^{2+}$ clusters (30). This assessment is confirmed by RR and EPR data discussed below. Iron quantitations combined with total amino acid analyses of NH-NsrR yield a ~ 2.8 iron atoms per protein. CH-

NsrR showed sub-stoichiometric iron content per NsrR protein, suggesting much poorer Fe-S cluster incorporation. CH-NsrR also revealed itself as prone to aggregation, presumably due to aberrant disulfide bond formation between free cysteine residues in protomers lacking the Fe-S cluster. Accordingly, the presence of 5 mM DTT could prevent the aggregation process. Attempts to increase Fe-S cluster occupation by anaerobic reconstitution of either CH-NsrR or NH-NsrR using $\text{Fe}(\text{SO}_4)$ and NaS_2 led to protein precipitation. Despite differences in the extent of Fe-S incorporation in the two constructs, their RR and EPR characterizations in all redox and coordination states investigated here were qualitatively identical (Figure S2 & S3). Thus, further discussions of spectroscopic data and iron:protein ratios are based on data obtained with NH-NsrR.

Assuming that all iron in NH-NsrR is present as 4Fe-4S clusters the iron quantitation leads to a $\epsilon_{412} = 14 \pm 1 \text{ mM}^{-1}\text{cm}^{-1}$ per 4Fe-4S cluster which is in good agreement with values observed for other 4Fe-4S proteins (31) such as FNR ($\epsilon_{420} = 13.3 \text{ mM}^{-1}\text{cm}^{-1}$) (32). Anaerobic gel filtration of holo-NsrR in the presence of 2.5 mM DTT suggests that, at $\sim 65 \mu\text{M}$, holo-NsrR exists exclusively as a dimer with an apparent molecular weight of 41 kDa (Figure 4). This value is in good agreement with the theoretical molecular weight of 39 kDa for the dimer. Apo-NsrR under the same conditions also elutes as a dimer while a small shoulder at 24 kDa is likely to represent a minor monomeric population. The predominance of the dimeric form in both holo- and apo-NsrR is also confirmed by chemical crosslinking experiments. Specifically, incubation of holo-NsrR and apo-NsrR at two different concentrations with the chemical cross-linker dimethyl suberimidate were analyzed by denaturing SDS-PAGE and showed the presence of the dimeric form, as indicated by a protein band approximately twice the size of the individual monomers (Figure 5). As expected for this protein concentration range (33), no evidence of a significant population of higher order oligomer is observed.

The [4Fe-4S] Cluster of NsrR Contains a Labile Ligand

The room temperature RR spectrum of anaerobically isolated NsrR obtained with a 488-nm excitation exhibits a strong $\nu(\text{Fe-S})$ bridging mode at 338 cm^{-1} consistent with a $[\text{4Fe-4S}]^{2+}$ cluster, and lacks an equally intense band near 290 cm^{-1} which would support a $[\text{2Fe-2S}]^{2+}$ cluster (Figure 6A) (31). The RR spectrum is similar to those obtained for oxidized *Clostridium pasteurianum* ferredoxin (*Cp Fdx*) and reduced *Chromatium vinosum* high potential iron-sulfur protein at the same excitation wavelength at 77 K (34) (see Figure S4 for low temperature RR spectrum of NsrR). It has been suggested that a high frequency of the totally symmetric $\nu(\text{Fe-S})$ bridging mode is characteristic of $[\text{4Fe-4S}]^{2+}$ clusters with a non-Cys terminal ligand, as in *Pyrococcus furiosus* ferredoxin where the bridging $\nu(\text{Fe-S}) = 442 \text{ cm}^{-1}$ in frozen solution (21). Although the frequency at room temperature for this mode in NsrR is not unusually high, it is interesting to note a -3 cm^{-1} shift in the presence of DTT (Figure 6B). A change in the UV-vis spectrum is also observed upon DTT addition (Figure 3). These observations suggest that DTT might bind to one of the Fe ions within the Fe-S cluster.

The as-isolated sample is EPR-silent, as expected for an antiferromagnetically coupled $[\text{4Fe-4S}]^{2+}$ cluster (data not shown). Treatment with excess dithionite at pH 8.5 yields a fast-relaxing EPR signal at $g = 2.04$ and 1.93 observable only below 40 K, as is typical for reduced $[\text{4Fe-4S}]^+$ clusters (Figure 7A). Attempts at reduction at pH 7.5 were unsuccessful as deduced from a lack of EPR activity in these samples. This is likely due to the increased midpoint potential of dithionite at low pH, particularly where the stock solution may be contaminated with sulfite (35) and may be indicative of a fairly low reduction potential of the $[\text{4Fe-4S}]^{2+}$ cluster in NsrR.

Quantitation of the reduced NsrR signal against Cu^{II} -EDTA yields 0.18 - 0.22 spin per 4Fe-4S cluster (as deduced from the UV-vis spectrum of starting material). At microwave powers saturating the $S = 1/2$ signal, another resonance at $g = 5.2$ is observed (Figure 7 inset) which

likely represents a population of $S = 3/2$ clusters (36) and explains, at least in part, the weak intensity of the $S = 1/2$ signal. Incubation of reduced NsrR with CN^- results in a disappearance of the $g = 5.2$ signal and produces a new $S = 1/2$ signal (Figure 7B) which now accounts for 0.64 spin per cluster. These observations are consistent with coordination of exogenous CN^- to convert the $[\text{4Fe-4S}]^+$ to a pure $S = 1/2$ species as observed for *Pyrococcus furiosus* ferredoxin (22). The remaining spin count missing in the EPR spectra may be due to chelation by the high concentration of CN^- (20 mM), incomplete reduction of the 4Fe-4S cluster, and/or an overestimation of the cluster concentration caused by adventitious iron in the preparation. In any case, the CN^- binding clearly demonstrates the presence of a labile Fe coordination site in NsrR.

The weak binding of an Fe ligand in NsrR is further illustrated by its reactivity toward O_2 . Exposure of anaerobically isolated NsrR to O_2 leads to a bleaching of the $[\text{4Fe-4S}]^{2+}$ absorption features (Figure 8A). Difference spectra of the oxygen-exposed samples minus anaerobic NsrR indicate a relative increase in absorbance around 500 nm and relative decrease at 410 nm, even as the overall absorbance decreases. These changes suggest the formation of a population of $[\text{2Fe-2S}]^{2+}$ clusters as previously observed upon exposure of FNR to O_2 (32). However, in our case the net decrease in absorbance around 500 nm over time suggests that neither the initial 4Fe-4S cluster nor the 2Fe-2S cluster is aerobically stable.

Interestingly, the presence of 4 mM DTT stabilizes the Fe-S cluster and allows the acquisition of transient UV-vis absorption after exposure to O_2 (Figure 8B). Although absorption features from DTT/Fe(III) complexes may form if iron(III) is released by NsrR and would contribute to these spectra (see Figure S5), the initial absorption increases observed between 450 and 600 nm are similar to those obtained in the absence of DTT. An EPR analysis of the resulting species yields a narrow spectrum with $g = 2.01$ and 1.96 observable up to 70 K (Figure 7C). This spectrum bears strong resemblance to a $[\text{3Fe-4S}]^+$ cluster as in oxidized *Desulfovibrio fructosovorans* [NiFe] hydrogenase, where the Ni atom is quantitatively lost from the $\text{NiFe}_3\text{-S}_4$ cluster upon oxidation (37). The same species is formed by anaerobic exposure of NsrR to excess ferricyanide (data not shown), but both methods yield only 0.04-0.05 spin per cluster. Again, these data are very similar to those obtained with FNR where only 5 - 8% of air or ferricyanide-oxidized samples could be accounted for by the $[\text{3Fe-4S}]$ EPR signal (38). The remainder may be accounted for by varying populations of EPR-silent $[\text{4Fe-4S}]^{2+}$ and $[\text{2Fe-2S}]^{2+}$ clusters and free iron for samples with longer incubation times. Our UV-vis data show no significant loss of absorbance at 410 nm after 30 min incubation with O_2 in the presence of 5 mM DTT and suggest that the majority of iron is present as $[\text{4Fe-4S}]^{2+}$ and $[\text{2Fe-2S}]^{2+}$ clusters.

Anaerobic exposure of NsrR to excess NO gas or dilution in NO-saturated buffer result in the formation of a new chromophoric species with a broad and intense near-UV absorption band at 363 nm (Figure 9A). This optical spectrum is consistent with those of dinitrosyl-iron complexes with extinction coefficients on the order of $\sim 4 \text{ mM}^{-1}\text{cm}^{-1}$ (39), and it suggests that the conversion of the 4Fe4S cluster into $[\text{Fe}(\text{NO})_2]$ species is efficient. The EPR spectrum displays a $g_{\text{av}} = 2.03$ (2.041, 2.034, 2.015) axial signal characteristic of $[\text{L}_2\text{Fe}(\text{NO})_2]^{-d^7}$ dinitrosyl-iron(I) complex (DNIC) (Figure 9B) (40-43). Exposures of NsrR to lower concentration of NO reveal mixtures of DNIC species with $g_{\text{av}} = 2.02$ (Figure 7D & 9B) which are gradually replaced with the more typical 2.033 DNIC signal at high NO concentration (Figure 9B). Quantitations of these EPR spectra range from 0.06 to 0.22 spin per 4Fe-4S cluster for the lowest and saturating NO concentrations, respectively. Thus, only a fraction of the DNIC clusters detected by UV-vis absorption is EPR-active, presumably because of antiferromagnetic coupling between $\text{Fe}(\text{NO})_2$ species in DNIC dimers. Similar observations have been reported on DNIC complexes in FNR, another NO sensing transcription factor (17, 44). The UV-vis spectra of NO-treated NsrR samples were unaffected by extensive

degassing (data not shown) and suggest that, at least *in vitro*, the DNIC formed in NsrR are irreversible complexes.

Discussion

Fe-S proteins function as versatile regulators by directly sensing O₂, NO, and iron [reviewed in (45)]. Previous studies in various bacteria have shown that NsrR is an NO-sensitive repressor but how NsrR responds to NO remained to be elucidated. The data presented here demonstrate for the first time that anaerobically isolated NsrR is a dimeric protein containing a [4Fe-4S]²⁺ cluster. RR characterization of this species yields a spectrum quite similar to those seen for other [4Fe-4S]²⁺ clusters in proteins such as certain bacterial ferredoxins and reduced high potential iron proteins (34). Reduction of NsrR with excess dithionite yields an EPR active species with relaxation properties consistent with those of a [4Fe-4S]⁺ cluster. The 4Fe-4S cluster of NsrR shows remarkable reactivity with exogenous ligands such as DTT and CN⁻, O₂ and NO. Cyanide binding to native Fe-S clusters has been observed only in *P. furiosus* ferredoxin (21) where one of the Fe atoms of the 4Fe-4S cluster is ligated by an Asp residue (46). Reports of ligation by exogenous thiols are also scarce but recruitment of β-mercaptoethanol as a sulfur ligand was observed in Cys/Ala and Cys/Gly mutants of the F_A and F_B clusters in photosystem I (47). In the absence of DTT, the labile Fe coordination site in NsrR is likely to be occupied by a non-Cys endogenous residue or an exogenous aqua or hydroxide ligand.

Exposure of NsrR to O₂ results in a gradual degradation of the 4Fe-4S cluster and formation of 3Fe-4S and 2Fe-2S clusters. The addition of DTT stabilizes the [4Fe-4S]²⁺ cluster although O₂-mediated decay and conversion to [2Fe-2S]²⁺ continue to occur. The reaction of NsrR with NO is also complex and results in the formation of multiple DNIC species. At saturating NO concentration, a typical EPR signal with g_{av} = 2.03 reveals the formation of one (Cys)₂Fe(NO)₂ per 4Fe-4S cluster. Examination of NO complexes formed at lower NO concentrations reveal more isotropic EPR signals with g_{av} = 2.02 that are also suggestive of DNIC species. These multiple EPR signatures for DNIC species in NsrR are in contrast with reports for FNR (17), WhiB3 of *Mycobacterium tuberculosis* (48) and SoxR of *E. coli* (16) where only a single EPR-active DNIC is observed regardless of NO concentration. While the DNIC species formation in NsrR appears irreversible *in vitro*, iron-sulfur repair systems may regenerate the 4Fe-4S cluster of holo-NsrR in *B. subtilis* (49-51).

The structural picture emerging from this spectroscopic study is that of a 4Fe-4S cluster anchored to a NsrR homodimer via three Cys ligands. Because there are three conserved Cys residues in NsrR, it is tempting to suggest that our construct is limited to “half-site” cluster loading. However, models where Cys residues from different subunits are recruited for the coordination of the 4Fe-4S cluster cannot be ruled out. Characterizations of Cys variants of NsrR are underway in our laboratories to address this issue.

This *in vitro* characterization of NsrR also provides insight into its function *in vivo*. It has been previously shown that NsrR plays a key role in NO signaling in *B. subtilis*, and that aerobically purified NsrR is still capable of repressing *hmp* and *nasD* expression *in vitro* (8). It was also shown that the *nsrR* mutation results in derepression of the ResDE-independent expression of *hmp* under aerobic conditions. Thus, apo-NsrR might retain its role as a transcription repressor under aerobic conditions. Possibly, all that is required for DNA binding and gene regulation is for NsrR to adopt a dimeric form, and our size-exclusion chromatography data demonstrate that the presence of Fe-S clusters is not required to stabilize the dimer. In this regard it is interesting that holo-IscR acts as a transcription repressor (52), whereas apo-IscR serves as a transcription activator (53), and DNA sequences recognized by the two forms of IscR are largely dissimilar. Alternatively, it is possible that within the cell the 4Fe-4S cluster is stabilized

against O₂-mediated decay and that the holo enzyme is responsible for aerobic repression *in vivo*. The *in vitro* stabilization of the 4Fe-4S cluster by DTT suggests that cysteine or other thiol donors may be able to play the same role within the cell; this labile ligand may even provide opportunities for other levels of regulation. Sensing of NO by NsrR occurs via conversion of the Fe-S cluster into a DNIC species. The presence of a labile Fe coordination site in NsrR may provide a simple mechanism to increase the cluster sensitivity to low NO concentration.

The Fe-S cluster of NsrR shows chemical characteristics similar to those of FNR. Both proteins contain a [4Fe-4S]²⁺ cluster, which upon exposure to O₂ converts to [2Fe-2S]²⁺. However, the [2Fe-2S]²⁺ cluster appears much less stable in NsrR than in FNR. *E. coli* FNR requires the [4Fe-4S]²⁺ cluster to form the transcriptionally active dimer (54). In contrast, our gel filtration and crosslinking experiments showed that NsrR is dimeric in both holo and apo forms. A recent work on *B. subtilis* FNR showed that holo- and apo-FNR are dimeric although a [4Fe-4S]²⁺ cluster is essential for DNA binding and transcription activation (55). Unlike *E. coli* FNR where four cysteine residues are essential for its activity (56), only three cysteines of *B. subtilis* FNR are indispensable for transcription activation. It was also shown that the three cysteine residues, together with non-cysteine ligand, serve as the ligands for the [4Fe-4S]²⁺ cluster formation. Therefore, *B. subtilis* NsrR shares more chemical features with the iron-sulfur cluster of *B. subtilis* FNR than that of *E. coli* FNR.

Our current study shows that *B. subtilis* NsrR, like *E. coli* FNR, interacts with NO and forms DNIC (17). DNA-binding activity of FNR is either decreased (17) or impaired (44) by nitrosylation. It remains to be examined whether NsrR-DNIC exhibits reduced DNA-binding activity, resulting in loss of the repressor activity. Alternatively, nitrosylation of NsrR might change specificity of DNA binding or affect formation of the transcription initiation complex. Moreover, since different DNIC species are observed depending on the concentration of NO, NsrR may exhibit different responses to different levels of NO concentration. Experiments aimed at testing these hypotheses are underway in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a grant GM74785 (P.M.-L.) from the National Institute of Health and MCB0818350 (M.M.N.) from the National Science Foundation. M.A.E. was supported by the International Fellowships Program from the Ford Foundation.

Abbreviations

DTT	dithiothreitol
EPR	electron paramagnetic resonance
IPTG	isopropyl-β-D-thiogalactopyranoside
RR	resonance Raman

References

1. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med* 2002;166:178–186. [PubMed: 12119230]

2. Nakano MM, Zuber P. Anaerobic growth of a “strict aerobe” (*Bacillus subtilis*). *Annu. Rev. Microbiol* 1998;52:165–190. [PubMed: 9891797]
3. Geng H, Nakano S, Nakano MM. Transcriptional activation by *Bacillus subtilis* ResD: tandem binding to target elements and phosphorylation-dependent and -independent transcriptional activation. *J. Bacteriol* 2004;186:2028–2037. [PubMed: 15028686]
4. Cruz-Ramos H, Boursier L, Moszer I, Kunst F, Danchin A, Glaser P. Anaerobic transcription activation in *Bacillus subtilis*: Identification of distinct FNR-dependent and -independent regulatory mechanisms. *EMBO J* 1995;14:5984–5994. [PubMed: 8846791]
5. Nakano MM, Hoffmann T, Zhu Y, Jahn D. Nitrogen and oxygen regulation of *Bacillus subtilis* *nasDEF* encoding NADH-dependent nitrite reductase by TnrA and ResDE. *J. Bacteriol* 1998;180:5344–5350. [PubMed: 9765565]
6. LaCelle M, Kumano M, Kurita K, Yamane K, Zuber P, Nakano MM. Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. *J. Bacteriol* 1996;178:3803–3808. [PubMed: 8682784]
7. Nakano MM. Induction of ResDE-dependent gene expression in *Bacillus subtilis* in response to nitric oxide and nitrosative stress. *J Bacteriol* 2002;184:1783–1787. [PubMed: 11872732]
8. Nakano MM, Geng H, Nakano S, Kobayashi K. The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. *J. Bacteriol* 2006;188:5878–5887. [PubMed: 16885456]
9. Rodionov DA, Dubchak IL, Arkin AP, Alm EJ, Gelfand MS. Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput. Biol* 2005;1:e55. [PubMed: 16261196]
10. Bodenmiller DM, Spiro S. The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. *J. Bacteriol* 2006;188:874–881. [PubMed: 16428390]
11. Bang IS, Liu L, Vazquez-Torres A, Crouch ML, Stamler JS, Fang FC. Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin *hmp*. *J. Biol. Chem* 2006;281:28039–28047. [PubMed: 16873371]
12. Overton TW, Whitehead R, Li Y, Snyder LA, Saunders NJ, Smith H, Cole JA. Coordinated regulation of the *Neisseria gonorrhoeae*-truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *J. Biol. Chem* 2006;281:33115–33126. [PubMed: 16954205]
13. Rock JD, Thomson MJ, Read RC, Moir JW. Regulation of denitrification genes in *Neisseria meningitidis* by nitric oxide and the repressor NsrR. *J. Bacteriol* 2007;189:1138–1144. [PubMed: 17122348]
14. Gilberthorpe NJ, Lee ME, Stevanin TM, Read RC, Poole RK. NsrR: a key regulator circumventing *Salmonella enterica* serovar Typhimurium oxidative and nitrosative stress *in vitro* and in IFN- γ -stimulated J774.2 macrophages. *Microbiology* 2007;153:1756–1771. [PubMed: 17526833]
15. Schwartz CJ, Giel JL, Patschkowski T, Luther C, Ruzicka FJ, Beinert H, Kiley PJ. IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc. Natl. Acad. Sci. U. S. A* 2001;98:14895–14900. [PubMed: 11742080]
16. Ding H, Dimple B. Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. *Proc. Natl. Acad. Sci. U. S. A* 2000;97:5146–5150. [PubMed: 10805777]
17. Cruz-Ramos H, Crack J, Wu G, Hughes MN, Scott C, Thomson AJ, Green J, Poole RK. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. *EMBO J* 2002;21:3235–3244. [PubMed: 12093725]
18. Beinert H, Kennedy MC, Stout CD. Aconitase as iron-sulfur protein, enzyme, and iron-regulatory protein. *Chem. Rev* 1996;96:2335–2373. [PubMed: 11848830]
19. Cairo G, Ronchi R, Recalcati S, Campanella A, Minotti G. Nitric oxide and peroxynitrite activate the iron regulatory protein-1 of J774A.1 macrophages by direct disassembly of the Fe-S cluster of cytoplasmic aconitase. *Biochemistry* 2002;41:7435–7442. [PubMed: 12044177]
20. Rouault TA. The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nat. Chem. Biol* 2006;2:406–414. [PubMed: 16850017]

21. Conover RC, Kowal AT, Fu WG, Park JB, Aono S, Adams MW, Johnson MK. Spectroscopic characterization of the novel iron-sulfur cluster in *Pyrococcus furiosus* ferredoxin. *J. Biol. Chem* 1990;265:8533–8541. [PubMed: 2160461]
22. Conover RC, Park JB, Adams MWW, Johnson MK. Exogenous ligand binding to the [Fe₄S₄] cluster in *Pyrococcus furiosus* ferredoxin. *J. Am. Chem. Soc* 1991;113:2799–2800.
23. Hu Y, Faham S, Roy R, Adams MW, Rees DC. Formaldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus*: the 1.85 Å resolution crystal structure and its mechanistic implications. *J. Mol. Biol* 1999;286:899–914. [PubMed: 10024458]
24. Miller, JH. Experiments in molecular genetics. Cold Spring Harbor Laboratory Cold Spring Harbor; N. Y.: 1972.
25. Guérout-Fleury AM, Shazand K, Frandsen N, Stragier P. Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* 1995;167:335–336. [PubMed: 8566804]
26. Nakano MM, Marahiel MA, Zuber P. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J. Bacteriol* 1988;170:5662–5668. [PubMed: 2848009]
27. Baruah A, Lindsey B, Nakano MM. Mutational analysis of the signal-sensing domain of ResE histidine kinase from *Bacillus subtilis*. *J. Bacteriol* 2004;186:1694–1704. [PubMed: 14996800]
28. Douglas JH, Reid RR, Smith FE, Thompson SL. Ferene--a new spectrophotometric reagent for iron. *Can. J. Chem* 1984;62:721–724.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]
30. Orme-Johnson, WH.; Orme-Johnson, NR. Iron-sulfur proteins. Spiro, TG., editor. Wiley-Interscience; New York: 1982. p. 67-96.
31. Duin EC, Lafferty ME, Crouse BR, Allen RM, Sanyal I, Flint DH, Johnson MK. [2Fe-2S] to [4Fe-4S] cluster conversion in *Escherichia coli* biotin synthase. *Biochemistry* 1997;36:11811–11820. [PubMed: 9305972]
32. Jordan PA, Thomson AJ, Ralph ET, Guest JR, Green J. FNR is a direct oxygen sensor having a biphasic response curve. *FEBS Lett* 1997;416:349–352. [PubMed: 9373183]
33. Davies GE, Stark GR. Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proc. Natl. Acad. Sci. U. S. A* 1970;66:651–656. [PubMed: 4913206]
34. Spiro, TG.; Czeruszewicz, RS.; Han, S. Biological applications of Raman spectroscopy. Spiro, TG., editor. Wiley-Interscience; New York: 1988. p. 523-553.
35. Mayhew SG. The redox potential of dithionite and SO₂⁻ from equilibrium reactions with flavodoxins, methyl viologen and hydrogen plus hydrogenase. *Eur. J. Biochem* 1978;85:535–547. [PubMed: 648533]
36. Lindahl PA, Day EP, Kent TA, Orme-Johnson WH, Munck E. Mossbauer, EPR, and magnetization studies of the *Azotobacter vinelandii* Fe protein. Evidence for a [4Fe-4S]¹⁺ cluster with spin S = 3/2. *J. Biol. Chem* 1985;260:11160–11173. [PubMed: 2993304]
37. Rousset M, Montet Y, Guigliarelli B, Forget N, Asso M, Bertrand P, Fontecilla-Camps JC, Hatchikian EC. [3Fe-4S] to [4Fe-4S] cluster conversion in *Desulfovibrio fructosovorans* [NiFe] hydrogenase by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U. S. A* 1998;95:11625–11630. [PubMed: 9751716]
38. Khoroshilova N, Popescu C, Munck E, Beinert H, Kiley PJ. Iron-sulfur cluster disassembly in the FNR protein of *Escherichia coli* by O₂: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. *Proc. Natl. Acad. Sci. U. S. A* 1997;94:6087–6092. [PubMed: 9177174]
39. Costanzo S, Menage S, Purrello R, Bonomo RP, Fontecave M. Reexamination of the formation of dinitrosyl-iron complexes during reaction of S-nitrosothiols with Fe(II). *Inorg. Chim. Acta* 2001;318:1–7.
40. McDonald CC, Phillips WD, Mower HF. An electron spin resonance study of some complexes of iron, nitric oxide, and anionic ligands. *J. Am. Chem. Soc* 1965;87:3319–3326.
41. Lee M, Arosio P, Cozzi A, Chasteen ND. Identification of the EPR-active iron-nitrosyl complexes in mammalian ferritins. *Biochemistry* 1994;33:3679–3687. [PubMed: 8142366]
42. Vanin AF, Serezhenkov VA, Mikoyan VD, Genkin MV. The 2.03 signal as an indicator of dinitrosyl-iron complexes with thiol-containing ligands. *Nitric Oxide* 1998;2:224–234. [PubMed: 9851363]

43. Foster HW, Cowan JA. Chemistry of nitric oxide with protein-bound iron sulfur centers. Insights on physiological reactivity. *J. Am. Chem. Soc* 1999;121:4093–4100.
44. Wu G, Cruz-Ramos H, Hill S, Green J, Sawers G, Poole RK. Regulation of cytochrome *bd* expression in the obligate aerobe *Azotobacter vinelandii* by CydR (Fnr). Sensitivity to oxygen, reactive oxygen species, and nitric oxide. *J. Biol. Chem* 2000;275:4679–4686. [PubMed: 10671497]
45. Kiley PJ, Beinert H. The role of Fe-S proteins in sensing and regulation in bacteria. *Curr. Opin. Microbiol* 2003;6:181–185. [PubMed: 12732309]
46. Sham S, Calzolari L, Wang PL, Bren K, Haarklau H, Brereton PS, Adams MW, La Mar GN. A solution NMR molecular model for the aspartate-ligated, cubane cluster containing ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochemistry* 2002;41:12498–12508. [PubMed: 12369841]
47. Jung YS, Vassiliev IR, Qiao F, Yang F, Bryant DA, Golbeck JH. Modified ligands to F_A and F_B in photosystem I. Proposed chemical rescue of a [4Fe-4S] cluster with an external thiolate in alanine, glycine, and serine mutants of PsaC. *J. Biol. Chem* 1996;271:31135–31144. [PubMed: 8940111]
48. Singh A, Guidry L, Narasimhulu KV, Mai D, Trombley J, Redding KE, Giles GI, Lancaster JR Jr. Steyn AJ. *Mycobacterium tuberculosis* WhiB3 responds to O₂ and nitric oxide via its [4Fe-4S] cluster and is essential for nutrient starvation survival. *Proc. Natl. Acad. Sci. U. S. A* 2007;104:11562–11567. [PubMed: 17609386]
49. Overton TW, Justino MC, Li Y, Baptista JM, Melo AM, Cole JA, Saraiva LM. Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulfur centers. *J. Bacteriol* 2008;190:2004–2013. [PubMed: 18203837]
50. Fontecave M, Ollagnier-de-Choudens S. Iron-sulfur cluster biosynthesis in bacteria: Mechanisms of cluster assembly and transfer. *Arch. Biochem. Biophys* 2008;474:226–237. [PubMed: 18191630]
51. Johnson DC, Dean DR, Smith AD, Johnson MK. Structure, function, and formation of biological iron-sulfur clusters. *Annu. Rev. Biochem* 2005;74:247–281. [PubMed: 15952888]
52. Giel JL, Rodionov D, Liu M, Blattner FR, Kiley PJ. IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O₂-regulated genes in *Escherichia coli*. *Mol. Microbiol* 2006;60:1058–1075. [PubMed: 16677314]
53. Yeo WS, Lee JH, Lee KC, Roe JH. IscR acts as an activator in response to oxidative stress for the suf operon encoding Fe-S assembly proteins. *Mol. Microbiol* 2006;61:206–218. [PubMed: 16824106]
54. Moore LJ, Mettert EL, Kiley PJ. Regulation of FNR dimerization by subunit charge repulsion. *J. Biol. Chem* 2006;281:33268–33275. [PubMed: 16959764]
55. Reents H, Gruner I, Harmening U, Bottger LH, Layer G, Heathcote P, Trautwein AX, Jahn D, Hartig E. *Bacillus subtilis* Fnr senses oxygen via a [4Fe-4S] cluster coordinated by three cysteine residues without change in the oligomeric state. *Mol. Microbiol* 2006;60:1432–1445. [PubMed: 16796679]
56. Melville SB, Gunsalus RP. Mutations in FNR that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli*. *J. Biol. Chem* 1990;265:18733–18736. [PubMed: 2229038]

Figure 1.

Western blot analysis of NsrR. Cleared lysates were prepared from *E. coli* BL21(DE3)/pLysS carrying pMMN740 (lane 1), *B. subtilis* strains LAB2854 (wild type, lane 2), ORB6188 (*nsrR* mutant, lane 3), ORB7284 (*nsrR* mutant carrying pMMN749, lane 4), and ORB7287 (*nsrR* mutant carrying pMMN750, lane 5). Molecular weight markers are shown on the lane marked with M.

Figure 2.

Anaerobic expression of *nasD-lacZ* in (A) LAB2854 (wild type), (B) ORB6188 (*nsrR* mutant), (C) ORB7284 (*nsrR* mutant carrying pMMN749), and (D) ORB7287 (*nsrR* mutant carrying pMMN750). *B. subtilis* strains were grown anaerobically in 2xYT supplemented with 1% glucose and 0.2% KNO_3 (circles) or with 0.5% glucose and 0.5% pyruvate (squares); open symbols correspond to growth and solid symbols to β -galactosidase activity. Time zero indicates the end of the exponential growth phase.

Figure 3. Absorption spectra of anaerobically purified NsrR (30 μM) at pH 8.5 in the absence (solid line) and presence (dashed line) of 5 mM DTT.

Figure 4.

Size exclusion chromatography elution profile of anaerobically purified holo-NsrR (65 μ M, solid line) and apo-NsrR (92 μ M, dashed line) at pH 7.5 monitored at 280 nm.

Figure 5. Chemical crosslinking of holo and apo-NsrR. The noted protein concentrations were anaerobically incubated in the presence of 1.0 mM dimethyl suberimidate for 1 h before quenching the reaction with acetic acid and subjecting samples to 12% SDS-PAGE analysis.

Figure 6. Room temperature RR spectra of (A) anaerobically purified NsrR (750 μM) and (B) anaerobically purified NsrR (750 μM) + 5.0 mM DTT ($\lambda_{\text{exc}} = 488 \text{ nm}$, 100 mW).

Figure 7.

EPR spectra of (A) anaerobically isolated NsrR chemically reduced with dithionite (200 μ M protein, 0.016 mW microwave power, 10 G modulation amplitude, $T = 10$ K), (B) reduced NsrR incubated with 20 mM NaCN (160 μ M protein, 0.016 mW microwave power, 10 G modulation amplitude, $T = 10$ K), (C) anaerobically isolated NsrR exposed to O_2 in the presence of 4 mM DTT (200 μ M protein, 0.005 mW microwave power, 10 G modulation amplitude) (D) NsrR in the presence of 1.5 molar equivalents of NO from NONOate (160 μ M protein, 0.005 mW microwave power, 10 G modulation amplitude) *Inset*: EPR spectrum of reduced NsrR highlighting $S = 3/2$ population. (200 μ M protein, 10 mW microwave power, 10 G modulation amplitude, $T = 5$ K). All spectra have been normalized according to microwave power and sample concentration.

Figure 8.

UV-vis spectra of (A) NsrR (30 μM) exposed to O₂, scans taken every 4 min. *Inset:* Difference spectrum of O₂-exposed NsrR at 14 min. minus anaerobic NsrR. (B) NsrR (30 μM) in the presence of 5 mM DTT exposed to O₂, scans taken every 4 min. Visible absorbance around 500 nm reaches its maximum at 6 min and slowly decays. *Inset:* Difference spectrum of O₂-exposed NsrR at 6 min minus anaerobic NsrR.

Figure 9.

(A) Absorption spectra of anaerobically isolated NsrR (24 μM) before (solid line) and after (dashed line) addition of NO gas. (B) EPR spectra of anaerobically isolated NsrR treated with either saturated NO solution or the indicated concentrations of NO via 1 h incubation with NONOate at pH 7.5 (0.4 mW microwave power, 2 G modulation amplitude, $T = 100\text{ K}$).