The Transcriptional Repressor Protein NsrR Senses Nitric Oxide Directly via a [2Fe-2S] Cluster

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Abstract

The regulatory protein NsrR, a member of the Rrf2 family of transcription repressors, is specifically dedicated to sensing nitric oxide (NO) in a variety of pathogenic and non-pathogenic bacteria. It has been proposed that NO directly modulates NsrR activity by interacting with a predicted [Fe-S] cluster in the NsrR protein, but no experimental evidence has been published to support this hypothesis. Here we report the purification of NsrR from the obligate aerobe Streptomyces coelicolor. We demonstrate using UV-visible, near UV CD and EPR spectroscopy that the protein contains an NO-sensitive [2Fe-2S] cluster when purified from E. coli. Upon exposure of NsrR to NO, the cluster is nitrosylated, which results in the loss of DNA binding activity as detected by bandshift assays. Removal of the [2Fe-2S] cluster to generate apo-NsrR also resulted in loss of DNA binding activity. This is the first demonstration that NsrR contains an NO-sensitive [2Fe-2S] cluster that is required for DNA binding activity.

Introduction

Nitric oxide (NO) is a highly reactive and toxic free radical gas that can freely diffuse into cells and attack the redox centers of proteins. Human macrophages produce NO as a very early line of defense against invading bacterial pathogens. Soil bacteria are exposed to NO produced by denitrifying microbes and by the NO synthases of plants and microbes. Bacteria have evolved specific NO sensor proteins that regulate the expression of enzymes involved in loss of DNA binding activity. This is the first demonstration that NsrR contains an NO-sensitive [2Fe-2S] cluster that is required for DNA binding activity.

NsrR homologues are encoded in many Gram-positive soil bacteria by genes that are usually linked to hmp [16]. To date, studies of NsrR have been restricted to the in vivo analysis of target genes in pathogenic bacteria such as N. meningitidis [14] or model organisms such as E. coli [9,10] and B. subtilis [12]. Multiple sequence alignments revealed three conserved cysteine residues in the primary sequences of NsrR proteins (Fig. 1A) that could potentially act as ligands for a [2Fe-2S] cluster. Despite this, the presence of an NO-sensing metal center in NsrR has yet to be shown experimentally.

Streptomyces is a genus of ubiquitous saprophytic soil bacteria best known for producing useful secondary metabolites [19]. The model organism for the genus, S. coelicolor, an obligate aerobe, encodes two Hmp homologues (HmpA1 and HmpA2), which presumably detoxify NO by oxidising it to NO3⁻ or reducing it to N2O. The hmpA1 gene is adjacent to a gene (SCO7427) encoding the only Rrf2 type protein encoded by the S. coelicolor genome (Fig. 1B). SCO7427 has previously been identified as an NsrR homologue using bioinformatics analysis [16] and alignment with...
other NsrR homologues shows that it contains the three conserved cysteine residues predicted to ligate the [2Fe-2S] cluster of NsrR (Fig. 1A). Here we report in vitro studies of S. coelicolor NsrR. We demonstrate that the protein can accommodate a [2Fe-2S] cluster, which is stable to atmospheric oxygen. We show that this form of the protein binds specifically to the promoter regions of hmpA1 and hmpA2. The [2Fe-2S] cluster reacts readily with NO, resulting in the formation of iron-nitrosyl species and a concomitant loss of specific DNA-binding activity.

Results and Discussion

NsrR contains an oxygen-insensitive [2Fe-2S] cluster

Over-expression of S. coelicolor NsrR in E. coli resulted in cell pellets that were dark brown in color. This color persisted during a two-step purification of NsrR by heparin affinity chromatography and gel filtration on Superdex 75, suggesting the presence of iron in the protein. The CD spectrum of the purified protein displayed bands in the region 260–750 nm with three positive features, \( \lambda_{\text{max}} 324, 445, 490 \text{ nm} \), together with two negative features, \( \lambda_{\text{max}} 375 \text{ and } 550 \text{ nm} \) (Fig. 2A). The bands were of similar energies and the same order of magnitude as those observed for other [2Fe-2S] cluster containing proteins [20,21,22], most notably the Rieske protein BphF from Burkholderia sp [20]. UV visible spectroscopy (Fig. 2B, solid line) of the purified protein also revealed features characteristic of an [2Fe-2S] protein, with major bands at 325 and 420 nm and shoulders at 460 and 550 nm [23]. Iron and sulfide analysis revealed the presence of 0.9 \( \pm 0.17 \) irons per sulfide, close to the expected ratio of 1:1 for a [2Fe-2S] cluster. Furthermore, protein analysis revealed that cluster incorporation was incomplete, with an average of 28% [2Fe-2S] cluster incorporation. This observation is commonly associated with over-expressed iron sulfur proteins. We note that aerobically purified B. subtilis NsrR was found to be brownish in color immediately following purification and had an absorption spectrum indicative of an iron sulfur protein. However, the color rapidly faded under aerobic conditions [12] and no further characterization was reported. Surprisingly, the [2Fe-2S] cluster of S. coelicolor was stable in the presence of atmospheric oxygen, although dithiothreitol was required to maintain stability. NsrR did not give rise to EPR signals (Fig. 2C, top), consistent with the presence of an oxidized [2Fe-2S] \(^2+\) cluster. The addition of sodium dithionite did not generate an EPR active species (data not shown). The addition of up to 20 mM sodium dithionite had little effect on the UV-visible absorbance spectrum of NsrR (data not shown), indicating that the cluster is stable in the presence of dithionite. We conclude that the
[2Fe-2S] NsrR reacts readily with NO

Exposure of the protein to NO resulted in an EPR spectrum featuring signals at g = 2.039, 2.0231 and 2.013 (Fig. 2C, bottom). These g-values are characteristic of a S = ½ dinitrosyl iron complex (DNIC) and, importantly, are essentially identical to those previously reported for a DNIC coordinated by cysteine thiolates, Fe(NO)2(Cys)2 [24,25,26]. Similar g-values were also reported following reaction with NO of the iron-sulfur containing proteinsaconitate, SoxR and FNR [2,3,23]. Quantification of the EPR signal in Fig. 2C revealed that only 8.89% of the iron in the sample was detected in the EPR experiments. This indicates that the majority of the iron was in an EPR-silent form.

Significant changes were also observed in the UV-visible spectrum upon treatment with NO such that features characteristic of the [2Fe-2S] cluster were lost (Fig. 2B, dashed line). Thioliigated DNIC species have characteristic absorbance properties: the mononuclear EPR-active DNIC has an absorption maximum at 397 nm while the EPR-silent dinuclear DNIC (in which two iron ions are each ligated by two terminal NO molecules and are bridged by two thiols) gives rise to absorption maxima at 310 and 362 nm [27]. The NO-treated NsrR spectrum (Fig. 2B) is consistent with a mixture of mononuclear and dinuclear thiol-coordinated DNIC species, with a shoulder at ~310 nm characteristic of the dinuclear DNIC, and broad absorbance out into the visible region consistent with a superposition of mononuclear and dinuclear DNIC absorption envelopes. A proportion of mononuclear DNIC species may be in an EPR-silent state [28]. We note that a similar mixture of DNIC species was observed for NO-treated FNR [2]. Together, these data demonstrate that NO nitrosylates the [2Fe-2S] cluster, resulting in the formation of cysteine thiolate-bound DNIC species. Both the NO treated and untreated NsrR proteins eluted at the same volume on gel filtration (data not shown) suggesting both proteins have the same hydrodynamic radius and that NsrR is not denatured upon treatment with NO.

Nitric oxide abolishes the DNA binding activity of NsrR

To identify potential NsrR target genes, the S. coelicolor genome was searched with the NsrR promoter matrix for Bacillales and Streptomyces spp (e-cutoff score = 10.7) [16]. A total of 322 genes have potential NsrR binding sites within 70 base pairs of DNA upstream of their translational start codons, including both the hmpA1 and hmpA2 genes (Fig. 1B). To investigate whether the purified NsrR protein can bind to these putative sites, bandshift assays were performed using radiolabelled DNA fragments carrying the hmpA1 and hmpA2 promoters. The probes were incubated with NsrR diluted in Tris buffer or Tris buffer saturated with NO and the reaction mixtures were separated on a non-denaturing polyacrylamide gel (Fig. 3A). The cluster has a reduction potential too low to be effectively reduced by this powerful reductant.
Figure 3A

Figure 3B

Figure 3C

Figure 3. DNA binding assays with the *hmpA1* and *hmpA2* promoters and purified NsrR protein. (A). Bandshift assay using 200 base pair restriction fragments (20 ng per reaction) carrying the *hmpA1* and *hmpA2* promoters, as indicated, and purified [2Fe-2S] NsrR in 50 mM Tris pH 7.0, 100 mM NaCl buffer saturated with NO (2 mM), as indicated. Binding is abolished by addition of NO saturated buffer to purified NsrR. (B). Bandshift assay using a 200 base pair restriction fragment (20 ng per reaction) carrying the *hmpA1* promoter with either EDTA:ferricyanide treated apo-NsrR or holo [2Fe-2S] NsrR as indicated in 50 mM Tris pH 7.0, 100 mM NaCl buffer. The apo-form of the protein is unable to bind to the *hmpA1* promoter indicating that the cluster is required for DNA binding activity. (C). Sedimentation equilibrium of oligonucleotides in the presence and absence of purified NsrR. The sedimentation of each sample was monitored at 260 nm and fitted to a single
demonstrate that NsrR can form a complex with the hmpA1 and hmpA2 promoters. NsrR was unable to bind to DNA fragments lacking an NsrR binding site (data not shown). Importantly, DNA binding to the hmpA1 and hmpA2 promoters was abolished by NO, demonstrating that NsrR DNA-binding activity is modulated in response to NO. Treatment of the protein with EDTA and ferricyanide [29] resulted in loss of the cluster (as judged from the complete loss of cluster-associated UV visible absorption features, data not shown). Bandshift assays demonstrated that apo-NsrR lacks DNA binding activity (Fig. 3B). A similar loss of DNA binding activity was observed upon addition of the iron chelator bipyridyl to holo NsrR (data not shown). Therefore, we conclude that it is the NO-reactive [2Fe-2S] cluster that is required for DNA binding. This cluster is sensitive to NO, reacting to form mononuclear and dinuclear DNIC species. The nitrosylated form of the protein is unable to bind DNA, clearly indicating a possible mechanism by which NsrR regulates gene expression in response to NO. Future work will be aimed at further characterizing NsrR and identifying its target genes in vivo in S. coelicolor.

**Components model as described in Materials and Methods. (Left) Lower panel: absorbance profile of 2 μM hmpA2 and 10 μM NsrR in 50 mM Tris-HCl pH 7.0, 100 mM NaCl after centrifugation at 16,000 (C), 18,000 (O) and 20,000 (A) rpm. Upper panel: Residual profile of the difference between the data and fitted curves. (Right) Upper panel: absorbance profile of 2 μM hmpA2 in 50 mM Tris-HCl pH 7.0, 100 mM NaCl after centrifugation at 16,000 (C), 18,000 (O) and 20,000 (A) rpm. Upper panel: Residual profile of the difference between the data and fitted curves.

**Materials and Methods**

**Purification of NsrR**

The nsrR gene was amplified from S. coelicolor genomic DNA using primers nsrRFor (5'-CATATGCCCTTGGCAAATGGTT-CAC) and nsrRRev (5'-TCATCCGGAGGGCCCAGG), cloned into pUC19 for sequencing and then sub-cloned into pET21a to construct plasmid pNsrR. *E. coli* strain BL21 was transformed with pNsrR and single transformant colonies were inoculated into 3×10 ml LB medium and grown overnight with shaking at 37°C. The overnight cultures were used to inoculate 3×1 liter LB in 2.5 liter flasks and cultures were grown with shaking at 37°C to mid log phase. Following induction with 0.1 mM IPTG, and further incubation with shaking at 30°C for 2.5 hours, cells were harvested, resuspended in buffer A (100 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM DTT) to a total volume of 40 ml and passed three times through a French pressure cell at 1000 Psi. The crude lysate was then centrifuged in a Sorvall SS-34 rotor at 18,000 rpm for 45 minutes to generate a clarified NsrR-containing extract. The clarified extracts were applied to a 5 ml Hitrap Heparin HP column (GE Healthcare) at room temperature and the column was washed in buffer A at 5 ml/min until the UV trace became stable. Proteins were eluted using a gradient of buffer B (100 mM Tris-HCl pH 8.0, 1 M NaCl, 5 mM DTT) equivalent to 20 column volumes. Fractions containing NsrR were identified by their dark brown color and by SDS-PAGE. The most concentrated fractions were applied to a Superdex 75 26/60 column (GE Healthcare) using a 2 ml superloop at a flowrate of 1.5 ml/min. Fractions (1.5 ml) were collected and analysed by SDS-PAGE; those containing pure NsrR were pooled and stored at −20°C in buffer B+5% glycerol until use. Apo-NsrR was prepared by incubating the purified protein with EDTA and ferricyanide in a molar ratio of protein:EDTA:ferricyanide (1:50:20) at 25°C for 20 minutes [29] before buffer exchanging the protein with buffer A. Loss of cluster was verified by measuring the UV-visible absorbance spectrum of the treated protein, as described below. Bipyridyl treatment of purified NsrR (10 μM) was carried out at a final bipyridyl concentration of 1 mM in DNA binding buffer (10 mM Tris-HCl, pH 7.5, 60 mM KCl) on ice for 30 minutes. The protein was then used directly in bandshift reactions (see below).
Near UV-CD, UV visible CD, UV-visible absorbance and EPR spectroscopy

NsrR was exchanged into buffer C (50 mM Tris pH 7.0, 100 mM NaCl) to remove excess DTT. A Jasco J-810 spectrophotometer, scanning at 200 nm min\(^{-1}\), was used to record the CD spectrum of NsrR using a standard 1 cm cuvette. The UV-visible absorbance spectrum of a 200 µL sample of NsrR was measured between 250 and 600 nm on a Hitachi V-3310 dual beam spectrophotometer. After recording the spectrum of the oxidised protein 10 µL of a 2 mM NO stock solution was added to give a final concentration of 100 µM NO in the cuvette. The spectrum of the NO incubated NsrR was immediately recorded and corrected. Absorbance traces were normalized to account for the small increase in volume after addition of NO.

X-band EPR spectra were recorded on a Bruker ELEXSYS 500 fitted with a Bruker Super-High-Q Cavities: ER 4122SHQE and the temperature was controlled using an Oxford Instruments ESR-9 flow cryostat. Spectra were recorded at 10 K, with a frequency of 9.437 GHz, power of 2 mW. 150 mL of NO decomposed 100 mM MAHMA-NONOate to ensure an excess of NO. The small increase in volume after addition of NO. and corrected. Absorbance traces were normalized to account for the small increase in volume after addition of NO.

Bandshift assays

DNA fragments carrying the hmpA1 and hmpA2 promoters were PCR amplified using S. coelicolor genomic DNA with primers phmpa1IF (5’-GACCGAGGCCTCCCTGGA), phmpa1IR (5’-GGGATCTACAGATGCTTGTTTTCC) and phmpa2IF (5’-TCCGGGCGCCTGCTTGCTCTG) and phmpa2IR (5’-GATGGTGCCGGAGCCAGGCT) and cloned into pUC19 for sequencing. The fragments were excised from pUC19 with EcoRI and HindIII, gel purified twice and ~1 µg of each fragment was radiolabelled using Klenow and a mixture of [\(^32\)P]dATP (Perkin Elmer) and cold dTTP, dGTP and dTTP for 15 minutes at room temperature. Radiolabelled probes were purified on QiaGene nucleotide removal columns and stored at ~20 °C. Purified NsrR protein was diluted in 50 mM Tris-HCl (pH 7.0) buffer or 50 mM Tris-HCl (pH 7.0) buffer saturated with NO gas (2 mM NO stock solution), to a final concentration of 500 nM. Bandshift reactions (10 µL) was either mixed with 50 µL of NO saturated buffer C or with 50 µL of decomposed 100 mM MAHMA-NONOate to ensure an excess of NO. In samples where NO was omitted, 50 µL of buffer C was added as a control. Spin quantification was carried out using the method of Aasaa and Vännigard as described previously, using 1 mM of aqueous Cu(II)/H₂O₂ as a concentration standard [32,33].

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