



BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH

www.bjournal.com.br

ISSN 0100-879X

Volume 45 (2) 93-178 February 2012

**BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION**

Braz J Med Biol Res, February 2012, Volume 45(2) 113-117

doi: 10.1590/S0100-879X2012007500006

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The Brazilian Journal of Medical and Biological Research is partially financed by



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Expression and characterization of an N-truncated form of the NifA protein of *Azospirillum brasilense*

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Abstract

Azospirillum brasilense is a nitrogen-fixing bacterium associated with important agricultural crops such as rice, wheat and maize. The expression of genes responsible for nitrogen fixation (*nif* genes) in this bacterium is dependent on the transcriptional activator NifA. This protein contains three structural domains: the N-terminal domain is responsible for the negative control by fixed nitrogen; the central domain interacts with the RNA polymerase σ^{54} factor and the C-terminal domain is involved in DNA binding. The central and C-terminal domains are linked by the interdomain linker (IDL). A conserved four-cysteine motif encompassing the end of the central domain and the IDL is probably involved in the oxygen-sensitivity of NifA. In the present study, we have expressed, purified and characterized an N-truncated form of *A. brasilense* NifA. The protein expression was carried out in *Escherichia coli* and the N-truncated NifA protein was purified by chromatography using an affinity metal-chelating resin followed by a heparin-bound resin. Protein homogeneity was determined by densitometric analysis. The N-truncated protein activated *in vivo nifH::lacZ* transcription regardless of fixed nitrogen concentration (absence or presence of 20 mM NH_4Cl) but only under low oxygen levels. On the other hand, the aerobically purified N-truncated NifA protein bound to the *nifB* promoter, as demonstrated by an electrophoretic mobility shift assay, implying that DNA-binding activity is not strictly controlled by oxygen levels. Our data show that, while the N-truncated NifA is inactive *in vivo* under aerobic conditions, it still retains DNA-binding activity, suggesting that the oxidized form of NifA bound to DNA is not competent to activate transcription.

Key words: Biological nitrogen fixation; *Azospirillum brasilense*; NifA protein

Introduction

The α -proteobacterium *Azospirillum brasilense* is a diazotroph found in association with important agricultural crops (1). This bacterium has been recently recommended as a plant growth-promoting inoculant for maize and wheat in Brazil. *A. brasilense* is able to reduce dinitrogen to ammonium by the nitrogenase enzyme complex, which consists of two proteins: dinitrogenase reductase and dinitrogenase, encoded by the *nifHDK* genes. In order to have an active nitrogenase enzyme, other *nif* gene products are necessary (2). In *A. brasilense*, as well as in many other diazotrophs, the *nif* genes are transcribed by the RNA polymerase holoenzyme containing the σ^{54} factor, which also requires a specific transcriptional activator, i.e., the NifA protein (1). NifA binds to an upstream activator sequence, characterized by a TGTN₁₀ACA motif, which is located about 100 bp upstream from the *nif* gene promoters

(3). The NifA then contacts the σ^{54} -RNA polymerase holoenzyme bound to the downstream promoter through a DNA loop and catalyzes open complex formation in an ATP-driven reaction (2). NifA activity is controlled by ammonium and oxygen in diazotrophic proteobacteria allowing *nif* expression when nitrogen fixation is required. In the α -proteobacterium *A. brasilense*, this regulation does not involve the anti-activator NifL, which controls NifA in most of the γ -proteobacteria such as *Klebsiella pneumoniae* and *Azotobacter vinelandii* (1,2).

The NifA proteins from many diazotrophs are structurally similar, showing three functional domains linked by two interdomains (2,4). The amino-terminal (N-terminal, GAF) domain is less conserved among the microorganisms, showing a variable number of amino acids, and in several diazotrophs this domain is involved in the autogenous control

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Received September 6, 2011. Accepted January 9, 2012. Available online January 27, 2012. Published February 17, 2012.

of NifA activity by fixed nitrogen (5-7). The central domain has about 240 amino acids, is highly conserved, and is involved in the interaction with the RNA polymerase σ^{54} factor, showing an AAA motif involved in ATP hydrolysis (8). The carboxy-terminal domain (C-terminal) has about 65 to 130 amino acids and contains a conserved helix-turn-helix motif responsible for DNA binding (2,4). The N-terminal and the central domains are linked by the Q interlinker (QL) sequence. The central and C-terminal domains are linked by an inter-domain region, named IDL. In *A. brasilense*, as well as in other members of the α -proteobacteria, at the end of the central domain and part of the IDL inter-domain there is a cysteine motif most probably involved in oxygen sensitivity (5).

In this study, we describe the expression, purification and *in vitro* and *in vivo* characterization of an N-truncated form of the *A. brasilense* NifA protein.

Material and Methods

Reagents

Chemicals were analytical or molecular biology grade and were purchased from Merck (Germany), J.T. Baker (Netherlands), Sigma (USA) or Invitrogen (USA). Restriction enzymes were from Fermentas/Thermo Scientific (USA) or Invitrogen. Oligonucleotides were from Invitrogen or IDT (USA). HiTrap chelating chromatographic columns and HiTrap heparin were furnished by GE Healthcare (Sweden).

Cloning of the *A. brasilense nifA* gene

The sequence encoding the central and C-terminal domains of the NifA protein was amplified using the primers Ab5'CCT (5'-CCCATATGAACGACGTGGTCTGCAC-3') and Ab3'CCT (5'-GGTGGATCCCGCTACATGAT-3'), which respectively introduced *Nde*I and *Bam*HI restriction sites (underlined). The amplified sequence corresponds to the 1038 to 2383 nucleotide according to the *A. brasilense nifA* sequence placed in GenBank (accession No. X60714.1). This sequence encodes the *A. brasilense* NifA protein lacking its first 201 amino acids, which include the N-terminal domain and part of the QL region. The amplified fragment (ΔN -*nifA*) was cloned into the vector pTZ57R/T (Thermo Scientific), yielding pCN57RCCT. This plasmid was then cleaved with *Nde*I and *Bam*HI and the released fragment was subcloned into the pET28a vector (Novagen, USA), generating the recombinant plasmid pCNpETCCT. To construct pCNK6CCT, ΔN -*nifA* was released from pCNpETCCT as an *Xba*I/*Hind*III fragment and cloned into pDK6 (9). The amplified fragment was sequenced to confirm gene integrity.

Expression and purification of the *A. brasilense* His-tagged N-truncated NifA protein (ΔN -NifA)

The recombinant plasmid pCNpETCCT was introduced

into *Escherichia coli* strain BL21(DE3) (10), and protein induction was carried out using 0.5 mM IPTG for 16 h at 18°C in 300 mL Luria Bertani (LB) medium. After induction, cells were harvested by centrifugation at 4700 *g* for 10 min at 4°C, and then resuspended in 1 mL sonication buffer (0.5 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM DTT) per gram of wet cells in the presence of 1 mM PMSF. The cells were lysed by sonication in 10 cycles of 20 s, and the crude extract (102 mg total protein) was centrifuged at 12,000 *g* for 15 min at 4°C to remove cell debris and insoluble proteins.

Protein purification was performed using a 1 mL HiTrap Ni²⁺ chelating column equilibrated with Tp buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, 0.1 mM DTT) containing 50 mM imidazole. After loading the soluble protein extract (48 mg protein), the column was washed with 2 volumes of Tp with 50 mM imidazole and 2 volumes of Tp with 100 mM imidazole. The protein was eluted by a stepwise increase in imidazole concentration (200, 250, 300, 400, 1000 mM) in Tp buffer with 1 column volume of each concentration. Fractions of 1 mL were collected and analyzed by SDS-PAGE (11). The ΔN -NifA protein was eluted at 250 and 300 mM imidazole. These fractions were pooled and diluted with 1 volume of 50 mM Tris-HCl, pH 8.0, and then loaded onto a 1 mL HiTrap heparin column previously equilibrated with Tp3 buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl). After washing the column with 3 volumes of Tp3, protein was eluted by a stepwise increase in NaCl concentration (500, 600, 700, 800, 900, 1000, 1500 mM) in 50 mM Tris-HCl, pH 7.5, with 1 column volume of the first two and the last concentrations and a 1/2 column volume of the other concentrations. Protein eluted with 700 mM NaCl was used for the DNA-binding electrophoretic mobility shift assay (EMSA).

Protein analysis

Protein was quantitated by the method of Bradford (12) using bovine serum albumin as standard. The protein homogeneity was determined by densitometric analysis of the protein bands on SDS-PAGE (11) stained with Coomassie blue R-250.

EMSA

A 345-bp DNA fragment corresponding to region 2510 to 2855 of the *Herbaspirillum seropedicae nifB* promoter (13) was amplified using primers labeled with fluorescent FAM and VIC. The reaction contained 0.1 μ M labeled-DNA (*H. seropedicae nifB* promoter region), DNA-binding buffer (10 mM Tris-acetate, pH 8.0, 8 mM MgCl₂, 10 mM potassium acetate, 1 mM DTT, 3.5% (w/v) PEG 8000) and increasing concentrations of the purified protein in a final volume of 15 μ L. The reactions were incubated for 10 min at room temperature. After incubation, 5 μ L 15% glycerol containing 0.01% bromophenol blue was added and the reaction was loaded onto a 4% non-denaturing polyacrylamide gel in

Tris-glycine buffer (25 mM Tris-base, 192 mM glycine) with 8 mM MgCl₂. Electrophoresis was performed at 60 V for 4 h at 4°C. The gel was visualized on a transilluminator and registered with a video-image- coupled system (UVP).

Transcriptional activation assay of *K. pneumoniae* *nifH::lacZ* fusion

E. coli JM109(DE3) (10) cells carrying plasmid pRT22 (*K. pneumoniae nifH::lacZ*) (14) were transformed with plasmid expressing the *A. brasilense* ΔN-NifA protein. Cells were grown overnight in nitrogen-free Davis and Mingioli (NFD) medium (15) supplemented with 5% LB medium, 20 mM NH₄Cl and antibiotics at 30°C. The cultures were then diluted to absorbance at 600 nm ≈0.2 in NFD containing 0.5 mM IPTG and in the presence or absence of 20 mM NH₄Cl. Cultures were incubated at 30°C for 6-7 h under anaerobiosis, with oxygen replaced with nitrogen or argon. β-galactosidase activity was determined using ONPG as described (16).

Results and Discussion

One major difficulty for *in vitro* analysis of the NifA protein is to obtain an adequate amount of purified protein since the protein is usually expressed at very low concentration under physiological conditions. Moreover, NifA tends to be insoluble when overexpressed, which also contributes to the difficulty of its *in vitro* characterization. The overexpression of the full-length *A. brasilense* NifA in *E. coli* also yielded an insoluble protein regardless of the different conditions used for expression and cell lysis (data not shown). Thus, in the present study, we removed the N-terminal domain of the NifA protein in order to improve solubility of the overexpressed protein. The N-terminal domain is not strictly required for the transcriptional activation, although it is involved in ammonium regulation (6).

The N-truncated NifA protein (ΔN-NifA) was first assayed for *in vivo* transcriptional activation activity in *E. coli* JM109(DE3) carrying plasmids pRT22 (*nifH::lacZ*) and pCNK6CCT (ΔN-NifA expressed from the *tac* promoter). These assays were carried out in the presence or absence of 20 mM NH₄Cl, and under air (21% O₂) or anaerobiosis (Table 1). β-galactosidase activity was only observed in cells carrying both plasmids, in the absence of O₂, indicating that the ΔN-NifA protein is able to activate *nifH* transcription under these conditions. Regulation by ammonium ion was not observed under these conditions, reinforcing the evidence that the N-terminal domain is involved in ammonium control but not required for transcriptional activation (6). On the other hand, regulation by O₂ was retained by the ΔN-NifA, most probably because the cysteine motif is still present in this form.

The purification of ΔN-NifA from *A. brasilense* fused to a

His-tag sequence was obtained using plasmid pCNpETCCT and expression in *E. coli* BL21(DE3). By performing protein induction at 18°C, about 50% of the expressed protein remained in the soluble fraction of the crude extract, allowing its purification. Two chromatography steps were used: a metal-binding affinity chromatography, using a HiTrap Ni²⁺ chelating column, followed by HiTrap heparin chromatography (Figure 1). This chromatographic procedure yielded 80% pure protein as determined by densitometric analysis

Table 1. Promoter activity of *Klebsiella pneumoniae nifH::lacZ* fusion in *Escherichia coli* JM109(DE3) cells.

Plasmid	β-galactosidase activity (Miller units)			
	+NH ₄ ⁺ /+O ₂	-NH ₄ ⁺ /+O ₂	+NH ₄ ⁺ /-O ₂	-NH ₄ ⁺ /-O ₂
pRT22	ND	38 ± 5	ND	ND
pRT22/pCNK6CCT	90 ± 8	131 ± 8	7450 ± 840	9416 ± 550

ND = activity not detected. β-galactosidase activity was determined as described in Material and Methods in cells carrying plasmid pRT22 (*nifH::lacZ*) and in the presence or absence of plasmid pCNK6CCT (which expresses ΔN-NifA upon a *tac* promoter). + and - indicate presence or absence, respectively, of 20 mM NH₄Cl (NH₄⁺) or air (O₂). Data are reported as means ± SD for at least three independent experiments.

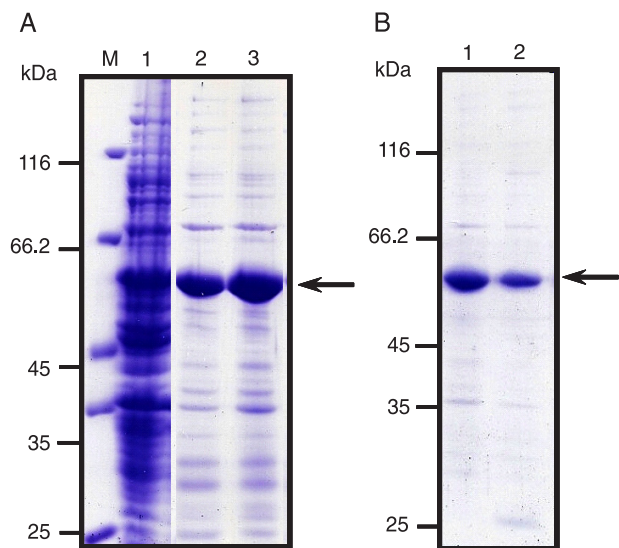


Figure 1. SDS-PAGE analysis of the *Azospirillum brasilense* ΔN-NifA protein expressed in *Escherichia coli* BL21(DE3) and purified as indicated in Material and Methods. *Panel A*, Crude extract (lane 1) and protein fractions eluted with 250 and 300 mM imidazole (lanes 2 and 3, respectively) from HiTrap Ni²⁺ chelating columns. *Panel B*, Protein fractions eluted with 700 and 800 mM NaCl (lanes 1 and 2, respectively) from HiTrap heparin columns. Arrows indicate the ΔN-NifA. Molecular masses are reported in kDa. Proteins were stained with Coomassie blue. Gels were 10% polyacrylamide in SDS-PAGE.

of Coomassie blue-stained SDS-PAGE.

The purified Δ N-NifA was assayed for *in vitro* DNA-binding activity to the *H. seropedicae nifB* gene promoter region using an electrophoretic mobility shift assay (Figure 2). The NifB protein is involved in the synthesis of the nitrogenase FeMo co-factor, and the *nifB* promoter contains a fully characterized NifA binding site (13). In this assay, the fluorescent labeled-DNA containing the *nifB* promoter showed a slower electrophoretic migration in the presence of increasing amounts of the purified *A. brasilense* Δ N-NifA, indicating that the protein was able to bind to the *nifB* promoter DNA. At a protein/DNA ratio of about 12 (1.2 μ M Δ N-NifA), a DNA-protein complex was clearly observed, and in the presence of 3.2 μ M Δ N-NifA, the free DNA band was not observed. This DNA-protein complex was also observed even in the presence of ten times mass excess of calf thymus DNA, although some of the DNA-protein complex was disrupted at higher concentrations of calf thymus DNA (data not shown). These results indicate that the purified Δ N-NifA protein from *A. brasilense* was able to bind to the promoter region of *nifB* from *H. seropedicae* in the presence of oxygen, implying that the DNA-binding activity of NifA is not strictly oxygen sensitive, although higher activity and/or specificity may depend on the oxygen level. Passaglia et al. (17) have previously shown that *A. brasilense* NifA was capable of binding *nifH* *in vitro* under aerobic conditions. In contrast to our study, these investigators used a crude extract of *E. coli* expressing a full-length *A. brasilense* NifA, and they also concluded that the DNA-binding activity was not oxygen sensitive. Nevertheless, to the best of our knowledge, no other report has shown *in vitro* DNA-binding activity using purified *A. brasilense* NifA.

Models for the regulation of NifA activity of *A. brasilense* and *H. seropedicae* in response to the levels of ammonium and oxygen have been proposed (6,7,18,19). According to these models, the N-terminal domain folds over the catalytic central domain inhibiting transcription activation under high ammonium conditions. In the presence of low ammonium concentrations, a PII protein (GlnB for *A. brasilense* or GlnK for *H. seropedicae*) interacts with the N-terminal domain, relieving the inhibition. Our results agree with these models since we did not observe ammonium regulation or dependence on the co-expression of the *A. brasilense* GlnB protein. Regulation by oxygen, in turn, has been proposed to rely on a probable Fe-S cluster located in the central domain and IDL linker (5). Monteiro et al. (7), using an N-truncated form of *H. seropedicae* NifA, also showed that an aerobically purified NifA retained DNA-binding activity. Moreover, Oliveira et al. (18) produced mutants for each of the four cysteine residues of the putative Fe-S cluster

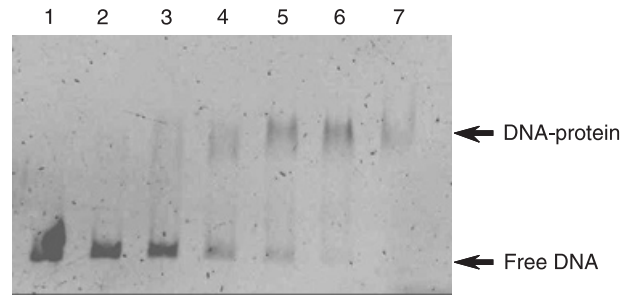


Figure 2. Electrophoretic mobility shift assay using the purified Δ N-NifA protein and *Herbaspirillum seropedicae nifB* promoter region. The procedure was performed as described in Material and Methods. The *nifB* promoter region was labeled with the fluorophores FAM and VIC and incubated with increasing concentrations of purified His-N-truncated NifA (lane 1, absence of Δ N-NifA; lanes 2 to 7, 0.16, 0.4, 0.8, 1.2, 1.6, 3.2 μ M Δ N-NifA, respectively). Arrows indicate unbound DNA and the DNA-protein complex.

binding motif in *H. seropedicae* NifA, and observed that the mutant proteins were still able to bind DNA although the ability to activate transcription was completely abolished. Oxidation of the Fe-S cluster may trigger conformational changes affecting nucleotide binding and/or hydrolysis necessary for transcriptional activation, as proposed (18,20). Therefore, under aerobic conditions, the oxidized Fe-S cluster would lead to a transcriptionally inactive form of NifA possibly compromising the catalytic activity of the central domain. It is possible that the affinity of the oxidized NifA for *nif* promoters is also decreased, but this possibility has not been experimentally tested. Our data show that while the N-truncated NifA is inactive *in vivo* under aerobic conditions, it still retains DNA-binding activity, suggesting that the oxidized form of NifA bound to DNA is not competent to activate transcription. A similar result was also reported for the *H. seropedicae* NifA, thus raising the possibility that a common mechanism of oxygen-dependent NifA activity control operates in both *A. brasilense* and *H. seropedicae*.

Acknowledgments

We are grateful to Roseli Prado, Julieta Pie, Marilza Lamour, Valter Baura, and Patrícia Sotomaio for technical assistance. We are also grateful to Dr. M. Geoffrey Yates (UK) for reading the manuscript. Research supported by INCT-Fixação Biológica de Nitrogênio, CNPq, CAPES, and Institutos do Milênio.

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