Expression and functional analysis of an N-truncated NifA protein of *Herbaspirillum seropedicae*

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**Abstract** In *Herbaspirillum seropedicae*, an endophytic diazotroph, *nif* gene expression is under the control of the transcriptional activator NifA. We have over-expressed and purified a protein containing the central and C-terminal domains of the *H. seropedicae* NifA protein, N-truncated NifA, fused to a His-Tag sequence. This fusion protein was found to be partially soluble and was purified by affinity chromatography. Band shift and footprinting assays showed that the N-truncated NifA protein was able to bind specifically to the *H. seropedicae* nifB promoter region. In vivo analysis showed that this protein activated the *nifH* promoter of *Klebsiella pneumoniae* in *Escherichia coli* only in the absence of oxygen and this activation was not negatively controlled by ammonium ions.

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**Key words:** NifA protein; Transcriptional activator; Nitrogen fixation; *Herbaspirillum seropedicae*

1. Introduction

*Herbaspirillum seropedicae* is an endophytic diazotroph found in association with important agricultural crops [1]. It is classified by rDNA analysis as a member of the β subclass of Proteobacteria [2]. Nitrogen fixation in this organism occurs under microaerobic conditions and the expression of the nitrogenase structural genes nif/HDK as well as the *nifB* gene is under control of the transcriptional activator NifA [3–5].

The NifA protein activates the transcription of σ^5^-dependent *nif* and other promoters [6] and it also activates the *Rhizobium meliloti* and *Bradyrhizobium japonicum* fix*ABC* genes [7,8]. The NifA proteins have a modular structure typical of σ^5^-dependent promoter-activating proteins. The N-terminal domain is involved in regulatory functions and has a low similarity among NifA proteins. The central domain has two potential nucleotide triphosphate-binding sites as revealed by sequence analysis and is responsible for interaction with the σ^5^-RNA polymerase complex [6]. The C-terminal domain contains a helix-turn-helix motif probably involved in DNA-binding. These domains are usually linked by the short interlinker sequences Q (N-terminal to central) and ID (central to C-terminal).

**Klebsiella pneumoniae** and *Azotobacter vinelandii* NifA pro-

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**Abbreviations:** DTT, dithiothreitol; ONPG, o-nitrophenyl-galactoside; IPTG, isopropyl β-thiogalactopyranoside; DMS, dimethyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW, molecular weight markers

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washed by a stepwise increase in imidazole concentration (10, 20, 30, 40, 50 mmol/l) in TP2 buffer (50 mmol/l Tris-Cl pH 6.3, 500 mmol/l NaCl) using 10 volumes of 10 mmol/l imidazole and 5 volumes of the other concentrations. The column was then washed with five volumes of TP3 buffer (50 mmol/l Tris-Cl pH 8.0, 100 mmol/l KCl; 10% glycerol; 1 mmol/l DTT) containing 50 mmol/l of imidazole. The His-Tag-N-truncated protein was eluted with one-two volumes of TP3 buffer containing 100 mmol/l of imidazole.

2.2. Protein analysis

The protein quantification was by the Bradford method [14] using bovine serum albumin as the standard. The protein purity was analyzed by SDS-PAGE [15] and the proteins were identified by staining with Coomassie blue R-250. The β-galactosidase activity was determined using ONPG as described by Miller [16].

2.3. Band shift assay

Binding of the N-truncated NifA to the H. seropedicae nifH promoter region was assayed in vitro as described [17]. A 345 bp DNA fragment corresponding to regions 2510 and 2855 of the H. seropedicae nifH promoter [3] was end-labelled with 32P using T4 polynucleotide kinase [18].

Each reaction mixture contained 6×10^{-11} mol of 32P-labelled nifH promoter region, 2×10^{-12} mol of the N-truncated NifA protein and the indicated amounts of calf thymus DNA or unlabelled nifH promoter in a DNA-binding buffer (10 mmol/l Tris-Acetate pH 8.0, 8 mmol/l MgCl2, 10 mmol/l potassium acetate, 1 mmol/l DTT and 3.5% (w/v) PEG 8000) in a total volume of 15 μl. The reaction was incubated for 10 min at room temperature (approximately 20°C). After incubation, Ficoll (final concentration of 6%, w/v) was added and the whole reaction was loaded onto a 4% non-denaturing polyacrylamide gel in TAE buffer [18] with 8 mmol/l MgCl2. The electrophoresis was at 60 V for 2 h at 4°C. Autoradiographs were developed on X-ray films. Densitometer analyses were carried out using Personal Densitometry SI of Molecular Dynamics.

2.4. Footprinting assays

These were conducted as described [19,20]. The reaction mixtures had the same composition as those for the band shift experiments. After 10 min incubation, DMS (0.05% final concentration) was added, incubated for a further 5 min and the reaction was stopped by adding 5 mmol/l ammonium acetate, 1 mol/l β-mercaptoethanol and 10 μg/ml yeast RNA (final concentrations). The reaction mixtures were precipitated with ethanol, washed with 95% ethanol and dissolved in 1 mol/l piperidine (30 μl). The DNA was cleaved at methylated guanine residues by heating at 90°C for 20 min, lyophilized twice and dissolved in formamide dye (Monteiro and Chubatsu, unpublished results). Since H. seropedicae NifA has domains structurally similar to those of K. pneumoniae NifA which were partially soluble when expressed separately as fusion proteins [10–12], we constructed a recombinant plasmid capable of expressing a N-truncated form of the NifA containing the central domain, the ID linker and the C-terminal domain of H. seropedicae fused to a hexa-histidine tag at its N-terminus.

The N-truncated NifA protein was tested in vivo for the capacity to activate the expression of the K. pneumoniae nifH promoter (nifH::lacZ fusion). E. coli strain JM109(DE3) transformants, hosting plasmids pET28a (vector), pRAM1 (His-Tag-NifA), pRAM2 (His-Tag-N-truncated NifA) or pRT22 (nifH::lacZ) alone or in combinations were analyzed for their β-galactosidase activity after induction in the presence or absence of oxygen or NH4Cl (Table 1). The His-tagged NifA protein (pRAM1) was unable to activate the K. pneumoniae nifH::lacZ fusion under the assay condition, confirming results by Souza et al. [23]. A β-galactosidase activity was only detected when pRAM2 was present, indicating that transcriptional activation was due to the expressed N-truncated NifA. SDS-PAGE analysis confirmed the expression of the proteins in all conditions tested (not shown). Promoter activation as revealed by β-galactosidase activity was detected only when cells were induced under a nitrogen gas phase, confirming the oxygen-sensitivity of H. seropedicae NifA [23]. These results are also consistent with the hypothesis that the oxygen-sensitivity does not involve the N-terminal domain of NifA but rather the central domain and the ID linker, probably involving the putative metal-binding and redox status-sensing cysteine motif, Cys-X1-Cys-X2-Cys-X3-Cys [6]. On the other hand, nifH expression was not inhibited by ammonia. This result also agrees with the previous observation [23] that the N-terminal domain of H. seropedicae NifA is involved in ammonia-sensing. The N-terminal domain of the NifA protein may interact with a nitrogen-sensing protein

Table 1

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristic</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>pET28a</td>
<td>Expression vector</td>
<td>1</td>
</tr>
<tr>
<td>pRAM1</td>
<td>His-Tag-NifA</td>
<td>1</td>
</tr>
<tr>
<td>pRAM2</td>
<td>His-Tag-N-truncated NifA</td>
<td>0</td>
</tr>
<tr>
<td>pRT22</td>
<td>nifH::lacZ</td>
<td>0</td>
</tr>
<tr>
<td>pET28a/pRT22</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>pRAM1/pRT22</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>pRAM2/pRT22</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

JM109 (DE3) cells with plasmids were induced with 0.5 mmol/l IPTG for 8 h at 30°C in the presence or absence of 20 mmol/l NH4Cl, under air or N2, and assayed for β-galactosidase activity. The data are averages of five experiments, with S.D.s of less than 10%.

*There was not a significant decrease in the NH4Cl concentration during the induction time.

3. Results and discussion

Attempts to purify NifA proteins from several microorganisms have been unsuccessful due to the insolubility of the over-expressed protein [21,6]. The H. seropedicae NifA protein also aggregated when over-expressed in E. coli as a fusion protein either with glutathione S-transferase or a His-Tag sequence (Monteiro and Chubatsu, unpublished results). Since H. seropedicae NifA has domains structurally similar to those of K. pneumoniae NifA which were partially soluble when expressed separately as fusion proteins [10–12], we constructed a recombinant plasmid capable of expressing a N-truncated form of the NifA containing the central domain, the ID linker and the C-terminal domain of H. seropedicae fused to a hexa-histidine tag at its N-terminus.

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or signal molecule which, in turn, controls the interaction between the central domain and the RNA polymerase. In *K. pneumoniae* and *A. vinelandii*, NifL senses the cellular redox and ammonia status indirectly, inhibiting the NifA activity possibly by an interaction with the central domain of the NifA protein [24,25]. In *H. seropedicae* and *Azospirillum brasilense* where NifL has not been found, regulation of the NifA activity by the ammonia may involve the PII protein, the product of the *glnB* gene [9,26] or possibly the PII parologue, the GlnK protein.

Since an active N-truncated NifA protein was expressed in vivo, a procedure to purify and analyze it in vitro was developed. A high level of N-truncated NifA expression was obtained after induction of the promoter by IPTG (Fig. 1) and, although most of the expressed protein was in an insoluble form, a fraction (5%) remained in the supernatant after low speed centrifugation. Induction with 0.5% lactose yielded essentially the same amount of the protein as 0.5 mmol/l IPTG, however, a higher percentage of soluble protein was obtained following induction by lactose (up to 40%). Presumably when induced by IPTG, the protein does not fold correctly and aggregates, whereas lactose, producing a slower induction, allows correct folding. The soluble His-Tag-N-truncated NifA protein was purified by affinity chromatography.

Changes in the buffer composition such as ionic strength, salt, presence or absence of DTT or glycerol and different concentrations of imidazole were made to eliminate contaminant proteins non-specifically bound to the Hi-Trap-Chelating-Ni\(^{2+}\) column. Heparin sepharose and ionic resins (SP-Sepharose and Q-Sepharose) were also tested but did not improve the purification. The conditions described here were essential to yield a highly purified protein as detected by SDS-PAGE (lane 4, Fig. 1).

The purified N-truncated NifA protein was assayed in vitro for DNA-binding activity. The protein bound to a \(^{32}\)P-labelled 345 bp DNA fragment of the *H. seropedicae nifB* promoter region [3], as revealed by a decrease in the migration rate of the DNA fragment (Fig. 2A). In this experiment, the binding of the DNA was observed using a molar protein/
DNA ratio of about 30 and the complex was stable even in the presence of a high excess of calf thymus DNA (2 μg, about 150 times excess in mass). The protein/DNA ratio needed to observe a minimal DNA-binding varied with the protein preparation, suggesting that some preparations were partially inactive. Competition with the same unlabelled nifB promoter DNA fragment titrated out the radioactively labelled DNA (Fig. 2B). Densitometric analysis showed a decrease of 77% of the N-truncated-NifA protein-[32P]nifB complex when 0.2 pmol (three times excess) unlabelled nifB DNA was present. These results indicate that the purified protein binds specifically to the nifB promoter region. The N-truncated NifA also bound the nifH promoter region of R. meliloti in the presence of calf thymus DNA (data not shown).

The nifB promoter region has two upstream activator sites (UAS), by sequencing analysis [3]. Both UAS sites were recognized by N. truncata NifA in vitro (Fig. 3). The guanine residues of the TGT motifs of both NifA-binding sites were protected from DMS-dependent methylation by the N-truncated NifA protein, similar to that observed in vivo with the K. pneumoniae NifA protein [5]. This result showed that the N-truncated NifA protein binds to both UAS sites present in the nifB promoter region, indicating that the N-truncated form of H. seropedicae NifA is capable of recognizing and binding the NifA-binding sites in vitro.

The DNA-protein interaction experiments as well as the protein purification were performed under air, with no precautions to exclude oxygen. Since in vivo experiments have shown that the H. seropedicae NifA protein is oxygen-sensitive ([23], Table 1), the observed DNA-binding might have been due to a transcriptionally inactive protein or that oxygen damage was partial and only a functionally active population of N-truncated NifA bound to the nifB promoter. DNA-binding of a functionally inactive protein would be consistent with the hypothesis that the C-terminal domain binds DNA and the oxygen-sensitivity is associated with the cysteine motif located in the central domain and ID linker and may not interfere with the DNA-binding activity. Attempts to visualize in vitro open complex formation [27] of the nifB promoter catalyzed by the purified N-truncated NifA were unsuccessful (data not shown), which suggests that this protein was transcriptionally inactive, although, capable of binding DNA. Another hypothesis is that the NifA protein requires another protein or co-factor for oxygen-sensibility in vivo, suggesting that the protein may be oxygen-sensitive in vivo but not in vitro at least for DNA-binding. If so, this factor is present in E. coli. We have also observed that the DNA-binding activity was lost when the protein was stored in liquid nitrogen (not shown) suggesting that the DNA-binding activity is unstable to freezing. Attempts to purify the His-Tag-N-truncated NifA protein under anaerobic conditions have been unsuccessful to date. The oxygen effect on the protein needs further investigation.

Recently, Passaglia and co-workers [28] published a report that crude extracts of A. brasilense cells, expressing a GST-NifA fusion protein, was capable of binding to the A. brasilense nifH promoter in the presence of oxygen. However, no transcription activation or DNA-binding was reported for the purified protein.

This report is the first to describe an in vitro analysis of a purified oxygen-sensitive NifA protein. Our results showed DNA-binding activity of the expressed protein as well as the functional significance of the nifB promoter NifA-binding regions. They also confirm that the central and C-terminal domains of NifA are involved in the oxygen sensitivity and the ammonia-sensing function is related to the N-terminal domain.

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References