Inter-domain cross-talk controls the NifA protein activity of *Herbaspirillum seropedicae*

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Abstract ***Herbaspirillum seropedicae*** **is an endophytic diazotroph, which colonizes sugar cane, wheat, rice and maize. The activity of NifA, a transcriptional activator of *nif* genes in *H. seropedicae*, is controlled by ammonium ions through a mechanism involving its N-terminal domain. Here we show that this domain interacts specifically in vitro with the N-truncated NifA protein, as revealed by protection against proteolysis, and this interaction caused an inhibitory effect on both the ATPase and DNA-binding activities of the N-truncated NifA protein. We suggest that the N-terminal domain inhibits NifA-dependent transcriptional activation by an inter-domain cross-talk between the catalytic domain of the NifA protein and its regulatory N-terminal domain in response to fixed nitrogen.** © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

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

1. Introduction

Herbaspirillum seropedicae is a member of the β-subclass of Proteobacteria [1] which colonizes internal tissues of sugar cane, wheat, rice and maize [2,3]. *Herbaspirillum* spp. cannot be isolated from soil samples inoculated with high cell numbers, but can be recovered from soil-growing plants suggesting a preferential endophytic association [4]. Recent reports showed that *H. seropedicae* stimulates plant growth although the contribution of biological nitrogen fixation is still unclear [5].

The NifA protein is the transcriptional activator of *nif* gene promoters of Proteobacteria. The regulatory mechanisms of *nifA* expression are variable and depend largely on the environmental availability of fixed nitrogen in free-living and associative diazotrophs or oxygen levels in symbiotic diazotrophs [6]. Oxygen and ammonium in the former group of organisms, or oxygen in the latter, also control the NifA activity by mechanisms not yet well understood. In the γ-Proteobacteria, the NifL protein controls NifA activity in response to ammonium and oxygen [7,8]. NifL is a flavoprotein which is capable of sensing the redox state of the cell and inactivates NifA under high oxygen levels [9,10]. In addition, NifL of *Klebsiella pneumoniae* and *Azotobacter vinelandii* senses the levels of fixed nitrogen via a PII-like protein [11,12]. The NifL protein has not been found outside the γ-Proteobacteria.

In the symbiotic nitrogen-fixing organisms *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, the NifA protein is inactivated in vivo by oxygen, possibly involving the cysteine motif present in the central/C-terminal linker region [6]. In *H. seropedicae* and *Azospirillum brasilense*, of the β- and α-Proteobacteria, respectively, the NifA activity is controlled by ammonium and oxygen levels. In these organisms oxygen inactivation is thought to be similar to that of rhizobia but ammonium control of NifA activity involves the N-terminal domain and the PII protein [13,14]. Insertional inactivation of the *glnB* gene of *A. brasilense*, which encodes the PII protein, produced a nitrogen fixation negative phenotype that was not complemented by constitutively expressed NifA, suggesting that PII is required for NifA activity. N-truncated forms of the NifA protein of *A. brasilense*, however, complemented the Nif^- phenotype of the *glnB* mutant although the activity control by ammonium was not observed [14]. The authors suggested that the N-terminal domain inhibits NifA-dependent transcriptional activation by interacting with the catalytic domain of the NifA protein and that the PII protein relieved this inhibition under low ammonium conditions. A *glnB* mutant of *H. seropedicae* was also Nif^- [15]. Other results suggested that the N-terminal domain of NifA protein may be involved in controlling transcriptional activation in the γ-Proteobacteria [16,17].

In this work, we present evidence that the N-terminal domain of the NifA protein of *H. seropedicae* interacts in vitro with an N-truncated form of the NifA protein producing an inhibition of ATPase and DNA-binding activities of NifA. Protection against proteolysis was observed suggesting direct physical interaction between the domains. These results indicate that the N-terminal domain controls NifA protein activity in *H. seropedicae* in response to fixed nitrogen in a manner similar to the effects of NifL on NifA in the γ-Proteobacteria.

2. Materials and methods

2.1. Plasmid construction and strains used

Plasmid pRAM2 expresses the central and C-terminal domains of the *H. seropedicae* NifA protein [18] and plasmid pRAM3 expresses the amino-terminal domain of NifA from the T7 promoter [19]. *Escherichia coli* strain BL21(DE3)pLysS was used for protein expression.

2.2. Protein purification

The N-truncated NifA of *H. seropedicae* was purified as described

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[18]. The purified protein was dialyzed (three buffer changes for 16 h) against TD1 buffer (50 mmol/l Tris-acetate pH 8.0, 100 mmol/l potassium acetate, 8 mmol/l magnesium acetate, 1 mmol/l dithiothreitol, 50% glycerol) and stored in liquid N_2.

Cell cultures expressing the N-terminal domain of NifA protein were lysed by sonication in TS1 buffer (50 mmol/l Tris–HCl pH 8.0, 500 mmol/l NaCl, 10% glycerol, 1 mmol/l dithiothreitol and 0.5% *N*-lauroyl-sarcosine). The soluble fraction of the cell lysate was loaded onto a Hi-Trap-Chelating-Ni^{2+} affinity column in TS1 buffer. The column was washed with a gradient of 10–50 mmol/l imidazole in TS2 buffer (same as TS1 but pH 6.3), and the protein was eluted with a gradient of 100–500 mmol/l of imidazole in TS1 buffer. The purified protein was dialyzed (three buffer changes for 20 h) against TD2 buffer (50 mmol/l Tris–HCl pH 8.0, 100 mmol/l KCl, 1 mmol/l dithiothreitol and 50% glycerol) and stored at −70°C.

Fractions containing more than 98% pure proteins were used in the experiments.

2.3. Protein analysis

The proteins were analyzed by SDS–PAGE [20] and stained with Coomassie blue R-250. The Bradford method [21] was used for protein quantification. Densitometric analyses were carried out using the Personal Densitometer SI from Molecular Dynamics.

2.4. DNA band-shift assays

The DNA-binding activity of N-truncated NifA was assayed in vitro as described [19]. In these reactions, 0.1 μmol/l of ^{32}P-labeled *H. seropedicae nifB* promoter, 0.5 μg of calf thymus DNA and DNA-binding buffer (10 mmol/l Tris-acetate pH 8.0, 8 mmol/l $MgCl_2$, 10 mmol/l potassium acetate, 1 mmol/l dithiothreitol and 3.5% (w/v) PEG 8000) were mixed in a final volume of 20 μl. The *H. seropedicae nifB* promoter (344 bp, corresponding to positions 2570 and 2850 of the *nifB* gene [22]) was obtained by PCR and contained two NifA-binding sites and the −24/−12 promoter element. The N-truncated NifA and the DNA were incubated together for 10 min at room temperature before the addition of the N-terminal domain. Complex formation was analyzed in a 4% polyacrylamide gel at 80 V using TBE buffer. Autoradiograms were obtained by contact exposure of Kodak BioMax MR film to dried gels for 24 h at −70°C.

2.5. ATPase activity

The ATPase activity of the N-truncated NifA protein was monitored as described by Weiss et al. [23]. The reaction mixtures contained TAP buffer (50 mmol/l Tris-acetate pH 8.0, 100 mmol/l potassium acetate, 8 mmol/l magnesium acetate, 27 mmol/l ammonium acetate, 1 mmol/l dithiothreitol, and 3.5% (w/v) PEG 8000) [24], 0.1 μCi (2 nmol/l) of [α-^{32}P]ATP or [γ-^{32}P]ATP and protein in a final volume of 15 μl. The reaction was initiated by the addition of the labeled ATP and incubated at 30°C (or 37°C where indicated) for 15 min. The reaction mixture (3 μl) was analyzed by thin-layer chromatography (TLC) on PEI-cellulose plates (F-254, 100 micron – Selecto Scientific, GA, USA) developed with 0.3 mol/l potassium phosphate pH 8.0. Autoradiograms were obtained by contact exposure as described above. To determine the effect of the N-terminal domain on ATPase activity of the N-truncated NifA, both proteins were incubated for 5 min in TAP buffer at 30°C prior to the addition of radiolabeled ATP. ATP hydrolysis was determined by densitometry of the autoradiograms.

2.6. Trypsin proteolysis

Proteolysis assays were performed at 30°C for 15 min in TA buffer (50 mmol/l Tris-acetate pH 8.0, 100 mmol/l potassium acetate, 8 mmol/l magnesium acetate and 1 mmol/l dithiothreitol) in a final volume of 20 μl, using 5 μmol/l of N-truncated NifA, 200 ng of trypsin and different amounts of the N-terminal domain. The reactions were stopped by the addition of loading buffer, boiled for 3 min and then loaded on an SDS–PAGE.

3. Results and discussion

The full length NifA protein of *H. seropedicae* is inactive in *E. coli* while an N-truncated form of the protein is transcriptionally active but lacked control by ammonium [13,18]. This control, however, is restored when the N-terminal domain is co-expressed with the N-truncated NifA protein [19]. These results indicate that the isolated N-terminal domain of *H. seropedicae* NifA inhibits the transcriptional activity of the N-truncated NifA in vivo in *E. coli* in response to fixed nitrogen. These observations prompted us to test whether the N-terminal domain could inhibit the DNA-binding and ATPase activities of the N-truncated NifA in vitro.

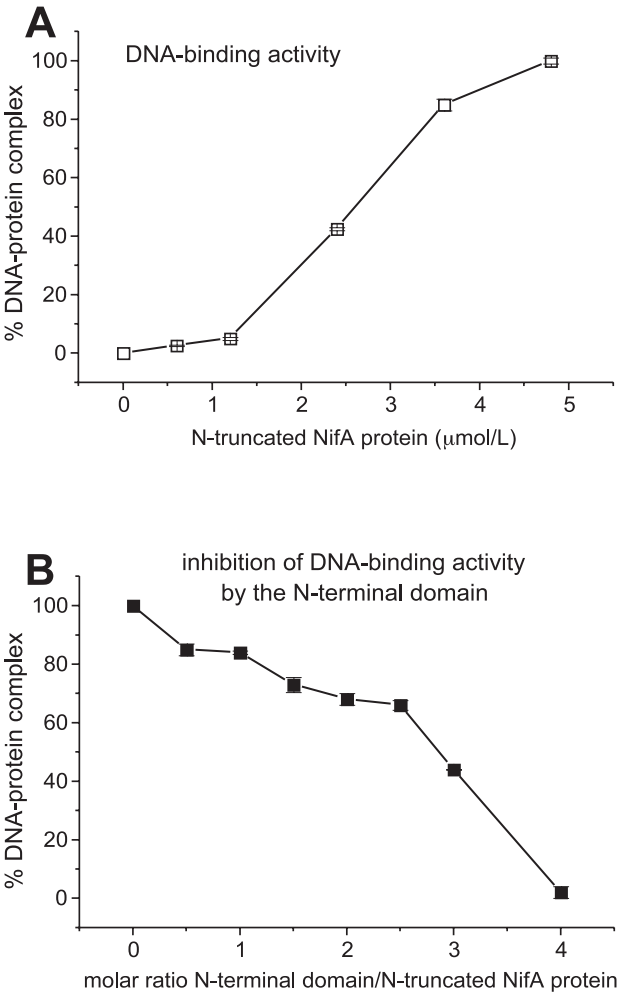

Fig. 1. DNA-binding activity of the N-truncated NifA protein. Band-shift assays were performed as described in Section 2 using *H. seropedicae nifB* promoter region labeled with ^{32}P. A: Labeled DNA was assayed with increasing amounts of purified N-truncated NifA in the presence of calf thymus DNA. B: *H. seropedicae nifB* promoter region, calf thymus DNA and 5 μmol/l of N-truncated NifA protein were assayed with indicated amounts of purified N-terminal NifA domain. The percentage of radioactivity of protein-bound DNA was analyzed by densitometry of the autoradiograms, and it indicates the percentage of the available template incorporated into the complex. Data are the average of three independent experiments.

The purified N-truncated NifA protein bound specifically under air to the *H. seropedicae nifB* promoter, with an apparent K_d of 2 μmol/l (Fig. 1A), as determined by DNA band-shift assays. This DNA-binding activity of the N-truncated NifA apparently exhibited positive cooperativity, a characteristic also observed in enteric NtrC [25]. DNA-binding was inhibited by the N-terminal domain since the DNA–NifA complex was progressively disrupted when the N-terminal domain of NifA was added (Fig. 1B) and about 50% disruption was observed with a three-fold molar excess of the N-terminal

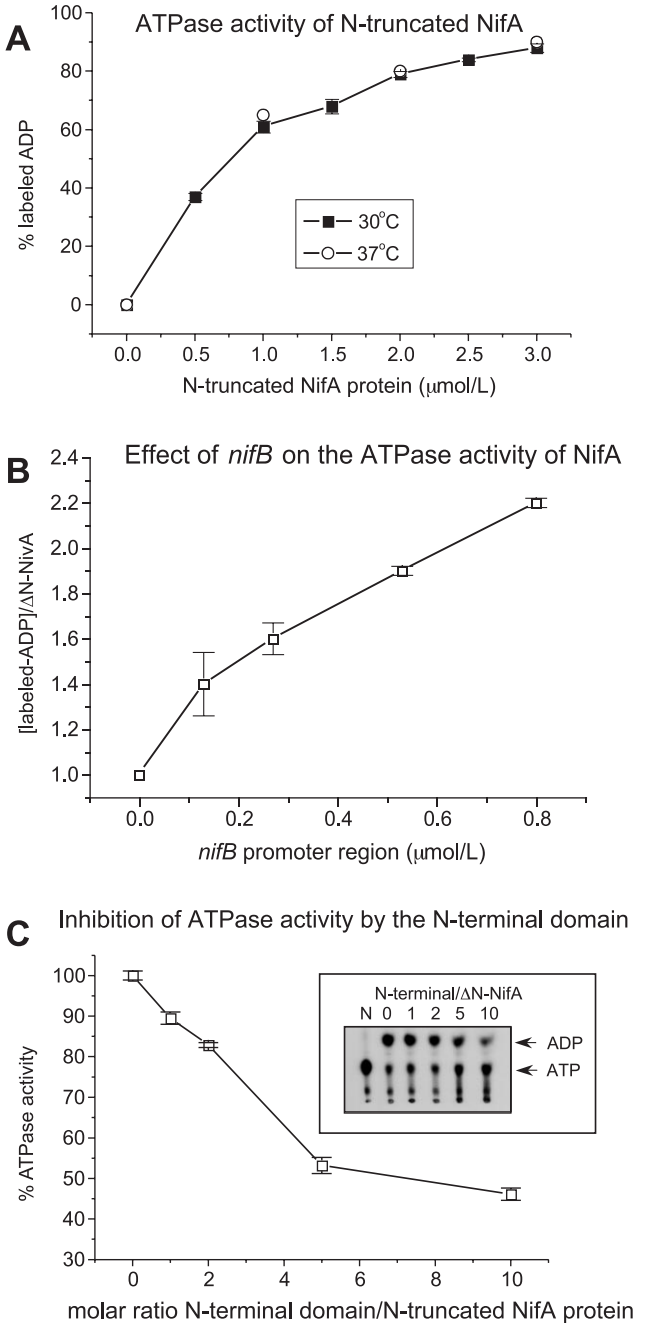

Fig. 2. ATPase activity of the N-truncated *H. seropedicae* NifA protein. A: The hydrolysis of [α-^{32}P]ATP by the N-truncated NifA protein was determined at 30°C or 37°C. B: ATPase activity by the N-truncated NifA protein was determined at 30°C in the presence of increasing amounts of the *H. seropedicae nifB* promoter region. C: ATPase activity was determined at 30°C in the presence of 1.5 μmol/l of N-truncated NifA and increasing amounts of the N-terminal domain; insert: a representative experiment where N indicates labeled ATP incubated without protein and the effect of the N-terminal domain on the ATPase activity of the N-truncated NifA (at molar ratio). Data were obtained by densitometric analysis of TLC autoradiograms and are the average of two (B) or three (A and C) independent experiments.

domain to the N-truncated NifA protein. Bovine serum albumin (BSA) had no effect on the DNA–NifA complex indicating that the disruption of this complex was a specific effect of the N-terminal domain.

The purified N-truncated NifA protein also showed ATPase activity in vitro (Fig. 2A). In contrast to the effect of temperature on the *K. pneumoniae* NifA protein [26], the *H. seropedicae* NifA showed activity also at 37°C (Fig. 2A). This is in agreement with in vivo data, which indicated that the *H. seropedicae* protein is thermostable [13]. The ATPase activity of the N-truncated protein was stimulated by the addition of the *H. seropedicae nifB* promoter (Fig. 2B). This activity increased two-fold when the *nifB* promoter was added to the N-truncated NifA protein at a molar ratio of 0.4 (DNA to protein). To eliminate the possibility of an unspecific effect, calf thymus DNA was added under the same conditions, but no increment in the ATPase activity was observed (data not shown). These results strongly indicated that the observed ATPase activity was due to the N-truncated NifA protein and not due to a contaminant. A positive effect on the ATPase activity by the presence of the DNA binding site has also been reported for the NtrC protein of *K. pneumoniae* [27].

As in the DNA-binding experiments, the addition of increasing amounts of the N-terminal domain, which had no ATPase activity, inhibited the N-truncated NifA ATPase activity (Fig. 2C). A five-fold molar excess of the N-terminal domain inhibited by 50% the ATPase activity of the N-truncated NifA. This inhibition of the ATPase activity was not observed either when 150 μmol/l of N-terminal domain of NifA was assayed with 1 U of commercial alkaline phosphatase or when a 10-fold excess was assayed with a purified *K. pneumoniae* NtrC protein (data not shown), reinforcing the suggestions that the N-terminal domain specifically interacts with the NifA protein producing a negative/inhibitory effect.

To verify physical interaction between the N-terminal domain and the N-truncated NifA protein a limited proteolysis protection assay was performed. When the 41 kDa N-truncated NifA protein was digested with trypsin, two major bands (migration rates of 20 and 25 kDa) were produced (Fig. 3). A change in the digestion pattern was observed in the presence of the N-terminal domain. By increasing the amount of the N-terminal domain in the reaction, a decrease in the proteolysis of the N-truncated NifA protein was observed, indicating resistance towards digestion by trypsin. The

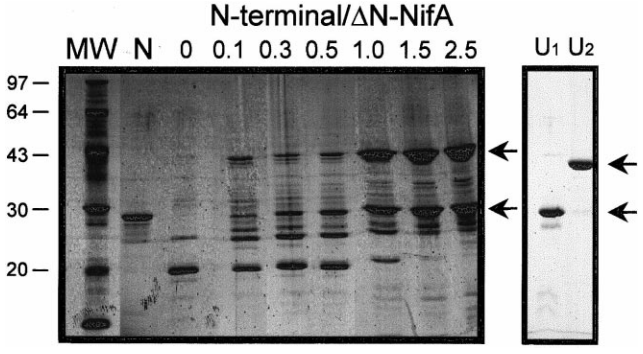

Fig. 3. Proteolysis protection of the N-truncated NifA protein by the N-terminal domain. The N-truncated NifA was incubated with trypsin for 15 min at 30°C in the presence of the indicated molar ratios of the N-terminal domain to N-truncated NifA. Lane N: N-terminal domain of NifA alone incubated with trypsin. Lane U_1: undigested N-terminal domain. Lane U_2: undigested N-truncated NifA. Arrows indicate the undigested proteins. MW indicates molecular weight (kDa).

same result was also obtained with higher levels of trypsin and different incubation periods indicating that the decrease in the proteolysis promoted by the N-terminal domain was real and not due to a lower protease specific activity. Similar results were obtained using proteinase K instead of trypsin (data not shown). On the other hand, the N-terminal domain did not protect BSA from proteolysis either with trypsin or with proteinase K (data not shown). These results indicate a physical interaction between the Central plus C-terminal domains and the N-terminal domain in vitro.

The NifA protein has a typical multidomain structure of an activator of σ^N-dependent promoters in this class of proteins. The central domain catalyzes ATP hydrolysis and interacts with the closed promoter complex, the C-terminal domain binds DNA and the N-terminal domain has regulatory functions [6]. Control of NifA activity in the γ-Proteobacteria involves interaction with NifL, apparently by the formation of a 1:1 inactive complex. Formation of such a complex depends on the levels of fixed nitrogen and oxygen in vivo, which apparently allow NifL to switch from a low affinity form for NifA to a high affinity form [9,25]. The NifL–NifA pair was suggested to be an atypical two component signal transduction system in which the phospho-transfer signaling pathway was substituted for a protein–protein interaction [28].

In the diazotrophs *A. brasilense* and *H. seropedicae*, ammonium control of NifA protein activity does not involve a NifL-like protein but it does require the NifA N-terminal domain [13,14].

In this work we show that the N-terminal domain interacts with the N-truncated NifA protein as demonstrated by proteolysis protection assay, indicating contact with the central and/or C-terminal of the NifA protein. This interaction between the N-terminal domain of NifA and the N-truncated form produces an inhibition of both ATPase and DNA-binding activities in vitro, suggesting that the regulatory N-terminal domain has a similar inhibitory function as the NifL protein in the γ-Proteobacteria. The N-terminal domain of *H. seropedicae* NifA also strongly inhibited ATP hydrolysis by *A. vinelandii* NifA but did not inhibit the *K. pneumoniae* NtrC (data not shown). Hence, the interaction of the N-terminal domain of *H. seropedicae* is specific inasmuch that did not interfere with the ATPase activity of the homologous NtrC, but the *A. vinelandii* NifA protein has sufficient similarity (42%) to allow negative interaction. The observation that the N-terminal domain negatively controls the NifA activity is in agreement with previous in vivo results showing that when the N-terminal domain was expressed at a higher level, the N-truncated NifA was transcriptionally inactive [19] and that may also explain why the full length form of NifA was inactive in *E. coli* [13].

Our results support the view that the N-terminal domain of the NifA protein from *H. seropedicae* controls the activity of the central and C-terminal domains by direct negative interaction, possibly triggered by a ligand. The PII protein has been suggested to be the signal molecule promoting ammonium-dependent NifA inhibition.

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