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Critical spacing between two essential cysteine residues in the interdomain linker of the *Bradyrhizobium japonicum* NifA protein

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A special sequence motif in the *Bradyrhizobium japonicum* NifA protein, consisting of two functionally essential cysteines separated by four other amino acids (Cys-aa$_4$-Cys), has been proposed to be part of a potential metal-binding site [(1988) Nucleic Acids Res. 16, 2207–2224]. Using the techniques of oligonucleotide-directed mutagenesis, we report here that several of the four intervening amino acids can be replaced by others without loss of NifA function. The deletion of one amino acid to give a Cys-aa$_3$-Cys motif renders the protein inactive whereas the creation of a Cys-aa$_5$-Cys motif (one amino acid inserted) still leads to a partially active NifA protein.

Gene regulation; Protein, NifA; Nitrogen fixation; Site-directed mutagenesis; *(Bradyrhizobium japonicum)*

1. INTRODUCTION

The NifA protein is a conserved transcriptional regulator occurring in many diazotrophic bacteria. Its function is to activate transcription from σ^{54}-dependent promoters of nitrogen fixation (*nif/fix*) genes [1]. Certain environmental conditions decide on whether or not these genes will be expressed: in the free-living, facultative anaerobe *Klebsiella pneumoniae* a low concentration of fixed nitrogen compounds (NH_4^+, amino acids) and anaerobiosis are the necessary prerequisites, whereas in the symbiotic root nodule bacteria belonging to the Rhizobiaceae a very low oxygen concentration alone is the major trigger. The regulatory response to these environmental conditions takes place in two ways. First, the expression of the *nifA* gene is subject to control and by this the amount of NifA protein made. Second, the activity of the NifA protein can be modulated. In *K. pneumoniae* the NifA protein is inhibited by the NifL protein in response to oxygen and intermediate concentrations of NH_4^+ [2] whereas in the rhizobia the NifA protein itself is oxygen responsive [3–5]. We have shown previously for the *Bradyrhizobium japonicum* NifA protein that it is active under microaerobic conditions but is irreversibly inhibited at high oxygen concentrations, when its activity was assayed in vivo in a heterologous *Escherichia coli* system [3,6]. The mechanisms by which oxygen-dependent inactivation is achieved are not known. However, since the activity of the *B. japonicum* NifA protein (BjNifA), and not that of the *K. pneumoniae* NifA protein (KpNifA), is dependent on a divalent metal ion, we have proposed that oxygen might interfere with BjNifA activity by changing the oxidation state of a bound metal [7]. This hypothesis is further substantiated by the fact that BjNifA has a potential metal-binding domain consisting of four cysteine residues essential for its function as transcriptional activator whereas three of these four cysteines are missing in KpNifA [7]. Moreover, two of the essential cysteines of BjNifA are located in the so-called interdomain linker region, an extra piece of protein (36 amino acids long) which is located between the central domain and the DNA-binding domain and is absent in KpNifA (see diagram in fig.1).

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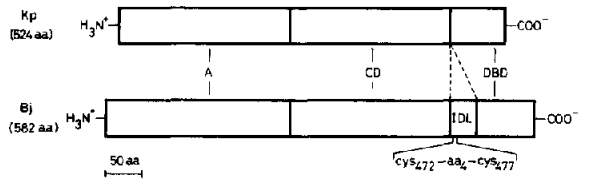

Fig.1. Scheme of the structural domains of two different types of NifA proteins from *K. pneumoniae* (Kp) and *B. japonicum* (Bj) [7,25]. The NH_2-terminal domain (A) with low sequence similarity, the highly conserved central domain (CD) and the COOH-terminal domain containing a DNA-binding motif (DBD) are present in both NifA types. The interdomain linker (IDL, 36 amino acids in length) is specific for the BjNifA type and shows a characteristic arrangement of two cysteine residues potentially involved in metal binding.

The two essential cysteines in the interdomain linker region of BjNifA are separated by four other amino acids. Not only the cysteines but also several amino acids between and adjacent to them are conserved in the NifA proteins from *Rhizobium meliloti* [8,9], *R. leguminosarum* biovar *viciae* [10], *Azorhizobium caulinodans* [11] and from the non-symbiotic, photosynthetic diazotroph *Rhodobacter capsulatus* [12] (see below in fig.3). The aim of the present work was to find out whether some of the four intervening amino acids might be funcationally important just like the cysteines, or whether it is the four amino acid spacing that might be important for a correct positioning of the essential cysteines.

2. MATERIALS AND METHODS

B. japonicum nifA mutants were constructed by oligonucleotide-directed mutagenesis as described previously [7,13]. Mutagenic oligonucleotides of 19 to 26 bp length were synthesized in an Applied Biosystems 380B DNA synthesizer (Foster City, CA, USA) and purified by preparative 12% polyacrylamide gel electrophoresis. The nucleotide exchanges together with the resulting alterations in the BjNifA amino acid sequence and the numbers of the corresponding mutant NifA proteins are shown in fig.2. To obtain the two mutant BjNifA pairs 7657/7658 and 7656/7678, in which single amino acids were replaced by one of two alternative amino acids, we used mixtures of oligonucleotides. Each mutation was verified by sequencing which included the adjacent *nifA* DNA on the fragment used for mutagenesis [7]. The mutant fragments were then substituted for the corresponding wild-type fragment in plasmid pRJ7553 [7]. and the *nifA*-internal *Eco*RI site used for cloning was checked by overlapped DNA sequence analysis.

NifA activity was assayed in an *E. coli* in vivo system by measuring NifA-dependent activation of a translational *nifD'-'lacZ* fusion located on plasmid pRJ1025 [14,15]. The test cultures were grown microaerobically in supplemented NFDM medium as described [3], and β-galactosidase activity was determined according to Miller [16]. Wild-type NifA synthesized constitutively from pRJ7553 was always included in the test series and its activity was defined as one hundred percent.

3. RESULTS AND DISCUSSION

As summarized in figs 2 and 3, four different classes of amino acid alterations were introduced into the BjNifA protein domain encompassing the two conserved cysteines at positions 472 and 477: (i) exchange of the cysteines; (ii) replacement of two of the four intervening amino acids; (iii) shortening of the spacing between the cysteines by three individual deletions each eliminating one of the intervening amino acids; (iv) enlargement of the spacing between the cysteines by introducing an additional amino acid between them. The relevant primary structures and the activities of the modified BjNifA proteins are presented in fig.3. Possible effects of the introduced mutations on mRNA or protein stability were not examined.

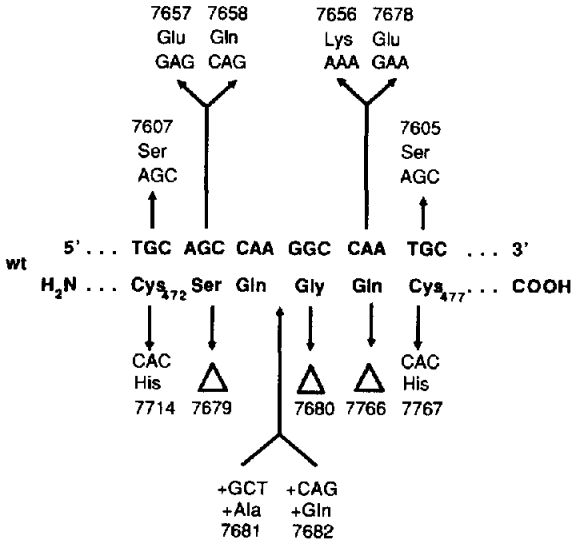

Fig.2. *B. japonicum nifA* point mutations constructed in this work. The wild-type (wt) nucleotide sequence and the deduced amino acid sequence around Cys-472 and Cys-477 in the BjNifA interdomain linker region are shown in bold letters [7,26]. Altered codons, corresponding changes in the protein sequence, and the numbers of the resulting mutant NifA proteins are indicated above or below the wild-type sequence. Deletions are indicated by Δ, insertions by +.

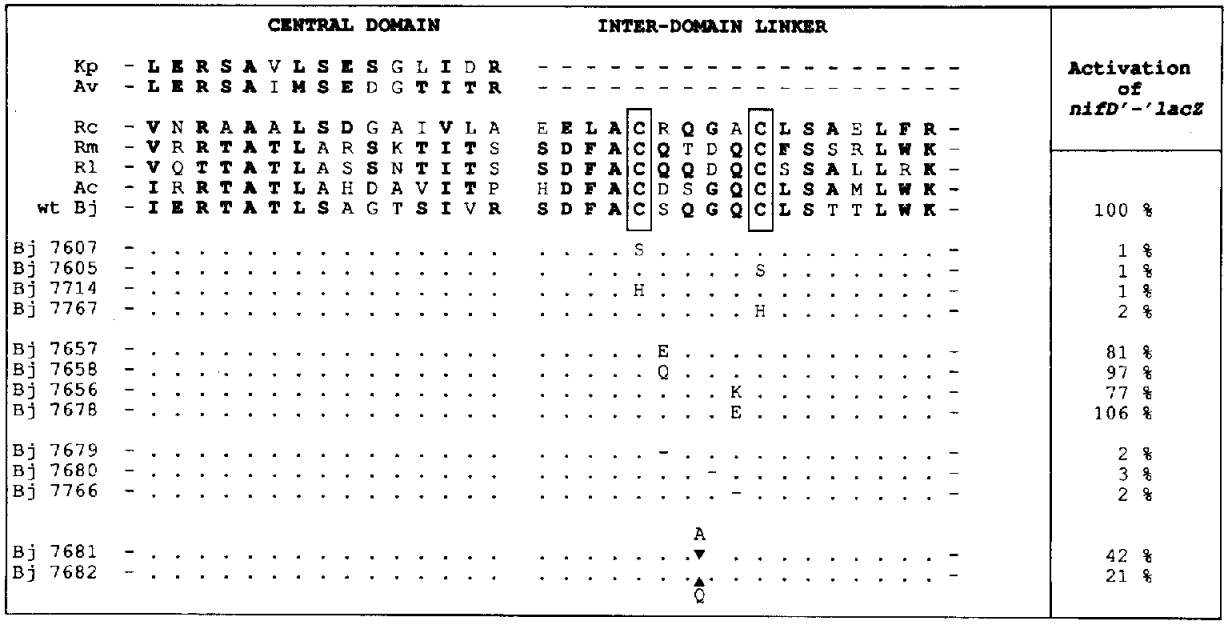

Fig.3. Primary structure of wild-type (wt, [7]) and mutated *B. japonicum* (Bj) NifA interdomain linker region, and activation of the *nifD'-'lacZ* fusion by the corresponding NifA proteins. For comparison, the relevant NifA amino acid sequences of *K. pneumoniae* (Kp [8,25]), *Azotobacter vinelandii* (Av [27]), *Rb. capsulatus* (Rc [12]), *R. meliloti* (Rm [8,9]), *R. leguminosarum* biovar *viciae* (Rl [10]) and *A. caulinodans* (Ac [11]) are shown above the wild-type BjNifA sequence. Functionally homologous amino acids present at corresponding positions in two or more of the sequences are indicated in bold letters. In the interdomain linker region, which is absent in the NifA proteins of *K. pneumoniae* and *A. vinelandii*, the conserved cysteine residues (positions 472 and 477 in BjNifA) are boxed. Activities of mutant BjNifA proteins are expressed as percentage of wild-type NifA activity; NifA-independent background expression of the *nifD'-'lacZ* fusion was ≤2%.

As reported previously [7], the replacement of either of the cysteines at positions 472 and 477 by stereochemically related serines rendered the BjNifA protein completely inactive. This suggested a functional role of the cysteine thiols, as they are absent in mutants 7607 and 7605.

It has been proposed for several potential metal-binding domains in numerous nucleic acid-binding proteins that not only cysteines but also histidines could be involved in metal binding [17]. Therefore, we tested whether Cys-472 and Cys-477 in the BjNifA protein could be replaced by histidines. This strategy has been applied successfully by Severne et al. [18] who demonstrated that in a presumptive metal ion complexing domain of the rat glucocorticoid receptor the replacement of two cysteines by histidines (but not by Ala or Ser) retained activity of the protein. In contrast to these findings, however, the corresponding mutant BjNifA proteins 7714 and 7767 did not exhibit any activity. If Cys-472 and Cys-477 of BjNifA take part in any metal binding their function cannot be taken over by histidines. Alternatively, the introduction of the more bulky histidines at any of these positions could have abolished NifA activity by steric hindrance or improper protein folding.

Next, the question was asked whether the four amino acids that are between Cys-472 and Cys-477 play a functional role similar to the cysteines of whether they just serve as 'spacer residues' to keep the cysteines at a proper distance. The latter hypothesis seemed more likely because in five NifA proteins known to harbour an interdomain linker these four intervening amino acids can differ quite significantly, both chemically and structurally (fig.3). Four mutant BjNifA proteins were constructed, two of them altered at amino acid position 473 and the others at position 476. Replacement of Ser-473 by Gln (7658) made the BjNifA protein more similar to the NifA proteins of *R. meliloti* and *R. leguminosarum* biovar *viciae* (fig.3). When the same Ser residue was replaced by

Glu (7657) the protein structurally resembled that of BjNifA 7658 but contained an altered local charge distribution due to the negatively charged side chain of Glu. Similarly, the Gln to Glu exchange at position 476 in mutant BjNifA 7678 probably affected predominantly the local charge distribution rather than the steric structure, whereas the exchange by Lys (7656) was assumed to alter both of these parameters. However, irrespective of which position was mutated and independent of the character of the replacing amino acid, all four mutant BjNifA proteins retained activity. If significant at all, only mutants 7657 (81%) and 7656 (77%) had slightly diminished activities, whereas mutants 7658 (97%) and 7678 (106%) were indistinguishable from wild-type NifA.

In the mutant BjNifA proteins 7679 and 7766 the same amino acids that were chosen for the replacements described before were now deleted. Both of these NifA derivatives were completely inactive (fig.3). This finding together with the results obtained from NifA mutants 7657, 7658, 7656 and 7678 provided strong support for the hypothesis that Ser-473 and Glu-476 do not play a functional role per se but rather a role as spacer amino acids. Mutant NifA 7680 in which Gly-475 had been deleted was inactive as well; however, this could not be attributed unambiguously to an altered spacing, because a potential inherent function of Gly-475 itself was not tested.

In contrast to the inactive, NifA proteins 7679, 7680 and 7766, each deleted for one amino acid, the introduction of an extra amino acid residue after Gln-474 resulted in at least partially active NifA proteins (7681 and 7682). Upon introduction of Ala (7681), the NifA activity was 42% compared to that of wild-type NifA, whereas an insertion of Gln at the same position (7682) lowered this value further down to 21% (fig.3). Based on these data it was not possible to distinguish whether the reduced NifA activities were due to the structural and chemical features of the newly introduced amino acid or, more indirectly, resulted from an altered spacing between the cysteines. Nevertheless, it became evident from these studies that a reduction of the spacing by one amino acid rendered the BjNifA protein inactive whereas an extension of this spacing by one residue still allowed the formation of a functional NifA protein. In this context, it is of interest that an alignment of potential metal-binding sites in numerous nucleic acid-binding proteins revealed a preference for two or four, but not three, spacing amino acids between the cysteines or histidines thought to be involved in complexing metal ions [17]. Interestingly also, some of these motifs showed a spacing of more than four amino acids similar to that in the partially active BjNifA mutants 7681 and 7682.

In spite of these apparent analogies it should be noted that the role of the postulated metal-binding domain in the rhizobial NifA proteins (fig.3) most likely is a different one than the DNA-binding function attributed to the 'metal-binding fingers' of the eukaryotic proteins listed in [17] and [19]. As has been proven for the KpNifA protein [20], the NifA proteins are believed to bind to their target DNA by means of their COOH-terminal helix-turn-helix motif in the DNA-binding domain (cf. fig.1); therefore, we suggest that the putative metal-bindinbg domain in BjNifA serves a different structural function, for example by positioning the central domain and the DNA-binding domain in a correct functional conformation relative to each other. A shift from anaerobiosis to aerobiosis might change the oxidation state of a metal ion bound to the active NifA protein and /or of the cysteines involved in metal binding, thereby inducing a switch to a non-functional conformation. Recently, a similar model has been presented for the *E. coli* Fnr protein, a transcriptional regulator of several anaerobic genes [21]. In this protein, the characteristic arrangement of cysteine residues, of which one had been shown to be essential [22], was proposed to be involved in metal binding, and the alkylation kinetics of cysteine thiol groups indicated that, depending on the oxygen concentrations used, the Fnr protein might exist in two different conformations [21]. Similarly, the MerR protein encoded by the Tn*501* mercury resistance operon is known to switch from a repressor to an activator form upon binding of Hg(II), and four cysteines in the COOH-terminal part of the protein are probably involved in Hg(II) binding [23]. However, the simple binding of MerR to its operator sequence on the DNA is independent of Hg(II).

Alternative to the aforementioned models the active BjNifA protein might exist in the form of a metal-linked dimer, as it was shown for a *trans*-acting protein of the human immunodeficiency

virus [24]. In such a case, the BjNifA protein would switch between an inactive monomer and an active dimer, depending on the redox state in the cell.

Our further characterization of the BjNifA protein will focus on its potential metal content and on the mechanism of control by oxygen. For this purpose, the mutant NifA proteins described in this report will serve as very valuable tools.

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