Volume 16 Number 5 1988 Nucleic Acids Research

Essential and non-essential domains in the *Bradyrhizobium japonicum* NifA protein: identification of indispensable cysteine residues potentially involved in redox reactivity and/or metal binding

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Received December 23, 1987; Revised and Accepted February 4, 1988

ABSTRACT

The amino acid sequence of the Bradyrhizobium japonicum nitrogen fixation regulatory protein NifA, as derived from the nucleotide sequence of the nifA gene, was aligned to the corresponding protein sequences from Klebsiella pneumoniae, Rhizobium meliloti and Rhizobium leguminosarum biovar viciae. High conservation was found in the central domain and in the COOH-terminal, putative DNA binding domain, whereas very little homology was present within the first 250 amino acids from the NH_2-terminus. Upon deletion of the first 218 amino acids (37% of the protein) and expression of the remainder as a Cat'-'NifA hybrid protein, a fully active, nif-specific transcriptional activator protein was obtained which also retained oxygen sensitivity, a characteristic property of the wild-type B.japonicum NifA protein. In contrast, an unaltered COOH-terminal domain was required for an active NifA protein. Between the central and the DNA binding domains, a so-called interdomain linker region was identified which was conserved in all rhizobial species but missing in the K.pneumoniae NifA protein. Two conserved cysteine residues in this region were changed to serine residues, by oligonucleotide-directed mutagenesis. This resulted in absolutely inactive NifA mutant proteins. Similar null phenotypes were obtained by altering two closely adjacent cysteine residues in the central domain to serine residues. Nif gene activation in vivo by the B.japonicum NifA protein, but not by the K.pneumoniae NifA protein, was sensitive to treatment with chelating agents, and this inhibition could be overcome by the addition of divalent metal ions. On the basis of these observations and previous data on oxygen sensitivity we raise the hypothesis that at least some, if not all, of the four essential cysteine residues may be involved in oxygen reactivity or metal binding or both.

INTRODUCTION

In all diazotrophic bacteria examined to date, the promoters of the nitrogen fixation genes (nif and fix) carry the characteristic -24/-12 nif consensus sequence which implies that they are recognized by an alternative holoenzyme form of RNA polymerase containing the product of the ntrA gene (glnF, rpoN) as sigma factor (σ^{54}) (see Refs. 1,2 for review). In addition, the product of the key regulatory gene, nifA, is required to activate transcription from the nif promoters. The NifA protein probably acts from promoter-upstream binding sites which function as transcriptional enhancers (3,4). Thus, the

NifA protein is believed to make contact to the upstream activator sequence and to the RNA polymerase-*nif* promoter complex. Moreover, in *Klebsiella pneumoniae* the NifL protein appears to interact with the NifA protein because NifL is known to function as an antagonist ('repressor') of the NifA-mediated *nif* gene activation in response to oxygen and intermediate concentrations of NH_4^+ (5). In other N_2 fixing bacteria such as the rhizobia, evidence for a *nifL*-like gene is lacking. For a better understanding of the molecular mechanisms of *nif* gene activation it is of particular interest to identify essential domains or specific amino acids in the NifA protein which play an important role in one of the aforementioned interactions.

A few predictions as to the potential function of specific protein domains have been made on the basis of amino acid sequence comparisons between the NifA proteins from *K.pneumoniae*, *Rhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* (6,7,8,9). The low homology in the N-terminal region between the *K.pneumoniae* and *R.meliloti* NifA proteins has prompted Drummond et al. to speculate that this is the domain which, in the *K.pneumoniae* protein, might interact with the NifL protein (7). Very high sequence conservation has been shown in a long central domain that was proposed to be responsible for the interaction with the RNA polymerase and/or with σ^{54} (7). Near the C-terminus a region of considerable homology has been found to contain a helix-turn-helix motif characteristic for DNA binding proteins (7). None of these structure/function predictions have been supported experimentally.

This is the first report assigning functional importance, or the lack of it, to specific domains or amino acids in the NifA protein. The experiments were carried out with the NifA protein from *Bradyrhizobium japonicum* (10), the nitrogen fixing root-nodule symbiont of soybean. An additional incentive for choosing this system was our recent observation that the *in vivo* activation of *nif* genes by the *B.japonicum* NifA protein, but not by the *K.pneumoniae* NifA protein (in the absence of NifL), was sensitive to aerobiosis (11), both in *E.coli* and in *B.japonicum* backgrounds. It was, therefore, also an attractive goal to identify essential regions in the *B.japonicum* NifA protein which were missing in the *K.pneumoniae* NifA protein.

MATERIALS AND METHODS

Materials and methods not explicitly mentioned here are described in a recent report by Thöny et al. (12). Position numbers of nucleotides in the *nifA* gene refer to Fig. 2 in Ref. 12.

Table 1. Plasmids used in this work

Plasmid	Relevant phenotype or genotype	Reference
pBR329	$Ap^R Cm^R Tc^R$	17
pACYC177	$Ap^R Km^R$	18
pACYC184	$Cm^R Tc^R$	18
pBR329Ω	$Ap^R Cm^R Spc^R$ tet::spc	this work
pJF118HE	Ap^R(pBR322) P_{tac}	19
pRJ1008	Ap^R(pMC1403) Bj-Φ(nifD'-'lacZ)hyb	14
pRJ1025	Tc^R(pRK290) Bj-Φ(nifD'-'lacZ)hyb	4
pRJ7562	Cm^R(pACYC184) Bj-Φ(nifD'-'lacZ)hyb	this work
pRJ7551	Km^R(pBR329) Bj-nifA^c tet::nptII	11
pRJ7553	Km^R(pACYC177) Bj-nifA^c	this work
pKP7648	Km^R(pBR329) Kp-nifA^c tet::nptII	this work
pRmW54-10	Cm^R(pACYC177-C) Rm-nifA^c bla::cat	8

Abbreviations: Bj = B.japonicum, Kp = K.pneumoniae, Rm = R.meliloti; c = constitutive nifA expression.

Plasmids

These are listed in Table 1, and derivatives are described in Fig. 2, Table 2 and in the legend of Fig. 3. Plasmid pRJ1025 previously constructed by Alvarez-Morales et al.(4) is a pRK290 derivative (13) which carries a translational nifD'-'lacZ fusion. For reasons of plasmid compatibility and simultaneous selection the same nifD'-'lacZ fusion was recloned into pACYC184 as follows: a 6.7 kb EcoRI-SalI fragment carrying the fusion was isolated from pRJ1008 (14) and, after conversion of the EcoRI site to a SalI site, ligated with SalI-digested pACYC184. To avoid expression of the nifD'-'lacZ fusion from the pACYC184 tet promoter, the fusion was orientated divergently from the direction of transcription of the tet gene. Plasmid pRJ7551 (11) is based on pBR329 and expresses the intact B.japonicum nifA gene from the cat promoter. Of this plasmid the 5' nifA deletion derivatives pRJ7517, pRJ7521 and pRJ7519 were generated by Bal31 exonuclease treatment. For the construction of pRJ7582 a 1304 bp Asp718-PstI fragment encoding the 3' part of nifA was cloned in frame (upon partial fill-in with Klenow DNA polymerase and nuclease S1 treatment of the Asp718 end) into PvuII- plus PstI-digested pBR329Ω. The cat'-'nifA fusion sites in all four plasmids pRJ7517, pRJ7519, pRJ7521 and pRJ7582 were analyzed by overlapped sequencing to determine (i) the exact position of the fusions and (ii) whether the two genes were fused in frame.

Thereby it was found that the fusion in pRJ7582 was in frame but occurred 3 codons further downstream than planned in the cloning strategy. This was probably due to an unexpected double-strand exonuclease activity of the S1 nuclease used during the cloning procedure.

A tetracycline-resistant, kanamycin-sensitive precursor plasmid of pRJ7551 was the source for the 3' nifA deletion derivatives pRJ7573 and pRJ7572 which were obtained thereof by Bal31 exonuclease treatment and precisely mapped by sequencing. These two plasmids directed the synthesis of mutant NifA proteins with native NH_2-terminal ends but deleted COOH-terminal ends.

In order to have available a K.pneumoniae nifA expressing plasmid with an identical vector as in pRJ7551, plasmid pKP7648 was constructed in the following way: pBR329 was linearized at its PstI site and after blunting of the 3' overhanging ends with T4 DNA polymerase EcoRI linkers were added. Subsequent digestion with EcoRI removed multiple EcoRI linkers and cut pBR329 at its unique EcoRI site. By recircularization, a pBR329 derivative lacking the 1396 bp PstI-EcoRI fragment was obtained. It was further modified by insertion of the 2347 bp XhoI fragment of Tn5 encoding kanamycin resistance (nptII) into the SalI restriction site of the tet gene in which the nptII gene was orientated divergently to the tet gene. Finally, the 606 bp BamHI-EcoRI fragment of this plasmid was replaced by the 3.4 kb BamHI-EcoRI fragment from pKP7533 (11) carrying the complete K.pneumoniae nifA gene plus the 3' end of nifL and the 5' end of nifB. In the final plasmid construct, pKP7648, expression of nifA is controlled by the cat promoter of pBR329 as it is the case for B.japonicum nifA on pRJ7551, and the replicon and antibiotic resistance marker are also the same as in pRJ7551.

Oligonucleotide-directed mutagenesis

For the mutagenesis of four cysteine codons into serine codons in the 3' part of the B.japonicum nifA gene the gapped duplex method was used (15). An 890 bp EcoRI-PstI fragment spanning the region of nifA to be mutagenized was subcloned into M13 vector mp9, and ssDNA obtained thereof was used for mutagenesis. From clones harbouring the desired point mutations, dsDNA was isolated, and suitable restriction fragments thereof were used to replace the corresponding fragments in pRJ7553. This plasmid was chosen as target for reasons of constructions; it carries the same transcriptional cat::nifA fusion as pRJ7551, however, it is based on vector pACYC177. The resulting point mutations in the constitutively expressed B.japonicum nifA genes on pRJ7609 ($G_{3208} \rightarrow C$), pRJ7622 ($T_{3243} \rightarrow A$), pRJ7607 ($T_{3303} \rightarrow A$) and pRJ7605 ($T_{3318} \rightarrow A$) were verified by sequencing. Also by oligonucleotide-directed mutagenesis, the

point mutation on pRJ7605 was reverted to wild-type nifA yielding pRJ7627 which thus was identical to pRJ7553. As additional control for the correct construction of the point mutations, expression plasmids of wild-type and point-mutated B.japonicum nifA genes were constructed using expression vector pJF118HE. To improve translation of the nifA genes they were fused in frame to the first 38 codons of the cat gene analogous to pRJ7517 shown in Fig. 2. The resulting cat'-'nifA cassettes were brought under the control of the inducible tac promoter on pJF118HE. This yielded plasmids pRJ7623 (derived from pRJ7517), pRJ7634 (from pRJ7609), pRJ7639 (from pRJ7622), pRJ7633 (from pRJ7607) and pRJ7632 (from pRJ7605) which were introduced into E.coli RR28 (20) for in vivo expression of wild-type and mutated B.japonicum nifA.

β-Galactosidase assays

Microaerobic cultivation of E.coli strains to be tested for β-galactosidase activity and measurement of enzyme activity have been described (10,11). In the experiments concerning the differential sensitivity of B.japonicum nifA- and K.pneumoniae nifA-mediated nifD'-'lacZ activation to the chelating agent o-phenanthroline, the E.coli strains to be assayed were grown in NFDM medium (21) lacking $FeSO_4$, and o-phenanthroline was added at different concentrations. Antibiotic concentrations for plasmid selection were as follows (μg/ml): ampicillin 100, chloramphenicol 20, kanamycin 30, spectinomycin 20, tetracycline 10.

Protein sequence comparison

The "GAP" program of the University of Wisconsin Genetics Computer Group (16) setting a gap weight of 5.0 and a gap length weight of 0.3 was used to align the B.japonicum NifA sequence individually to the R.meliloti, R.leguminosarum and the K.pneumoniae NifA sequences.

RESULTS AND DISCUSSION

Alignment of amino acid sequences of NifA proteins: Identification of a conserved interdomain linker region specific to *Bradyrhizobium* and *Rhizobium*

We have recently presented the complete nucleotide sequence of the nifA-containing operon, fixRnifA, of B.japonicum (12). Two potential translation initiation sites for the nifA gene were found, one at position 1821 (ATG) and the other at position 1890 (ATG), resulting in open reading frames of either 605 or 582 codons which both end at position 3636 (TGA) (see Fig. 2 in Ref. 12). The second start codon is preceded by a well-conserved Shine-Dalgarno sequence at a proper distance of 8 nucleotides (12) so that we assume this to be the translational start used in vivo. The nifA gene thus defined encodes a

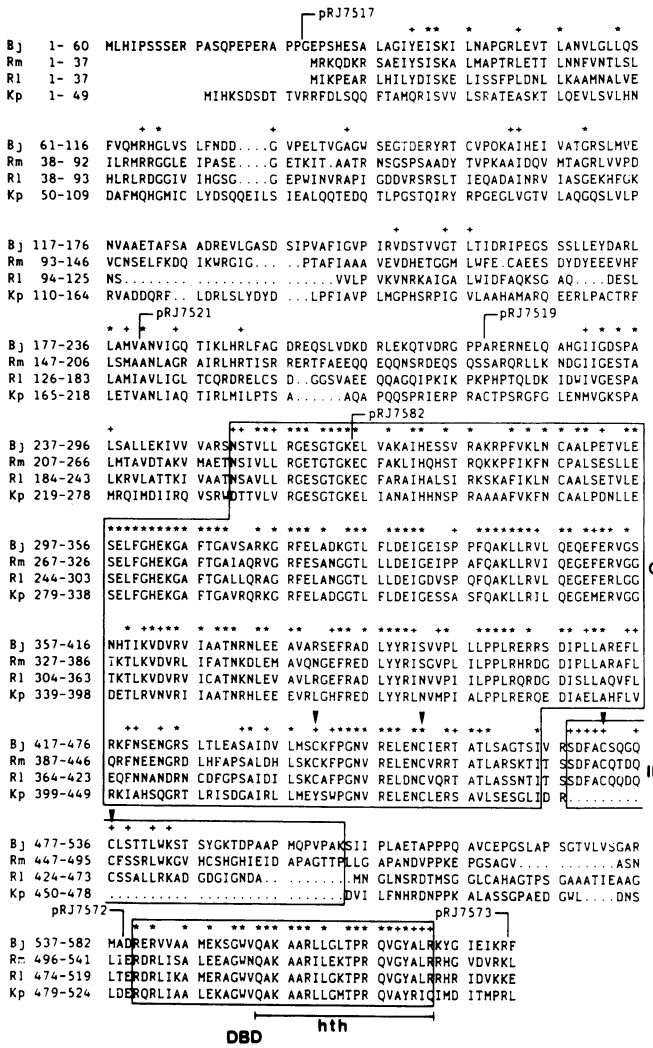

Figure 1. Comparison of the deduced amino acid sequences of the B.japonicum (Bj), R.meliloti (Rm), R.leguminosarum biovar viciae (Rl) and K.pneumoniae (Kp) NifA proteins. For references see text. Identical amino acids in all four proteins are marked by an asterisk (*) and identical amino acids occurring only in the rhizobial NifA proteins by the symbol (+). Functional domains originally proposed by Drummond et al. (7) were modified to boxes termed central domain (CD), interdomain linker (IDL) and DNA binding domain (DBD). The conserved helix-turn-helix (hth) motif common to many DNA binding proteins is denoted by a horizontal bar within DBD. The endpoints of the truncated NifA proteins encoded by the plasmids shown in Fig. 2 are indicated by the respective plasmid numbers. In the Bj NifA protein sequence the cysteine residues that were converted to serine residues by site-directed mutagenesis (see also Table 2) are marked with vertical arrowheads.

predicted NifA protein of 582 amino acids and a molecular weight of 63084. The B.japonicum NifA protein is the largest of all NifA proteins described to date: the R.meliloti (6,8), R.leguminosarum (9) and K.pneumoniae (7) NifA proteins have 541, 519 and 524 amino acids, and molecular weights of 59864, 56131 and 58631, respectively.

Figure 1 shows a computer-assisted alignment of the B.japonicum NifA protein sequence with corresponding sequences from R.meliloti, R.leguminosarum and K.pneumoniae. Over its entire length it shares 193 (= 33%) identical amino acids with the Rhizobium sequences, and 139 of these amino acids (= 24%) are conserved in all four sequences shown in Fig. 1. The distribution of these positional identities, however, is quite uneven. In the amino-terminal region until position 250 of the B.japonicum NifA sequence, the four sequences share only 12 identical amino acids, and several gaps had to be introduced to make this similarity visible. The very low degree of similarity in this region does not allow the formulation of two distinct domains "A" and "C" as proposed by Drummond et al. (7), and it is impossible, therefore, to attribute any common function to the N-terminal region of NifA solely on the basis of sequence comparisons. By contrast, extensive similarity is found in the so-called central domain ("CD", Fig. 1) ranging from asparagine-251 to isoleucine-465 of the B. japonicum NifA sequence. In this domain, 108 amino acids are identical in all four proteins which amounts to a 50% similarity, and 133 identical amino acids (= 62%) result out of a comparison of only the rhizobial sequences. The conserved central domain as depicted in Fig. 1 corresponds to domain D in the model of Drummond et al. (7) who proposed that this region of the protein may function in the interaction between the RNA polymerase or the sigma factor or both. Another region with significant amino acid sequence similarity exists between arginine-540 and arginine-573 of the B.japonicum NifA protein. As shown previously (7) this domain contains a helix-turn-helix motif characteristic for DNA binding proteins and is thus called the DNA binding domain ("DBD", Fig. 1); it corresponds to domain E as defined by Drummond et al. (7). Between the central and the DNA binding domains the four sequences shown in Fig. 1 share no overall similarity. However, the rhizobial NifA proteins possess additional 27 to 36 amino acids which exhibit significant similarity and which are not present in the K.pneumoniae sequence. We call this region the interdomain linker ("IDL", Fig. 1). Between serine-468 and lysine-484 of the B.japonicum NifA polypeptide there are 10 identical amino acids in the R.meliloti and R.leguminosarum NifA sequences, including two conserved cysteine residues at positions 472 and 477.

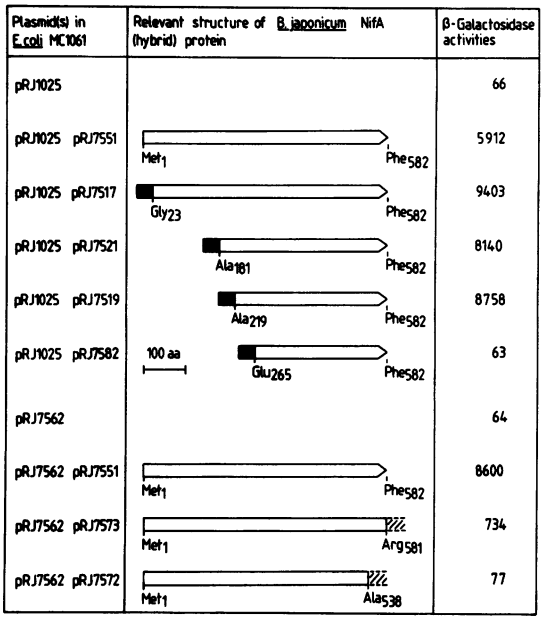

Figure 2. Functional analysis of NH_2- and COOH-terminal deletion derivatives of the B.japonicum NifA protein. NifA-mediated activation was measured as β-galactosidase activity expressed from the nifD'-'lacZ fusion plasmid pRJ1025 (upper part) or pRJ7562 (lower part) in the E.coli MC1061 background. Open bars denote B.japonicum NifA protein sequences, filled bars represent 38 NH_2-terminal amino acids of chloramphenicol acetyltransferase (pBR329) fused in frame to NifA proteins deleted at the NH_2-terminal end. Amino acids derived from pBR329 sequences fused to the COOH-terminal NifA deletions are shown as hatched open bars (for further details see Materials and Methods).

Based on the results obtained from these sequence comparisons we wished (i) to obtain experimental evidence whether or not the non-conserved N-terminal region was required to constitute an active NifA protein, (ii) to obtain proof for the necessity of the DNA binding domain in the function of the NifA protein, and (iii) to find out whether the interdomain linker and, in particular, the conserved cysteine residues might play a functional role in the B.japonicum NifA protein, despite the fact that the K.pneumoniae NifA protein is active without having this interdomain linker.

The amino-terminal region is not required for the function of the *B.japonicum* NifA protein

Using exonuclease Bal31 a set of deletions in the 5' end of the B.japonicum nifA gene was created, the truncated genes expressed, and the ability of the shortened NifA proteins to activate the B.japonicum nifD promoter was

tested (Fig. 2). Translation of 5'-deleted nifA genes on plasmids pRJ7517, pRJ7521, pRJ7519 and pRJ7582 (Fig. 2) was forced by fusing the truncated nifA genes in frame to the cat gene of pBR329 whose promoter was used for expression. The hybrid Cat'-'NifA proteins derived from the fused genes contained at their NH_2-terminal end the first 38 amino acids of chloramphenicol acetyltransferase. The exact sites within the NifA protein to which the Cat segment was fused are indicated in Fig. 1. The reference plasmid pRJ7551 contained the complete B.japonicum nifA gene which was transcribed from the cat gene promoter of pBR329 and translated from its own translational start signal because extension of the reading frame of the cat gene into the inserted B.japonicum DNA of pBR7551 ends in a translational stop codon 84 bp upstream of the ATG initiation codon of nifA (not shown). All plasmids were transformed into E. coli MC1061 carrying the nifD'-'lacZ fusion on plasmid pRJ1025. The β-galactosidase activities resulting from activation of the nifD promoter are shown in Fig. 2 (upper part). In microaerobically grown cells the intact NifA protein encoded by pRJ7551 led to a 50-fold activation of the nifD promoter on pRJ1025. The significantly increased activation by plasmids pRJ7517, pRJ7521 and pRJ7519 as compared to pRJ7551 was probably due to the elevated expression of cat'-'nifA hybrid genes as opposed to the expression of the native nifA on pRJ7551. (Evidence for increased nifA expression by the cat'-'nifA fusion plasmids was also obtained in E.coli minicell experiments [not shown]). Deletion of up to 218 amino acids from the NH_2-terminal end of NifA (pRJ7519) did not affect activation of the nifD promoter whereas deletion of 264 amino acids (pRJ7582) resulted in a completely inactive NifA protein.

Since the pRJ7582-encoded NifA protein is deleted for the first 14 amino acids of the conserved central domain rendering the protein inactive, this supports the essential role of the central domain in NifA function. Surprisingly, the entire N-terminal region at least up to proline-218 is not required for NifA to function as transcriptional activator. The corresponding region in the K.pneumoniae NifA protein has been suggested to potentially function as a domain interacting with the NifL protein (7), a negative effector of NifA activity in response to intermediate levels of NH_4^+ and to oxygen (5). However, such an interaction is unlikely to occur in the case of the B.japonicum NifA protein: firstly, evidence for a nifL-like gene in B.japonicum is lacking (10, 11,12), and secondly, all active NifA proteins encoded by pRJ7517, pRJ7521 and pRJ7519 are still sensitive to oxygen like the wild-type NifA protein (11; data not shown). The question remains open why during evolution the N-terminal region has been kept despite the tremendous sequence divergence in this region

even between closely related species (Fig. 1).

Activity of the *B.japonicum* NifA protein is sensitive to fusions near the C-terminal, DNA binding domain

Deletions in the 3' end of B.japonicum nifA were created by bidirectional exonuclease Bal31 treatment starting from a PstI site downstream of nifA (12). The reading frames of the shortened nifA genes thus continued into random reading frames of the vector DNA which led to hybrid NifA proteins with tails of foreign peptide sequences near the carboxy-terminal end. In pRJ7573 the last nifA codon for phenylalanine-582 was deleted, whereas in pRJ7572 the fusion was after codon 538 (for alanine), i.e. the putative DNA binding domain was missing (Figs. 1 and 2). The altered NifA proteins, like the wild-type protein encoded by pRJ7551, were expressed from the cat promoter and their ability to activate the nifD promoter was tested. For reasons of plasmid construction and simultaneous antibiotic selection for the maintenance of two plasmids, the nifD'-'lacZ tester plasmid was pRJ7562 and not pRJ1025. Figure 2 (lower part) shows that in microaerobically grown E.coli cells the pRJ7573-encoded NifA protein resulted in only 9% of the β-galactosidase activity obtained with the wild-type NifA protein while the pRJ7572-encoded NifA protein was completely inactive. Although this result appears to support the essential role of the putative DNA binding domain in NifA function, we cannot rule out the possibility that the carboxy-terminal protein tail fused to the NifA protein might interfere with other functional domains of the protein.

Requirement of four conserved cysteine residues for *B.japonicum* NifA activity

We have previously obtained evidence that the activity of the B.japonicum NifA protein, but not that of the K.pneumoniae NifA protein, was sensitive to oxygen (11). We also learned recently that the R.meliloti NifA protein was sensitive to oxygen, too (W. Klipp et al., manuscript in preparation). The latter finding was confirmed in our laboratory: we found that the R.meliloti NifA protein encoded by plasmid pRmW54-10 (8) could activate the B.japonicum nifD'-'lacZ fusion (on pRJ1025) in E.coli MC1061 resulting in about 6000 to 9000 units of β-galactosidase activity under microaerobic growth condition but only about 20% of these values in aerobically grown cells. Since cysteine residues in proteins are very often involved in redox-reactive processes we were prompted to alter the two cysteines in the interdomain linker of the B.japonicum NifA protein (positions 472 and 477) which are conserved in all rhizobial species but are absent in K.pneumoniae (Fig. 1). We anticipated that this would yield information on whether or not the interdomain linker, and in particular these cysteines, were essential for NifA activity. The protein sequence compa-

Table 2. Functional analysis of four B.japonicum NifA proteins, each mutated in a single conserved Cys residue, by activation of a nifD'-'lacZ fusion in E.coli MC1061

Plasmids in E.coli MC1061		Relevant NifA phenotype	β-Galactosidase activity (Units)
--	--		22
pRJ1025	--		66
pRJ1025	pRJ7553	wild-type	3324
pRJ1025	pRJ7609	$Cys_{440} \rightarrow Ser_{440}$	32
pRJ1025	pRJ7622	$Cys_{452} \rightarrow Ser_{452}$	52
pRJ1025	pRJ7607	$Cys_{472} \rightarrow Ser_{472}$	41
pRJ1025	pRJ7605	$Cys_{477} \rightarrow Ser_{477}$	32
pRJ1025	pRJ7627	Ser_{477}(pRJ7605) $\rightarrow Cys_{477}$ (Reversion to wild-type)	3007

rison as shown in Fig. 1 also revealed the presence of another cysteine residue (cysteine-440) near the end of the central domain which is conserved only in the rhizobial species but is replaced by a tyrosine in K.pneumoniae. Furthermore, there was one cysteine residue (position 477) at the end of the central domain which was conserved in all species (Fig. 1). The four critical cysteine residues within the B.japonicum NifA protein were mutated via oligonucleotide-directed mutagenesis into stereochemically similar serine residues, and the ability of the modified NifA proteins to activate the nifD promoter was tested. The results are given in Table 2. The data clearly show that none of the mutated nifA gene products was able to activate the nifD'-'lacZ fusion. The resulting β-galactosidase activities were in the range of 1 to 2% of the value obtained in the presence of wild-type nifA on pRJ7553 and indistinguishable from background β-galactosidase activity observed in the absence of any activating plasmid.

Three controls were done to make sure that the point mutations were responsible for the observed phenotypes:

(i) After mutagenesis, the complete DNA fragment that was the target in the gapped-duplex procedure was sequenced, and in all four cases only the desired nucleotide exchange was found (see Materials and Methods).

(ii) The mutated nifA gene on pRJ7605 was reverted to wild-type nifA, again by oligonucleotide-directed mutagenesis. The resulting plasmid, pRJ7627, activated the nifD'-'lacZ fusion similarly as the original nifA plasmid

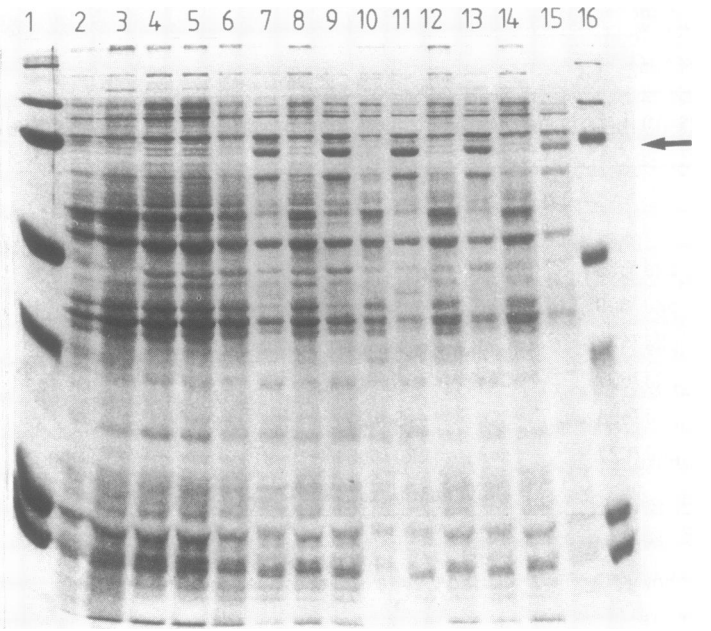

Figure 3. Expression ofwild-type (pRJ7623) and point-mutated (pRJ7634, pRJ7639, pRJ7633, pRJ7632) B.japonicum nifA genes in E.coli RR28. Crude extracts of induced (5h after addition of IPTG) and non-induced (-IPTG) cultures were separated on a 10% polyacrylamide-SDS gel, and the gel was subsequently stained with Coomassie brilliant blue. The lanes contain the following samples: protein molecular weight markers (lanes 1, 16; molecular weights from top to bottom: 200, 97.4, 68, 43, 25.7, 18.4, 14.3 kilodalton), and SDS extracts (lanes 2 to 15) from uninduced (even numbers) and induced cells (uneven numbers), i.e. RR28 alone (2,3), containing vector pJF118HE (4,5), or the nifA plasmids pRJ7623 (6,7), pRJ7634 (8,9), pRJ7639 (10,11), pRJ7633 (12,13) and pRJ7632 (14,15). See Materials and Methods for the type of nifA present on these plasmids. The arrow (right margin) points to the 65.2 kilodalton Cat'-'NifA hybrid protein present only in extracts from induced cultures of E.coli RR28 harbouring the nifA plasmids.

pRJ7553 (Table 2). Analogous controls with the other three point mutations were not done.

(iii) We demonstrated that the nifA open reading frame was not destroyed during the mutagenesis and subsequent cloning procedures. For this purpose, efficiently translatable fusions between the 38th codon of cat and the 23rd codon of nifA were constructed using nifA DNA derived from the wild-type (pRJ7553) and the mutant plasmids (pRJ7609, pRJ7622, pRJ7607 and pRJ7605). These constructs were brought under the control of the inducible tac promoter by cloning suitable fragments into the expression vector pJF118HE (19) resul-

ting in the corresponding plasmids pRJ7623 ("wild-type" nifA) and pRJ7634, pRJ7639, pRJ7633 and pRJ7632 (containing the point mutations). E.coli cells harboring these plasmids were grown under non-inducing (-IPTG) and inducing conditions (+IPTG) and total cell extracts were analyzed by SDS polyacrylamide gel electrophoresis. A protein band of the expected size of 65.2 kilodalton for the Cat'-'NifA hybrid protein was detectable only in extracts from induced cultures of all nifA plasmid-containing strains (Fig. 3, lanes 7,9,11,13,15). This proves the integrity of the nifA open reading frames in the original mutant plasmids pRJ7609, pRJ7622, pRJ7607 and pRJ7605. Figure 3 also reveals no obvious instability (degradation) of the mutant proteins during the 5h period after induction by IPTG as compared with the wild-type NifA protein.

The results of Table 2 have shown that all four cysteine residues investigated were absolutely essential for B.japonicum NifA activity. Moreover, this result implies that the interdomain linker present in the rhizobial NifA proteins must play an indispensable role in the function of these proteins, a surprising fact if one recalls that the K.pneumoniae NifA protein is an active transcriptional effector even without such an interdomain linker. A striking feature of all four mutant NifA proteins is the absolute null phenotype associated with them. We consider it unlikely, therefore, that the cysteines are simply involved in hydrogen bond formation or van-der-Waals interactions either within the NifA protein or with proteins or DNA of the nif transcription complex, because in such a case one would conceive that the replacing serines could at least partly substitute the function of the cysteines. Rather, we favor a role of these cysteines in one of the following two ways:

(i) As in many proteins they may be involved in the formation of either intra- or intermolecular disulfide bridges. For example, an intramolecular disulfide bridge between the two cysteines of the interdomain linker could be involved in the correct positioning of the DNA binding domain for optimal function. Alternatively, intermolecular disulfides could be involved in the dimerization of the NifA protein. Numerous DNA binding proteins have been shown to be dimers in their active form (see Ref. 22 for review).

(ii) Another potential role of the cysteines might be the binding of a cofactor which is essential for NifA activity of Rhizobium and Bradyrhizobium, such as a covalently bound heme, a specific metal ion, or a complex $[Fe_x:S_x]$-cluster. In the amino acid sequence of the K.pneumoniae NifL protein (23) the presence of a putative heme binding motif similar as in c-type cytochromes has been postulated (24), but neither this sequence nor the sequences around the essential cysteines of the B.japonicum NifA protein fulfil the require-

Table 3. Examples of proven or putative metal-binding domains[a] in DNA binding proteins, and their comparison to similar sequences from the B.japonicum and R.meliloti NifA proteins

Transcriptional regulator protein + (Me[II]), and organism in which it occurs	Metal-binding domain[b]	Reference
TFIIIA (Zn[II]) Xenopus laevis	$C-X_{2-5}-C-X_{12}-H-X_{2-3}-H$	27,28
GAL4 (Zn[II]) S.cerevisiae	$C-X_2-C-X_{13}-C-X_2-C$	29
ADR1 (Zn[II]) S.cerevisiae	$C-X_2-C-X_{12}-H-X_{3-4}-H$	30
MerR (Hg[II]) Tn501, P.aeruginosa	$C-X-CH-X_7-C$	31
Fur (Fe[II]) E.coli	(i) $C-X_4-C-X_4-H-X-H$ (ii) $HHH-X-H-X_2-C-H_2-C$	32,33
NifA (?) B.japonicum (Bj) and R.meliloti (Rm)	(i) Bj,Rm $C-X_{11}-C-X_{19}-C-X_4-C$ (ii) Bj $C-X_4-C-X_9-SYGK$ Rm $C-X_4-C-X_9-HCSH$	this work

[a] Evidence that these domains bind Me[II] is available for TFIIIA (27,28) and GAL4 (29) whereas the metal-binding function of the other sequences is hypothetical.

[b] Amino acids are given in the one-letter code; X stands for any amino acid. Two alternative variants, (i) and (ii), as deduced from the complete amino acid sequences are proposed in the cases of the Fur and NifA proteins.

ments as they are known for the covalent binding of a c-type heme (25). The direct binding of a metal ion to some of these cysteines appears to be a more attractive hypothesis (see next paragraph).

Do the rhizobial NifA proteins contain a metal-binding site?

A great number of nucleic acid binding proteins contain potential metal-binding domains (26). For some of them it is established that cysteine thiols (Cys) and histidine imidazoles (His) serve as coordinates in the binding of divalent metals (Me[II]). The most well-known domains are the "zinc fingers", e.g. in the Xenopus laevis 5S RNA transcription factor IIIA (27,28) or in the positive regulatory proteins GAL4 (29) and ADR1 (30) of Saccharomyces cerevisiae (see Table 3). In the interdomain linker region of the R.meliloti NifA protein the sequence $Cys-X_4-Cys-X_9-His-Cys-X-His$ perfectly fulfills the criteria by which Berg (26) has defined a potential metal-binding site in a variety of nucleic acid binding proteins (Table 3). In the corresponding B.ja-

Table 4. Differential influence of o-phenantrholine (OP) on K.pneumoniae (Kp), B.japonicum (Bj) and R.meliloti (Rm) nifA-mediated nifD'-'lacZ activation in E.coli MC1061

Growth medium	β-Galactosidase activities[a] derived from nifD'-'lacZ on pRJ1025 activated by		
	Kp nifA (pKP7648)	Bj nifA (pRJ7551)	Rm nifA (pRmW54-10)
NFDM[b]	10131 ± 1546	8289 ± 596	8657 ± 988
NFDM -Fe	11378 ± 1035	8161 ± 1047	8416 ± 606
NFDM +0.05 mM OP[c]	10069 ± 1817	5996 ± 387	8928 ± 1295
NFDM -Fe +0.01 mM OP	11960 ± 933	8158 ± 833	9371 ± 1131
NFDM -Fe +0.03 mM OP	15988 ± 1152	145 ± 9	1749 ± 528
NFDM -Fe +0.05 mM OP[c]	17361 ± 1254	137 ± 13	1877 ± 702

[a] Mean values from four independent cultures assayed in duplicate.

[b] NFDM medium contains 0.107 mM Fe^{2+} (as $FeSO_4$).

[c] In contrast to the E.coli MC1061/pRJ1025 strains containing the rhizobial nifA plasmids (pRJ7551 or pRmW54-10), the addition of o-phenanthroline to the growth medium of E.coli MC1061/pRJ1025+pKP7648 resulted in a reduction of the final cell density (at 600 nm) by maximal 58% of cultures without o-phenanthroline.

ponicum sequence some of these amino acids are replaced by tyrosine or lysine which could also serve as Me[II]-binding coordinates. Alternatively, the other two essential cysteines near the end of the central domain (Cys-440, Cys-452) could participate in that function (Table 3).

These observations prompted us to test the possibility that the B.japonicum NifA activity might depend on metal ions. We could in fact show that the in vivo activation of a nifD'-'lacZ fusion in E.coli by the B.japonicum NifA protein, but not by the K.pneumoniae NifA protein, was strongly sensitive to the addition of ≳3mM EDTA to the growth medium (NFDM medium; 21). Upon addition of Me[II] to the medium the inhibitory effect of EDTA was overcome either fully (Fe[II]) or partly (Cd[II]), Cu[II], Mg[II], Mn[II], Ni[II], Zn[II]) (data not presented). Similarly, using the chelator o-phenanthroline we found that a concentration as low as 0.03 mM strongly inhibited NifA activity in NFDM medium without added $FeSO_4$ but not in normal NFDM medium containing iron (Table 4). Again, the B.japonicum NifA activity and, less severely, also the R.meliloti NifA activity were selectively inhibited in contrast to the K.pneumoniae NifA activity which was not inhibited at all (Table 4). These results suggest that the formation of active rhizobial NifA proteins requires the presence of divalent metal ions. However, since the experiments were done in vivo,

they neither do rigorously prove a binding of Me[II] to NifA nor do they prove that this metal is Fe[II] because another unknown Me[II] in the medium could have been depleted by the chelators used. Furthermore, if the rhizobial NifA proteins are indeed metalloproteins, the presence of Me[II]-binding domains does not necessarily imply that these structures, like the zinc fingers (26, 27), are involved in DNA binding. Rather, the situation with NifA would be more reminiscent of the MerR and the Fur proteins in which the presence of both a helix-turn-helix motif and a metal-binding motif (for Hg[II] and Fe[II], respectively; Table 3) has been proposed (31,32,33).

Conclusion and possible mechanism

The data presented in this paper and in a previous report (11) have led to several interesting parallel observations:

(i) The in vivo activities of the NifA proteins of B.japonicum and, to a lesser extent, also of R.meliloti, but not of K.pneumoniae, are sensitive to oxygen.

(ii) The in vivo activities of the NifA proteins of B.japonicum and, to a lesser extent, also of R.meliloti, but not of K.pneumoniae, are sensitive to treatment with metal chelators.

(iii) The NifA proteins of B.japonicum and, even more pronounced, that of R.meliloti, but not that of K.pneumoniae, have a potential, cysteine-containing metal-binding motif forming part of the interdomain linker region between the central domain and the DNA binding domain. In the case of the B.japonicum NifA protein this region was proven to be essential by creating an inactive protein after having converted the cysteines to serines.

On the basis of this parallelism we are tempted to propose a working hypothesis that provides a causal connection between these observations. One might conceive a mechanistic model in which a metal (e.g. Fe[II]) would bind in its reduced state to the interdomain linker thus aiding the correct positioning of the DNA binding domain in order to form an active NifA protein. Conversely, under aerobic conditions the metal ion may be oxidized and unable to bind to NifA with the result that the protein is now inactive. Furthermore, it may be possible that this metal is more firmly bound to the R.meliloti NifA protein than to the B.japonicum NifA protein because the activity of the former is less sensitive to both oxygen and chelators. The relatively pronounced residual oxygen insensitivity of the R.meliloti NifA protein may also be the reason why the expression of this protein from the nifA promoter needs to be additionally controlled by oxygen (34), in contrast to B.japonicum (12).

In other oxygen-reactive transcriptional regulators the mechanism of oxy-

gen sensing is also not known. Nevertheless, there are some interesting similarities. For instance, the E.coli Fnr protein, a positive regulator of the genes for the anaerobic fumarate and nitrate reductases, contains the sequence Cys-X_2-His-Cys-X_2-Cys which has been considered as a potential oxygen-reactive domain (35). The second cysteine in this row has recently been converted to a serine resulting in an inactive Fnr protein (unpublished data cited in Ref. 36). The ferric uptake regulation protein of E.coli, called Fur (see also Table 3), has recently been purified and shown to regulate aerobactin operon expression in vitro in the presence of Fe[II] but not Fe[III], and most interestingly, anaerobic conditions were necessary to demonstrate regulation of the operon by iron (33). We hope that our future studies with the NifA protein will not only help to unravel the mechanism of oxygen control of nif gene expression in B.japonicum but will also contribute to an understanding of the mechanism of oxygen sensing in other oxygen regulated systems.

ACKNOWLEDGEMENTS

We gratefully appreciate the expert technical assistance of S. Hitz and B. Pfluger. We also thank W. Klipp and A. Pühler for plasmid pRmW54-10 and H. Paul for typing the manuscript. This work was supported by a grant from Lubrizol Genetics Inc./Agrigenetics Research Associates.

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