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A defined amino acid exchange close to the putative nucleotide binding site is responsible for an oxygen-tolerant variant of the *Rhizobium meliloti* NifA protein

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Summary. In *Rhizobium meliloti* the NifA protein plays a central role in the expression of genes involved in nitrogen fixation. The *R. meliloti* NifA protein has been found to be oxygen sensitive and therefore acts as a transcriptional activator only under microaerobic conditions. In order to generate oxygen-tolerant variants of the NifA protein a plasmid carrying the *R. meliloti nifA* gene was mutagenized *in vitro* with hydroxylamine. About 70 mutated *nifA* genes were isolated which mediated up to 12-fold increased NifA activity at high oxygen concentrations. A cloning procedure involving the combination of DNA fragments from mutated and wild-type *nifA* genes allowed mapping of the mutation sites within the central part of the *nifA* gene. For 17 mutated *nifA* genes the exact mutation sites were determined by DNA sequence analysis. It was found that all 17 mutated *nifA* genes carried identical guanosine – adenosine mutations resulting in a methionine – isoleucine exchange (M217I) near the putative nucleotide binding site within the central domain. Secondary structure predictions indicated that the conformation of the putative nucleotide binding site may be altered in the oxygen-tolerant NifA proteins. A model is proposed which assumes that at high oxygen concentrations the loss of activity of the *R. meliloti* NifA protein is due to a conformational change in the nucleotide binding site that may abolish binding or hydrolysis of the nucleotide. Such a conformational change may be blocked in the oxygen-tolerant NifA protein, thus allowing interaction with the nucleotide at high oxygen concentrations.

Key words: Nitrogen fixation – Nucleotide binding site – Oxygen regulation – Random mutagenesis

Introduction

The NifA protein plays a central role in diazotrophic organisms by activating genes involved in the nitrogen fixation process (Gussin et al. 1986). For *Klebsiella pneumoniae* it has been shown that the NifA protein acts as a transcriptional activator by binding to upstream activator sequences (UAS) of *nif* promoters (Buck et al. 1986). These *nif* promoters are characterized by a $-12/-24$ consensus sequence which requires the gene product of the *ntrA* gene as an alternative σ-factor (σ^{54}) in the RNA polymerase holoenzyme (Hirschman et al. 1985; Hunt and Magasanik 1985). The interaction between the NifA protein and RNA polymerase results in the formation of a transcriptionally active open promoter complex (Morett and Buck 1989; Morett et al. 1991). In *K. pneumoniae* the NifA activity is controlled by the NifL protein, which negatively regulates the activity of NifA under conditions of high oxygen concentration or nitrogen excess (Hill et al. 1981; Merrick et al. 1982).

In the symbiotic nitrogen-fixing soil bacterium *Rhizobium meliloti,* the *nifA* gene was first described as *fixD* (Weber et al. 1985). It was shown that in *R. meliloti* the activity of NifA is influenced only by the oxygen concentration and that this regulation is mediated by the NifA protein itself (Beynon et al. 1988; Klipp et al. 1989). By comparison of the protein sequences of known NifA proteins, a general structure for this molecule, consisting of distinct protein domains, was developed. Three domains, each with a high degree of amino acid homology and putative specific functions were identified. The central domain is assumed to interact with the σ^{54}-RNA polymerase complex (Drummond et al. 1986). It contains a putative nucleotide binding site and represents the transcriptional activator unit since NifA variants consisting only of this domain show transcriptional activation ability (Huala and Ausubel 1989; Morett et al. 1988). The C-terminal DNA-binding domain (DBD) is characterized by a helix-turn-helix motif typical for proteins that interact directly with DNA. For *K. pneumoniae* NifA it was shown that this domain is responsible for the binding of NifA to the upstream activator sequences and thereby contributes to rapid formation of the transcriptional activator complex (Morett et al. 1988). The central and the DNA binding domains are separated by an

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additional interdomain linker region (IDL) in oxygen-sensitive NifA proteins found in *Rhizobium meliloti, Rhizobium leguminosarum, Bradyrhizobium japonicum, Azorhizobium caulinodans, Herbaspirillum seropedicae* and *Rhodobacter capsulatus* (Weber et al. 1985; Buikema et al. 1985; Grönger et al. 1987; Roelvink et al. 1989; Iismaa and Watson 1989; Thöny et al. 1987; Nees et al. 1988; Souza et al. 1991; Masepohl et al. 1988). Cysteine residues of the interdomain linker, together with cysteine residues from the central domain, form a Cys-X_{11}-Cys-X_{19}-Cys-X_4-Cys motif resembling metal-binding sites found in other proteins (Berg 1986). In the current model of oxygen-dependent regulation of NifA activity this sequence is responsible for recognizing the oxygen concentration based on the redox state of a bound metal ion. For *B. japonicum* NifA it has been shown that alteration of the conserved cysteines to serines or changing the distance between the cysteine residues results in an inactive form of NifA, indicating that the correct structure of this domain is essential for the function of oxygen-sensitive NifA proteins (Fischer et al. 1988; 1989).

In this paper we describe the isolation of oxygen-tolerant mutants of the *R. meliloti* NifA protein. We show that these mutants are due to a defined amino acid exchange close to the putative nucleotide binding site. In addition, a model is presented which explains the oxygen-dependent regulation of NifA activity.

Materials and methods

Bacterial strains and growth conditions. E. coli ET8894 (MacNeil 1981) was used to determine the β-galactosidase activities of *lacZ* fusions. *E. coli* 294 (Weber and Pühler 1982) was the host for plasmid propagation. *E. coli* strains were grown in LB medium (Miller 1972) at 37° C. In view of the heat sensitivity of the NifA protein, strains expressing the *nifA* gene were grown at 30° C. Antibiotics used for plasmid selection were chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), and gentamicin (10 μg/ml). In minimal medium (M63; Miller 1972), used for β-galactosidase assays, the antibiotic concentration was reduced 4-fold. The plasmids used in this study are listed in Table 1.

β-Galactosidase assay. E. coli ET8894 strains harbouring the appropriate plasmids were grown overnight in M63 medium supplemented with 20 mM glutamine. Samples of 10 ml were washed twice and resuspended in 20 ml M63 medium lacking ammonia and with a reduced glutamine concentration (1.4 mM). The samples were divided into two portions and incubated for 5 h in screw-capped test tubes without agitation (microaerobic conditions) or in 100 ml Erlenmeyer flasks with vigorous shaking (aerobic conditions). β-Galactosidase activity was determined by the SDS/chloroform method as described by Miller (1972).

DNA manipulations and sequencing. DNA isolation, restriction enzyme analysis, agarose gel electrophoresis and cloning procedures were performed using established techniques (Maniatis et al. 1982). Plasmid DNA for sequencing analysis was isolated by the method of Birnboim and Doly (1979) and was purified by buoyant density centrifugation in a cesium chloride gradient. The DNA was 10-fold concentrated and samples were sequenced directly from the original plasmids by the dideoxy method (Sanger et al. 1977) using a commercial T7 polymerase sequencing kit (Pharmacia) and various specific oligonucleotides as *nifA* internal primers. Oligo-

Table 1A. Plasmids

Designation	Characteristics[a]	Source or reference
pACYC177-C	Km^r, Cm^r	Pühler et al. 1983
pUC8	Ap^r	Vieira and Messing 1982
pWKR189	Gm^r, Ap^r	Moreno-Vivian et al. 1989
pWK131	*K. pneumoniae* $nifA^c$ cloned into pACYC177-C	Pühler et al. 1983
pRmW541-10	*R. meliloti* $nifA^c$ cloned into pACYC177-C	Weber et al. 1985
pGW7	P_{nifH}-*lacZ*	Weber et al. 1985

Table 1B. Recombinant plasmids constructed for this work

Designation	Characteristics[a]
pRKB2	pRmW541-10 carrying a P–S–B–P linker
pRKB3.1-38	pRmW541-10 derivatives encoding oxygen-tolerant NifA mutants
pRKB4.1-9	Gm^r derivatives of pRKB3.1-9
pRKB5.1-9E	Plasmids carrying $nifA_{EcoRI}$ hybrids
pRKB5.1-9S	Plasmids carrying $nifA_{SalI}$ hybrids
pRKB5.1-9B	Plasmids carrying $nifA_{BamHI}$ hybrids
pACYC177-Gm[b]	Gm^r derivative of pACYC177-C

[a] Only characteristics relevant for this study are listed. Antibiotic resistances: Ap^r, ampicillin; Cm^r, chloramphenicol; Gm^r, gentamicin; Km^r, kanamycin; $nifA^c$, constitutively expressed *nifA* gene

[b] A P-Gm^r-P fragment from pWKR189 was exchanged against the P-Cm^r-P fragment in pACYC177-C

Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I

nucleotides used as DNA sequencing primers were synthesized on an Applied Biosystems DNA synthesizer (Model 380B) by using the phosphoamidite method (Beaucage and Caruthers 1981; Matteucci and Caruthers 1981).

Hydroxylamine treatment. Plasmid DNA for hydroxylamine treatment was isolated as described for DNA sequencing. 200 µl DNA (0.1 µg/µl) were mixed with 800 µl 1 M NH_2OH pH 6.0 and 1 ml sodium phosphate buffer pH 6.0 and incubated at 75° C for 2.5 h. The incubation time was determined in preeliminary experiments using vector plasmid pACYC177-C to yield a surviving fraction of 10%. The hydroxylamine was removed either by dialysis followed by ethanol precipitation or by using the commercial Geneclean kit (BIO101).

Isolation of oxygen-tolerant nifA mutants. Samples of hydroxylamine-treated DNA were transformed into *E. coli* ET8894 (pGW7) using standard techniques. The cells were spread on M63 plates (Miller 1972) supplemented with 20 mM glutamine and 40 mg/l X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Samples of 20 plates were placed in a 15 l box closed with a rubber-sealed lid and flushed with pure oxygen. The plates were incubated for 3 days at 30° C, replenishing the box twice a day with pure oxygen. Colonies carrying oxygen-tolerant *R. meliloti* NifA were identified by their deep blue colour.

Construction of plasmids for fragment exchange experiments. To combine different parts of wild-type and mutated *nifA* genes, a gentamicin resistance gene was first linked to the mutated *nifA* genes. The 2.75 kb *Hind*III-*Pst*I fragment carrying a gentamicin resistance gene (Gm^r) from plasmid pWKR189 (Moreno-Vivian et al. 1989) was cloned into the *Pst*I site of pRmW541-10 derivatives encoding oxygen-tolerant NifA proteins using a *Pst*I-*Hind*III linker containing the appropriate restriction sites. The resulting plasmids, pRKB4.1-9, allowed the cloning of *Eco*RI, *Sal*I and *Bam*HI fragments carrying different parts of the *nifA* structural gene linked to the Gm^r gene. A plasmid which can be used as a source of different *nifA* wild-type fragments was constructed by inserting a *Pst*I-*Sal*I-*Bam*HI-*Pst*I linker into partially digested pRmW541-10. The resulting plasmid, pRKB2, was used to exchange *Eco*RI, *Sal*I and *Bam*HI fragments of wild-type *nifA* for the corresponding fragments of mutated *nifA* genes. Following the scheme presented in Fig. 1A three types of *nifA* hybrids (designated E, S or B) were constructed for each of the *nifA* mutants tested.

Secondary structure analysis. Secondary structure predictions were generated by using the Chou and Fasman method ($1974_{a,b}$) with an extended data base (Argos et al. 1978). The Chou and Fasman program is part of the ANALYSEP program run on a Hewlett Packard HP360 computer system.

Results

Oxygen-tolerant mutants of the R. meliloti *NifA protein can be isolated in* E. coli *following* in vitro *hydroxylamine mutagenesis of* $nifA^+$ *DNA*

To isolate oxygen-tolerant *R. meliloti* NifA proteins a system for the rapid screening of a large number of mutants was developed. The *R. meliloti* $nifA^+$ gene is constitutively expressed from the *aphI* promoter in plasmid pRmW541-10 (Weber et al. 1985). To determine the NifA activity in *E. coli* we used plasmid pGW7 (Weber et al. 1985), which contains the *R. meliloti nifH* promoter fused to the *E. coli lacZ* gene. Due to microaerobic conditions in the centres of *E. coli* colonies the oxygen-sensitive *R. meliloti* wild-type NifA protein is able to activate the *nifH* promoter even under normal atmospheric conditions (20% oxygen), resulting in colonies with blue centres on X-Gal-containing media. This precludes discrimination between colonies carrying the oxygen-sensitive *R. meliloti* wild-type NifA protein and colonies carrying oxygen-tolerant NifA mutant proteins. However, *E. coli* strains carrying the oxygen-sensitive *R. meliloti* wild-type NifA form white colonies on X-Gal-

Table 2. Activation of the *R. meliloti nifH* promoter by oxygen-tolerant *R. meliloti* NifA mutants in *E. coli*

Plasmids[a]	β-Galactosidase activity[b]	
	Aerobic	Microaerobic
→ pRKB3.1	88.1± 3.6	150.0± 4.5
→ pRKB3.2	78.5±11.4	144.3± 7.3
→ pRKB3.3	90.1±10.2	148.5±31.6
→ pRKB3.5	80.2±11.5	153.3±13.7
→ pRKB3.8	79.4±17.3	133.0±26.3
→ pRKB3.9	68.9±17.2	134.3±38.2
pRKB3.20	94.7±21.3	136.7± 5.1
pRKB3.21	94.0±16.7	139.0± 0.9
pRKB3.29	83.0± 0.3	159.7± 8.3
pRKB3.35	84.3± 6.1	159.4± 6.0
pRKB3.37	85.9±15.2	166.3±21.4
pRKB3.38	81.6±23.5	168.3±37.7
→ pRKB3.6	92.2±11.1	159.5±14.9
→ pRKB3.7	76.1±18.1	158.4±33.5
→ pRKB3.4	95.6±16.1	152.5± 7.1
pRKB3.22	49.4± 8.3	111.0±12.5
pRKB3.23	64.4± 8.0	144.8± 5.6
pRmW541–10[c]	8.1± 0.9	88.2± 2.2
pWK131[d]	275.0± 1.6	185.3± 0.1
pACYC–177C[e]	1.5± 0.1	0.2± 0.1

[a] *E. coli* ET8894 (pGW7) is the host for these plasmids; plasmids marked by arrows were used for the construction of hybrid *nifA* genes

[b] β-Galactosidase activity was determined as described in Materials and methods. The standard deviations were calculated from assays of three independent colonies

[c] Plasmid pRmW541-10 contains *R. meliloti* wild-type *nifA*

[d] Plasmid pWK131 contains the oxygen-tolerant *K. pneumoniae nifA*

[e] Plasmid pACYC177-C was used as a negative control since this plasmid serves as the vector for pRmW541-10 and pWK131

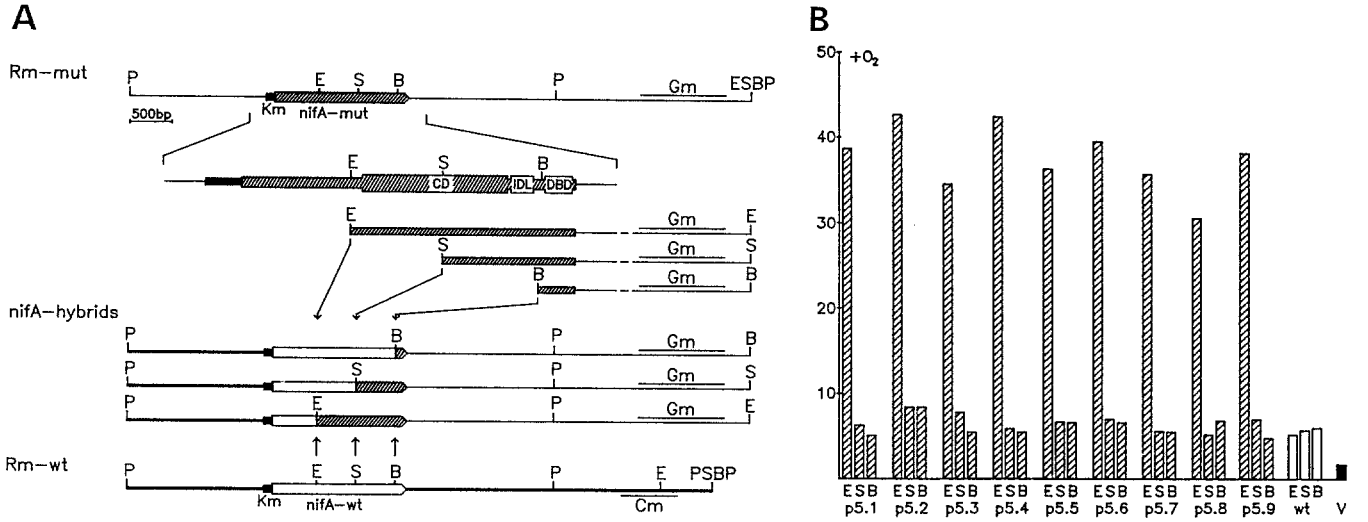

Fig. 1A, B. Construction and analysis of *R. meliloti nifA* hybrid genes to map mutation sites in the *nifA* coding region leading to oxygen tolerant NifA proteins. **A** Construction scheme for *nifA* hybrid genes containing different parts of the wild-type (Rm-wt) and mutated *nifA* genes (Rm-mut). All *nifA* hybrid genes are constitutively expressed from the kanamycin resistance gene promoter (Km). Restriction sites relevant for the construction of these plasmids are shown (B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I). The domain structure of the NifA protein is also presented (CD: central domain, IDL: interdomain linker, DBD: DNA binding domain). Chloramphenicol (Cm) and gentamicin (Gm) resistance markers were used to differentiate between wild-type *nifA* and mutated *nifA* genes. **B** β-Galactosidase activities of *E. coli* ET8894 containing a *nifH-lacZ* fusion on plasmid pGW7 and different *nifA* hybrid genes on plasmids pRKB5.1 to pRKB5.9. *Hatched bars* show the activity of *nifA* hybrids containing the *Eco*RI, *Sal*I or *Bam*HI fragments of 9 different oxygen-tolerant *nifA* mutants, respectively (pRKB5.1–9E,S,B). The β-galactosidase activity of *E. coli* ET8894 (pGW7) was also determined for the *R. meliloti* wild-type *nifA* gene (wt; *open bars*) and the vector plasmid pACYC177-Gm (V; *closed bar*)

containing plates when exposed to a pure oxygen atmosphere. Therefore, it was assumed that it should be possible to identify *E. coli* cells expressing an oxygen-tolerant NifA mutant protein by assaying for formation of dark blue colonies on X-Gal-containing plates under these conditions. To prevent low-level activation of the *R. meliloti nifH* promoter by the *E. coli* NtrC protein (Ausubel 1984) the NifA activation experiments were all performed in the *lac*$^-$ *ntrC*$^-$ strain ET8894 (Mac Neil 1981).

To generate oxygen-tolerant NifA mutants, plasmid pRmW541-10 was treated *in vitro* with hydroxylamine as described in Materials and methods. The mutagenized DNA was then introduced into *E. coli* ET8894 carrying the reporter plasmid pGW7. Subsequently, the transformants were plated on X-Gal-containing agar and incubated in a pure oxygen atmosphere. By this method 73 oxygen-tolerant NifA mutants were isolated from 9 independent hydroxylamine mutagenesis experiments. To exclude mutations in the reporter plasmid or in the chromosomal background of the host, the mutated *nifA* plasmids were isolated, reintroduced into *E. coli* ET8894 (pGW7) and retested for the expression of oxygen-tolerant NifA activity. The activation of a *nifH-lacZ* fusion in plasmid pGW7 by 17 of these oxygen-tolerant NifA mutants was analyzed in detail (Table 2). Compared to the *R. meliloti* wild-type NifA (pRmW541-10), which was able to activate the *nifH-lacZ* fusion only under microaerobic conditions, the oxygen-tolerant NifA mutants mediated up to a 12-fold increase in β-galactosidase activity under high oxygen tensions. A slightly increased activity (1.5–2-fold) was found for all oxygen-tolerant NifA mutants under microaerobic conditions.

Mutations leading to oxygen-tolerant forms of the R. meliloti *NifA protein are located in the central part of the* nifA *gene*

To map the sites of mutation within the *nifA* coding region and to exclude promoter mutations or copy number effects, a procedure was developed that allowed the construction of hybrid *nifA* genes consisting of different parts of the wild-type and the mutated *nifA* genes (Fig. 1A). To facilitate the combination of the different parts, the mutated gene was first linked to a gentamicin resistance gene whereas the wild-type *nifA* gene was linked to a chloramphenicol resistance gene. Depending on the restriction enzyme used, DNA fragments coding only for the DNA binding domain (*Bam*HI), the DNA binding domain, the interdomain linker and the C-terminal part of the central domain (*Sal*I) or a fragment encoding all three domains of the *nifA* gene product (*Eco*RI), were exchanged between wild-type and mutated *nifA* genes.

Nine oxygen-tolerant *nifA* mutant genes were chosen and the corresponding *nifA* hybrid genes were constructed according to the scheme presented in Fig. 1A. The resulting 27 hybrid plasmids were introduced into *E. coli* ET8894 carrying the reporter plasmid pGW7 and the activities of the resulting NifA proteins were determined under aerobic conditions. As shown in Fig. 1B only those

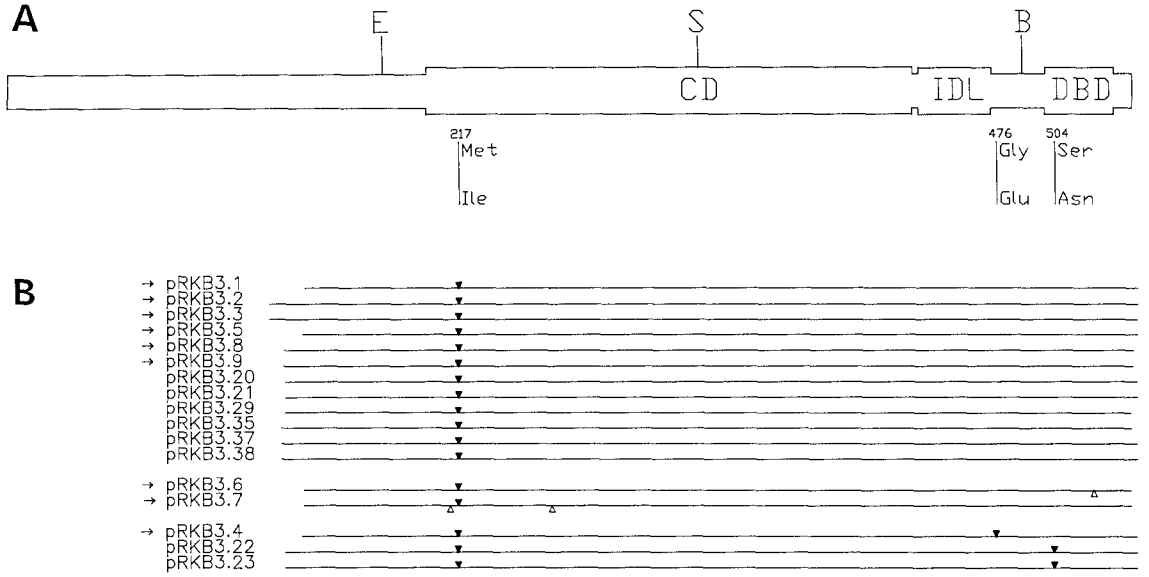

Fig. 2A, B. DNA sequence analysis of mutated *R. meliloti nifA* coding regions leading to expression of oxygen-tolerant NifA proteins. **A** Domain structure of the NifA protein. The amino acid replacements in the oxygen-tolerant NifA mutants are indicated below the corresponding wild-type residue. The numbers correspond to the amino acid position in the wild-type *R. meliloti* NifA protein. **B** The segments of the mutated NifA coding regions sequenced and the position and type of mutation found are indicated. *Closed triangles* mark DNA mutations that cause amino acid exchanges and those which do not alter the amino acid sequence are symbolized by *open triangles*. Plasmids marked by an *arrow* were used for the construction of *nifA* hybrids (Fig. 1). Abbreviations are as in Fig. 1

nifA hybrid genes generated by the *Eco*RI fragment exchange mediated the oxygen-tolerant mutant phenotype. The inability of the *Sal*I and *Bam*HI fragments to transfer the oxygen-tolerant phenotype indicated that the mutation sites responsible for oxygen tolerance in all 9 mutants tested were localized within the *Eco*RI-*Sal*I fragment carrying the N-terminal part of the central domain. These experiments also ruled out the possibility that the oxygen tolerance of the NifA mutants might be due to higher levels of NifA gene expression associated with promoter mutations or copy number effects.

A methionine – isoleucine exchange (M217I) close to the putative nucleotide binding site in the central domain of the R. meliloti *NifA protein results in oxygen tolerance*

The construction of *nifA* hybrid genes indicated that the mutations resulting in oxygen-tolerant mutants of the *R. meliloti* NifA protein were located in the N-terminal part of the central domain. To localize the exact mutation site, the DNA sequences of these 9 mutant *nifA* genes were determined. In addition, 8 further oxygen-tolerant *nifA* mutant genes were chosen for DNA sequence analysis, which was performed by the dideoxy method using appropriate primers and the original *nifA* mutant plasmids without further subcloning steps.

Surprisingly all 17 mutant genes carried identical mutations (Fig. 2). A guanosine/adenosine mutation resulted in a methionine (AU*G*)/isoleucine (AU*A*) exchange at position 217 in the amino acid sequence of the *R. meliloti* NifA protein. The M217I exchange is located

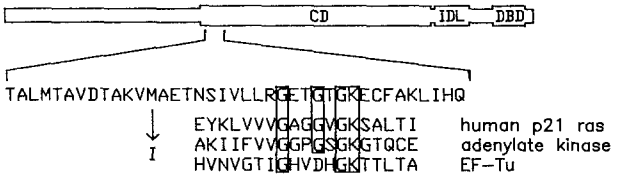

Fig. 3. Location of the M217I amino acid exchange of oxygen-tolerant *R. meliloti* NifA mutants in relation to the putative nucleotide binding site. The position of the methionine-isoleucine exchange (M217I) is shown relative to the putative nucleotide binding site. The alignment of the putative *R. meliloti* nucleotide binding site with nucleotide binding sites of the human p21 *ras* protein (DeFeo-Jones et al. 1983), the rabbit adenylate kinase (Kuby et al. 1984) and the *E. coli* elongation factor EF-Tu (Laursen et al. 1981) is also presented. The conserved amino acid residues of the nucleotide binding consensus sequence are *boxed*. Abbreviations are as in Fig. 1

only 10 residues upstream of the highly conserved G-X_2-G-X-G-K motif found in nucleotide binding sites of different proteins (Fig. 3). No further mutations were found within the sequenced region of 12 *nifA* mutant genes (Fig. 2) confirming the finding that mutations within the *Eco*RI-*Sal*I fragment are responsible for the oxygen-tolerant phenotype. Two mutant genes located on plasmids pRKB3.6 and pRKB3.7 carried additional mutations, which, however, did not alter the amino acid sequence. Three mutant proteins encoded by plasmids pRKB3.4, pRKB3.22 and pRKB3.23 contained additional amino acid exchanges. The construction of *nifA* hybrid genes showed, that the G476E exchange in the interdomain linker region of mutant pRKB3.4 is not able to generate an oxygen-tolerant NifA protein, when separated from the M217I exchange.

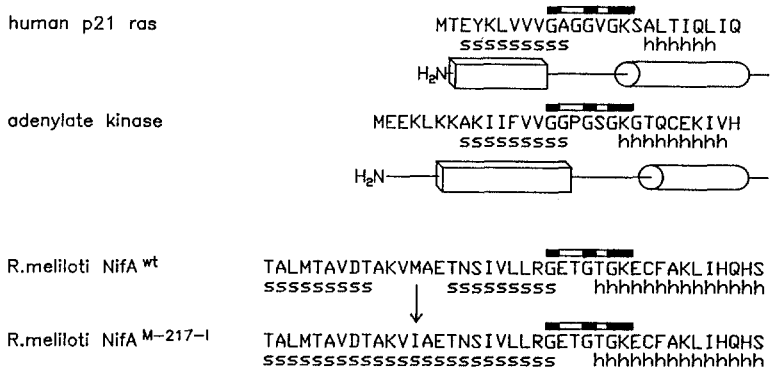

Fig. 4. Comparison of predicted secondary structures in the nucleotide binding site of the *R. meliloti* wild-type NifA protein, the *R. meliloti* NifA^{M217I} mutant and proteins with known three-dimensional structures. The amino acid sequences in the vicinity of the nucleotide binding site in the human p21 *ras* protein (DeFeo-Jones et al. 1983), the rabbit adenylate kinase (Kuby et al. 1984), the *R. meliloti* wild-type NifA protein and the *R. meliloti* NifA^{M217I} mutant are given. The conserved amino acids in the glycine-rich loop are marked by *filled bars*. A high probability for β-sheet (s) and α-helix formation (h) predicted by the Chou and Fasman method are shown below the amino acid sequences. *Blocks* and *cylinders* indicate the positions of the β-sheet and the α-helical segments determined by X-ray analysis of the human p21 *ras* protein (Pai et al. 1989) and the rabbit adenylate kinase (Fry et al. 1986)

The methionine – isoleucine exchange M217I alters the predicted secondary structure of the putative nucleotide binding site of the R. meliloti *NifA protein*

All oxygen-tolerant *R. meliloti* NifA mutants carried a M217I mutation near the putative nucleotide binding site, suggesting that this protein domain might play a major role in the oxygen-dependent regulation of NifA activity. Nucleotide binding sites have been identified at the protein sequence level in different ATP and GTP binding proteins (Saraste et al. 1990). The conserved amino acid sequence consisting of a glycine-rich sequence flanked by a conserved lysine residue is also present in all NifA proteins so far identified (Bennett et al. 1988; Buikema et al. 1985; Drummond et al. 1986; Grönger et al. 1987; Iismaa and Watson 1989; Masepohl et al. 1988; Nees et al. 1988; Roelvink et al. 1989; Souza et al. 1991; Thöny et al. 1987; Weber et al. 1985). In ATP and GTP binding proteins with known 3–D structures the nucleotide binding pocket is formed by a glycine-rich flexible loop and two flanking regions with defined secondary structures: a β-sheet on the N-terminal site and an α-helix on the C-terminal site of the loop (Fry et al. 1986; Jurnak 1985; Pai et al. 1989).

To ascertain whether similar secondary structures are also present in *R. meliloti* NifA and whether these secondary structures are altered in the NifA^{M217I} mutant, we performed a secondary structure analysis by computer. Figure 4 shows the predicted secondary structures of the *R. meliloti* NifA protein, the *R. meliloti* NifA^{M217I} mutant protein and of proteins with known 3–D structures. In contrast to the secondary structure prediction method of Garnier (1978), the secondary structures of the nucleotide binding sites for the human p21 *ras* protein and rabbit adenylate kinase predicted by the Chou and Fasman method (1974a, b) with an extended data base (Argos et al. 1978) showed nearly perfect agreement with the structure determined by X-ray analysis (Fig. 4). Therefore we presume that the NifA secondary structures predicted by this method are good approximations of the true structures. The calculated probability for β-sheet and α-helix formation in the regions flanking the glycine-rich flexible loop in *R. meliloti* NifA is in accordance with those found for the human p21 *ras* protein and adenylate kinase. The methionine to isoleucine exchange in the oxygen-tolerant NifA mutant changes the predicted secondary structure. The probability for β-sheet formation is increased and it is likely that the two distinct β-sheets found in the wild-type NifA protein are connected to form one large β-sheet in the NifA^{M217I} mutant.

Discussion

The *R. meliloti* NifA protein is able to activate *nif* consensus promoters only under microaerobic conditions. By *in vitro* hydroxylamine mutagenesis mutant *nifA* genes were created, which encode oxygen-tolerant NifA proteins. These mutants were identified by their ability to activate the promoter of a *nifH-lacZ* fusion even in the presence of pure oxygen. Construction of *nifA* hybrid genes consisting of different parts of the wild-type and the mutated *nifA* genes revealed that the mutations are located within the *nifA* coding region. Oxygen-sensitive NifA proteins are characterized by an interdomain linker region, which is missing in the oxygen-tolerant NifA proteins of *K. pneumoniae* and *A. vinelandii*. Therefore, the interdomain linker was thought to be responsible for the oxygen sensitivity and we expected that the mutation sites should be localized within this domain of the *R. meliloti* NifA protein. However, the construction and analysis of *nifA* hybrid genes demonstrated that the mutations resulting in oxygen-tolerant NifA mutants were not located in the interdomain linker region nor in the DNA binding domain but in the N-terminal part of the central domain.

DNA sequence analysis showed that all 17 independently isolated NifA mutants are characterized by the same methionine – isoleucine exchange at amino acid position 217. The large number of mutants carrying the same amino acid exchange indicates that the methionine

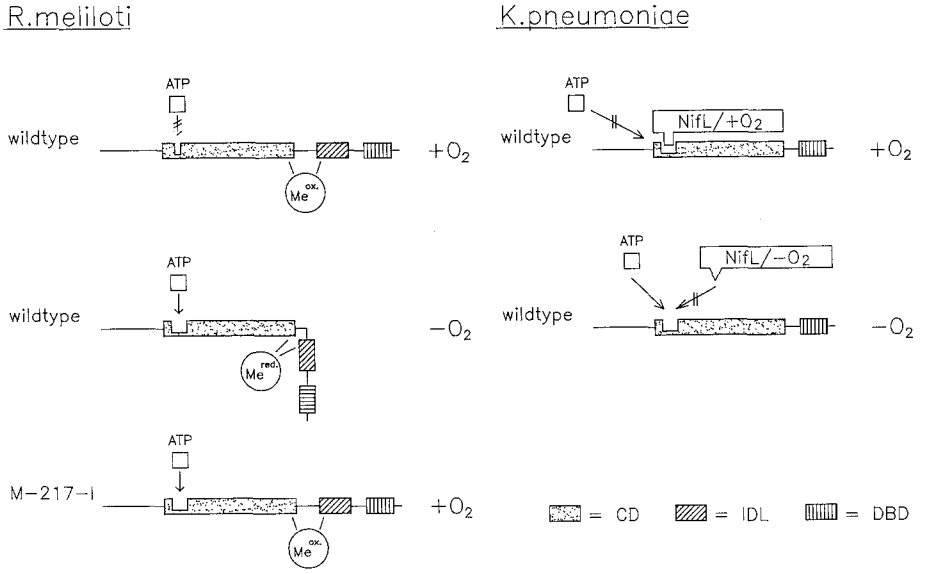

Fig. 5. Models that account for the influence of the ATP binding site on the regulation of oxygen-sensitive and oxygen-tolerant NifA proteins. High oxygen concentrations may influence the redox state of a metal ion (Me^{ox}/Me^{red}) bound by cysteine residues in the interdomain linker region (IDL) and the C-terminal part of the central domain (CD) of oxygen-sensitive NifA proteins like *R. meliloti* NifA. The resulting conformational change could be responsible for alterations in the nucleotide binding site which prevent ATP binding or hydrolysis. The M217I mutation in oxygen-tolerant NifA mutants could be responsible for a conformational change in the nucleotide binding site allowing the nucleotide to be bound or hydrolyzed even under aerobic conditions. Under aerobic conditions the activity of oxygen-tolerant NifA proteins like *K. pneumoniae* NifA is regulated by the NifL gene product. Protein – protein interaction between NifL and NifA might block the nucleotide binding site or prevent hydrolysis of the nucleotide at high oxygen concentrations or in nitrogen excess

affected is involved in the oxygen-dependent regulation of NifA activity. Since this methionine residue is not conserved in oxygen-sensitive NifA proteins from various organisms it is unlikely that the methionine itself plays a direct role in oxygen regulation. Therefore, we believe that the oxygen-tolerant phenotype of the $NifA^{M217I}$ mutant is due to a conformational change in the central domain.

Since it has been shown that protein degradation mediated by the *lon/sno* system participates in the aerobic inactivation of *R. meliloti* NifA in *E. coli* (Huala et al. 1991), the conformational change in the $NifA^{M217I}$ mutant may influence the stability of the *nifA* gene product. To test this hypothesis we have analyzed NifA-LacZ and $NifA^{M217I}$-LacZ fusions. The *Bam*HI site within the *R. meliloti nifA* gene, which is located downstream of the interdomain linker region (Fig. 1A), was used to construct translational fusions with *E. coli* LacZ. The analysis of these two fusion proteins revealed no differences in β-galactosidase activity under aerobic conditions (NifA-LacZ: 2503 ± 88 β-galactosidase units; $NifA^{M217I}$-LacZ: 2636 ± 111 β-galactosidase units; data not shown). However, these experiments could not demonstrate conclusively that there are no differences in degradation rates between the wild-type NifA and the oxygen-tolerant $NifA^{M217I}$ mutant, since these fusion proteins may themselves have stabilities different from that of wild-type NifA or might retain β-galactosidase activity even if the N-terminal NifA portion were degraded.

For *B. japonicum* NifA it has been shown that a shift to aerobic conditions results in loss of protection of both the upstream activator sequence and the promoter, indicating that DNA binding and activation functions of NifA are both controlled by oxygen (Morett et al. 1991). Therefore it is more likely that the M217I mutation in *R. meliloti* NifA interferes with one of these steps rather than influencing only the stability of the protein. The methionine in position 217 is only 10 amino acid residues away from the putative nucleotide binding site, which is highly conserved in all NifA proteins. Nucleotide binding sites are characterized by three distinct structural elements (Möller and Amons 1985; Fry et al. 1986; Saraste et al. 1990): the phosphate binding loop (P-loop) formed by a glycine-rich sequence linked to a conserved lysine; an α-helix structure at the C-terminal part of the P-loop and a β-sheet structure at the N-terminal part of the P-loop. Amino acid sequence homologies and secondary structure predictions indicate the presence of the same three structural elements within the nucleotide binding site of the *R. meliloti* NifA protein. Interestingly, the secondary structure prediction for the *R. meliloti* oxygen-tolerant NifA mutant differed from that for the wild-type NifA protein. The methionine to isoleucine exchange at position 217 increases the probability of β-sheet formation in this area. It is worthy of note that hydroxylamine mutagenesis is able to create 9 different amino acid exchanges within the 8 amino acid residues separating the two predicted β-sheets in front of the glycine-rich P-loop (Fig. 4). Secondary structure predictions for all these possible mutants revealed that only the M217I exchange results in a sharply increased probability for β-sheet formation; all other mutants result only in minor changes (data not shown). This might explain why all mutants in the ATP binding site resulting in oxygen-tolerant NifA

mutants carry the same methionine to isoleucine exchange. Since the β-sheet linked to the P-loop is necessary for the correct three-dimensional structure of the nucleotide binding site, we suggest that alterations in the NifA^{M217I} protein structure are more likely to influence the function of the nucleotide binding site than to change other functions of the central domain, such as interaction with the σ^{54}-RNA polymerase complex. A central role for the ATP binding site in transcriptional activator proteins has been demonstrated in experiments using the *K. pneumoniae* NtrC protein, which shows structural and functional similarities to NifA. It was shown that ATP is required for the NtrC-dependent activation of *ntr* promoters and that a mutation in the ATP binding site results in an inactive NtrC protein (Popham et al. 1989; Austin et al. 1991). Binding of this mutant protein to upstream activator sequences and binding of the σ^{54}-RNA polymerase complex to *ntr* promoters was not affected. However, the NtrC mutant lacked the ability to catalyze isomerization of a closed complex comprising σ^{54}-RNA polymerase and the *ntr* promoter into a transcriptionally active open promoter complex.

Recently it has been demonstrated that NtrC proteins harbouring mutations in the vicinity of the ATP binding site show increased activity. The activity of these NtrC mutants was no longer dependent on phosphorylation and the protein showed intrinsic ATPase activity, which is not observed in the wild-type protein (Dixon et al. 1991). Our results indicate that the nucleotide binding site might also be involved in the oxygen-dependent regulation of *R. meliloti* NifA activity. It seems likely that conformational changes near the ATP binding site of NifA and NtrC may uncouple ATP binding or hydrolysis from negative regulation. Therefore, we prospose the following model for the oxygen-dependent regulation of *R. meliloti* NifA activity. The oxygen concentration may be responsible for conformational changes in the interdomain linker region mediated via the redox state of a metal ion. This conformational change may be transduced to the central domain, resulting in alterations of the nucleotide binding site. Under high oxygen tensions ATP binding or hydrolysis is prevented and therefore isomerization of the closed promoter complex cannot occur. The conformational change in the nucleotide binding site of the oxygen-tolerant NifA^{M217I} mutant may uncouple this intramolecular signal transduction and allow ATP binding or hydrolysis even in the presence of oxygen (Fig. 5). The altered structure of the nucleotide binding site might also enhance the accessibility for ATP under microaerobic conditions, resulting in increased activity under these conditions.

An analogous regulatory role of the nucleotide binding site can be assumed for the oxygen-tolerant NifA proteins of *K. pneumoniae* and *A. vinelandii*, which lack the interdomain linker region. In these organisms the NifL protein is responsible for oxygen regulation. It has been shown that *K. pneumoniae* NifL and NifA form a protein complex under repressing conditions (Henderson et al. 1989) and it was concluded by Drummond et al. (1990) that the NifL protein must act directly upon the central domain of NifA. According to our model it is possible that this protein-protein interaction between NifL and NifA might either block the nucleotide binding site or prevent hydrolysis of the nucleotide (Fig. 5) and therefore might substitute for the conformational change proposed for the oxygen-sensitive type of NifA.

The analysis of oxygen-tolerant *R. meliloti* NifA mutants has shown that the nucleotide binding site may play a central role in the regulation of the NifA activity. Further experiments are now necessary to elucidate the regulatory role of this protein domain previously thought not to be involved in oxygen regulation.

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