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Reduced Level of NADH-dependent Nitrate Reductase Activity in Rice Mutant M819 Due to Deletion of a Valine Residue in Heme Domain

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Summary

It was previously reported that the rice nitrate reductase (NR)-deficient mutant M819 (wild type: cv. Norin 8) lacks the functional heme domain of NADH-NR polypeptide possibly due to a point mutation or small deletion. In this paper we were able to locate the mutation in the NR apoenzyme gene. Northern blot analysis using a rice NADH-NR genomic clone as a probe showed that the size of NR mRNA of M819 was the same as that of the wild type, suggesting that the mutation did not involve a large deletion or insertion in the NADH-NR coding sequence. The DNA fragment containing the coding region of NADH-NR heme domain was amplified by PCR from M 819 and Norin 8. The alignment of nucleotide sequences of the domain revealed that 3 nucleotides (GTC) encoding one valine residue (Val-561 or Val-562) were deleted in M819. Thus, it was demonstrated that there was a point mutation in the coding region of NADH-NR heme domain in the mutant M819.

Key Words : Nitrate reductase, Heme domain, *Oryza sativa*, Mutant.

Introduction

Nitrate is a major nitrogen source for higher plants. In plants, the reduction of nitrate to nitrite by nitrate reductase (NR; NADH, EC 1.6.6.1 and NAD(P)H, EC1.6.6.2) is considered to be a rate-limiting step of nitrate assimilation, because the reduction occurs in the first step of the nitrate assimilation pathway (Caboche and Rouzé 1990, Srivastava 1992). NR from plants is a homodimeric enzyme and each subunit (100-120 kDa) contains three prosthetic groups: FAD, heme (cytochrome b_{557}) and a molybdenum cofactor (MoCo) (Caboche and Rouzé 1990, Srivastava 1992, Hoff *et al.* 1992). In the order, these prosthetic groups transfer two electrons from NAD(P)H to nitrate. By limited proteolysis of spinach NR with trypsin and *Staphylococcus aureus* V8 protease, Kubo *et al.* (1988) suggested that the NR subunit is organized into three discrete domains containing each of the three prosthetic groups, that is, 28 kDa FAD domain, 14 kDa heme domain and 75 kDa MoCo domain. In addition to the physiological activity, the enzyme can also display a variety of *in vitro* partial activities using either artificial electron donors (MV, $FMNH_2$ and BPB) to reduce nitrate to nitrite or artificial electron acceptors (cytochrome c and ferricyanide) (reviewed in Caboche and Rouzé 1990, Meyer *et al.* 1991, Hoff *et al.* 1992).

In order to enhance nitrate assimilation in crops, it is necessary to analyze both regulation mechanism and structure/function relationships of NR at the molecular level. The application of recombinant DNA technology has improved our understanding of transcriptional regulation of the enzyme (Vincentz and Caboche 1991, Vaucheret *et al.* 1992, Jensen *et al.* 1996). Furthermore in spinach and *Arabidopsis,* a functional serine residue has been recently identified in the NR molecule as a post-translational regulatory site (Bachmann *et al.* 1996, Su *et al.* 1996). Several essential amino acid residues required for NR activity have been identified by sequence analysis of NR apoenzyme-deficient mutants (*nia* mutants) as well as by site-directed mutagenesis (Dwivedi *et al.* 1994, Meyer *et al.* 1991, 1995, Wilkinson and Crawford 1993, LaBrie and Crawford 1994).

Hasegawa *et al.* (1991) isolated a rice *nia* mutant M 819 as a chlorate-resistant plant. Biochemical characterization showed that in the mutant M819 NADH-NR activity and all the partial activities involving the heme domain (MV- and $FMNH_2$-NR activities) were impaired, while the mutant showed a normal level of both NADPH- and BPB-NR activities (Hasegawa *et al.* 1992). Thus, Hasegawa *et al.* (1992) suggested that a point mutation or small deletion was located in the heme domain-coding region of the NADH-NR structural gene in M819. In this paper we analyzed the structural change of a NR gene in M819 by northern blotting and sequencing to investigate the structure/function relationship of the enzyme at the molecular level.

Materials and Methods

Plant materials

A rice *nia* mutant line M819 and the wild type cv.

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'Norin 8' were used. M819 was isolated as one of the chlorate-resistant mutant lines from γ-ray-induced mutant lines maintained at the Institute of Radiation Breeding, Japan (Hasegawa *et al.* 1991, 1992). Seedlings were cultured for 14 days at 28 °C in IRRI's culture medium (Yoshida *et al.* 1972). Shoots were harvested, frozen in liquid nitrogen and stored at -80 °C to isolate DNA. For RNA analysis, seedlings were grown in Kimura's B solution (Yoshida *et al.* 1972) at 30 °C. Fourteen-day-old seedlings were transferred onto a 10 mM potassium nitrate solution to induce the expression of NR gene. Three hours after the transfer, shoots were harvested, frozen in liquid nitrogen and stored at -80 °C until use.

Northern blot hybridization

Total RNA was extracted from the shoot of each genotype as described by Kuipers *et al.* (1994). Ten μg RNA was used for northern blotting. RNA fractionation by formaldehyde gel electrophoresis and transfer to nylon membrane (Nylon membrane positively charged, Boehringer Mannheim) were performed according to the method of Watanabe (1989).

The blot was probed with a 3.4 kb *Eco*RV-*Bgl* II fragment from the rice genomic NADH-NR clone, pHBH1 (Hamat *et al.* 1989) kindly provided by Dr. A. Kleinhofs (Washington State U.) and with a 360 bp *Not* I fragment from pN8 plasmid DNA cloned by PCR as described later. Labeling of the probes and hybridization were carried out according to the manufacturer's instructions (DIG-AMPPD system, Boehringer Mannheim).

DNA isolation and oligonucleotide primers synthesis

Total DNA was extracted from the shoots of the *nia* mutant M 819 and wild type Norin 8 according to the modified CTAB method (Murray and Thompson 1980), and used for PCR amplification. Four oligonucleotide primers: 5 A, 5'-CAAGTACGGCAAGCACTGGT-3' (forward, nucleotides 3621-3640); 3 A, 5'-ACGGAGTT GTAGGAGCTGTA-3' (reverse, nucleotide 4317-4298); V 2, 5'-GACTCGGCGTGGATCGTCGT-3' (forward, nucleotide 4100-4119) and 3 B, 5'-GACCTCGTCGACC ATGCTGCTCG-3' (reverse, nucleotide 4591-4572) were designed from the sequence of rice *nia* 1 NADH-NR gene (Choi *et al.* 1989). Synthesis of 5 A and 3 A primers was entrusted to the Funakoshi co., LTD and the remaining two primers (V 2 and 3 B) were ordered to Biologica Co., LTD.

Cloning and sequencing of NADH-NR heme domain.

PCR amplification with 5 A and 3 A primers was performed using 2.5 U *Pfu* DNA polymerase (STRATAGENE) in a total volume of 25 μl of the native *Pfu* buffer # 1 (1 ×, STRATAGENE) containing 0.2 mM dNTPs, 10 % DMSO, 12.5 ng genomic DNA and 20 pmol of each primer. Cycling conditions for PCR were 1 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 58 °C and 2 min at 72 °C followed by 4 min at 72 °C for final extension.

The approximately 700 bp products from M819 and Norin 8 that was amplified were cloned into the *Eco*RV site of pBluescript II SK$^+$ (STRATAGENE) using the DNA ligation kit ver. 2 (Takara). The recombinant plasmid DNAs derived from M819 and Norin 8 were designated as p819 and pN 8, respectively. Three different clones of p819 and pN 8 were used for subcloning and sequencing. The approximately 330 bp *Eco*RI-*Not* I fragment of the p819 and pN 8 containing the heme domain was then subcloned into pBluescript II SK$^+$ (STRATAGENE), and the plasmids were used as templates for the sequencing reactions which were performed using the dye primer sequencing kit (Applied Biosystems) and then analyzed by a 377A automatic DNA sequencer (Applied Biosystems). Sequencing was performed three times for each strand. DNA sequences were analyzed by the GENETYX software (Software-Kaihatsu, Japan) and the secondary structure of the predicted proteins was analyzed using the PHD neural network at the EMBL (Heidelberg, Germany).

Simple detection of the mutation by PCR

V 2 and 3 B primers were used for the PCR. V 2 primer was designed at the position of mutation in M819. PCR amplification with 2.5 U *Taq* DNA polymerase (TOYOBO) was performed in 25 μl of the reaction buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 0.1 % Triton X-100, 2.5 mM magnesium chloride, 0.2 mM dNTPs, 50 ng genomic DNA and 20 pmol of each primer. Cycling conditions were the same as those described above, except for the change of the annealing temperature at 64 °C.

Southern blot hybridization

One μg pN 8 plasmid DNA was digested with *Apa*I follows by *Bam*HI. Thirteen ng digest was electrophoresed in 0.8 % agarose gel and transferred onto the nylon membrane positively charged (Boehringer Mannheim) with 0.4 M sodium hydroxide solution. The blot was probed with a 3.4 kb *Eco*RV-*Bgl*II fragment from the pHBH1 clone. Labeling of the probe and hybridization were performed with the ECL systems (Amersham).

Results

Northern blot analysis of NR mutant

Initial northern blot analysis of the mutant M819 and wild type was performed using a rice NADH-NR genomic clone pHBH 1 as a probe. The probe identified an about 3 kb band in both M819 and Norin 8 (Fig. 1). The size of the NR mRNA of M819 was approximately the same as that of the wild type Norin 8, which suggests that the mutation did not involve a large deletion or insertion in the NADH-NR coding sequence.

PCR-based cloning of NADH-NR heme domain

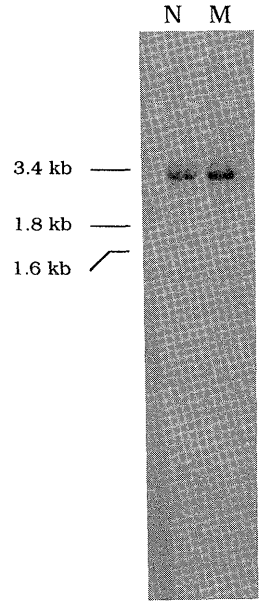

Fig. 1. Northern blot analysis of NR transcripts in total leaf RNA from M819 with putative mutant in the heme domain and wild type Norin 8. Hybridization was performed with the 3.4 kb *Bgl*II-*Eco*RV fragment of rice NADH-NR clone, pHBH1. Molecular size was determined based on the location of rice rRNAs (25 S, 17 S and 16 S). N : Norin 8, M : M819.

An approximately 700 bp fragment (consistent with the length we had designed) was amplified from genomic DNAs of M819 and Norin 8 by PCR (Fig. 2 A, B). No size difference was observed between the mutant and wild type in the PCR products, thus supporting the

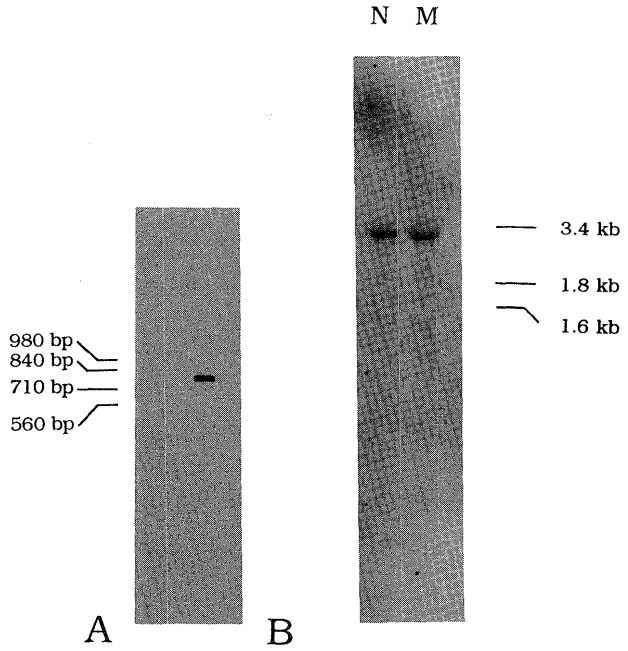

Fig. 3. Homology analysis between pN 8 and NR gene using Southern and northern blot hybridization. A; Southern blot hybridization between pN 8 and pHBH 1. The pN 8 plasmid was digested with *Apa* I and *Bam* HI, and hybridized to the 3.4 kb *Bgl* II-*Eco*RV fragment of pHBH1. The size of the hybridizing band was deduced from the lambda DNA digested with *Hin*dIII and *Hin*cII. B; Northern blot analysis of NR transcripts in M819 and Norin 8. Hybridization probe was the 360 bp *Not* I fragment of pN 8 clone. Molecular size was determined as described in the legend to Fig. 1. N: Norin 8, M: M819.

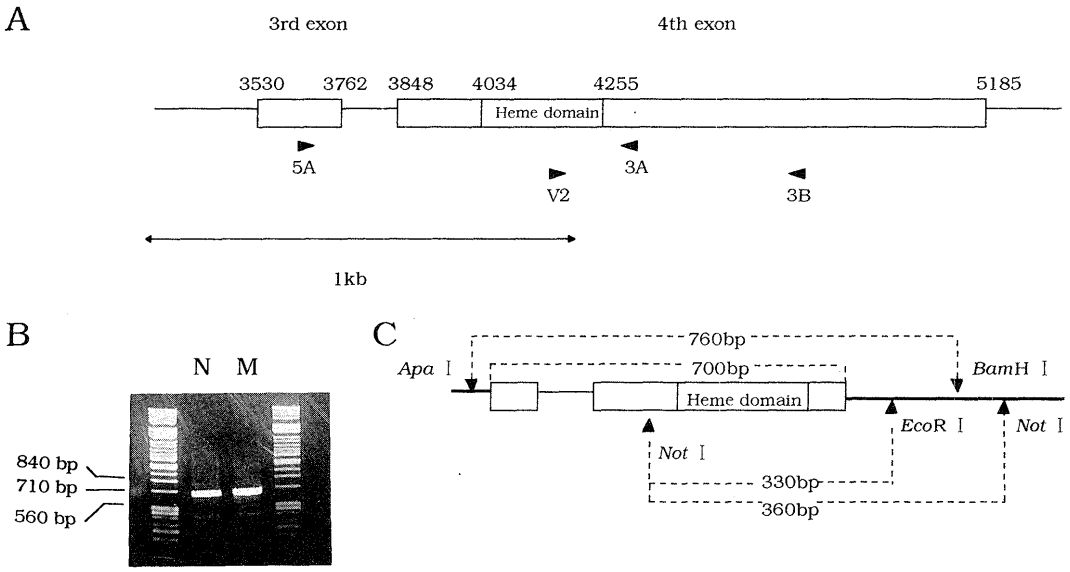

Fig. 2. PCR-based cloning of NADH-NR heme domain from M819 and wild type Norin 8. A; Position and orientation of the oligonucleotide primers used in the study. The numbering of the nucleotides and the border of exon/intron are taken from rice *nia* 1 genomic sequence (Choi *et al.* 1989). B; PCR products in M819 and Norin 8 using 5 A and 3 A primers described in A. Molecular weight markers consisted of lambda DNA digested with *Hin*d III and *Hin*cII. N: Norin 8, M: M819. C; Schematic representation of plasmid pN 8 and p819. Bold line indicates the vector region (pBluescript II SK^+).

northern blot results. The PCR products from M819 and Norin 8 were cloned into the *Eco*RV site of pBluescript II SK$^+$ and designated as p819 and pN 8, respectively (Fig. 2 C).

In order to confirm that the PCR products were homologous to the NADH-NR gene, Southern and northern blot analyses were performed with the pN 8 clone. The pN 8 clone digested with *Apa*I and *Bam*HI was electrophoresed on a 0.8 % agarose gel and probed with the 3.4 kb *Eco*RV-*Bgl* II fragment from the pHBH 1 clone. The Southern blot analysis showed that a 760 bp band containing the 700 bp PCR product hybridized to the NR probe, indicating that the PCR product was indeed a part of the NADH-NR gene (Fig. 3 A). We then performed the northern blot analysis. The 360 bp *Not*I-digested pN8 fragment labeled with DIG detected the NR mRNA, about 3 kb, in Norin 8 and M819 (Fig. 3 B), which was identical with the result obtained using the pHBH 1 probe (Fig. 1) and suggested that we had indeed cloned the NADH-NR heme domain.

Identification of the mutation

Both p819 and pN8 clones were digested with *Eco*RI and *Not* I, and the resulting fragments of approximately 330 bp which contained the heme domain were subcloned into pBluescript II SK$^+$ and sequenced, respectively. The DNA sequence of the domain of Norin 8 was completely identical with that of the rice *nia* 1 NADH-NR gene derived from cv. M201 (Choi *et al.* 1989). However, sequencing of the domain of M819 revealed the presence of a deletion of 3 nucleotides (GTC) encoding one valine residue (Val-561 or Val-562) (Fig. 4).

To confirm the sequence data, we designed the V2 primer (5'-GACTCGGCGTGGATCGTC*GT*-3') at the position of mutation in M819 (Fig. 2 A and Fig. 4) and carried out PCR with the primer and 3 B reverse primer. The expected size (490 bp) of the band was amplified in Norin 8, but not in M819 (Fig. 5), which indicated that the mutation in M819 was accurately located at the position of the NADH-NR gene. Thus, it was demonstrated that the mutant M819 contained a point mutation in the coding region of NADH-NR heme domain.

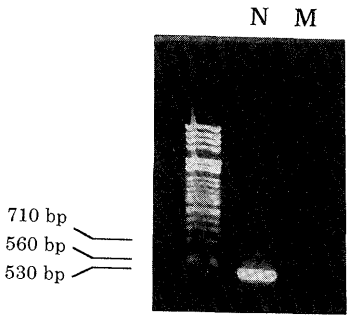

Fig. 5. Simple detection of the mutation in M819 by PCR. The V 2 and 3 B primers were used for PCR amplification. Primer position was indicated in Fig. 2 A and Fig. 4. Molecular weight marker consisted of the lambda DNA digested with *Hin*dIII and *Hin*c II. N: Norin 8, M: M819.

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        4034                                                                    4093
Norin8      CCG TTC ATG AAC ACC ACC GAC GGC AAG CAG TTT ACC ATG TCC GAG GTG CGC AAG CAC TCG
        534 P   F   M   N   T   T   D   G   K   Q   F   T   M   S   E   V   R   K   H   S  553
M819        CCG TTC ATG AAC ACC ACC GAC GGC AAG CAG TTT ACC ATG TCC GAG GTG CGC AAG CAC TCG
             P   F   M   N   T   T   D   G   K   Q   F   T   M   S   E   V   R   K   H   S

        4094    (5'-GAC TCG GCG TGG ATC GTC GT-3': V2 primer)                   4153
Norin8      TCG CAG GAC TCG GCG TGG ATC [GTC] [GTC] CAC GGT CAC GTC TAC GAC TGC ACG GCC TTC CTC
        554 S   Q   D   S   A   W   I   V   V   H   G   H   V   Y   D   C   T   A   F   L  573
M819        TCG CAG GAC TCG GCG TGG ATC GTC ... CAC GGT CAC GTC TAC GAC TGC ACG GCC TTC CTC
             S   Q   D   S   A   W   I   V   .   H   G   H   V   Y   D   C   T   A   F   L

        4154                                                                    4213
Norin8      AAG GAC CAC CCC GGC GGC GCC GAC AGC ATC CTC ATC AAC GCC GGC ACC GAC TGC ACC GAG
        574 K   D   H   P   G   G   A   D   S   I   L   I   N   A   G   T   D   C   T   E  593
M819        AAG GAC CAC CCC GGC GGC GCC GAC AGC ATC CTC ATC AAC GCC GGC ACC GAC TGC ACC GAG
             K   D   H   P   G   G   A   D   S   I   L   I   N   A   G   T   D   C   T   E

        4214                                                        4255
Norin8      GAG TTC GAC GCC ATC CAC TCC GAC AAG GCC AAG GCG CTC CTC
        594 E   F   D   A   I   H   S   D   K   A   K   A   L   L  607
M819        GAG TTC GAC GCC ATC CAC TCC GAC AAG GCC AAG GCG CTC CTC
             E   F   D   A   I   H   S   D   K   A   K   A   L   L
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Fig. 4. Nucleotide sequences and deduced amino acids sequences of the NADH-NR heme domain of M819 and wild type Norin 8. Gaps in the alignment are represented by dots. One of the two boxed codons in Norin 8 was involved in the mutation in M819. The position of V 2 primer is indicated in parenthesis.

Discussion

The mutation inside the NR apoenzyme gene could be located accurately by measuring the partial activities of the enzyme in the *nia* mutant (Chérel *et al.* 1990). Hasegawa *et al.* (1992) had determined the partial NR activities (MV-NR, $FMNH_2$-NR and BPB-NR) of the rice NADH-NR deficient *nia* mutant M819. However, since the partial activities involving the heme domain (MV-NR and $FMNH_2$-NR activities) in M819 were about one-tenth of those of the wild type, they assumed that the mutation was probably located in the heme domain of NADH-NR (Hasegawa *et al.* 1992). Heme domain deficiency in M819 has been recently confirmed by another experiment in which the reduced level of cytochrome c activity of the mutant was observed (Ichii *et al.* 1995). Attempts to detect structural change of the NR gene in M819 by Southern blot analysis was not successful because the modification was not sufficiently large (Yatou 1991). Thus, it is reasonable to assume that a point mutation or small deletion is involved in the mutation in M819 (Hasegawa *et al.* 1992).

In this paper, we initially compared the size of NR mRNA from M819 with that of the wild type, but no difference was observed between them (Fig. 1), which supported the previous Southern blot results (Yatou 1991). In M819 and the wild type Norin 8, we cloned and sequenced the NADH-NR heme domain which was determined by the homology with the NR heme domain of *Nicotiana plumbaginifolia* described by Meyer *et al.* (1991). The DNA sequence of Norin 8 was completely identical with that of the rice *nia* 1 NADH-NR gene (Choi *et al.* 1989), but sequence data revealed that the 3 nucleotides corresponding to one of the two tandem valines (Val-561 and Val-562) in the heme domain were indeed deleted in M819 (Fig. 4). Thus, the validity of the previous data and location of the mutation proposed by Hasegawa *et al.* (1992) were confirmed. Since we did not determine the complete NADH-NR coding sequence for the mutant, we could not exclude the presence of mutations in the other domains, i. e. FAD and MoCo domains. However, it is reasonable to assume that the frequency of double mutation at a single locus is very low compared to a single mutation event (Meyer *et al.* 1995).

It has been reported that there may be at least three different NR genes in rice (Hamat *et al.* 1989). Therefore, we did not rule out the possibility that p819 was not a part of the NADH-NR gene, but another NR gene, e. g. NAD(P)H-NR gene and NR-pseudogene. Therefore, we designed a V 2 primer at the 3 nucleotide-deficient point in M819 and performed PCR (Fig. 5). The band of expected size (490bp) was amplified in Norin 8, but no band was detected in M819, which demonstrated that p819 is a part of the NADH-NR gene and that the point mutation is located in the gene.

Recently, the essential amino acid residues for NR activity have been determined in plants by sequence analysis of *nia* mutants as well as site-directed mutagenesis. In corn, five "essential" cysteines in the FAD domain were identified by site-directed mutagenesis (Dwivedi *et al.* 1994). Sequencing of the *nia* mutant in *Nicotiana plumbaginifolia* in relation to the heme domain, indicated that one of the heme-binding histidines was converted to asparagine, which resulted in the inactivation of NR (Meyer *et al.* 1991). Similarly, several critical residues in the MoCo domain in which mis-sense mutations reduced the NR activity have also been identified by sequence analysis of *nia* mutants in *Arabidopsis* and *Nicotiana plumbaginifolia* (LaBrie and Crawford 1994, Wilkinson and Crawford 1993, Meyer *et al.* 1995). In our experiments, sequencing of the rice *nia* mutant M819 revealed the presence of a deletion of either Val-561 or Val-562 in the heme domain (Fig. 4). Unlike Val-561, Val-562 was completely conserved among the 12 NRs derived from higher plants for e. g., tobacco, bean, tomato, *Arabidopsis,* barley and rice (see review: Hoff *et al.* 1992). Therefore, it is possible that the conserved Val-562 is essential for the expression of the NR activity and, that the critical residue is deleted in M819. Prediction of the secondary structure of the heme domain by the PHD neural network (Rost and Sander 1993, 1994) based on multiple sequence alignment suggested that both the probability for Val-561 and Val-562 to occur in the β-sheet extending from Trp-559 to His-563 exceeded 90 %. Thus, we conclude that the deletion of a valine residue in M819 may locally change the secondary structure of NR, which may affect the NR activity and, therefore, the valine residue (possibly, conserved Val-562) may be critical residue for the enzyme activity. In order to identify Val-562 as a critical residue accurately, site-directed mutagenesis will be employed and Val-562 will be substituted for another amino acid.

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