Biochemical and Immunological Characterization of Nitrate Reductase Deficient nia Mutants of Nicotiana plumbaginifolia

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ABSTRACT

Sixty-five Nicotiana plumbaginifolia mutants affected in the nitrate reductase structural gene (nia mutants) have been analyzed and classified. The properties evaluated were: (a) enzyme-linked immunosorbent assay (two-site ELISA) using a monoclonal antibody as coating reagent and (b) presence of partial catalytic activities, namely nitrate reduction with artificial electron donors (reduced methyl viologen, reduced flavin mononucleotide, or reduced bromphenol blue), and cytochrome c (Cyt c) reduction with NADH. Four classes have been defined: 40 mutants fall within class 1 which includes all mutants that have no protein detectable in ELISA and no partial activities; mutants of classes 2 and 3 exhibit an ELISA-detectable nitrate reductase protein and lack either Cyt c reductase activity (class 2: fourteen mutants) or the terminal nitrate reductase activities (class 3: eight mutants) of the enzyme. Three mutants (class 4) are negative in the ELISA test, lack Cyt c reductase activity, and lack or have a very low level of reduced methyl viologen or reduced flavin mononucleotide-nitrate reductase activities; however, they retain the reduced bromphenol blue nitrate reductase activity. Variations in the degrees of terminal nitrate reductase activities among the mutants indicated that the flavin mononucleotide and methyl viologen-dependent activities were linked while the bromphenol blue-dependent activity was independent of the other two. The putative positions of the lesions in the mutant proteins and the nature of structural domains of nitrate reductase involved in each partial activity are discussed.

In higher plants, NR\(^\text{1}\) (EC 1.6.6.1) has been found to be a homodimer of about 110 kD, containing FAD, heme, and MoCo as prosthetic groups that mediate the electron transfer from NADH to nitrate (3, 14); in Chlorella, NR is an homotetramer dissociable into two active dimers similar to higher plant NR (25). Biochemical analyses on the purified squash NR led to a structural model in which the two identical subunits are organized into domains containing each of the three prosthetic groups: FAD, heme, and MoCo (22). By limited proteolysis, heme and FAD domains have been isolated from Neurospora crassa (17) and Chlorella (26) enzymes, respectively; using the same technique, Kubo et al. (15) have recently released the MoCo domain from spinach NR. Thus, NR appears to be composed of three distinct domains linked by protease-sensitive hinge regions. From the recently obtained NR cDNA and genomic clones sequences, a sequential arrangement, from N to C termini, of MoCo, Cyt b, and NADH:Cyt b reductase domains in the structure of the NR apoenzyme has been found (1, 8, 28).

In addition to the physiological reduction of nitrate with NAD(P)H as electron donor, NR can display partial activities in vitro: reduction of nitrate to nitrite with artificial electron donors (reduced viologen dyes, reduced flavin nucleotides [FMNH\(_2\) and FADH\(_2\)], or BPB), and reduction of electron acceptors such as Cyt c, ferricyanide, or ferric citrate. The former (terminal) activities require the presence of an active molybdenum cofactor, whereas the latter involve at least the FAD domain of the molecule (3, 14, 29). These partial activities can be associated with proteolytic fragments of the enzyme: an FAD and heme-containing fragment of spinach NR displays ferricyanide and CcR activities (15), but the isolated FAD-containing fragment of Chlorella (26) or spinach (15) NR retains only the ferricyanide reductase activity, thus strongly suggesting that the heme domain is needed for the expression of NADH-CcR activity; the MV and FMNH\(_2\)-NR activities are associated with oligomers containing the MoCo and heme domains (15, 26). The BPB-NR activity has recently been described by Hoarau et al. (12) and has been shown to differ from the FMNH\(_2\)- and MV-NR activities by the sites involved on the NR molecule and probably also the electron pathway (2, 19).

Nitrate reductase-deficient mutants are powerful tools for studying both enzyme structure and the regulation of nitrate assimilation. Collections of mutants have been selected from different fungi and plant species (for review see refs. 14 and 29). In higher plants, two kinds of mutations affecting the enzyme structure have been characterized: they can be located either in the apoprotein gene(s) (nia mutants) or in one of the genes which control the synthesis or integration of the MoCo (cnx mutants). In fungi, mutations affecting several genes involved in the regulation of nitrate assimilation have been found (7), but no regulatory mutants have been identified among the extensive collections of higher plant NR\(^*\) mutants (10, 14, 29). Biochemical characterizations of the inactive NR protein have been performed in nia mutants of barley and tobacco species, by measuring the partial activities (13, 18) and the amounts of immunochemically cross-reacting material (16, 23, 27). Two Nicotiana plumbaginifolia mutants have

\(^{1}\) Abbreviations: NR, nitrate reductase; BPB, reduced bromphenol blue; CcR, Cyt c reductase; MV, reduced methyl viologen; NR\(^*\), nitrate reductase-deficient mutants; MoCo, molybdenopterin; FAD, flavin adenine dinucleotide.
already been characterized; one retained CcR activity and the other one was negative in all partial activities tests (9).

The collection of *N. plumbaginifolia* NR− mutants described by Gabard *et al.* (10) is the largest so far obtained in higher plants, and thus is likely to give the most complete picture of possible mutations affecting NR catalytic activity. This paper presents a biochemical classification of 65 *nia* mutants on the basis of partial catalytic activities and reactivity with a monoclonal antibody. In addition to FMNH$_2$ and MV as classical electron donors, we have also used BPB to detect NR activity. In our study, we have also taken advantage of the isolation of monoclonal antibodies against NR, and of the use of *N. plumbaginifolia* as plant material: in this species, a single gene encodes the NR apoenzyme (10), so that a missense mutation affecting this gene will lead to the expression of a single type of modified polypeptide chain. On the other hand, the situation is more complex for most of the other species used as models for the genetic analysis of nitrate reduction: tobacco is an amphidiploid, *Arabidopsis thaliana* apparently carries two functional NR structural genes (4), and barley and soybean synthesize, in addition to the classical NADH-NR, isofoms of NR that can use both NADH and NADPH as electron donors (29).

**MATERIALS AND METHODS**

**Plant Material**

Mutant plants were regenerated from haploid mutagenized protoplasts of *Nicotiana plumbaginifolia* (cv Viviani), after selection on the basis of chloride resistance (10). Mutagenesis conditions have been described in detail in (10). Mutant A7 was induced by UV treatments and the other mutants were either of spontaneous origin or induced by gamma ray treatments. The seeds obtained by selfing of either grafted mutant plants or back-crosses of these plants with the wild type were sown on a medium containing nitrate as nitrogen source (BNO$_3$ medium) (10). The NR− plantlets were then propagated *in vitro* on an ammonium-containing medium and grafted onto tobacco (*Nicotiana tabacum* cv Wisconsin) stocks. The grafted plants were grown under an 8 h dark/16 h light (200–300 μmol·m$^{-2}$·s$^{-1}$) regime; they were watered with a nutrient solution containing nitrate and ammonium (10). The green young leaves were harvested before flowering, after 6 to 8 h of the light period, and stored at ~80°C until extracted. Wild type-grafted plants were used as references.

**Extraction and Purification of NR**

Soluble proteins of the leaves were extracted and ammonium sulfate precipitated as previously described (6). Some of the tests were carried out after an affinity purification step, which was performed either by AMP-Sepharose chromatography or by an immunoaffinity chromatography technique that preserves the enzymatic activity (20). For the immunoaffinity purification step, the monoclonal antibody ZM 96(9)25, directed against maize NR (5), was used as ligand.

**Immunocchemical Tests**

The antigenicity of the mutant NR proteins has been estimated by a two-site ELISA using the monoclonal antibody ZM 96(9)25 as coating reagent, and a polyclonal antiserum raised against maize NR as second antibody (6). In some cases, when specifically indicated, the monoclonal antibody NP 19(19)26 directed against *N. plumbaginifolia* NR (C Meyer, J Grosclaude, T Moureaux, P Rouzé, unpublished data) has also been used. The ELISA reactivity, which is an estimation of the amount of NR cross-reacting material detected in the conditions of the test, has been calculated by the distance between the ELISA curve of the mutant and the wild-type curve used as reference.

The western blot was performed after SDS-PAGE, as previously described (5), using an acrylamide concentration of 10%; the nitrocellulose sheet was allowed to react successively with an antiserum directed against maize NR and an anti-rabbit IgG-peroxidase conjugate (Biosys).

**Enzymatic Assays**

Nitrate reduction with NADH (175 μM), MV (50 μM), FMNH$_2$ (180 μM), or BPB (250 μM) was measured by nitrite accumulation in 0.1 m potassium phosphate (pH 7.5), containing 12 mM KNO$_3$; when artificial electron donors were used, the reaction was initiated by the addition of 7 mM sodium dithionite dissolved in NaHCO$_3$, 50 mM. The reaction was stopped as described by Hoarau *et al.* (12), after 5 min incubation for BPB-NR activity and 30 min for the three other NR activities.

The CcR activity was measured according to Wray and Filner (30). Ammonium sulfate-precipitated crude extracts or affinity-purified extracts were used as source of material for this measurement. Because these extracts may contain NADH-CcR activities that are not linked to NR, the NR-associated NADH-CcR-activity was systematically estimated by the difference between the total CcR activity of the extract measured in the presence of a control monoclonal antibody and the residual activity after inhibition by a saturating amount of inhibitory antibodies. The monoclonal antibody 96(9)25 was used as inhibitory antibody for all the mutant proteins it could recognize. For the mutants in which the monoclonal antibody did not detect an NR protein, the inhibition was performed with the polyclonal antiserum directed against maize NR.

**RESULTS**

**Enzymatic and Immunological Tests of Mutants**

Because of the large number of *nia* mutants, it was necessary to use rapid tests to establish a classification. The two-site ELISA tests and measurements of terminal activities can be performed on ammonium sulfate-precipitated crude extracts, simultaneously for several samples; they were thus used routinely for the characterization of all the mutants. The antibody ZM 96(9)25 was chosen for the ELISA test because of its high affinity for all plant NADH-NRs; it completely inhibits both NADH-CcR and terminal MV and FMNH$_2$-NR activities, but slightly activates the BPB-NR activity (19). In the terminal activities assays, some loss of nitrite due to its reduction by nitrite reductase may occur during incubation. The loss of nitrite due to residual nitrite reductase activity
remaining after ammonium sulfate precipitation has been estimated, by measurement of nitrite disappearance in the absence of nitrate, to represent from 8 to 33% for the MV-NR activity; this loss was very low for the BPB-NR activity. Unlike MV and BPB, FMNH₂ is not used as an electron donor by nitrite reductase, and the assay with FMNH₂ is not biased by nitrite consumption (18). The method of determination of CcR activity after purification of the NR protein was highly reliable; the background level was zero or very low, and the inhibition of NADH-CcR activity was complete when it was performed with the monoclonal antibody ZM 96(9)25. For the mutants which did not react in ELISA with this monoclonal antibody and for which the NR protein could not be purified, the test was less sensitive and less reliable, since the polyclonal antiserum inhibited only 60 to 70% of the N. plumbaginifolia NR CcR activity.

The results of the different tests are presented in Figures 1 and 2 and summarized in Figure 3. As expected, the wild type exhibited a good reactivity in the ELISA test (Fig. 1), CeR activity, and a ratio of about 2:1:1:3 for the NADH, MV, FMNH₂, and BPB-NR activities, respectively (Fig. 2). We verified the complete lack of NADH-NR activity in the mutants; this is in agreement with the observed inability of mutants to grow on nitrate as sole nitrogen source. The nia mutants were each deficient for at least one of the partial activities examined and their properties are discussed below.

**nia Mutants Classification**

**nia** mutants have been classified into four classes, according to their immunological and enzymatic properties (Figs. 1, 2, and 3). Most of the mutants we have tested (40 out of 65) were negative in ELISA and enzymatic tests (class 1 of Fig. 3). As the ELISA involved the use of a monoclonal antibody, we envisaged the possibility that some of these mutants might produce an NR protein not recognized by this antibody; indeed ELISA experiments, using the monoclonal antibody NP 19(19)26 instead of ZM 96(9)25, showed that some of the class 1 mutants, such as I2 or H20, contained cross-reacting material. Some of the class 1 mutants (including mutant I2) produced an mRNA, whereas no mRNA could be detected in others such as E23 or K13 (S Pouteau, I Chérel, H Vauchéret, M Caboché, unpublished results). This class is therefore not homogeneous. It can include mutants in which the NR protein is either not synthesized at all, is present in the extract but not recognized by the antibody ZM 96(9)25, or is extensively degraded. It is expected that a lack of a part of the polypeptide chain, due for instance to premature termination of translation, would result in preferential recognition by the degradation machinery of the cell.

For 14 mutants (class 2), the NR protein was well recognized by the monoclonal antibody ZM 96(9)25 (Fig. 1) and exhibited the three terminal activities in ratios similar to those of the wild type (Fig. 2). This suggests a good integrity of the functional domain of nitrate reduction. On the other hand, no CcR activity could be detected in these mutants. The shapes of the ELISA curves for these mutants are identical to that of the wild type, which shows that the affinity of the
monoclonal antibody and polyclonal antiserum for the NR proteins is not significantly modified (Fig. 1). The relative amounts of protein can therefore be roughly estimated from the distance between different parallel ELISA curves. The terminal activities are dependent on the presence of a functional MoCo (30); the MoCo is therefore expected to be integrated in the apoprotein of the class 2 mutants. As the MoCo seems to be also involved in the dimerization of the enzyme (18), the enzyme would be dimeric. This has been confirmed for one mutant (D51) by gel filtration in HPLC; the NR protein was eluted in the same fractions as the wild-type enzyme (our unpublished data). When an AMP-Sepharose affinity chromatography was performed, no specific elution by NADH could be achieved for the mutants of this class that we have tested (D51, D57, D64, E83, H29) suggesting that their NADH-binding site is affected.

Eight mutants (class 3) have been identified as being affected in the last steps of the electron transfer; they have lost the terminal activities but retain the NADH-CrR activity (Fig. 2). One of them (E87) has been tested by Western blot after an AMP-Sepharose affinity chromatography and showed no alteration of the subunit size (Fig. 4). All of the class 3 mutants share flattened ELISA curves, with lower slope and plateau (Fig. 1). These curve shapes resemble those obtained for most groups of cnx mutants (A Marion-Poll, I Chérèl, M Gonneau, MT Leydecker, F Pelsy, M Caboche, unpublished data) and may thus result from a general phenomenon related to a modification of the domain of the enzyme involved in the terminal activities. This lowering of the titration curves could indicate either a modification of the epitope recognized by the monoclonal antibody ZM 96(9)25 or a reduction of the binding of the polyclonal antiserum. This modified antigenicity might result from a monomeric status of the protein related to a modification of the MoCo binding domain. It might also result from partial proteolytic degradation, related or not to monomerization, of a certain proportion of the NR molecules. In this case, at least part of the degraded forms would be eliminated by the AMP-Sepharose chromatography (Fig.
4). The high NADH-CcR activities obtained in the mutants of class 3, as compared to the wild type on the basis of ELISA signals (Fig. 2), could result from either a better efficiency of catalysis in these mutants, or a bias in the measurement of the NR protein amount.

The last class (4) is represented by only three mutants: E56, A1, and K21. The NR protein of these mutants could reduce nitrate but almost exclusively with BPB as electron donor. The E56 mutant displayed MV and FMNH₂-NR activities but in a ratio of only 1/50 compared to the BPB-NR activity (Fig. 2). As was the case for the other mutants which retained terminal activities, E56 and A1 did not express NADH-CcR activity (Fig. 2). Since in the A1 and K21 mutant extracts the BPB-NR activity was low, it is possible that the two other terminal activities were below the detection limit. These three mutants have lost the epitope recognized by the monoclonal antibody ZM 96(9)25 (Figs. 1 and 2). However, the NR protein of the E56 mutant could be purified by AMP-Sepharose affinity chromatography and visualized by Western blot; since it has the same subunit size as the wild-type enzyme (Fig. 4), the E56 mutant probably carries a missense mutation.

**DISCUSSION**

We report here a biochemical characterization of 65 nia mutants. The most abundant mutants are the class 1 mutants. In this class, very different mechanisms and locations of mutations are possibly involved, including mutations preventing the correct transcription or splicing of the transcript and frameshift mutations. The molecular analysis of the mutated gene will be required to characterize these mutants.

Despite the high frequency of class 1 mutants, the proportion of mutants which retain partial activities is rather high. We obtained 14 class 2 mutants out of a total of 65 that were able to reduce nitrate with artificial electron donors. Among 9 barley and 11 tobacco mutants (13, 18), only one mutant of this type was found in each collection. The percentage of mutants with NR-associated NADH-CcR activity could not be precisely estimated, since the test has been performed for a limited number of mutants. We did not find any mutant with detectable CcR activity among 19 out of 40 class 1 mutants. It is therefore unlikely that NADH-CcR activity expressing mutants will be found in the class 1. Thus, the number of 8 class 3 mutants retaining this activity is probably not greatly underestimated. In other species, the number of such mutants is highly variable: an inducible NADH-CcR activity was detectable in 4 out of 9 barley mutants (13), but in none of the 11 tobacco mutants described by Mendel and Müller (18). We did not find any mutant which exhibited an ELISA reactivity and lacked all NR-associated activities. When all partial catalytic activities are simultaneously lost, the enzyme, if synthesized, is probably in a conformation too altered to be recognized by the monoclonal antibody ZM 96(9)25; conversely, such a mutation could also lead to the expression of an unstable apoenzyme, which might be degraded as soon as it is synthesized. Using a polyclonal antiserum, Somers et al. (27) found only one barley mutant, out of four lacking FMNH₂-NR and CcR activities, in which cross-reacting material could be detected. The mutations leading to a class 4 phenotype, which have never been described in other collections of mutants, seem to be rare events.

As we found independent mutants with similar enzymological and antigenic properties, we looked for correlations between the biochemical type of mutation and other characteristics such as protein amount and conformation. No clear-cut rule concerning the relationship between the biochemical type of mutant and the ELISA estimation of the accumulation of the protein is apparent. The D51 mutant, which belongs to the class 2 expressing terminal NR activities, is the only one which clearly overproduces the NR protein (2.5 to 5 times the wild-type level); this observation is reproducible and is also valid for plantlets propagated in vitro on their roots (data not shown). A clear correlation is observed, however, between the partial catalytic activities pattern and the ELISA profile with the ZM 96(9)25 as coating antibody: this profile is similar to that of the wild type for all class 2 mutants, flattened for class 3 mutants, and close to the X axis in the case of classes 1 and 4 mutants.

The different types of altered NRs that have been characterized also provide information concerning domains that may be involved in partial catalytic activities. The measurement of BPB-NR activity in mutants, in addition to allowing us to identify a new type of mutant, showed that the electron pathway was different for this activity and the two other terminal activities: for example, the FMNH₂ and MV-NR activities were always linked and expressed with the same ratio, whereas the BPB-NR activity could be dissociated from these two activities in the class 4 mutants. On the other hand, the parallelism between the FMNH₂ and MV-NR specific activities suggests that they involve the same domain(s) of the molecule. Sherrard and Dalling (24) observed different thermal stabilities for these two activities, suggesting that the two electron donors interact with the NR molecule at two different sites; in addition, measurements of initial reaction rates for these two activities in the Chlorella enzyme were quite different (25), which seems contradictory with the hypothesis of a common electron pathway. In Chlorella, the FMNH₂-NR activity is supposed to involve both the heme and MoCo domains (25), but not the MV-NR activity, for which the electrons would be directly transferred to the MoCo. However, the inactivation kinetics during limited proteolysis of spinach NR by trypsin and Staphylococcus aureus V8 protease were the same for the MV and FMNH₂-NR activities (15), and these two activities were inhibited by the same monoclonal antibodies, directed against spinach (21) or maize NR (I Cherel, unpublished results). The situation may be different in Chlorella and higher plants; the MV-NR activity could also be affected by a mutation in the heme domain without involving the heme for the electron transfer. Several observations indicate that the heme domain might be involved in the full expression of these two activities, at least in higher plants. In the proteolysis experiments described by Kubo et al. (15), the loss of FMNH₂ and MV-NR activities was apparently correlated with the cleavage between the heme and MoCo domains of spinach NR observed during a treatment by S. aureus V8 protease (15). In agreement with this hypothesis is a recent experiment made in our laboratory to map the epitope recognized by the monoclonal antibody ZM 96(9)25:
cDNA fragments coding for the structural FAD and heme domains of NR were cloned in a plasmid expression vector and expressed independently in Escherichia coli. ELISA measurements showed that the antibody ZM 96(9)25 could specifically recognize the heme domain of NR (M. Kavanagh, unpublished data). Two independent phenomena—the binding of the monoclonal antibody ZM 96(9)25 and the E56 mutation, which prevents this binding—lead to the same alterations of partial catalytic activities: inhibition of CcR, FMNH2-NR, and MV-NR activities, but no inhibition, or even activation, of BPB-NR activity. These observations suggest that a common part of the NR molecule, probably in the heme domain, is required for the expression of the first three activities. Conversely, the BPB-NR activity would only need an intact MoCo domain. A NR structural scheme is proposed on this basis (Fig. 5). This representation does not take into account possible interactions between domains or subunits, which may modulate the expression of partial catalytic activities.

This scheme can also be used to explain the origin of the different classes of mutants as follows: the class 4 mutants, which had lost the ZM 96(9)25 antibody epitope but retained the NADH-binding site and a terminal NR activity, would most likely be mutated in the heme domain. The class 3 mutants, which retained the CcR activity supposed to involve both the FAD and heme domains (15, 26), would carry a mutation in the MoCo domain. For the class 2 mutants, their modification of the NADH-binding site suggests that the mutation would be located in the FAD domain; this is further supported by their loss of CcR activity and their retention of all terminal NR activities, even the FMNH2-NR activity (Fig. 6).

If the probability of mutation in a domain increases with its size, the MoCo domain, which occupies about 75 kD out of 110 kD of the NR subunit (15, 28), should have been preferentially mutated. A frameshift mutation in the MoCo domain (5' end of the DNA) would result in early termination of the translation and loss of all NR-associated activities, resulting in a class 1 phenotype. The expression of NADH CcR activity needs correct transcription and translation of the FAD domain which is located in the C terminus (1, 8); this would be obtained only for mutations preserving the reading frame. This would explain why we obtained few mutants retaining NADH-CcR activity.

The present classification indicates that studies on the structure of the NR protein in mutants should be helpful in defining the structure-function relationships. It will be interesting to locate accurately the mutations in the nucleotide sequence to confirm the interpretation of the present data.

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LITERATURE CITED


