Characterization of Nitrate Reductase Deficient Mutants of Chlorella sorokiniana

Otto Knobloch and Rudolf Tischner*
Institut für Pflanzenphysiologie, University of Göttingen, Untere Karspüle 2, 3400 Göttingen, West Germany

ABSTRACT

After x-ray irradiation, 13 mutants of Chlorella sorokiniana incapable of using NO₃⁻ as N source were isolated using a pinpoint method. Using immunoprecipitation and Western blot assays, no nitrate reductase was found in five strains while in eight mutants the enzyme was detected. The latter strains contained different patterns of nitrate reductase partial reactions. All isolates were of the nia-type as indicated by the inducibility of purine hydroxylase I and by complementation of nitrate reductase activity in the Neurospora crassa mutant Nit-1. A restoration of NADP-nitrate reductase in Nit-1 was also obtained with NH₄⁺-grown cells indicating that Mo-cofactor is constitutive in Chlorella. Complementation experiments among the Chlorella mutants resulted in restoration of NADH-nitrate reductase activity. The characteristics of some of the Chlorella mutants are discussed in view of an improper orientation of Mo-cofactor in the residual nitrate reductase protein.

In assimilation of nitrate, the main N source of plants, NR₁ is considered as the rate limiting step (1). This enzyme contains three different domains: FAD, Cyt b₅₅₇/heme and Mo-cofactor, forming a short redox chain (1). The initial electron donor is NAD(P)H and the electrons are transferred via FAD and Cyt b₅₅₇/heme to Mo-cofactor, where nitrate is reduced. NR is also believed to play an important role in the iron supply of plants by the reductive cleavage of iron siderophores (2). Several unphysiological reactions can be measured via the FAD-domain using one electron acceptors like Cyt c or ferricyanide (dehydrogenase activity). With FADH₂, the Cyt b₅₅₇/heme domain and Mo-cofactor are employed to reduce nitrate, while with MVred as electron donor only the Mo-domain is used (see Solomonson and Barber (24)).

In this paper we present characterization of NR mutants of Chlorella sorokiniana, an organism which contains an easy to isolate and very stable NR (in contrast to NR in higher plants [1, 24]). These mutants are suggested to be useful for the analysis of the relationship between structure and function of NR at the molecular level. NR-deficient mutants of barley (3) and Neurospora crassa (7) have been isolated and used as described here.

1 Abbreviations: NR(A), nitrate reductase (activity); NiR(A), nitrite reductase (activity); FAD, flavine adenine dinucleotide; MV, methyl viologen; PH, purine hydroxylase I (xanthine oxidase); NC, nitrocellulose; HRP, horseradish peroxidase.

MATERIALS AND METHODS

Plant Material

The experiments were carried out with Chlorella sorokiniana, strain 211-8k of the Algae Collection of the Institut für Pflanzenphysiologie of the University of Göttingen (SAG). Complementation experiments were done using Neurospora crassa and its mutant Nit-1 (20) obtained from O. Meyer (Institut für Mikrobiologie, University of Göttingen).

Cultivation Procedures

Nitrogen-free nutrient solution was prepared according to Galling (11) and with nitrate as N source as reported by Kuhl and Lorenzen (14). In nutrients with nitrite as N source, the KNO₃ (10 mM) was replaced by 100 mM KNO₂ and when NH₄⁺ was used as N source, the pH of the medium was adjusted to 8.0 and 5 mM (NH₄)₂SO₄ was supplied. When hypoxanthine and xanthine were used as N sources, they were added to N-free nutrient solutions at their maximum solubility. Media for N. crassa and Nit-1 with NH₄Cl (4.3 g/L) instead of KNO₃ were prepared according to Garrett (12).

Cultivation of algae was performed according to the method of Kuhl and Lorenzen (14), that of the fungi after Meyer (Microbiological Institute, University of Göttingen, personal communication). For solid media, a 1.7% agar was used containing the N sources as indicated.

Methods

Nitrate uptake measurements were performed after (27). Induction of NR and NiR in the fungi was completed 6 h after a transfer of NH₄⁺-grown cells into NO₃⁻-containing medium. PH (xanthine oxidase) of the algae was induced by a transfer of NH₄⁺-grown cells into hypoxanthine (5.25 mM) containing medium for 4 h. Cell-free extracts of the algae cells were prepared after Tischner (28) and of the fungi by grinding the cells with pestle in a mortar with sea sand and some crystalls of DNase in the homogenization buffer (0.5 mM EDTA, 1 mM PMSF, 1 mM DTI in 100 mM KH₂PO₄-NaOH, pH 7.4) and subsequent removing of cell debris at 40,00× g.

Protein Content

Protein content was estimated as described by Lowry et al. (16).
Enzyme Assays

NADH:NR of *Chlorella sorokiniana* was tested after Tischner (28), dehydrogenase reaction (NADH:Cyt c) after Solomonon and Vennesland (26), FADH2:NR and MV:NR after Funkhouser and Ramadoss (10). NADPH:NR of *N. crassa* and restored Nit-l-NR was tested after Meyer (Microbiological Institute, University of Göttingen, personal communication). PHI-test was performed according to Franco et al. (9).

For intraspecies complementation of algal-NADH:NR-activity the corresponding extracts were mixed in an appropriate ratio and stored on ice over night before the enzyme assay.

For SDS-mediated liberation of Mo cofactor from NR, algal extracts were diluted 10-fold with SDS (1%) and stored on ice for 10 min.

Immunoochemical Assays

For Ouchterlony double diffusion assays and Western blotting a monoclonal monospecific antibody was used, raised in rabbits against a purified NR according to Tischner (28). Ouchterlony double diffusion assays, with sodium azide (0.05%) for prevention of microbial growth, were performed after Clausen (4) on 5 × 5 cm glass plates.

PAGE

PAGE was performed according to Laemml (15) with or without SDS. The Mₚ was estimated after SDS-gel electrophoresis and comparison to the electrophoretic mobility of standard proteins subunits (thyroglobulin 330 kD, ferritin 220 kD, phosphorylase b 94 kD, BASE 67 kD, catalase 60 kD). Western blotting was carried out after Towbin el al. (29); immunodetection of NR on NC sheets was performed as follows. After blotting the air-dried NC sheet was treated with blocking solution (20 mM Tris/HCl (pH 7.5), 500 mM NaCl, 1% BSA) for 1 h and then incubated in anti-NR (1:1000)-containing buffer (blocking solution with only 0.33% BSA) for 2 h. The NC membrane was washed twice (30 min each) in buffer 1 (blocking solution without BSA) and once (15 min) in buffer 2 (buffer 1 plus 0.05% (v/v) Tween 20). Then it was transferred into HRP-conjugated secondary antibody 1:1000, same buffer as for anti-NR) for 1 h. The bands containing NR were developed after a further washing procedure by adding a mixture of 30 mg chloronaphthol in 10 mL ice cold MeOH and 40 μL H₂O₂ (30%) in buffer 1. Interference with *Chlorella* peroxidase was not observed using crude extracts from nitrate and ammonium grown cells. Autoclaving the NC Membrane to destroy peroxidase (23) did not change the result of the Western blot.

RESULTS

Isolation of NO₃⁻-Assimilation Mutants

An axenic suspension of *Chlorella* autospores (15 mL) was irradiated with x-rays (1000 rad/min). Samples were periodically collected and plated on agar. The colony number obtained after 4 to 6 days of incubation in constant light at 35°C revealed the rate of surviving cells compared to the untreated suspension (Fig. 1). Irradiated cell suspensions were plated on agar with NO₃⁻ (10 mM) as N source. While wild-type cells grew up to colonies corresponding in size to the amount of nitrate available, other cells grew up only to pinpoints. Those were cut out and separately cultivated in 15 mL Bellco screw-cap tubes with 4 mL of sterile NH₄⁺ (10 mM) and glucose (0.5%)-containing nutrient solution in constant light at 35°C for 4 d. The cells from the dark green suspensions were centrifuged and resuspended in a small amount of N-free medium and streaked out on agar plates with NO₃⁻, NO₂⁻ or NH₄⁺ as N sources. Among those cell lines derived from pinpoint colonies 3 to 5% proved to be mutants defective in growth on nitrate. In total we obtained 13 mutants, which grew only with ammonia or nitrite.

Characterization of the Mutants

For further characterization of the 13 mutants derepression experiments were employed. The derepression resulted in an optimum NRA (Fig. 2), NiR(A), and nitrate uptake rate (13) 80 min after a transfer of NH₄⁺-grown wild-type cells into NO₃⁻- or N-free medium (Fig. 2). This experimental design was therefore also employed for the further characterization of the mutants. The nitrate uptake capacity and the presence of NR-protein (using both Ouchterlony double diffusion assay and Western blotting) were analyzed. The NR partial reactions were estimated. Growth of the mutants was also tested with NO₃⁻ (+100 μM molybdate), hypoxanthine and xanthine as N sources. The assay for PH I was used as an indicator for a functional Mo cofactor synthesis. The induction of PHI was strictly substrate dependent. No induction was observed with

![Figure 1. Survival curve of Chlorella sorokiniana cells after x-ray irradiation.](image-url)
uric acid, urea, NO$_3^-$ and NH$_4^+$. This result is different to that reported for *Chlamydomonas* (9). The data of these experiments are summarized in Table I.

None of the isolated mutants was able to take up nitrate. However, the mutational defect is in the enzyme itself, not in the uptake system (see “Discussion”). In all of the isolates we found a functioning Mo-cofactor synthesis. Eight of them contained NR residues with different partial reactions, therefore representing nia-type mutants (1). In the remaining five mutants the synthesis of NR-apoprotein is prevented according to the negative results from the Ouchterlony assay and the Western blot (data not shown). The strains containing residual NR display different partial activities: (a) strain 6 lacks NADH:Cyt c activity, while both FADH$_2$:NR and MV:NR were 6 to 8 times higher than that of the wild-type cells. A mutation in the FAD-region seems to be a reasonable interpretation; (b) strain 12 shows only FADH$_2$:NR and the mutation cannot be sufficiently explained up to now; and (c) strains 1, 3, 5, 7, 9, and 10 contain only NADH:Cyt c activity which is doubled (strains 3, 5, and 10) or slightly reduced (strains 1, 7, and 9) compared to that in the wild-type cells.

From Western blots of polyacrylamide gels no difference in the $M_r$ of the NRs between mutants and wild-type cells was detected (Fig. 3). Always two NR-bands were found, which cannot be explained sufficiently up to now. An interference with cellular peroxidases can be neglected (see “Materials and Methods”). However, for the aspect stressed here, it is important that identical NR-bands resulted for both mutants and wild-type cells. The partial reaction of strain 6 and that of strains 3, 5, and 10 formally recombine to NADH:NR(A).

The complementation was successfully achieved by mixing the extracts in the ratio of the corresponding partial activities (i.e. strain 6: strains 3, 5, or 10 = 2:3). This resulted in 12% of the specific NADH: NR(A) in the wild-type cells. A complementation was also obtained with the *Neurospora crassa* mutant *Nit−* (lacking Mo-cofactor) and extracts of strains 3, 5, 6, and 10 restoring the NADPH:NR(A) in *Nit−* (Table II). The complementation was more effective (6–10 times) if the extracts were treated with SDS (0.1%) before the experiment.

**DISCUSSION**

The isolation of our mutants using a pinpoint method is different from the way mutants in nitrate assimilation have been isolated before which employed chlorate sensitivity (e.g., *Arabidopsis* [6] or *Aspergillus* [5]). Our *Chlorella* strain did not respond to chlorate concentrations up to 200 mm. Pre-cultivation on N limitation, NH$_4^+$ or NO$_3^-$ did not affect the sensitivity toward chlorate (data not shown). This result is contrary to that reported by Solomonson and Vennesland (26) for *Chlorella vulgaris* Beijerinck (Berlin strain). In favor of *Chlorella* is the high amount of NR (up to 1% of total soluble protein) in the cells (25), the high activity and stability of this enzyme. A comparison between wild-type NR and mutant NR can help to understand the molecular structure. However, a genetic analysis is not possible with this haploid and asexual organism.

The mutants presented are NR mutants despite the lack in nitrate uptake. NR is a derepressible enzyme in *Chlorella* as the *de novo* synthesis of NR occurred after the depletion of ammonia. That means, NO$_3^-$ is not an inducer (compare Fig. 2). Therefore, even in mutants lacking a nitrate uptake system

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**Table I. Characterization of NR Mutants of *C. sorokiniana***

<table>
<thead>
<tr>
<th>Mutant No.</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
<th>NO$_5^-$</th>
<th>Hx/X</th>
<th>Western Blot</th>
<th>NO$_3^-$ Uptake</th>
<th>PHI</th>
<th>Specific Activities of NR$^b$</th>
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<td></td>
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<td></td>
<td></td>
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<td>+</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>5.8</td>
</tr>
</tbody>
</table>

$^a$ Values given as percentage of the activity of wild type cells. $^b$ Brackets mean poor growth on hypoxanthine (Hx) or xanthine (X). $^c$ Cyt c reductase.
NR should be synthesized. Thus in all our mutants the enzyme is mutated, otherwise we should have found strains with active NR but unable to grow on NO₃⁻. Furthermore it should be mentioned, that the nutrient contained 10 mM nitrate. One can expect, that therefore a certain amount of nitrate enters the cells by diffusion. This should allow growth of mutants defect in nitrate uptake but with complete NR. This, however, was never observed.

None of the mutants presented grew with NO₃⁻ as N source even in the presence of 100 μM molybdate. This indicates a conserved affinity between NR-apoprotein and Mo-cofactor as it has been discussed for NR mutants of different species (5, 18, 19). Five of our mutant strains do not synthesize NR protein (no band in Western blots). It seems as if the mutation affected a (still speculative) regulator gene of NR.

In the mutant strains 3, 5, 6, and 10 a high activity of the remaining partial reactions was found. This might be due to an unbalanced NR-residue synthesis due to a lack of end product repression. The actual NR amount in the cell is a result of both derepression/repression by low and high concentrations of an end product and enzyme degradation. In our mutants no endproduct was formed and this resulted in an overshoot production of NR defect in special domains. Such an effect has been shown for Chlorella (30) and for glutamate grown and N-starved Cyanidium caldarium cells (22). However, the NADH:Cyt c activity was reduced in mutant strains 1, 7, and 9 which also do not contain the other partial reactions. On the other hand one can speculate, that the NR structure has been modified in the mutants blocking one of the partial activities and probably enhancing the other partial activities. This interpretation would not need a higher amount of NR protein in the mutants.

The induction of PHI is a common procedure to check for Mo-cofactor, which is part of all molybdenum-containing multicenter-redox-enzymes (1, 21). From our results we conclude that none of our mutants suffered from Mo-cofactor deficiency. A mutational defect in Mo-cofactor synthesis would affect all Mo-cofactor containing enzymes. Therefore our NR-mutants are of the nia-type (1).

The restoration of NADPH:NRA in Neurospora crassa mutant Nit-1 by extracts from our Chlorella mutants confirmed, that the Mo-cofactor synthesis is unaffected in all of

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**Table II. Complementation of NADPH:NR in N. crassa Mutant Nit-1 with Crude Extracts from C. Mutants (Strains 3, 5, 6, and 10) and Wild-Type Cells**

<table>
<thead>
<tr>
<th>Complementation</th>
<th>Strains</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nit-1 plus untreated crude extracts</td>
<td>3 5 6 10</td>
<td>NH₄⁺ NO₃⁻</td>
</tr>
<tr>
<td>Nit-1 plus SDS-(0.1%) treated crude extracts</td>
<td>+++ +++ +++ +++</td>
<td>+ +++</td>
</tr>
</tbody>
</table>

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Figure 3. The M₉ of NR is the same in mutants and wild-type cells as demonstrated with Western blots of SDS-gels. The subunit M₉ was about 90 to 95 kD. Two wells were used for each mutant, their numbers indicated correspond to Table I.
the mutants presented. A small portion of Mo-cofactor is always present in the cells, but the bulk is released after SDS-treatment of NO₃⁻-grown cells. The wild-type cells (and also the mutants) produce a small amount of Mo-cofactor even in NH₄⁺-medium (Table II). This has been reported for Mo-cofactor in *N. crassa* (20), squash (1), tobacco (17), and *Escherichia coli* (19).

The *in vitro* restoration of the homomultimeric Chlorella-NR (24) must be different to that reported for the heteromultimeric NR of *Chlamydomonas* (8, 9). For the restoration using several of our mutants two speculative interpretations are possible: (a) the exchange of domains between complementarily damaged subunits can produce functioning NR; and (b) subunits defect in different reactions aggregate thus that the electron flow from NADH to NO₃⁻ is restored.

The characteristics of strain 6 are suggested as: FADH₂:NR and MV*:NR are active indicating a functional heme-domain and Mo-cofactor. Dependent on the lack of NADH:Cyt c activity and NADH:NR we suggest the mutational defect at the FAD-domain rather than at the heme domain. This interpretation fits the NR model by Campbell and Smarrelli (1) where Cyt c-reduction only requires the FAD-domain. However it is contrary to that presented by Solomonson and Barber (24) where both FAD and heme domain are necessary for Cyt c reduction.

The mutational changes in strains 3, 5, and 10 can be interpreted as follows. The FAD-domain, the electron flow between FAD/Cyt b₅₅ and Cyt b₅₅/Mo-cofactor and the binding site for MV₅₅ were not specifically altered as both reductase activities drop simultaneously. Therefore, the Mo-domain must have been changed. However, Mo-cofactor is synthetized, incorporated into the NR-apoprotein and can be used for complementation of *Nit-I*-NR, especially after being released from the NR-apoprotein by SDS-treatment (Table II). Moreover, Mo-cofactor is correctly produced during PHI induction. Therefore, we suggest that the tertiary structure of NR was changed resulting in an improper orientation of Mo-cofactor in the NR-apoprotein.

For this reason experiments in our laboratory are in progress to approach the structure of the Mo-domain and the incorporation of Mo-cofactor into the protein backbone. This aim can be achieved using methods of molecular biology in the comparison of the intact and the mutationally changed enzymes.

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LITERATURE CITED


