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Communication

nit 7: A New Locus for Molybdopterin Cofactor Biosynthesis in the Green Alga *Chlamydomonas reinhardtii*[1]

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ABSTRACT

Two new nitrate reductase-deficient mutants from *Chlamydomonas reinhardtii* have been genetically and biochemically characterized. Both H1 and F23 mutants carry single recessive allelic mutations that map at a new locus designated *nit*-7. This locus is unlinked to the other six *nit* loci related to the nitrate assimilation pathway in *C. reinhardtii*. Both mutant alleles H1 and F23 lack an active molybdopterin cofactor, the activity of which is restored neither *in vitro* nor *in vivo* by high concentrations of molybdate. Nitrate reductase subunits in these mutants seem to assemble, although not in a stable form, in a high molecular weight complex and, as in other molybdenum cofactor-defective mutants of *C. reinhardtii*, they cannot reconstitute nitrate reductase activity with an active molybdenum cofactor source from extracts of ammonium-grown cells. The results suggest that *nit*-7 mutants are defective in molybdopterin biosynthesis. They do produce some precursor(s) that are capable of binding to nitrate reductase subunits.

Eukaryotic NR[2] (EC 1.6.6.1–3) catalyzes the first step of the nitrate assimilation pathway, the reduction of nitrate to nitrite. The NR enzyme complex in most systems is a dimer of about 220 kD that exhibits two partial activities: diaphorase activity, supported by the prosthetic groups FAD and Cyt b_{557}, and terminal NR activity, which requires a MoCo (4). Eukaryotic MoCo is a complex of molybdenum and a pterin derivative named molybdopterin (14). MoCo plays an important role in catalytic activity, assembly mechanism, and structural stability of molybdoenzymes (12, 20).

The biosynthesis of MoCo remains mainly unknown in eukaryotes. The instability of MoCo and its precursors makes it difficult to establish a MoCo biosynthetic pathway through the direct detection and analysis of actual intermediates. A first approach to elucidating this pathway has been made by the identification of structures of MoCo and a molybdopterin precursor, compound Z. Guanine has been proposed as the precursor compound of MoCo (13, 14).

The isolation and subsequent analysis of mutant strains defective in the synthesis of active MoCo are very important tools for elucidating its biosynthetic pathway. The *cnx* mutants found in several fungi and higher plants—five loci have been described in *Neurospora crassa* (19), six in *Aspergillus nidulans* (3), and six in *Nicotiana plumbaginifolia* (9)—are MoCo deficient. Biochemical characterizations of these mutant strains have allowed their classification into several clearly different phenotypical groups corresponding to the different steps of MoCo biosynthesis.

In *Chlamydomonas reinhardtii*, six genes are involved in the synthesis of an active NR: *nit*-1, coding for NR apoprotein; *nit*-2, for the regulation of NR synthesis; and four (*nit*-3, *nit*-4, *nit*-5, and *nit*-6) for the synthesis of active MoCo (4, 6, 7). Mutants defective at *nit*-5 and *nit*-6 are cryptic because only double mutants at both loci lack active MoCo (6). *nit*-3 seems to be involved in the synthesis of active MoCo, and *nit*-4, *nit*-5, and *nit*-6 in the processing of molybdate (4). The cloning of the *nit*-1 gene has shown that the *C. reinhardtii* NR seems to be like the other eukaryotic NR and that the previous proposals on two different subunits should be interpreted in light of intragenic complementation events either *in vivo* or *in vitro* (4, 5). In fact, efficient intragenic complementation between NR structural mutants has been demonstrated in *N. plumbaginifolia* (16). Taking into account the size of the *nit*-1 transcripts, *C. reinhardtii* NR subunits of about 100 kD would be expected (5).

In the present work, two new mutant strains of *C. reinhardtii* have been biochemically and genetically characterized and are shown to be defective at a new locus, *nit*-7, related with molybdopterin biosynthesis. The mutants showed high M_r NR diaphorase activity, corresponding to assembled NR subunits.

MATERIALS AND METHODS

Cells and Growth Conditions

Wild-type strain 6145c mt$^-$ of *Chlamydomonas reinhardtii* was supplied by Dr. Ruth Sager (Sydney Farber Cancer Center, Boston). *nit*$^-$ mutants 305 mt$^-$ (*nit*-1), *nit*-2–137c mt$^-$ (*nit*-2), 307 mt$^-$ (*nit*-3), 104 mt$^-$ (*nit*-4), and 102d mt$^-$ (*nit*-5, *nit*-6) have been described elsewhere (6–8). Mutant strains H1 and F23 (kindly provided by Dr. Rogene Schnell, Department

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[2] Abbreviations: NR, nitrate reductase; DPIP, 2,4-dichlorophenol indophenol; MoCo, molybdenum cofactor; FAD, flavin adenine dinucleotide.

of Genetics and Cell Biology, University of Minnesota) were isolated as spontaneous chlorate-resistant mutants from *C. reinhardtii* parental strain A_{54} containing mutations *sr*-1, and *ac*-17 (11). F23d mt$^-$ was isolated from *nit*$^-$ segregants of the cross F23 mt$^+$ × 6145c mt$^-$.

Cells were grown in culture rooms at 25°C under continuous saturating illumination in either liquid minimal media with 5% (v/v) CO_2-enriched air or solid Tris-acetate-phosphate media, both containing 10 mM NH_4Cl as the only nitrogen source, as detailed elsewhere (11).

Mutant *nit*-1 of *Neurospora crassa* was cultured aerobically in Fries basal medium (10), in an orbital shaker at 27°C with 10 mM NH_4Cl as the only nitrogen source. After growing for 2 to 3 d, mycelia were collected by filtration, washed with distilled water, and transferred to fresh medium containing 8 mM KNO_3 for 5 h to induce aponitrate reductase. Afterwards, mycelia were collected as above, washed, and frozen at −40°C until use.

Cell Disruption and Preparation of Extracts

Cells from wild-type 6145*c* and mutant strains were harvested at mid-logarithmic phase of growth and frozen at −40°C. Extracts were obtained by thawing frozen cell pellets in 2 mL buffer A (25 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM PMSF) per g fresh weight. When required, extracts were obtained in anaerobiosis by using buffer A and a high-purity oxygen-free nitrogen atmosphere before and during resuspension of cell pellets.

Mycelia from *nit*-1 were disrupted by grinding on a precooled mortar with acid-washed sand (1.7 g/g of mycelium). Ground material was extracted with 5 mL of buffer B (25 mM potassium-phosphate buffer, pH 7.5, containing 25 mM sodium molybdate and 1 mM EDTA) per g of mycelium. The suspension was centrifuged at 20,000*g* for 10 min, and the resulting supernatant used for the MoCo assay.

MoCo Activity Determination

MoCo was assayed by measuring reconstituted NR activity in a mixture of 200 μL of *N. crassa nit*-1 extracts and 100 μL of a MoCo source preincubated at 15°C for 1 h (1).

Enzyme Assays

Reconstituted NR from *N. crassa nit*-1 was determined by adding to the complementation mixtures 300 μL of buffer C (200 mM potassium phosphate buffer, pH 7.2, containing 20 μM FAD, 0.7 mM NADPH, and 60 mM KNO_3) and carrying out the reaction at 25°C for 1 h. One unit of MoCo activity is defined as the amount of MoCo that yields one unit of reconstituted NR activity, expressed as the amount of enzyme that catalyzes the reduction of 1 μmol of nitrate/min.

NADPH-DPIP reductase (NR-diaphorase) activity was spectrophotometrically assayed as reported previously (8), except that DPIP reduction was measured at 660 nm. One unit of diaphorase activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of DPIP/min.

Analytical Methods

Nitrite was determined colorimetrically according to Snell and Snell (18), and protein according to Smith *et al.* (17) using BSA as standard.

Molecular Exclusion Chromatography

Molecular exclusion chromatography was performed on a Sephacryl S-300 column (1.6 × 60 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM DTE, 1 mM EDTA, 10 μM FAD, and 100 mM NaCl. Chromatography was run at 30 mL/h. Samples of 3 mL supplemented with 2.5% glycerol were applied, and fractions of 2.2 mL were collected. Exclusion volume (V_o) and total volume (V_t) were determined with dextran blue and potassium ferricyanide, respectively. Horse spleen apoferritin (443 kD), sweet potato β-amylase (200 kD), yeast alcohol dehydrogenase (150 kD), and bovine erythrocyte carbonic anhydrase (29 kD) were used as protein markers.

In Vivo Complementation and Genetic Analyses

The new mutant strains were analyzed by *in vivo* complementation with other *nit*$^-$ mutants already characterized (6, 7). Phenotypically wild-type diploids were isolated as detailed elsewhere (7). Genetic analysis was performed by the random spore plating method as reported previously (6, 11).

RESULTS AND DISCUSSION

The two new mutant strains H1 and F23, incapable of growing on nitrate medium, were first analyzed in crosses with the wild-type 6145c. The ratio of segregants *nit*$^+$:*nit*$^-$ was close to 1:1, which indicates that both H1 and F23 carry a single *nit* mutation designated *nit*-x and *nit*-y, respectively (Table I). The 290 segregants analyzed from the cross H1 × F23 were all *nit*$^-$, which indicates that these mutants are either allelic or defective at two closely linked genes. Crosses with mutant alleles of *nit*-1, *nit*-2, *nit*-3, and *nit*-4 gave rise to a *nit*$^+$:*nit*$^-$ ratio of meiotic products close to 1:3, as would be expected for independent *nit* mutations. In the crosses H1 ×

Table I. *Crosses between Wild-Type and NR-Deficient Mutants from C. reinhardtii*

Cross	Genotype for NR	Progeny		Ratio
		nit$^+$	*nit*$^-$	*nit*$^+$:*nit*$^-$
H1 × 6145*c*	*nit*-x × +	60	40	1:0.7
H1 × 305	*nit*-x × *nit*-1	48	123	1:2.6
H1 × *nit*-2-137c	*nit*-x × *nit*-2	31	69	1:2.2
H1 × 307	*nit*-x × *nit*-3	54	136	1:2.5
H1 × 104	*nit*-x × *nit*-4	45	138	1:3.1
H1 × 102	*nit*-x × *nit*-5 *nit*-6	58	105	1:1.8
F23 × 6145*c*	*nit*-y × +	55	45	1:0.8
F23 × 305	*nit*-y × *nit*-1	48	144	1:3.0
F23 × *nit*-2-137c	*nit*-y × *nit*-2	29	71	1:2.5
F23 × 307	*nit*-y × *nit*-3	29	71	1:2.5
F23 × 104	*nit*-y × *nit*-4	48	130	1:2.7
F23 × 102	*nit*-y × *nit*-5 *nit*-6	63	94	1:1.5
H1 × F23d	*nit*-x × *nit*-y	0	290	0:1.0

102 and F23 × 102, an excess of wild-type recombinants did appear (Table I) (ratios 1:1.8 and 1:1.5, respectively). Because the unlinked mutant alleles at *nit*-5 or *nit*-6 from mutant 102 separately render a wild-type phenotype (10), the results indicate that *nit*-x and *nit*-y are unlinked to *nit*-5 and *nit*-6.

In vivo complementation analyses confirm the above results. Each of the previously characterized mutants defective at *nit*-1, *nit*-2, *nit*-3, *nit*-4, and *nit*-5/*nit*-6 (strains 305, *nit*-2–137c, 307, 104, and 102, respectively) complemented mutants H1 and F23 in the corresponding heterozygous diploids, as expected for diploids carrying different recessive mutant alleles. Negative complementation between H1 and F23d indicate that recessive mutations at *nit*-x and *nit*-y are allelic. We will refer to the new locus hereafter as *nit*-7.

Mutant strains H1 and F23 lack both NAD(P)H-NR and terminal-NR activities (not shown). In addition, mutants were tested for active MoCo content by means of the *in vitro* complementation assay with *N. crassa nit*-1 mutant. Wild-type strain 6145*c* of *C. reinhardtii* grown in ammonium medium showed low MoCo levels (1.2 munit/mg protein) that increased after nitrate treatment for 6 h (9.4 munit/mg protein), in agreement with previously reported data (1). The presence of 10 mM molybdate in extracts doubled the amount of detected MoCo activity (2.4 and 21.0 munit/mg protein for ammonium-grown and nitrate-induced cells, respectively). Active MoCo was not detected in either of the mutant strains, even after molybdate addition to the assay mixtures. The lack of MoCo activity in extracts of both F23 and H1 strains suggests that an intact molybdopterin is absent, because addition of external molybdate is known to enhance MoCo activity by restoring the molybdopterin-molybdate complex (15).

Genes whose defect can be obviated by high concentrations of molybdate are thought to be involved in molybdate processing (8, 15). Similarly to mutant 307 (*nit*-3) from *C. reinhardtii* (8), growth of H1 and F23 mutants on nitrate was not restored by high concentrations of molybdate (1, 3, 5, 10, 20, and 30 mM were tested). This failure in the growth restoration by molybdate, together with the absence of MoCo activity *in vitro* even in the presence of 10 mM molybdate, suggests that the new locus *nit*-7 codes for some step of molybdopterin biosynthesis.

The assembly of subunits to form the NR complex appears to depend on molybdopterin, the organic moiety of MoCo (12, 15, 20). However, active MoCo is not required for NR complex formation, as has been shown in *A. nidulans, N. plumbaginifolia,* and *C. reinhardtii* (8, 15). The ability of the apparently defective MoCo species to induce assembly of apoproteins was tested in strains H1 and F23 by measuring the appearance of the high M_r diaphorase activity of the NR complex. Extracts from wild-type and mutant strains were subject to molecular exclusion chromatography in Sephacryl S-300, and diaphorase activity was assayed in eluted fractions. Elution profiles of extracts from ammonium-grown wild and mutant cells showed a major peak of diaphorase activity corresponding to low M_r constitutive diaphorases (Fig. 1). In extracts from nitrate-induced cells, diaphorase activity was also detected in fractions corresponding to higher M_r proteins. In the wild-type strain, the high M_r peak of diaphorase activity (about 200,000) coeluted with NR activity and corresponded

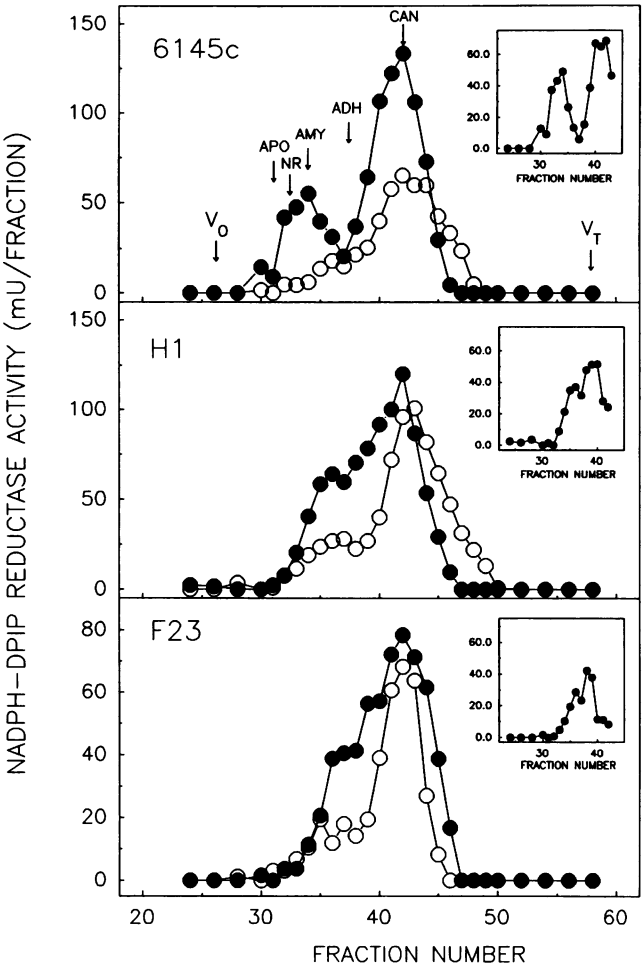

Figure 1. Elution profiles of constitutive and NR-related NADPH-diaphorases from extracts of *C. reinhardtii* wild-type and mutant strains in Sephacryl S-300. Extracts of the indicated strains, grown on ammonium (○) or derepressed in nitrate (●), were filtered through a Sephacryl S-300 (1.5 × 60 cm) column, previously calibrated with marker proteins whose elution volumes are indicated with arrows: APO, apoferritin; AMY, β-amylase; ADH, alcohol dehydrogenase; CAN, carbonic anhydrase. Fractions of 2.2 mL were collected for NADPH-DPIP reductase activity assays. Insets, the curves are obtained by subtracting for each fraction NADPH-DPIP activity of ammonium extracts from those of nitrate extracts.

to the diaphorase activity of the complex, whereas the increased levels of low M_r diaphorases from cells induced with nitrate were those of NR subunits (4, 8). However, in nitrate-induced cells of mutants H1 and F23, the peak of diaphorase activity associated with the NR complex is shifted to a lower M_r and appears as a shoulder of the lower M_r peak of diaphorase (Fig. 1). The insets (Fig. 1) show more clearly both the peak of diaphorase activity related to NR subunits and the shift to lower M_r of the NR-associated diaphorase activity in the mutants. The aggregation of NR subunits to form the complex appears to involve various types of factors, including interactions between MoCo and thiol groups of the apoprotein (20). Weak interactions between cofactor and apoprotein, and conformational changes of the subunits, have been also sug-

gested (2, 12). This could explain why MoCo-defective algal mutants H1 and F23 (*nit*-7), like strain 307 (*nit*-3) (8), contain high M_r diaphorases. In addition, the size of the nitrate-inducible high M_r diaphorase of mutants in *nit*-7 and *nit*-3 (8), smaller than that of the native NR complex, could be explained by a labile assembly of NR subunits containing a defective MoCo, which dissociates more easily into subunits than does the NR complex of the wild type. A *C. reinhardtii* $MoCo^-$ mutant lacking high M_r NR-diaphorases and similar to the *N. crassa nit*-1 mutant has not been reported so far.

Because active NR subunits with diaphorase activity are present in nitrate-induced *nit*-7 mutants, we tested the ability of this diaphorase to reconstitute NR activity with an external source of active MoCo. Concentrated extracts of ammonium-grown wild-type cells containing 0.7 munit active MoCo/mL were used as the MoCo source in complementation assays, in the presence of 10 mM molybdate, with extracts from any of the $MoCo^-$ mutant strains 102, 104, 307, H1, and F23, previously induced with nitrate during 6 h. These complementation assays were carried out under the same conditions used for MoCo determinations by the complementation assay with the *N. crassa nit*-1 mutant. In all the cases, a reconstituted NADPH-NR activity was not detected. The same negative results were also obtained by using free MoCo released by heat treatment (80°C for 90 s) of milk xanthine oxidase as a more concentrated MoCo source (12.1 munit active MoCo/mL) in complementation assays with the above $MoCo^-$ mutant strains of *C. reinhardtii* (results not shown). The failure to reconstitute NR by *in vitro* complementation between NR subunits from *C. reinhardtii* MoCo mutants and a source of active MoCo strongly suggests that the MoCo binding site in NR subunits is occupied by a defective MoCo nonexchangeable by an active one.

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