

# Nitrate Reductase from a Mutant Strain of *Chlamydomonas reinhardtii* Incapable of Nitrate Assimilation

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Nitrate reductase from mutant 305 of *Chlamydomonas reinhardtii* has been purified about 90-fold and biochemically characterized. The enzyme can use reduced flavins and viologens as electron donors to reduce nitrate but, unlike the nitrate reductase complex from its parental wild strain, lacks NAD(P)H-nitrate reductase and NAD(P)H-cytochrome *c* reductase activities, does not bind to Blue-Agarose or Blue-Sepharose and exhibits a significantly lower molecular weight (177.000 vs. 241.000), whereas its kinetic characteristics and its sensitivity against several inhibitors and treatments are very similar to those of the terminal nitrate reductase activity of the wild strain complex. Spectral studies and antagonistic experiments with tungstate show the presence of cytochrome *b*<sub>557</sub> and molybdenum. These facts lead us to propose that nitrate reductase from mutant 305 has a protein deletion which affects the pyridine nucleotide binding region of the diaphorase protein but without any effect on the terminal nitrate reductase activity.

## Introduction

Assimilatory NAD(P)H-nitrate reductase (EC 1.6.6.2) of *C. reinhardtii* is an enzyme complex which, like that of other eukaryotes, consists of two partial activities which can be separately assayed: 1) a diaphorase or NAD(P)H-cytochrome *c* reductase, and 2) a terminal or reduced flavin or viologen-nitrate reductase [1–3].

Mutant strains of *C. reinhardtii* only possessing one of the two partial activities of the complex have been recently isolated and characterized [4–11]. Two of these mutants, 104 and 305, which have solely NAD(P)H-cytochrome *c* reductase and terminal nitrate reductase, respectively, are specially interesting due to their ability to reconstitute the entire NAD(P)H-nitrate reductase complex by *in vitro* complementation [7]. The existing data on molecular and kinetic characteristics of mutant 104 as well as on the complementation system [6, 7], together with the existence of mutant 301 with diaphorase activity and molybdenum cofactor [10], suggest for the enzyme complex of *C. reinhardtii* a

quaternary structure very different from that described for the fungal nitrate reductase [12].

Nitrate reductase mutants with solely terminal nitrate reductase activity have been reported in fungi [12–14], algae [4, 15, 16] and higher plants [17, 18], although only in the case of mutant *nit-3* of *Neurospora crassa* the enzyme has been adequately purified and characterized [19].

In this work, the biochemical characterization of purified nitrate reductase from mutant 305 of *C. reinhardtii* is presented and its properties are discussed in comparison with those of the terminal nitrate reductase of its parental wild strain 6145 *c*. The 305 enzyme activity is associated with a protein of lower molecular weight which lacks diaphorase activity, has enzymatic and kinetic properties very close to those of the native complex and contains molybdenum and cytochrome *b*<sub>557</sub>, thus probably possessing altered residues of diaphorase protein.

## Experimental

### Chemicals

Blue-Agarose, Blue-Sepharose, Coomassie Brilliant Blue G-250, DTE\*, FAD, FMN and *p*-HMB

\* Abbreviations: BV, benzyl viologen; DTE, dithioerythritol; MV, methyl viologen; *p*-HMB, *p*-hydroxymercuribenzoate.

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were purchased from Sigma (St. Louis, Mo., USA), NADH, NADPH and cytochrome *c* were obtained from Boehringer (Mannheim, FRG), BV and MV were from Serva (Heidelberg, FRG), and DEAE-Sephadex and Sephadex G-25 from Pharmacia (Uppsala, Sweden). All other chemicals were of the highest quality available.

#### *Growth conditions and preparation of extracts*

*C. reinhardtii* mutant 305 [4] and its parental strain 6145 *c* were cultured with ammonia and derepressed with nitrate under conditions previously reported [7]. Cell-free extracts were prepared by freezing and thawing as described [9].

#### *Enzyme purification*

Nitrate reductase from either mutant 305 or 6145 *c* was purified by a procedure which included the following steps: 1. 2% protamine sulfate, pH 7.0, was added dropwise to cell-free extracts (1 ml/25 mg protein) and gently stirred during 15 min. Then the suspension was spun down at  $27.000 \times g$ , 10 min, and the resulting pellet dissolved with 0.2 M potassium phosphate buffer, pH 7.0, 0.1 mM DTE, 0.1 mM EDTA. 2. The enzyme solution was made 25 mM potassium phosphate, pH 7.0, 0.1 M NaCl, and applied to a DEAE-Sephadex A-50 column ( $2 \times 20$  cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 0.1 M NaCl, 0.1 mM DTE, 0.1 mM EDTA, and the enzyme eluted with the same buffer but 0.25 M in NaCl. 3. Fractions containing nitrate reductase were pooled and saturated with ammonium sulfate up to 55%, the suspension was centrifuged at  $27.000 \times g$ , 10 min, and the pellet dissolved in 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM DTE and 0.1 mM EDTA. 4. After desalting by passage through a Sephadex G-25 column, the enzyme solution was applied to a Bio-Gel HTP column ( $2 \times 10$  cm) equilibrated with the buffer of step 3. The enzyme was eluted with a linear gradient of potassium phosphate buffer (0.01–0.4 M), pH 7.0, at 0.1 M phosphate. 5. Fractions with nitrate reductase were concentrated and treated as in step 3, and the resulting solution was used as source of purified enzyme.

#### *Enzyme assays*

Nitrate reductase was determined by measuring the nitrite formed from nitrate with chemically

reduced flavins or viologens as electron donors [20]. Alcohol dehydrogenase was measured by following the increase in absorbance at 340 nm of NADH formed by ethanol oxidation [21], and catalase by measuring the absorbance decrease at 240 nm due to  $H_2O_2$  decomposition [22]. Ovalbumin was estimated by measuring the absorbance at 280 nm.

Spectrophotometric determinations and absorption spectra were obtained in a PYE-UNICAM SP-8-100 recording spectrophotometer. Activity units are expressed as  $\mu$ mol of substrate transformed per min, and specific activity as units per mg protein.

#### *Analytical methods*

Protein was measured according to a modified Lowry's procedure using bovine serum albumin as standard [23]. Nitrite was estimated by the diazo-coupling colorimetric assay of Snell and Snell [24] with formaldehyde to eliminate interferences caused by products of dithionite decomposition [25].

#### *Sedimentation coefficient determination*

Sedimentation coefficients were calculated from sucrose density gradient centrifugation of enzymes and standards [26]. Linear gradients of 5–20% (w/v) sucrose in 50 mM Tris-HCl buffer, pH 7.5, 0.1 mM DTE, 0.1 mM EDTA, and 10  $\mu$ M FAD were used. 0.2 ml samples containing the enzyme and/or the marker proteins were centrifuged at 45,000 r.p.m., 12 h, in a SW-56Ti rotor. 3-drops fractions were collected from the bottom of tubes and activities and proteins determined as indicated. Sedimentation coefficients of standards used in calculations were those given by Brewer *et al.* [27].

#### *Electrophoresis*

Analytical disc gel electrophoresis was performed as detailed previously [8], according to Jovin *et al.* [28]. Proteins were located on the gels by staining with 1% Coomassie Brilliant Blue G-250 in 7% (v/v) acetic acid, 1 h. Nitrate reductase activity was detected directly on the gels as described by Solomonson *et al.* [29].

## **Results**

#### *Purification of nitrate reductase of mutant 305*

All the attempts made to purify nitrate reductase of mutant 305 by affinity chromatography with

Table I. Nitrate reductase purification of mutant 305 from *C. reinhardtii*.

| Purification step      | Total Protein (mg) | BVH-nitrate reductase |                           |           | FADH <sub>2</sub> -nitrate reductase |                           |           |
|------------------------|--------------------|-----------------------|---------------------------|-----------|--------------------------------------|---------------------------|-----------|
|                        |                    | Total activity [U]    | Specific activity [mU/mg] | Yield [%] | Total activity [U]                   | Specific activity [mU/mg] | Yield [%] |
| 1. Initial extract     | 4864               | 53                    | 11                        | 100       | 11.8                                 | 2                         | 100       |
| 2. Protamine sulfate   | 370                | 27                    | 73                        | 50        | 5.6                                  | 15                        | 47        |
| 3. DEAE-Sephadex A-50  | 108                | 13                    | 118                       | 24        | 2.5                                  | 23                        | 21        |
| 4. Ammonium sulfate I  | 51                 | 10                    | 193                       | 18        | 1.9                                  | 46                        | 16        |
| 5. Bio-Gel HTP         | 11                 | 7                     | 654                       | 13        | 1.6                                  | 143                       | 14        |
| 6. Ammonium sulfate II | 6                  | 6                     | 947                       | 11        | 1.3                                  | 216                       | 11        |

Details of the procedure are given in Experimental.

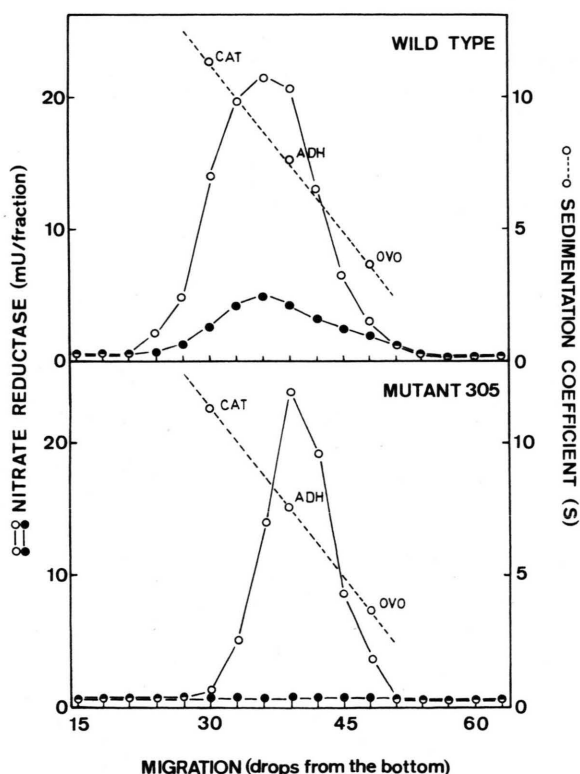


Fig. 1. Sucrose density gradient profile of *C. reinhardtii* wild and mutant 305 strains nitrate reductases. Samples of purified nitrate reductase of either wild type 6145 *c* or mutant 305 were centrifuged under conditions described in Experimental. NADPH-nitrate reductase (●—●) and BVH-nitrate reductase (○—○) activities and standards (---○---) were determined in the corresponding fractions. Standards used were: CAT, bovine catalase, 11.3 S; ADH, yeast alcohol dehydrogenase, 7.6 S; and OVO, ovalbumin, 3.55 S.

either Blue-Agarose or Blue-Sepharose in different conditions of buffer, pH and ionic strength were unsuccessful. By following the procedure described in Experimental the BVH-nitrate reductase was purified 90-fold (0.95 U/mg) (Table I).

FADH<sub>2</sub>-nitrate reductase paralleled always BVH-nitrate reductase along all the purification steps and the ratio between the two activities remained constant along the whole process. Purified preparations of nitrate reductase lacked NAD(P)H-cytochrome *c* reductase activity.

#### Molecular weight determination of nitrate reductase from 305 and wild strains

Both NADPH- and BVH-nitrate reductase activities from wild strain are associated with a protein of  $S_{20,w}$  8.8 S, whereas BVH-nitrate reductase from mutant 305 has a  $S_{20,w}$  of 7.6 S (Fig. 1). By considering Stokes radii values of 6.9 and 5.55 nm [7], and assuming apparent specific volumes of  $0.725 \text{ cm}^3 \cdot \text{g}^{-1}$  for both wild and mutant enzymes, molecular weights of 250.000 and 174.000, and frictional ratios of 1.66 and 1.51, indicative of non-spherical shapes, have been calculated [30] for 6145 *c* and 305 nitrate reductases, respectively. Molecular weights of 232.000 and 180.000, for native and mutant nitrate reductases, have been also calculated electrophoretically by using polyacrylamide gels containing 4.5, 5.5, 6.5 and 7.5% (w/v) acrylamide [31] (Fig. 2). From the above results, average molecular weights of 241.000 and 177.000 for the two enzymes are proposed.

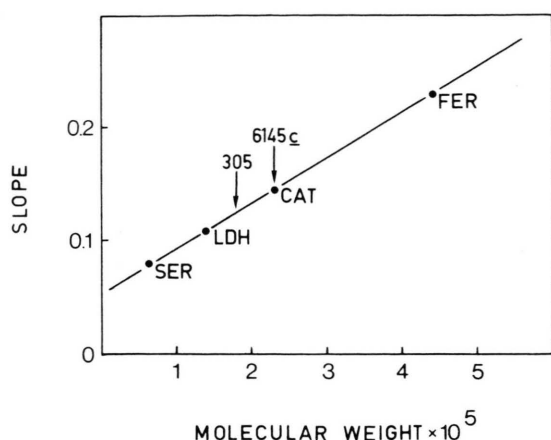


Fig. 2. Molecular weight determination of *C. reinhardtii* wild and mutant 305 strains nitrate reductases. Extracts from 6145 *c* and 305, derepressed with nitrate, and standard proteins were run in parallel in gels with different total concentration of acrylamide, and, after staining, their corresponding  $R_F$  were calculated. Slopes were obtained by plotting  $\log(100 \times R_F)$  vs. the different acrylamide concentrations. Standards were: FER, ferritin, 440,000; CAT, catalase, 232,000; LDH, lactate dehydrogenase, 140,000; and SER, bovine serum albumin, 67,000.

#### Optimum pH and donors and acceptors specificity

Terminal nitrate reductase of both 6145 *c* and 305 exhibited maximal activity at pH 7.5, using either reduced flavins or viologens, with phosphate, Tris-HCl or phosphate/borate buffers (Results not shown).

Both NADH and NADPH were equally effective as electron donors for nitrate reductase of *C. reinhardtii* 6145 *c* (Table II). Addition of FAD stimulated the activity of this enzyme whereas reduced pyridine nucleotides with or without FAD were incapable of nitrate reduction with the 305 mutant enzyme. Viologens and flavins chemically reduced with dithionite, but not dithionite alone, acted as electron donors with both native and mutant 305 nitrate reductases. Apparent  $K_m$ s for several donors and acceptors of enzymes from 6145 *c* and 305 are presented in Table III. Values were very similar for both enzymes but those for reduced viologens (2–6  $\mu$ M) were markedly lower than for reduced flavins (180–500  $\mu$ M).

#### Inhibitors

Both FMNH<sub>2</sub>-nitrate reductases of wild and mutant 305 strains were similarly affected by low concentrations of cyanide, azide and cyanate,

Table II. Electron donors of nitrate reductase of *C. reinhardtii* wild and mutant 305.

| Donor   | Concentration<br>[mM] | Nitrate reductase activity (nmol NO <sub>2</sub> <sup>-</sup> formed/10 min) |     |
|---|-----------------------|--|-----|
|   |                       | wild   | 305 |
| NADH  | 0.3                   | 50   | 0   |
| NADPH   | 0.3                   | 60   | 0   |
| NADH, FAD   | 0.3, 0.02             | 90   | 0   |
| NADPH, FAD  | 0.3, 0.02             | 100  | 0   |
| S <sub>2</sub> O <sub>4</sub> <sup>2-</sup>       | 4.6                   | 0  | 0   |
| S <sub>2</sub> O <sub>4</sub> <sup>2-</sup> , FMN | 4.6, 0.5              | 24   | 36  |
| S <sub>2</sub> O <sub>4</sub> <sup>2-</sup> , FAD | 4.6, 0.5              | 30   | 51  |
| S <sub>2</sub> O <sub>4</sub> <sup>2-</sup> , MV  | 4.6, 0.1              | 170  | 130 |
| S <sub>2</sub> O <sub>4</sub> <sup>2-</sup> , BV  | 4.6, 0.1              | 190  | 240 |

The reaction mixture contained in a 1 ml final volume: Tris-HCl, pH 7.5, 100  $\mu$ mol; enzyme preparation, 30  $\mu$ g; KNO<sub>3</sub>, 10  $\mu$ mol; and the donor system at the indicated concentrations.

Table III. Apparent  $K_m$  values of different donors and acceptors for nitrate reductase of *C. reinhardtii* wild and mutant 305.

| Donor or acceptor            | $K_m$ [ $\mu$ M] |     |
|------------------------------|------------------|-----|
|                              | wild             | 305 |
| FMNH <sub>2</sub>            | 180              | 300 |
| FADH <sub>2</sub>            | 400              | 500 |
| MVH                          | 6                | 4   |
| BVH                          | 2                | 3   |
| NO <sub>3</sub> <sup>-</sup> | 120              | 75  |

$K_m$  values were calculated from Woolf's plots of  $[S]/v$  vs.  $[S]$  by following initial rates of reaction with nitrate as saturating substrate and 50–100  $\mu$ g of enzyme. In the case of NO<sub>3</sub><sup>-</sup>, FMNH<sub>2</sub> was used as electron donor. Other experimental conditions were as described in Experimental.

whereas *p*-HMB only inhibited partially even at concentrations as high as 2 mM (Table IV). Similar results were found using BVH as electron donor (Results not shown).

#### Thermal stability

Nitrate reductase activity levels from mutant 305 remained unaltered after storage at –20 °C, 3 months. The half-life of the activity was shortened with increasing incubation temperatures (Table V). After heating at 50 °C, 25 min, FADH<sub>2</sub>-nitrate reductase was almost undetectable whereas BVH-nitrate reductase dropped only to 50% of its initial value. The terminal nitrate reductase of the complex

Table IV. Effect of different inhibitors on the FMNH<sub>2</sub>-nitrate reductase activity of *C. reinhardtii* wild and mutant 305.

| Inhibitor        | Concentration [M] | Activity [%] |     |
|------------------|-------------------|--------------|-----|
|                  |                   | wild         | 305 |
| None             | —                 | 100          | 100 |
| <i>p</i> -HMB    | $2 \cdot 10^{-4}$ | 69           | 70  |
| <i>p</i> -HMB    | $2 \cdot 10^{-3}$ | 61           | 61  |
| KCN              | $5 \cdot 10^{-5}$ | 34           | 21  |
| KCN              | $5 \cdot 10^{-4}$ | 8            | 3   |
| NaN <sub>3</sub> | $10^{-5}$         | 84           | 73  |
| NaN <sub>3</sub> | $10^{-4}$         | 37           | 26  |
| KCNO             | $5 \cdot 10^{-5}$ | 73           | 70  |
| KCNO             | $5 \cdot 10^{-4}$ | 24           | 21  |

The reaction mixture contained in 1 ml final volume: Tris-HCl, pH 7.5, 100  $\mu$ mol; enzyme preparation, 50–100  $\mu$ g; KNO<sub>3</sub>, 10  $\mu$ mol; FMN, 0.5  $\mu$ mol; Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 4.6  $\mu$ mol; and the indicated concentration of inhibitor.

Table V. Thermic stability of nitrate reductase activities of *C. reinhardtii* mutant 305.

| Temperature [°C] | Half-life [min]                      |                       |
|------------------|--------------------------------------|-----------------------|
|                  | FADH <sub>2</sub> -nitrate reductase | BVH-nitrate reductase |
| 25               | 132                                  | 450                   |
| 37.5             | 52                                   | 186                   |
| 50               | 8                                    | 26                    |
| 62.5             | 1                                    | 6                     |

Half-life was calculated from the slope of  $\ln(a/a_0)$  vs. time linear plot, where  $a$  is the crude extracts activity measured after incubation at a fixed temperature for a determined time and  $a_0$  the activity of the corresponding control maintained at 0°C for the same time.

from 6145 *c* behaved very similarly in this respect (Results not shown).

#### Molybdenum and heme content

When cells from 6145 *c* and 305, grown with ammonia, were transferred to nitrate media supplemented with increasing amounts of tungstate (up to 500  $\mu$ M), a progressive decline in the terminal nitrate reductase activity was observed (Results not shown).

The absorption spectrum of purified 305 nitrate reductase exhibited a peak at 413 nm insensitive to the addition of either NADH or NADPH. Sodium dithionite shifted the Soret band to 424 nm and induced the appearance of two new maxima at 527 nm ( $\beta$ ) and 557 nm ( $\alpha$ ) indicative of a *b*-type cytochrome (Fig. 3). Preparations of 6145 *c* purified

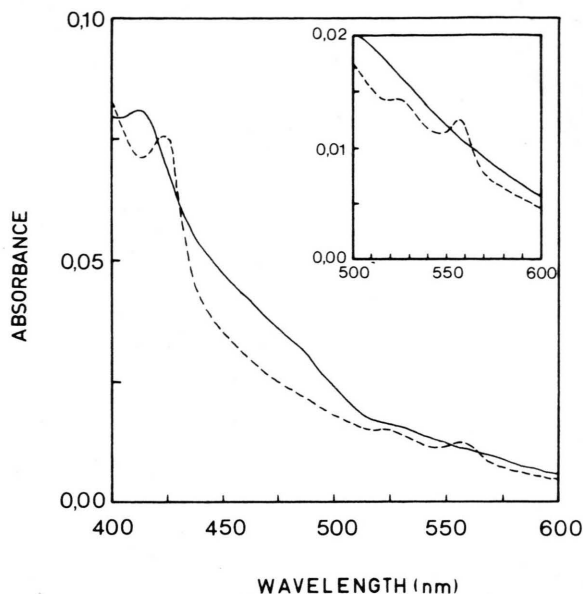


Fig. 3. Absorption spectra of purified nitrate reductase of *C. reinhardtii* mutant 305. Solid line: absorption spectrum of the oxidized enzyme (2 mg/ml) dialyzed against 10 mM potassium phosphate buffer, pH 7.0. Dashed line: absorption spectrum of the enzyme reduced with small amounts of solid Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>. Spectra of reduced enzyme were recorded immediately after the addition of reductant at room temperature.

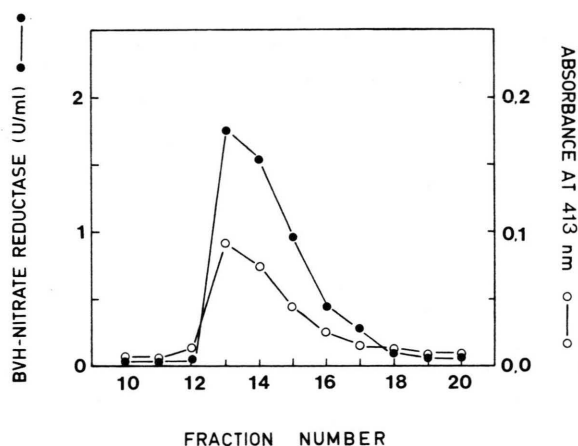


Fig. 4. Elution profile of nitrate reductase of *C. reinhardtii* mutant 305, through a Bio-Gel HTP column. The 305 enzymatic preparation (step 4, Table I) was applied to a Bio-Gel HTP column and eluted as described in Experimental. As indicated, BVH-nitrate reductase activity and absorbance at 413 nm were determined in each fraction.

by following the same method described for the 305 nitrate reductase showed identical spectral properties, except that the oxidized enzyme could be reduced by NAD(P)H (Results not shown). The cytochrome *b*<sub>557</sub> is associated with the nitrate reductase of 305 as deduced from the parallel elution pattern of activity and absorption at 413 nm of samples subjected to chromatography on Bio-Gel HTP (Fig. 4).

## Discussion

Nitrate reductase of mutant 305 of *C. reinhardtii*, purified 90-fold, is unable to reduce with NAD(P)H either cytochrome *c* (diaphorase activity) or nitrate (total nitrate reductase activity), does not attach to Blue-Agarose or Blue-Sepharose and has an average molecular weight (177.000) lower than that of the native complex (241.000) which indicates that the mutant enzyme lacks an essential protein portion probably enclosing the binding region for pyridine nucleotides. A markedly higher molecular weight (500.000) has been reported for the NADH-nitrate reductase complex of *C. reinhardtii* strain 1132 *c*, based on gel filtration data [1]. Likewise, the sedimentation coefficient values found for nitrate reductase of 6145 *c* (8.8 S) and mutant 305 (7.6 S) are lower than those reported by Sosa *et al.* [4], 10 S and 8.3 S, respectively.

The close similarities of both terminal nitrate reductases from 6145 *c* and 305 in electron donors and cofactors, *K<sub>m</sub>*s values, pH optimum and response to inhibitors and heat treatment suggest that mutation has not affected the protein structure responsible for the terminal nitrate reductase activity. The lack of effect of *p*-HMB on terminal nitrate reductase is indicative of the absence of functional -SH groups which, by contrast, play an essential role in the diaphorase activity of nitrate reductase [1, 19, 32, 33]. It is noteworthy that the effectiveness of the electron donors for nitrate reductase of wild type 6145 *c* is opposite to that of wild strain 1132 *c* previously described [1, 5], since the 6145 *c* enzyme uses NADPH, FADH<sub>2</sub> and BVH, over NADH, FMNH<sub>2</sub> and MVH which are utilized preferentially by 1132 *c* nitrate reductase.

The different stability of FADH<sub>2</sub>- and BVH-nitrate reductase activities against heat treatment suggests that reduced flavin nucleotides and viologens donate their electrons at different points,

distinguishable by their different thermosensitivity, of the nitrate reduction electron chain. A similar conclusion has been reached for the *nit-3* enzyme of *N. crassa* [19].

Molybdenum is an essential constituent of all known nitrate reductases and has been described as responsible, in form of a dialyzable cofactor, for both catalytic reduction of nitrate and assembling of subunits which integrate the nitrate reductase complex [7, 8, 33–35]. The presence of molybdenum in *C. reinhardtii* nitrate reductase had been demonstrated by using Mo<sup>99</sup> and W<sup>185</sup> [2]. Tungsten incorporation inactivates terminal nitrate reductase of both 6145 *c* and mutant 305 which indicates that molybdenum plays an essential role in catalytic activity [7]. In addition, an active dialyzable heat-labile molybdenum cofactor, shared by nitrate reductase and xanthine dehydrogenase, has been found in *C. reinhardtii* [7, 8, 10].

The presence of cytochrome *b*<sub>557</sub> has been reported in all eukaryotic nitrate reductases described up to now [3, 33]. The purified nitrate reductase of 305 contains cytochrome *b*<sub>557</sub> as deduced from its spectral properties (peaks at 413 nm in oxidized enzyme and at 424, 527 and 557 nm in reduced enzyme), identical to those of the entire complex of 6145 *c* [6], and from the parallelism between cytochrome *b* content and activity found during the last steps of enzyme purification. Very recently, the presence of cytochrome *b*<sub>557</sub> in a purified protein with NAD(P)H-cytochrome *c* reductase activity considered as a true subunit of *C. reinhardtii* nitrate reductase has been also found [6]. Whether or not, this cytochrome *b* plays a role in terminal activity remains to be ascertained.

Mutant *nit-3* of *N. crassa* resembles markedly mutant 305 in enzymatic and physicochemical properties as well as in their metal constituents [19], although the *in vitro* complementation system and the characteristics of reported mutant strains of *N. crassa* [12, 13, 36, 37] and *C. reinhardtii* [6–8, 10] are notably different.

The presented results lead us to propose that 305 nitrate reductase contains residues of altered diaphorase subunits smaller than the native ones of molecular weight 44.500 [6, 7, 9–11]. These altered subunits have a significant protein deletion probably located at the pyridine nucleotides binding region, retain to a certain extent the heme domain of cytochrome *b*<sub>557</sub>, and are able to assemble with

terminal subunits to form a deficient complex which lacks the diaphorase activity and is endowed with terminal nitrate reductase activity indistinguishable from that of *C. reinhardtii* wild type. On the basis of the available biochemical data from different nitrate reductase mutants of *C. reinhardtii* [6–11], a heteropolymeric character has been attributed to the nitrate reductase complex of this alga. The native enzyme would consist of two kinds of subunits, separately responsible for NAD(P)H-cytochrome *c* reductase and terminal nitrate reductase, assembled for full activity by a molybdenum cofactor which besides is an integral part of

the terminal moiety [6–8, 10]. Thus, 305 nitrate reductase would be constituted by altered diaphorase subunits containing the heme domain and non-altered subunits of the other kind assembled by the molybdenum cofactor.

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