Nitrate Reductase Deficient Mutants of *Chlamydomonas reinhardtii*. Biochemical Characteristics

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Two mutants of *Chlamydomonas reinhardtii* that cannot grow with nitrate as nitrogen source have been examined biochemically. Each mutant differs from the wild-type strain by a single mutation in a Mendelian gene. One mutant (14/15) carries a mutation in a gene designated *nitB*. This organism took up nitrate and converted nitrate-nitrogen to insoluble-nitrogen; nevertheless no enzymic activities associated with nitrate reductase (NADH-nitrate reductase, reduced benzyl viologen nitrate reductase or inducible NADH-cytochrome *c* reductase) could be demonstrated in frozen/thawed cells or cell-free extracts. At high nitrate concentrations (5 mM) the rate of nitrate uptake by this mutant [$V_{\max}$ 59 nmol (mg dry wt)⁻¹ h⁻¹] was considerably lower than that of wild-type [$V_{\max}$ 920 nmol (mg dry wt)⁻¹ h⁻¹]. It is concluded that *nitB* is probably a regulatory gene for nitrate reductase. The other mutant (17/4) has a mutation in a gene designated *nitA*. This organism did not take up nitrate and had no NADH-nitrate reductase or inducible NADH-cytochrome *c* reductase activities. Frozen/thawed cells and cell-free extracts had reduced benzyl viologen nitrate reductase activity. The enzyme from the *nitA* mutant eluted from a Sepharose 4B column later than wild-type enzyme; it was therefore probably of lower molecular weight. The *nitA* mutation of *C. reinhardtii* closely resembles the *nit-3* mutation of *Neurospora crassa* and, like this mutation, it probably results in loss of the NADH combining sub-unit of nitrate reductase. Strain 137c of *C. reinhardtii* had no enzymic activities associated with nitrate reductase and did not take up nitrate. This organism may have mutations in both the *nitA* and *nitB* loci.

INTRODUCTION

The preceding paper (Nichols & Syrett, 1978) described the isolation, from the alga *Chlamydomonas reinhardtii*, of mutants which cannot grow with nitrate as nitrogen source. Two of these mutants (17/4 and 14/15) were selected for more detailed study and it was shown that each differed from the wild-type strain from which it was derived by a single mutation in a Mendelian gene. The genes carrying these mutations were designated *nitA* (in 17/4) and *nitB* (in 14/15). We also examined strain 137c of *C. reinhardtii*. This organism, which has been used by Levine and his colleagues for extensive studies on the genetics of the photosynthetic mechanism (Levine & Goodenough, 1970), was shown to have two mutations with respect to ability to grow on nitrate (Nichols & Syrett, 1978).

In this paper we describe some of the biochemical characteristics of these organisms in relation to their ability to take up nitrate and to produce functional nitrate reductase.

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METHODS

Growth of organisms. Studies were carried out with *Chlamydomonas reinhardtii*, strain 2192 (wild-type), mutant 17/4 (*nitA* mutant), mutant 14/15 (*nitB* mutant) and strain 137c. Only strain 2192 grew with nitrate as nitrogen source (Nichols & Syrett, 1978). The liquid medium used for growing cultures contained, per litre of double-distilled water, 0.72 g KH_2PO_4 , 1.44 g K_2HPO_4 , 0.02 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 ml trace element solution (Sueoka, 1960) and 132 mg N supplied as either KNO_3 , urea, KNO_3 plus urea (equal N in each) or NH_4Cl . Cultures were grown, usually for 48 h, at 25 °C in wash bottles fitted with sintered glass bubblers. They were aerated vigorously with air containing 0.5 % (v/v) CO_2 and illuminated by daylight fluorescent tubes giving a light intensity, at the cultures, of 30 W m^{-2} . For enzyme purification, larger volumes of culture were grown in 10 l aspirators.

Determination of rates of nitrate uptake. Organisms grown on urea medium were harvested, washed and suspended in nitrogen-free medium at about 3 mg dry wt ml^{-1} . The suspension was illuminated at 25 °C and aerated with CO_2 -enriched air. Nitrate was added at zero time and samples of the culture were removed at suitable intervals (usually 15 or 30 min). Some samples were centrifuged immediately and the supernatants were retained for analysis. Others were heated in boiling water for 10 min and then allowed to stand for 2 h before centrifuging; this treatment released any nitrate present in the cells. Nitrate in the supernatants was determined by the frozen/thawed *Chlorella* cell method of Hipkin & Syrett (1973).

Uptake of $^{15}\text{NO}_3^-$. Uptake of nitrate was measured as described above, 40 μM - $\text{Na}^{15}\text{NO}_3$ (99 % atom excess) being added at zero time. At zero time and at 4 h after the addition of $^{15}\text{NO}_3^-$, 10 ml samples of culture were removed, centrifuged and the cells were washed twice with nitrogen-free medium. Some samples of cells were then digested for total-nitrogen determination. Others were extracted with methanol until colourless before being digested; measurements on these samples gave the insoluble-nitrogen. All samples and treatments were in duplicate. Digestion was by a Kjeldahl method using 5 M-sulphuric acid containing sodium selenate (20 g l^{-1}) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (20 g l^{-1}). After digestion, ammonium was collected from each digest in boric acid in a Conway unit (Conway, 1947) and titrated with 0.5 M-HCl. The dried samples of ammonium chloride were analysed for ^{15}N content by Dr J. W. Millbank of Imperial College, London. Nitrogen gas was generated from the ammonium chloride samples by means of alkaline hypobromite in an apparatus similar to that described by Ross & Martin (1970). It was analysed by means of an AEI MS2 mass spectrometer.

Preparation of frozen/thawed cells and of cell-free extracts. Organisms were harvested by centrifuging (1000 g for 5 min), washed twice with nitrogen-free medium and suspended in cold 0.05 M-potassium phosphate buffer, pH 7.5, containing 5 μM -FAD, at about 5 mg dry wt cells ml^{-1} . For the preparation of frozen/thawed cells, portions of this suspension were placed in polypropylene tubes, frozen in liquid nitrogen and stored at -20 °C. Suspensions were thawed for use without allowing the temperature to rise above 3 °C.

For the preparation of cell-free extract, cell suspension at 3 °C was passed through a French press (Milner *et al.*, 1950) at 100 MPa and then centrifuged either at 17000 g for 20 min or at 90000 g for 60 min, at 3 °C. The resultant supernatants were used as crude cell-free extracts. Protein was determined by the method of Hartree (1972) using bovine serum albumin as a standard.

Enzyme assays.

(a) *NADH-nitrate reductase (NADH-NR).* The reaction mixture (1.3 ml) contained either frozen/thawed cells (about 1 to 3 mg dry wt) or cell-free extract (about 500 μg protein), 10 μmol KNO_3 and 0.05 M-phosphate buffer, pH 7.5, containing 5 μM -FAD. Incubation was at 30 °C and the reaction was started by adding 0.6 μmol NADH. It was stopped, after 15 min, by adding 0.2 ml 1 M-zinc acetate and 1 ml 95 % (v/v) ethanol, and cooling in ice; this treatment removed residual NADH (Medina & Nicholas, 1957). The tubes were left standing for 10 min and then agitated and centrifuged. Nitrite in the supernatants was determined spectrophotometrically after addition of Griess-Ilosvay reagent.

(b) *FMNH₂-nitrate reductase (FMNH₂-NR).* The reaction mixture was similar to that for assay of NADH-NR but it contained 3.2 μmol FMN and the reaction was started by adding 0.25 ml sodium dithionite [8 mg ml^{-1} in 1 % (w/v) sodium bicarbonate] instead of NADH. The reaction was terminated after 15 min at 30 °C by vigorous agitation to oxidize residual dithionite and addition of zinc acetate and ethanol. Nitrite formed was then determined.

(c) *Reduced benzyl viologen nitrate reductase (BVH-NR).* The method was similar to that of Thacker & Syrett (1972) but frozen/thawed cells were used and 0.05 M-phosphate/FAD buffer, pH 7.5, was used instead of Tris/HCl buffer.

(d) *NADH-cytochrome c reductase (NADH-CR).* Two methods of assay were used. (i) This assay was similar to that of Cove & Coddington (1965). The assay mixture (3.0 ml) contained 2 mg cytochrome c (horse heart, Grade III), 0.5 to 1.0 ml 90000 g cell-free supernatant and 0.05 M-phosphate/FAD buffer, pH 7.5; after incubation for 5 min at 30 °C the reaction was started by adding 2.1 μmol NADH and the

increase in absorbance at 550 nm was followed. (ii) (Shehata, 1977). The assay mixture (3.0 ml) contained 8 mg cytochrome *c*, 0.1 to 0.5 ml 17000 *g* cell-free supernatant and 0.05 M-phosphate buffer, pH 7.5, without FAD; after incubation at 30 °C, the reaction was started by adding 0.4 μ mol NADH and the reduction of cytochrome *c* was followed at 550 nm. The quantity of cytochrome *c* reduced was calculated from the increase in light absorption at 550 nm using the value for the molar extinction coefficient of cytochrome *c* (21000) given by Massey (1959).

It was established for all enzyme assays that the reaction was linear with time over the period of measurement and that the rate was proportional to the quantity of enzyme preparation added. Controls with enzyme inactivated by heat were always run.

Purification of nitrate reductase. Cultures grown for 48 h on urea plus nitrate medium were harvested and cell-free extracts were prepared as described above. The supernatant from centrifugation at 17000 *g* was centrifuged again at 100000 *g* for 60 min at 4 °C. The protein which precipitated from this solution at between 30 and 50 % saturation with ammonium sulphate contained most of the nitrate reductase activity. This precipitate was collected by centrifugation at 5000 *g*, dissolved in 0.05 M-phosphate buffer, pH 7.5, containing 5 μ M-FAD and used as a partially purified source of the enzyme. Further purification of the enzyme from wild-type strain 2192 was achieved by affinity chromatography on a blue dextran Sepharose 4B column (Solomonson, 1975). The column (90 $\times$ 15 mm) was equilibrated with buffer A (0.08 M-potassium phosphate, pH 7.3, containing 0.15 mM-dithiothreitol, 0.1 mM-EDTA and 5 μ M-FAD). The dissolved ammonium sulphate precipitate was applied to the column and the column washed with buffer A and then with buffer B (of similar composition to buffer A but containing 0.4 M-phosphate, pH 7.3). Nitrate reductase was then eluted with 0.1 mM-NADH dissolved in buffer A. When cytochrome *c* reductase activity was to be determined in the eluates, FAD was omitted from the elution buffer; in this case, FAD was added to fractions before determination of NADH-NR or FMNH₂-NR activities.

Nitrate reductase from mutant 17/4 did not bind to the blue dextran affinity column presumably because, as it cannot react with NADH, it lacks the dinucleotide fold (Thompson *et al.*, 1975). Some purification of the enzyme from strain 17/4 was obtained by elution from columns (500 $\times$ 25 mm, bed volume about 240 ml) of Sephadex G-200 or Sepharose 4B. These columns were prepared and used following the instructions of the manufacturer (Pharmacia). About 10 mg protein (ammonium sulphate precipitate dissolved in 3 ml phosphate buffer) was applied to the Sephadex column and about 5 mg (in 2 ml buffer) to the Sepharose column. Fractions of 3 ml were collected from the Sephadex column and of 6 ml from the Sepharose column. Extracts from strain 2192 were also run on these columns.

Chemicals. All biochemicals were purchased from Sigma; Na¹⁵NO₃ was from Prochem, British Oxygen Company.

RESULTS

Ability to assimilate nitrate and to grow with nitrate as nitrogen source

The *nitA* mutant (strain 17/4), the *nitB* mutant (strain 14/15) and strain 137c could not grow in normal medium with nitrate as nitrogen source but all grew well when urea or ammonium was provided. It was possible that, although unable to grow on nitrate, the mutants might accumulate this ion. Therefore washed suspensions of organisms (2192, 137c and the *nitA* and *nitB* mutants) which had been grown for 48 h on urea were supplied with nitrate and its disappearance was measured. Samples of the suspensions were removed at intervals and some were heated to release nitrate in the cells into the external medium before analysis. The *nitA* mutant and strain 137c showed negligible uptake of nitrate but the wild-type strain 2192 and the *nitB* mutant took up nitrate (Fig. 1). The nitrate taken up was not retained in the cells because none was released into the medium when the cells were killed by heating. Three-d-old cultures of the *nitB* mutant had little ability to assimilate nitrate.

Clearly, the *nitB* mutant took up nitrate but this did not accumulate as nitrate in the cells. This result led to renewed attempts to demonstrate growth of this mutant on nitrate albeit at a slow rate; these were unsuccessful. Nevertheless, convincing evidence that the nitrate-nitrogen taken up by the *nitB* mutant was assimilated into organic nitrogen was obtained by supplying ¹⁵NO₃⁻ at a concentration of 40 μ M. Figure 2 shows the uptake of nitrate by urea-grown organisms of strain 2192 and the *nitA* and *nitB* mutants during the experiment. Table 1 shows the incorporation of ¹⁵N into insoluble-nitrogen and total-nitrogen after 4 h.

Fig. 1. Nitrate uptake by strain 2192, the *nitB* and *nitA* mutants and strain 137c. Cultures were grown with urea as nitrogen source for 48 h. The initial nitrate concentration was 20 μM and the cell density was about 3 mg dry wt ml^{-1} for each experiment. Nitrate was determined in the supernatants from unheated (○) and heated (●) samples.

Table 1. Assimilation of ^{15}N from $^{15}\text{NO}_3^-$ into total-nitrogen and insoluble-nitrogen

Each value is the mean from determinations on two separate samples; replicate values did not differ by more than 4%. All differences between 'initial' and 'after 4 h' values for total-nitrogen and insoluble-nitrogen are significant at the 5% probability level including those for the *nitA* mutant.

Strain	Nitrogen determination		Atom % $^{15}\text{N}_2$	Atom % excess
2192	Total:	initial	0.375	0
		after 4 h	1.405	1.033
	Insoluble:	initial	0.371	0
		after 4 h	1.368	0.996
<i>nitA</i> mutant	Total:	initial	0.371	0
		after 4 h	0.426	0.054
	Insoluble:	initial	0.376	0
		after 4 h	0.427	0.055
<i>nitB</i> mutant	Total:	initial	0.374	0
		after 4 h	1.033	0.661
	Insoluble:	initial	0.374	0
		after 4 h	0.991	0.617
Checks	(i) Laboratory air		0.369	0
	(ii) Analar $(\text{NH}_4)_2\text{SO}_4$		0.367	0

Both 2192 and the *nitB* mutant incorporated ^{15}N from $^{15}\text{NO}_3^-$; the *nitA* mutant showed little incorporation.

Figure 3 shows the relationships between rate of nitrate uptake and nitrate concentration in the medium for strain 2192 and the *nitB* mutant. At low nitrate concentrations the strains did not differ greatly in their ability to assimilate nitrate but at higher concentrations, e.g. 5 mM, the difference was marked. From curves fitted to the points in Fig. 3 by the computer procedure of Bliss & James (1966), the following kinetic values were obtained: for the *nitB* mutant, K_s 255 μM , $V_{\max}$ 59 nmol (mg dry wt) $^{-1}$ h $^{-1}$; for strain 2192, K_s 674 μM , $V_{\max}$ 920 nmol (mg dry wt) $^{-1}$ h $^{-1}$; where K_s is the nitrate concentration supporting half the maximum rate of uptake.

Fig. 2

Fig. 3

Fig. 2. Uptake of $^{15}\text{NO}_3^-$ by strain 2192 (○), the *nitB* mutant (△) and the *nitA* mutant (●). Cultures were grown on urea medium for 48 h and suspensions of washed cells (about 1 mg dry wt ml^{-1}) were supplied with $\text{Na}^{15}\text{NO}_3$ at an initial concentration of 40 μM . Samples were removed at zero time and after 4 h for measurement of ^{15}N incorporation (see Table 1).

Fig. 3. The relationship between rate of nitrate uptake and nitrate concentration for strain 2192 (○) and the *nitB* mutant (●). Conditions were similar to those for the experiments of Fig. 1. Replicate samples of each culture were supplied with nitrate to give the initial concentrations shown on the abscissa. Nitrate was determined in samples removed at intervals (over 8 h for the lower nitrate concentrations, and over 24 h for the higher ones). The graphs of nitrate concentration against time were reasonably linear and the rates plotted in the figure were obtained from their slopes. The hyperbolae drawn were fitted by the iterative computer procedure of Bliss & James (1966). They give the following kinetic values: for the *nitB* mutant, K_s 255 μM , $V_{\max}$ 59 $\text{nmol (mg dry wt)}^{-1} \text{h}^{-1}$; for strain 2192, K_s 674 μM , $V_{\max}$ 920 $\text{nmol (mg dry wt)}^{-1} \text{h}^{-1}$.

Table 2. *NADH-nitrate reductase and BVH-nitrate reductase activities in frozen/thawed cell preparations of wild-type (strain 2192) and three mutant strains*

Cultures were grown for 48 h on urea medium, and then organisms were harvested, washed and incubated for 5 h in medium containing the nitrogen sources shown before being taken for enzymic analysis. Activities are expressed as $\text{nmol nitrite formed min}^{-1} (10 \text{ mg cell dry wt})^{-1}$. Mean values are given based on the number of separate determinations indicated in parentheses.

Strain	Nitrogen source	Enzyme activity	
		BVH-NR	NADH-NR
2192	Urea	1.8 (2)	0.6 (3)
	Urea + nitrate	26.7 (3)	18.6 (3)
	None	8.9 (2)	2.3 (3)
	Ammonium	0.9 (2)	ND
	Ammonium + nitrate	6.6 (1)	ND
137c	Urea	* (2)	* (2)
	Urea + nitrate	* (2)	* (2)
	None	3.3 (2)	0.2 (2)
<i>nitB</i> mutant	Urea	0.3 (2)	0.6 (2)
	Urea + nitrate	* (2)	0.2 (2)
	None	6.4 (2)	0.4 (2)
<i>nitA</i> mutant	Urea	1.7 (3)	0.1 (3)
	Urea + nitrate	46.6 (4)	0.1 (3)
	None	69.8 (3)	0.3 (3)
	Ammonium	* (1)	ND
	Ammonium + nitrate	22.8 (1)	ND

* No activity detected.

ND, Not determined.

Table 3. *Enzymic activities in cell-free extracts*

Cultures were grown for 48 h in medium containing the nitrogen source shown and then cell-free extracts were prepared. These extracts contained about 1 mg protein ml⁻¹. NADH-CR activity was measured by assay (ii). Specific activities are expressed as nmol product formed min⁻¹ (mg protein)⁻¹.

Strain	Nitrogen source	Enzyme specific activity		
		NADH-NR	FMNH ₂ -NR	NADH-CR
2192	Nitrate	80	65	21
	Nitrate + urea	80	68	55
	Urea	3	1.0	16
	Ammonium	1	0.3	9
<i>nitB</i> mutant	Urea + nitrate	0	0	18
<i>nitA</i> mutant	Urea + nitrate	0	2.3	18

Presence or absence of nitrate reductase activity

Nitrate reductase catalyses three enzymic activities, namely (i) NADH-NR, reduction of nitrate with NADH as electron donor, (ii) BVH-NR or FMNH₂-NR, reduction of nitrate with reduced benzyl viologen or FMNH₂ as electron donor, and (iii) NADH-CR, reduction of cytochrome *c* with NADH as electron donor (Beevers & Hageman, 1969). Table 2 gives nitrate reductase activities measured with cells grown on urea for 48 h, incubated with nitrogen source for 5 h and then frozen/thawed. Where low apparent activities were recorded they have been included in the table but little significance is attached to these values. Cells of wild-type 2192 contained detectable NADH-NR and BVH-NR activities when incubated with urea. These activities were much higher when nitrate was present in the incubation medium but lower with ammonium; the activities also increased when urea-grown cell were incubated for 5 h in the absence of a nitrogen source. In strain 137c and in the *nitB* mutant, activities were always low; there is a suggestion that a low BVH-NR activity developed in both during nitrogen starvation but this may not be real. The *nitA* mutant had no NADH-NR activity but it had substantial BVH-NR activity which increased during incubation with nitrate and even more so during 5 h incubation with no nitrogen source; the activity was lower when the medium contained ammonium in addition to nitrate. Similar results were obtained from less extensive assays using cell-free extracts from cells grown on urea/nitrate medium (Table 3).

NADH-cytochrome c reductase activity

Nitrate reductase catalyses the reduction of cytochrome *c* with NAD(P)H as electron donor, but this reaction can also be catalysed by other enzymes (MacDonald *et al.*, 1974). Crude cell-free extracts from 2192 and from both mutants had this activity (Table 3). Cells of 2192 incubated for 5 h with urea plus nitrate contained much more NADH-CR activity than cells incubated with urea alone (Table 4). In contrast, the presence of nitrate during the incubation did not affect the activity of cells of strain 137c or the *nitA* and *nitB* mutants; it appears probable, therefore, that these organisms lack the NADH-CR activity that is specifically associated with nitrate reductase.

Purification of nitrate reductase from strain 2192 and the nitA mutant

Table 5 records the partial purification of nitrate reductase from strain 2192 using a blue dextran affinity column. The specific activities of NADH-NR and FMNH₂-NR were increased about 200-fold during purification. The purified enzyme retained NADH-CR activity.

Table 4. *NADH-cytochrome c reductase activity in cell-free extracts of wild-type (strain 2192) and three mutant strains*

Cultures were grown for 48 h on urea medium, and then organisms were harvested and incubated for 5 h in medium containing the nitrogen source shown before preparation of cell-free extracts for assay. NADH-CR activity was measured by assay (i) and is expressed as nmol cytochrome *c* reduced min⁻¹ (mg protein)⁻¹. Mean values are given based on the number of separate determinations indicated in parentheses.

Strain	Nitrogen source	NADH-CR activity	Induced CR
			Non-induced CR
2192	Urea	19.8 (6)	2.9
	Urea + nitrate	56.9 (6)	
137c	Urea	13.0 (5)	1.0
	Urea + nitrate	13.6 (5)	
<i>nitB</i> mutant	Urea	14.6 (4)	0.6
	Urea + nitrate	8.7 (4)	
<i>nitA</i> mutant	Urea	27.3 (2)	0.8
	Urea + nitrate	22.2 (2)	

Using a Sepharose 4B column, nitrate reductase from strain 2192 was purified about 30-fold; again the partially purified preparation retained NADH-CR activity. Table 6 summarizes the purification of the FMNH₂-NR enzyme from the *nitA* mutant on Sepharose 4B. Two points are noteworthy. Firstly, a marked increase in FMNH₂-NR activity occurred during ammonium sulphate precipitation; this has been noted several times and presumably results from an activation phenomenon. A similar, but less marked, increase was found with extracts from 2192. Secondly, in our system the nitrate reductase from strain 2192 eluted from the Sepharose column in fractions 8 to 11, very close to the blue dextran marker, whereas the FMNH₂-NR activity from the *nitA* mutant eluted later, in fractions 20 to 22. The later elution indicates that the enzyme in this mutant is of lower molecular weight than that from 2192.

Apparent K_m values for nitrate reduction

The relationship between nitrate concentration and rate of nitrate reduction was investigated using enzymes partially purified by ammonium sulphate precipitation and either NADH or FMNH₂ as electron donors. During assays, incubations were for 10 min and the quantity of nitrite formed was determined; even at the lowest nitrate concentrations the quantity of nitrate reduced was less than 30% of the total present in the reaction mixture. Hyperbolae were fitted to the plots of rate of nitrite formation against nitrate concentration by the computer procedure of Bliss & James (1966). The computer also gave the following apparent *K_m* values for nitrate: for enzyme from strain 2192, 412 ± 89 μM with NADH as electron donor and 1900 ± 244 μM with FMNH₂; for enzyme from the *nitA* mutant, 284 ± 33 μM with FMNH₂ as electron donor.

DISCUSSION

The three strains we have examined differ from each other and from wild-type both genetically (Nichols & Syrett, 1978) and biochemically. Unlike wild-type, none of them is able to grow with nitrate as nitrogen source.

The *nitA* mutant differs from wild-type by a mutation in a single Mendelian gene. Biochemically it has lost NADH-NR and NADH-CR activities but retains BVH(FMNH₂)-NR activity. This enzymic activity, however, confers no ability for intact cells to utilize nitrate. They do not take up nitrate and do not assimilate ¹⁵N from ¹⁵NO₃⁻ to any appreciable

Table 5. Summary of purification of nitrate reductase from strain 2192 by affinity chromatography

Assays were set up as described in Methods except that buffers without FAD were used. FAD was added for the NADH-NR and FMNH₂-NR assays. NADH-CR activity was measured by assay (ii). Activities are expressed as nmol product formed min⁻¹ and specific activities as nmol product formed min⁻¹ (mg protein)⁻¹.

Step	Vol. (ml)	Protein (mg)	NADH-NR			FMNH ₂ -NR			NADH-CR		
			Total activity	Recovery (%)	Specific activity	Purifica- tion factor	Total activity	Recovery (%)	Specific activity	Purifica- tion factor	Purifica- tion factor
Crude extract (17000 g supernatant)	15	114	4300	—	40	1.0	7100	—	60	1.0	1.0
30 to 50 % (NH ₄) ₂ SO ₄ ppt.	8	40	2300	54	60	1.5	3500	50	90	1.5	1.4
Affinity column											
Buffer B	20	0.74	450	10	600	15.0	450	6	600	10.0	16.0
Buffer A plus NADH	20	0.14	800	19	7100	178.0	800	11	7600	125.0	300.0

Table 6. Summary of the purification of FMNH₂-NR and NADH-CR activities from the *nitA* mutant on Sepharose 4B

Cultures were grown on nitrate/urea medium for 48 h, and then organisms were harvested, washed and resuspended in nitrogen-free medium. Cell-free extract was prepared as described in Methods. For assay of FMNH₂-NR, FAD (5 μM) was added to the assay buffer. NADH-CR activity was measured by assay (ii). Activities are expressed as nmol product formed min⁻¹, and specific activities as nmol product formed min⁻¹ (mg protein)⁻¹.

No NADH-cytochrome c reductase activity could be detected in the fractions collected from the column.

Step	Total protein (mg)	FMNH ₂ -NR			NADH-CR		
		Total activity	Recovery (%)	Specific activity	Total activity	Recovery (%)	Specific activity
Crude extract (17000 g supernatant)	71.4	120	—	2	264	—	4
30 to 50 % (NH ₄) ₂ SO ₄ ppt.	34.3	307	256	9	233	88	7
Sepharose column							
Tube 20	1.1	15	12.5	14	0	0	0
Tube 21	1.2	20	15.0	17	0	0	0
Tube 22	1.2	14	11.7	11	0	0	0

extent. This mutant grows well on hypoxanthine and this finding supports the view that the BVH(FMNH₂)-NR activity is associated with the molybdenum-containing sub-unit of nitrate reductase (Coddington, 1976). The nitrate reductase from the *nitA* mutant elutes later than nitrate reductase from wild-type from a Sepharose column. It is therefore presumably of lower molecular weight than the wild-type enzyme and it is reasonable to suggest that it has lost the sub-unit of the enzyme which combines with NADH (Barea *et al.*, 1976). Phenotypically our *nitA* mutant corresponds closely to the *nit-3* mutant of *Neurospora crassa* (Coddington, 1976) which also produces a BVH-nitrate reductase of lower molecular weight than enzyme from wild-type and which has also lost NAD(P)H-NR and NAD(P)H-CR activities. In both mutants, BVH-nitrate reductase activity, under inducing conditions, was higher than that of wild-type under similar conditions and in both, the nitrate reductase, like that of the wild-type strains, was repressed in the presence of ammonium. It is probable, therefore, that the *nitA* mutation in *C. reinhardtii* is similar to that in *N. crassa nit-3* and is a mutation in a structural gene for nitrate reductase.

The *nitB* mutant also differs from wild-type by a single mutation. Biochemically this organism has no enzymic activities associated with nitrate reductase that can be assayed except possibly a little BVH-NR activity in nitrogen-starved cells. Nevertheless this organism can take up nitrate. At high nitrate concentrations the rate of uptake is considerably lower than that of wild-type, but at low nitrate concentrations their rates of uptake are not too dissimilar; there is no doubt that this mutant, like wild-type, can assimilate ¹⁵N from ¹⁵NO₃⁻ into insoluble-nitrogen. Therefore the *nitB* mutant must reduce nitrate. Since the BVH(FMNH₂)-NR activity in the *nitA* mutant does not allow intact cells of this organism to assimilate nitrate, we conclude that the *nitB* mutant must possess some NADH-reductase activity although we have failed to find any despite several attempts with concentrated cell-free extracts. Similarly the failure of the *nitB* mutant to grow, albeit slowly, with nitrate as a nitrogen source is puzzling. This mutant clearly requires further investigation. At present we suggest that *nitB* is a regulatory gene and that a mutation in this gene has depressed nitrate reductase formation in strain 14/15.

Strain 137c is the strain which has been much used by Levine and his colleagues (Levine & Goodenough, 1970). It differs from wild-type 2192 with respect to growth on nitrate by two mutations (Nichols & Syrett, 1978). Biochemically it lacks all the nitrate-inducible activities associated with nitrate reductase and does not take up nitrate. It does grow with hypoxanthine as nitrogen source and this suggests that it can produce a normal molybdenum-containing sub-unit which is believed to be necessary for NADH-NR, BVH(FMNH₂)-NR and xanthine dehydrogenase (Pateman *et al.*, 1964). Crosses of 137c with either the *nitA* or *nitB* mutants did not show any wild-type recombinants (Nichols & Syrett, 1978). Possibly, then, strain 137c has mutations in both the *nitA* and *nitB* genes.

The work described in this and the preceding paper (Nichols & Syrett, 1978) is, to our knowledge, the first that has attempted to study nitrate reduction in a eukaryotic alga by the methods of biochemical genetics. Our information is, as yet, much more limited than that available from studies with fungi, but one interesting difference is now apparent. Biochemically, the *nitA* mutant of *C. reinhardtii* closely resembles the *nit-3* mutant of *N. crassa* in that both have BVH-nitrate reductase activity but both are unable to assimilate nitrate. The *nit-3* mutant of *N. crassa* does, however, take up and accumulate nitrate (Schloemer & Garrett, 1974). Our initial experiments with the *nitA* mutant of *C. reinhardtii* were based on the expectation that it, too, would accumulate nitrate. It does not, and experiments using radioactive chlorate as an analogue of nitrate (Shehata, 1977) have failed to show any uptake into cells of *C. reinhardtii* that lack a functional nitrate reductase. It may be that *C. reinhardtii* is unlike *N. crassa* in that the nitrate reductase protein is responsible not only for nitrate reduction but also for nitrate uptake. The suggestion that this is so in some organisms has been put forward by Butz & Jackson (1976). One might then take this idea further and suggest that our *nitB* mutant, which lacks any detectable

nitrate reductase activity, may also lack an uptake system for nitrate. Certainly this view would be consistent with the low maximum rate of nitrate uptake by this strain.

Note added in proof. Sosa, Ortega & Barea (*Plant Science Letters*, 1978, **11**, 51–58) have recently isolated nitrate reductase deficient mutants of *Chlamydomonas reinhardtii*. The mutants were not genetically analysed but their biochemical characteristics were determined. One group of mutants was similar to our *nitA* mutant and another may be the same as our *nitB* mutants. They did, however, isolate two further groups of mutants that differ biochemically from those we describe.

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