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Studies of enzyme activities in nitrogen-limited cells of *Prasinocladus marinus*

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ABSTRACT

Prasinocladus marinus displays significant levels of glutamine synthetase (GS), glutamate dehydrogenase (GDH), glutamate synthase (GOGAT) and aspartate: 2-oxoglutarate transaminase (AOT). The effects of nitrogen deficiency on enzyme activities in nitrogen-limited continuous cultures were investigated. Increasing nitrogen deficiency was accompanied by increase in the activity of GS and decrease in chlorophyll and total cell protein, but cell dry weight was not altered significantly. Cells growing on both NH_4 and NO_3 had significant levels of NADPH-GDH activity, but the levels of NADH-GDH were low under all the nitrogen concentrations tested. However, the activity of AOT was generally unaffected.

The present investigation suggests that some of these enzymes may primarily function under conditions of low nitrogen availability and this may facilitate the efficient utilization of available nitrogen under these conditions. This would also clearly be of considerable benefit to the alga, to maximize its growth in changing conditions.

INTRODUCTION

Although there have been many investigations on the effects of nitrogen deficiency on the cellular composition and rates of nitrogen assimilation in unicellular algae (e.g. Strickland *et al.* 1969; Thomas & Dodson 1972; Healey 1973; Eppley & Renger 1974; Harrison *et al.* 1977; Syrett 1981), there have been few studies of the accompanying changes in the activities of assimilatory enzymes. Such studies usually employed freshwater algal cells (Morris & Syrett 1965; Hipkin & Syrett 1977).

The effects of N deprivation and darkness on composition of free amino acids in *Phaeodactylum tricornutum* Bohlin were studied in some detail (Flynn & AL-Amoudi 1988). There are few studies of the accompanying changes in the activities of the assimilatory enzymes. These usually employed a unicellular algae grown in batch culture on excess nitrogen and then subjected to nitrogen starvation, i.e. transferred to a medium lacking a nitrogen source (Morris & Syrett 1965; Oesterheld 1971; Hipkin & Syrett 1977). Investigations of enzyme activities in microalgae grown in continuous culture on low concentrations of nitrogen, i.e. subjected to intermediate levels of nitrogen deficiency, were carried out by Everest *et al.* (1986). The long term

effects of nitrogen starvation and refeeding on some enzymes involved in nitrogen assimilation in *Platymonas* were reported by Edge & Ricketts (1978).

In the marine environment, the growth of algae is often considered to be limited by the availability of nitrogen (Ryther & Dunstan 1971). The metabolic states induced under these conditions might differ from those brought about by nitrogen starvation.

The aim of this investigation is to examine some of the changes in the enzymes involved in primary nitrogen assimilation which result from growth with low levels of nitrogen.

MATERIALS AND METHODS

CONTINUOUS CULTURE

Axenic cultures of *Prasinocladus marinus* (Plymouth collection No. 308 kindly provided by Dr T. R. Ricketts, University of Nottingham, were maintained and grown in modified ASP medium (Provasoli *et al.* 1957) without vitamins, but supplemented with tris (1 gl^{-1}) and minor elements. The nitrogen source was either KNO_3 or NH_4Cl, added to give concentrations of 0, 10, 20, 40 and 80 μg nitrogen ml^{-1} medium. The pH was adjusted to 8·0 and the media sterilized by autoclaving. Cultures were maintained in volumes of 4 l at 14°C in a continuous light regime (AL-Amoudi 1988). Light intensity averaged 23 $W m^{-2}$, and was provided by a fluorescent strip light in the continuous culture apparatus (LKB 2023 minicoldlab). The cultures were gassed continuously with sterile 95% O_2 containing 5% CO_2 at a flow rate of 3·5 ml min^{-1}. The alga was grown for up to 3 weeks. Typically, it grew from an initial cell density of 0·4 $\times$ 10^6 cells ml^{-1} to mid-exponential growth phase (1·0 — 1·5 $\times$ 10^6 cells ml^{-1}).

PREPARATION OF CELL-FREE EXTRACTS

Cells were harvested aseptically by centrifugation at 1200 *g* (RC5C Sorvall Instruments) for 12 min and resuspended in 0·2 M imidazol buffer, pH 7·2, containing 1 M dithiothreitol. Cells were treated in an MSE Soniprep 150 for 4 min periods at 2·5 μ amplitude with constant cooling in ice/salt bath to disrupt the cells completely. Sonicated cell suspensions were clarified by centrifugation at 28000 *g* for 30 min at 4°C.

CELL COUNTS

Triplicate 2 ml aliquots of cultures were diluted with 1 ml aliquots of 10% v/v formalin. Cell concentration was determined using a haemocytometer.

ANALYTICAL METHODS

Protein and chlorophyll determination

Protein was measured in cell-free extracts by a modified Lowry method (Hartree 1972). Chlorophyll was extracted in methanol (Hipkin & Syrett 1977), measured spectrophotometrically and determined as described by Jensen (1978).

Nitrogen determination

Triplicate 10 ml aliquots of cultures were centrifuged at 1000 *g* for 15 min; the supernatants were decanted and retained for cell counts, since loss of cells takes place during decantation. The deposits were dried over a boiling-water bath under a stream of filtered air and digested with 1 ml of concentrated H_2SO_4, using a $CuSO_4 \cdot K_2SO_4 \cdot SeO_2$ catalyst. The ammonia released from the digest was collected in a Markham apparatus and distilled into 2% boric acid solution before titration with 0·001 N HCl.

Dry weight determination

Fifty-ml volumes of cell suspension were filtered through dried, pre-weighed 4·0 cm diameter Sartorius glass-fiber filter-paper discs. The cells were washed with 10 ml distilled water and dried on the filter papers at 80°C until there was no further change in weight.

Determination of enzyme activities

Glutamate dehydrogenase and glutamine synthetase activities were determined as described by Everest & Syrett (1983). Glutamate synthase activity was measured by the method of Matoh *et al.* (1980). L-Aspartate: 2-oxoglutarate transaminase was assayed according to the method of Balkow & Wildner (1982). The reaction mixture contained (per ml): 25 μmol aspartate, 25 μmol 2-oxoglutarate, 0·22 μmol NADH,

Table 1. Nitrogen content, total cell protein, cell number and enzyme activities measured in cell-free extracts from material grown in continuous culture with nitrate-nitrogen. Results are means of two sets of data. The range of duplicates was within (*) ± 0·2–1·0, (**) ± 0–1·0.

Nitrate (μg N/ml)	Nitrogen content (* pg/cell)	Protein content (* pg/cell)	Cell number (per ml culture) ($\times 10^6$)	** Enzyme activities (nmol product/min/mg protein) GS	GDH NADH	GDH NADPH	AOT
10	5·5	35	0·88	6·7	0·05	3·2	7·5
20	7·2	38	0·95	9·8	0·03	3·3	7·0
40	9·2	40	1·23	2·6	0·07	2·2	9·7
80	11·3	44	1·35	2·0	0·04	1·8	8·2

Table 2. Nitrogen content, total cell protein, cell number and enzyme activities measured in cell-free extracts from material grown in continuous culture with ammonium-nitrogen. Results are means of two sets of data. The range of duplicates was within (*) ± 0·3–2·0, (**) ± 0·001–0·15.

Ammonium (μg N/ml)	Nitrogen content (* pg/cell)	Protein content (* pg/cell)	Cell number (per ml culture) ($\times 10^6$)	** Enzyme activities (nmol product/min/mg protein) GS	GDH NADH	GDH NADPH	AOT
10	6·7	39	0·4	5·9	0·02	5·1	8·7
20	7·0	43	0·9	3·8	0·03	4·9	7·3
40	11·2	46	1·1	2·9	0·04	3·5	7·7
80	15·2	52	1·3	0·3	0·09	2·2	8·6

and 20 nmol malate dehydrogenase. The reaction mixture was initiated by the addition of enzyme, and preincubated for 15 min at 30°C. The change in optical density at 334 nm was recorded.

RESULTS

The effects of limitation of ammonia and nitrate nitrogen on cell density, cell nitrogen, total cell protein and enzymic activities are shown in Tables 1 and 2. Cell density, cell nitrogen and total cell protein decreased as the nitrogen input was decreased from 80 to 10 $\mu g\,ml^{-1}$. Also, the ability of the cells to acquire nitrogen from the medium is markedly improved by increasing the concentration of either nitrogen source. Ammonium-grown cells exhibited an increase in cell protein and nitrogen content when the NH_4^+ concentration was raised from 10 to 80 μg nitrogen ml^{-1}.

The activity of the enzymes glutamine synthetase, NADH and NADPH-glutamate dehydrogenase showed a general increase as the nitrogen input was decreased. Nitrate-grown cells showed a 69 to 79% decrease in the levels of GS activity when the nitrate concentration of the medium was increased from 10 and 20 to 80 $\mu g\,N\,ml^{-1}$. A much pronounced decrease in the levels of GS activity occurred when the alga was grown on increasing concentrations of NH_4^+. On the other hand, growth on NH_4^+ resulted in high levels of NADPH-GDH activity. An induction of this activity was noticeable in cells grown on low concentration (10 $\mu g\,N\,ml^{-1}$ of NH_4^+). The levels of NADPH-GDH activity decreased on increasing either NH_4^+ or NO_3^- concentrations. Cells grown on 10 to 80 $\mu g\,N\,ml^{-1}$ NH_4^+ and those grown on nitrate showed little change in their levels of NADH-GDH. The activity of the enzyme aspartate: 2-oxoglutarate transaminase did not alter significantly when the concentration of nitrogen in the medium was changed. A reduction of the nitrogen input from 80 to 10 μg ml resulted in a decrease of 70 to 81% in cell chlorophyll (Fig. 1a) but cell dry weight did not alter significantly (Fig. 1b).

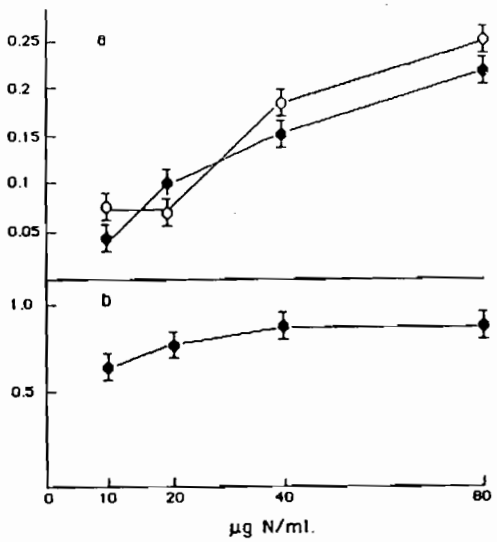

Fig. 1. Ammonium-grown cells (open circles) or nitrate grown cells (shaded circles) were sampled during nitrogen limitation. Where possible, two or more sets of measurements were made with cells grown on each concentration of nitrogen. (a) The optical density of 10^6 cells/ml as a measure of chlorophyll content at 650 nm. (b) mg dry weight of 10^6 cells/ml.

DISCUSSION

Nitrogen deficiency caused decrease in the number of cells per ml medium, and in the protein and chlorophyll content of cultures of *P. marinus*. Similar effects have been reported in many other algal species (Yentsch & Vaccaro 1958; Healey 1973; Belyanin *et al.* 1975; Thomas *et al.* 1976; Harrison *et al.* 1977; Everest *et al.* 1986).

In common with the majority of other algal species investigated (e.g. Kessler & Oesterheld 1970; Rigano & Violante 1973; Syrett & Hipkin 1973), the induction of nitrate reductase does not appear to require nitrate, since enzyme activity could be measured in cells grown on low concentrations of ammonium-nitrogen. However, enzyme activity measured in nitrate-limited cultures was much higher than in ammonium-limited cultures.

Examination of the results in Table 1 and those in Table 2 indicates that nitrogen-deficient algae often exhibit elevated levels of glutamate dehydrogenase and glutamine synthetase activity as in other algae (Eppley & Rogers 1970; Hipkin & Syrett 1977; Shatilov *et al.* 1978; Dortch *et al.* 1979; Cullimore & Sims 1981; Rigano *et al.* 1981). In most species, glutamate dehydrogenase activity is higher in ammonium-grown cells (Hipkin & Syrett 1977; Paul & Cooksey 1981) and is often absent in nitrate-grown cells (Kretovich *et al.* 1970; Shatilov *et al.* 1970).

Conversely, glutamine synthetase activity is often higher in nitrate-grown cells than in ammonium-grown cells (Rigano *et al.* 1981; Slawyk & Rodier 1988). However, this effect was not observed in *P. marinus*. It appears that in this species, both glutamate dehydrogenase and glutamine synthetase are partially repressed during growth on an excess of nitrate or ammonium, and derepressed during growth on low concentrations of nitrogen, when intracellular levels of repressors are low. Recent work has shown that increasing nitrogen deficiency was accompanied by increases in both NADPH-glutamate dehydrogenase and glutamine synthetase activities (Everest *et al.* 1986). Furthermore, the levels of NADPH-GDH activities in *Chlorella autotrophica* were dependent upon the source and concentration of nitrogen and salinity of the medium. This was not the case for NADH-GDH which showed little change in response to either nitrogen source or external salinity (Ahmad & Hellebust 1984). Evidence that ammonium-grown plants have lower GS activity than those grown on nitrate has already been obtained for *Lemna minor*, where the ammonium-grown plants were characterized by a higher endogenous glutamine (feedback inhibitor of GS) pool than nitrate-grown plants (Rhodes *et al.* 1975).

Although the results suggest that GS and GDH are regulated in *P. marinus*, yet they do not indicate how these activities are regulated. The changes in chlorophyll demonstrate a close relation between levels of GS and GDH activities on one hand and nitrogen content on the other hand. This presents itself as an interesting inter-relationship between the energy-yielding process of photosynthesis and nitrogen metabolism.

In the case of GOGAT activity measurements no study was made of the response of this enzyme to nitrogen deficiency, but activities in the range of 5–7 n mol glutamate mg^{-1} protein min^{-1} were observed in cells of *P. marinus* grown on ASP medium (nitrate nitrogen source in the exponential phase). With normal nutrition, this alga has an average cellular nitrogen content of about 7 pg and a generation time of about 24 h (cells double their numbers). Thus the overall rate of nitrogen uptake and assimilation in normal nutrition is about 0·30 pg per average cell per hour. GS and

GOGAT would be able to sustain nitrogen assimilation rates of this order.

As with several other microalgae such as *Platymonas striata* (Edge & Ricketts 1978) and *Chlamydomonas reinhardii* (Cullimore & Sims 1981), only the activity of GOGAT was found to be sufficient to carry out the required rate of nitrogen assimilation. Furthermore, GOGAT activity has been shown to be dependent on ferredoxin in *Chlorella* (Lea & Miflin 1975). This has led Ahmad & Hellebust (1984) to suggest that the GS-GOGAT cycle in *Chlorella autotrophica* grown on 12 : 12 LD cycle may operate only for half of the growth period. It would appear, therefore, that under these conditions the GS-GOGAT pathway is important for nitrogen assimilation and that a mechanism operates to ensure a strictly balanced synthesis in this alga.

Little is known about the regulation of transaminases in algae although the enzymes are believed to play a key role in carbon as well as nitrogen assimilation of plants (Balkow & Wildner 1982). Transaminase reactions are freely reversible, and the direction of the reaction is solely dependent on the actual level of the amino donor and amino acceptor substrates. However, whether grown on nitrate or ammonia, the activity of the aspartate 2-oxoglutarate aminotransferase, is generally unaffected. Moreover, the presence of the enzyme probably depends on *de novo* protein synthesis. Earlier work has shown that alanine: 2-oxoglutarate aminotransferase produced by refeeding cells of *Platymonas striata* on ammonium ion was prevented by the presence of the inhibitor of protein synthesis, cycloheximide (AL-Amoudi 1983).

It is suggested that any of the substrates studied can enter the cells at rates adequate to satisfy any requirement of the assimilatory systems under the conditions employed, and that it does not matter which nitrogen source is used since all sources can ultimately give rise to amino group feeding into general nitrogen metabolism (Ricketts 1988).

It is well known that the rate of uptake of nitrogen compounds by algae increases after a period of nitrogen deprivation (Syrett 1981). The increase in the activity of the nitrogen-assimilating enzymes in nitrogen-deficient algae may be an adaptation which facilitates the efficient utilization of available nitrogen under the conditions employed (Everest *et al.* 1986). Under conditions of increasing nitrogen deficiency, the activities of the nitrogen-assimilating enzymes increase at the time when total cell protein decreases. At least some of this enzyme protein must originate from existing cellular nitrogen which becomes redistributed. As a result, the levels of essential enzymes are maintained or increased at the expense of other protein fractions and pigments which become partially redundant when growth rate is reduced (Everest *et al.*, 1986).

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دراسات على النشاطات الانزيمية في محدودية المصدر النتروجيني لخلايا پراسينوكلادس مارينس

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خلاصـــة

أظهرت الدراسة على طحلب پراسينوكلادس مارينس نشاطات أنزيمية ملحوظة بـالنسبة لـ قلوتامين سنثيتيز (جي اس) ، قلوتاميت دهيدروجينيز (جي دي اتش) ، قلوتاميت سنثيز (جي او جي اى تي) وأسبرتايت ـ٢ ـ أكسوقلوتاريت ترانسا مينيز (اى او تي) .

فقد تم دراسة تأثير نقص المصدر النتروجيني في المزارع الطحلبية على النشاطات الانزيمية . ووجد أنه كلما نقص المصدر النتروجيني صحبته زيادة في نشـاط (جي اس) ونقصان كميـات الكلوروفيل والبروتين ، رغم أن الوزن الجاف للطحلب لم يتغير بدرجة محسوسة . كما أوضحت الدراسة أن الخلايا النامية في وجود كل من الأمونيا والنترات أظهرت نشاطات محسوسة لـ (ان اى دي بي اتش و جي دي اتش) ولكن من ناحية اخرى فان مستوى النشاط لكل من (ان اى دي اتش ـ جي دي اتش) كان منخفضا في هذا الطحلب . اما بالنسبة لنشاط (اى او تي) فانه لم يتأثر .

وتشير الدراسة إلى أن بعضا من هذه الانزيمات يعمل بصفة أساسية تحت تركيزات منخفضة من النتروجين ، مما يمكن الطحلب من الاستفادة بكفاءة من كميات النتروجين الضئيلة المتاحة . وهذه القدرة تعتبر ذات فائدة للطحلب للوصول لأعلى معدل نمو تحت الظروف المتغايرة .