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Ammonium assimilation in the red microalga *Porphyridium purpureum*

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ABSTRACT

Cells of *Porphyridium purpureum* contain levels of glutamate synthase (GOGAT) and NADP-glutamate dehydrogenase (GDH) activity which are equivalent, but lower than that of glutamine synthetase (GS), indicating that the GDH pathway may share with the GS-GOGAT path a role in ammonium assimilation and regulation of nitrogen metabolism in this alga. Cells grown with different concentrations of nitrate and ammonium contained sufficient GS activity to carry out the required rate of nitrogen assimilation under autotrophic as well as heterotrophic conditions. Methionine sulphoximine (MSO) inhibited GS activity to a greater degree *in vitro* than *in vivo*, but MSO inhibition of ammonium assimilation was much enhanced in nitrogen-starved cells. A role for GDH in nitrogen assimilation by *P. purpureum* is supported by the observation that the alga grows normally in the presence of MSO, when GS activity is almost completely inhibited. The activity of GS is high in nitrate-grown cells.

INTRODUCTION

Until about 1974, it seemed well established that the principal route of entry of ammonium nitrogen into amino acids was reductive amination of α-ketoglutarate catalyzed by the enzyme glutamate dehydrogenase (GDH: E.C.1.4.1.3). The discovery of glutamine oxoglutarate aminotransferase, usually referred to by its trivial name glutamate synthase (GOGAT: E.C.1.4.7.1), led Lea & Miflin (1974) to suggest an alternative route for nitrogen assimilation in higher plants via glutamine synthetase (GS: E.C.6.3.1.2) coupled with glutamate synthase (GOGAT). Since then, evidence for the GS/GOGAT pathway for ammonium assimilation has been obtained from most members of the plant kingdom (Lea & Miflin 1974). In higher plants and some microalgae such as *Platymonas striata* (Edge & Ricketts 1978) and *Chlamydomonas reinhardii* (Cullimore & Sims 1981a), the GS-GOGAT cycle appears to be the primary pathway of ammonium assimilation, and GDH plays only a minor role (Ahmad & Hellebust 1984; Miflin & Lea 1980). However, many microalgae show high levels of GS and GDH activity (Rigano *et al.* 1979; Hipkin *et al.* 1982; Ahmad & Hellebust 1984), indicating a potential role for these enzymes in ammonium assimilation. Studies with the GS-inhibitor, methionine sulphoximine (MSO), have established conclusively that ammonium assimilation in *Chlamydomonas reinhardii* occurs exclusively via the GS/GOGAT pathway (Cullimore & Sims

1981b). But ammonium assimilation by the soil alga, *Stichococcus bacillaris*, after growth on nitrate, is only incompletely inhibited by MSO and the organism contains much GDH activity which is probably responsible for ammonium assimilation (Everest & Syrett 1983; Ahmad & Hellebust 1986). Moreover, with some green algae, e.g. *Chlorella autotrophica* (Ahmad & Hellebust 1984), HSO has little effect on growth and nitrogen assimilation of the alga in the presence of ammonium.

This paper describes a study of nitrogen assimilation of the red microalga *P. purpureum*, which can grow with either nitrate or ammonium as a nitrogen source. The activities of GS and NADP-GDH enzymes were measured after photoautotrophic and heterotrophic growth, as well as after the addition of MSO to the batch cultures.

MATERIAL AND METHODS

Organism and growth conditions

Axenic cultures of *P. purpureum*, (CCAP.U.K.1380/1a; Rhodophyceae) were maintained and grown in modified ASP-medium (Provasoli *et al.* 1957) buffered with Tris (1g/l) at pH 8. Cultures were maintained in volumes of 3 l at 25°C on a 12 : 12 h light/dark cycle at 70 μmol m^{-2} s^{-1} in an LKB 2023 minicoldlab. The nitrogen source was 2 or 20 mM of either potassium nitrate or ammonium chloride. Dark-grown cells were obtained by transferring phototrophically-growing cells to the heterotrophic medium (normal medium plus 10 mM glucose) for two days in the dark. In MSO studies, freshly prepared, filter-sterilized solutions of MSO were added to autoclaved growth media to a final concentration of 400 μM. Cultures were supplied with sterile air and continuously stirred by a magnetic stirrer as described by Al-Amoudi (1988).

Preparation of cell extracts

Log phase cells were collected by centrifugation at 3000 g for 15 min. (RC5C Sorval with a GS 3 rotor). Cells growing autotrophically were harvested 3 to 4 h after the start of the light period. The cell pellet was suspended in 4 ml of imidazole buffer (50 mM, pH 7·1) containing 10 mM mercaptoethanol. The cell suspensions were sonicated 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. The resulting preparation was used for determination of enzyme activities.

Enzyme assays

Glutamine synthetase (GS) was measured as described by Slawyk & Rodier (1988). Samples and blanks were centrifuged (10 min at 4000 g) and the absorbance of the supernatent was measured at 540 nm using an LKB Ultrospec 4050 spectrophotometer with 1 cm cell. Enzyme activities are expressed in terms of nmol-glutamyl hydroxamate/mg protein/min.

NADP-glutamate dehydrogenase (GDH): (i) Oxidative deamination was measured by following the reduction of NAD(P), and (ii) Reductive amination was measured by following the oxidation of NAD(P)H. Both activities were assayed by the method of Everest & Syrett (1983). Glutamate synthase (GOGAT) activity was mea-

sured by following the rate of NADPH oxidation at 340 nm according to the method of Matoh *et al.* (1980).

Cell counts

Triplicate 1 ml aliquots of cultures were taken and cell concentration determined using a haemocytometer.

Protein determination

Protein was measured in cell-free extracts by a modified Lowry method (Hartree 1972).

Nitrogen determination

Cell nitrogen was determined in sulphuric acid digests according to the procedure described by Ricketts (1985). Ammonium in the medium was determined as described in Parsons *et al.* (1984).

RESULTS

EFFECTS OF NITROGEN SOURCE AND CULTURE CONDITIONS ON GROWTH AND ENZYME ACTIVITY

Growth, cell protein content, nitrogen content per average cell, and enzyme activity in *P. purpureum*, growing photoautotrophically or heterotrophically, are shown in Tables 1 and 2. Cells grow exponentially with 2 and 20 mM of ammonium or nitrate from an initial cell density of $0{\cdot}5 \times 10^6$ to a cell density of 2×10^6 to 4×10^6 cells/ml. The alga showed little change in growth rate when the concentration of ammonium or nitrate was increased from 2 to 20 mM. There was also little change in cell nitrogen in response to increased nitrogen concentration. However, cells growing heterotrophically (Table 2) had a somewhat higher protein content than those growing under photoautotrophic conditions. The growth rates under heterotrophic conditions were lower than those under photoautotrophic conditions (presumably due to the failure of the divided cells to escape from the parental theca in darkness and which therefore remained together and were enumerated as single cells). The maximum activities of GS found in the crude extracts of photoautotrophic and heterotrophic cells were 36·6 and 6·10 nmol/mg protein/min, respectively, i.e.

Table 1. Growth characteristics and enzyme activity of photoautotrophic cells of *Porphyridium purpureum*

Nitrogen source	Growth constant (day)	Protein content (pg/cell)	Nitrogen content (pg/cell)	N assimilation rate (pg/cell/day)	Enzyme activity (nmol/mg protein/min)	
					GS	NADP-GDH
02 mM nitrate	0·29	22·3	3·6	1·00	36·6	8·7
20 mM nitrate	0·34	26·0	4·2	1·40	28·2	7·4
02 mM ammonium	0·27	20·0	3·2	0·86	27·4	5·4
20 mM ammonium	0·33	22·3	3·6	1·20	19·5	4·9

Table 2. Growth characteristics and enzyme activity of heterotrophic cells *of P. purpureum.* Growth constants, protein content and enzyme activity are given as means of two to three experiments. The protein content is that of the cell-free extract (see Methods). Nitrogen assimilation rates are estimated from growth constants and nitrogen content

Nitrogen source	Growth constant (day)	Protein content (pg/cell)	Nitrogen content (pg/cell)	N assimilation rate (pg/cell/day)	Enzyme activity (nmol/mg protein/min)	
					GS	NADP-GDH
02 mM nitrate	0·19	23·6	3·8	0·72	5·3	12·3
20 mM nitrate	0·14	28·0	4·5	0·63	4·5	9·8
02 mM ammonium	0·13	22·3	3·6	0·47	6·1	10·2
20 mM ammonium	0·16	27·3	4·4	0·70	4·6	4·5

the activity was about six times higher in photoautrophic than in heterotrophic cells. This decrease in GS activity under heterotrophic conditions was accompanied by little change in cell protein content. The alga showed decreased GS activity when the ammonium concentration was increased from 2 to 20 mM and this decrease was more marked under photoautotrophic than under heterotrophic conditions. The alga exhibited substantial NADP-GDH activity but no NADPH-GDH activity. The level of NADP-GDH was dependent upon the nitrogen source and the growth conditions. The activity of GOGAT in cells growing photoautotrophically with 20 mM nitrate or ammonium was 6·2 and 3·4 nmol/mg protein/min, respectively.

IN VITRO INHIBITION OF GLUTAMINE SYNTHETASE BY MSO

Substantial glutamine synthetase activity was measured in extracts from 2 mM nitrate-grown cultures. Pre-incubation of extracts with 0·4 mM MSO in the presence of ATP and Mg resulted in significant inhibition of the activity (83%); pre-incubation with MSO alone resulted in 90% inhibition. The absence of both ATP and Mg (without MSO addition) resulted in a loss (79%) of the GS activity (Table 3).

The time course of GS inhibition in cultures growing with either nitrate or ammonium following the addition of 0·4 mM MSO to the medium is shown in Table 4. Little change was observed in GS activity when the alga was assayed immediately after the addition of MSO, indicating that the centrifugation procedure used

Table 3. *In vitro* inhibition of glutamine synthetase in *P. purpureum* by 0·4 mM methionine sulphoximine (MSO). Cell-free extracts, prepared from cells grown photoautotrophically with 2 mM nitrate were pre-incubated with the stated additions for 5 min prior to the initiation of the assay. The concentrations of $MgSO_4$, ATP and MSO were 80, 6 and 0·4 mM, respectively

Additions to assay	Glutamine synthetase activity (nmol/mg protein/min)	(%) Inhibition
Complete	27·2	
− (ATP + Mg)	5·7	79
+ MSO − (ATP + Mg)	2·6	90
+ MSO + (ATP + Mg)	4·7	83

Table 4. *In vitro* inhibition of glutamine synthetase (GS) by MSO. Cultures grown photoautotrophically with 2 mM nitrate or ammonium were concentrated by centrifugation and cells resuspended in the same medium. The cultures were then split into four portions, two of which were treated with 0·4 mM MSO. At each sampling time, a 100 ml aliquot was used for enzyme assay. At time zero, cells were washed at 4°C immediately after the addition of MSO

Time (h)	Glutamine synthetase activity (nmol/mg protein/min)					
	Nitrate-grown cells (%)			Ammonium-grown cells (%)		
	Without MSO	With MSO	Inhibition	Without MSO	With MSO	Inhibition
Pre-MSO	32·8	33·5	0	27·3	26	0
0	33·6	28·7	15	28·1	25·3	10
1	35·2	24·6	30	26·5	20·4	23
2	36·3	8·5	77	27·2	10·8	60
3	34·5	6·7	80	28·0	8·2	71
24	35·0	2·6	93	27·4	2·3	92

was adequate for removing MSO from the extracellular space. Subsequent inhibition of GS, therefore, must be due to MSO taken up by the cells. Inhibition of GS in MSO-treated cultures growing with nitrate or ammonium was rapid, about 77% and 60%, respectively within 2 h, and MSO-treated cells growing with either nitrate or ammonium contained only little GS activity after 24 h incubation with MSO. Cells grown in the presence of MSO for several days maintained a low residual GS activity (result not shown), which could be sufficient to provide glutamine for protein and nucleic acid synthesis, and for other essential functions.

THE EFFECT OF MSO ON INORGANIC NITROGEN ASSIMILATION

Table 5 shows the effect of addition of 0·4 mM MSO to the culture on the rate of assimilation of ammonium by the cells. Two separate effects were observed. Firstly, the extent of the inhibition of ammonium assimilation by MSO was much greater in the culture that was pre-incubated with MSO and nitrogen-starved for 24 h, compared with those pre-incubated with MSO and nitrogen-starved for 2 h. Secondly,

Table 5. Effect of MSO on ammonium disappearance from *P. purpureum* cultures. Nitrate-grown cultures were resuspended in N-free medium and received 0·2 mM ammonium chloride at zero time. Samples were taken at 15 min intervals for one hour and ammonium was estimated in the culture medium

Culture conditions	Rate of ammonium disappearance nmol/NH_4^+/10^7 cells/h	(%) Ammonium assimilation
(a) Culture pre-incubated for 2 h in N-free medium prior to NH_4^+ addition		
−MSO	42·2	100
+MSO	27·3	65
(b) Culture pre-incubated for 24 h in N-free medium prior to NH_4^+ addition		
−MSO	182·2	100
+MSO	21·5	12

pre-incubation without MSO (nitrogen starvation) resulted in a greatly increased capacity to assimilate ammonium. Under all conditions there was some ammonium assimilation. It should be noted that the rate of ammonium disappearance of 42·2 nmoles/10^7 cells/h (Table 5) is equivalent to 70 pmoles N/10^6 cells/min, which is not too different from the value of 50 pmoles N/10^6 cells/min calculated (see Table 1 and Discussion).

DISCUSSION

Porphyridium purpureum contains high levels of GS activity when grown either photoautotrophically or heterotrophically. Similarly, high levels of GS were found in *Chlorella stigmatophora* (Everest *et al.* 1986) and in *Porphyridium cruentum* and in a range of algal species (Casselton *et al.* 1986). Furthermore, GS in *P. purpureum* appears to be strongly regulated by the form and concentration of nitrogen in the medium. Under both photoautotrophic and heterotrophic conditions, maximum activities were found in cells grown with low levels of either ammonium or nitrate. Increasing the supply of nitrate to 20 mM had little effect on the level of GS. However, a similar increase in ammonium concentration decreased the level of this enzyme considerably. Similar results have also been observed in *Prasinocladus marinus* (Al-Amoudi 1991). These results may indicate that the activity of GS in *Porphyridium purpureum* is regulated by the availability of ammonium in an intracellular pool rather than the concentration of nitrogen in the medium (Hipkin *et al.* 1990). In media with 2 mM or 20 mM nitrate or ammonium, the ratio GS : NADP-GDH was about 4 : 1 and 5 : 1 for nitrate and ammonium respectively under photoautotrophic conditions; it decreased to 1 : 2·3 and 1 : 1 for nitrate and ammonium respectively when the culture was under heterotrophic conditions. Thus, the role played by GS pathway in this alga appears to be more associated with conditions of restricted nitrogen supply and illumination. Changes in the activity of GS during light/dark transitions of *Chlamydomonas* cultures grown phototrophically and mixotrophically have been reported by Cullimore & Sims (1981a). They showed that the increase in light assimilation following the dark period was correlated with the increased activity of GS. It should be noted that the rate of ammonium assimilation required by the alga at mid-exponential growth phase (Table 1, with 2 mM nitrate in photoautotrophic cells) can be calculated from the cell nitrogen content and growth constant (Ahmad & Hellebust 1986, 1988), which is equivalent to 1·0 pg N/cell/day (= 49·6 pmoles N/10^6 cells/min). Cell-free extracts of this organism grown under the same conditions (Table 1) contain about 0·0223 mg protein from 10^6 cells. An enzyme in that extract responsible for cellular ammonium assimilation should therefore have an activity of at least 50 pmoles ammonium per 0·0223 mg protein or 2·2 nmoles ammonia per mg protein. This value can be compared with the activities of GS and GDH given in Tables 1 and 2. But, in making this comparison, it must be remembered that GS activity was measured by the transferase assay method (it was the formation of glutamylhydroxymate not that of glutamine that was determined). Everest & Syrett (1983) showed that transferase activity is generally 10–15 times higher than the corresponding synthetase activity, so the values for GS activity in Tables 1 and 2 must be divided by at least 10 before comparing them with the calculated value of 2·2 nmoles ammonia/mg protein. Such comparison then suggests that, in photoauto-

trophic cells (Table 1), the measured activity of GS and GDH is adequate to account for the observed rate of N-assimilation. In heterotrophic cells (Table 2), rates of N-assimilation are lower at about 30 pmoles $N/10^6$ cells/min and the required enzyme activity in cell-free extracts must be at least 1·3 nmoles ammonia/mg protein. Making allowance for lower GS synthetase activity as compared to transferase activity, the GS values in Table 2 appear to be inadequate whilst the GDH values are sufficient. In the MSO-treated cells grown on nitrate, the GS activity was only 10% and 17% of the corresponding GS activity (see Table 3). Therefore the residual GS activity in the MSO-treated cells grown on nitrate is less but still allowed nitrogen assimilation. Consequently, it is most probable that in the MSO-treated cells grown on nitrate, GDH also participates in ammonium assimilation. However, the GDH activity can make a significant contribution to ammonium assimilation in the presence of an active GS such as in the green alga *Stichococcus bacillaris*, where the GS-GOGAT cycle and GDH pathway participate together in ammonium assimilation under both autotrophic and heterotrophic conditions (Everest *et al.* 1986). High levels of GOGAT activity were present in both nitrate- and ammonium-grown alga, indicating an important role for the GS/GOGAT cycle in both cells. The activity of GOGAT was about 12% of that of GS and less than 53% of that of GDH in cells grown photoautotrophically on 20 mM nitrate; in ammonium-grown cells, GOGAT activity was about 32% of that of GS and more than 21% of that GDH. In *P. purpureum*, GDH levels were regulated by the form and concentration of nitrogen in the medium under both photoautotrophic and heterotrophic conditions. In ammonium-grown cells, assimilation must be almost exclusively via GS/GOGAT, because GDH level is low. The evidence presented in this study is that GDH can function in ammonium assimilation under certain circumstances, but its role appears to be limited, and is considerably less important than that of GS. The study with MSO clearly demonstrates the ability of *P. purpureum* to assimilate ammonium via the GS pathway. *In vivo* the activity of GS was inhibited partly, but not completely, by MSO. Since MSO treatment at 0·4 mM ammonium only resulted in a 58% and 89% fall in GS after 3 and 24 h respectively after the addition of MSO, it seems likely that the cells of *P. purpureum* studied here are either unable to transport the inhibitor, or are not completely tolerant to MSO. However, extracts incubated with MSO *in vitro* in the absence of ATP and Mg caused 90% loss of activity. Under both *vivo* and *vitro* conditions, the protein content of cells treated with MSO is remarkably similar to that observed for MSO-free cells (not shown). In some cases the low amount of protein observed may be attributable to lack of glutamine following the deactivation of GS.

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تمثيل الأمونيا في الطحلب الأحمر پورفيريديم پرپوريم

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خلاصة

تشير نتائج هذا البحث إلى أن خلايا الطحلب پورفيريديم پرپوريم تحتوي على تراكيز متكافئة من القلوتاميت سنثيز (GOGAT) وقلوتاميت دهيدروجينيز (GDH). لكن هذه التراكيز تقل عن تركيز قلوتامين سنثيتيز (GS). وهذا يوضح بجلاء مشاركة (GDH) مع (GS-GOGAT) بنفس المسار في تمثيل الأمونيا وتنظيم الأيض النتروجيني في الطحلب. كذلك أظهرت الدراسة أن الخلايا النامية في تراكيز متباينة من الأمونيا والنترات تحتوي على قدر كاف من نشاط (GS) يؤهلها للوصول إلى معدل التمثيل النتروجيني المطلوب تحت ظروف التغذية الذاتية والتغذية غير الذاتية. وأوضحت النتائج أن مركب ميثايونين سلفوكسيمين (MSO) قد ثبط نشاط (GS) بدرجة أكبر في المستخلص الخلوي في وعاء الإختبار، منه في داخل الخلية الحية، كما أن تأثيره المثبط لتمثيل الأمونيا قد ازداد في حالة الخلايا النامية في وسط خال من النتروجين. وتشير الدراسة إلى أن دور (GDH) في عملية التمثيل النتروجيني للطحلب تعززه كفاءة النمو في وجود (MSO) عندما يتوقف نشاط (GS) بصورة شبه كاملة. وكان نشاط (GS) هذا عاليا في الخلايا النامية في وسط يحتوي على النترات.