Regulation of Asparaginase, Glutamine Synthetase, and Glutamate Dehydrogenase in Response to Medium Nitrogen Concentrations in a Euryhaline *Chlamydomonas* Species

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**ABSTRACT**

The ammonium assimilatory enzymes glutamine synthetase (EC 6.3.1.2) and glutamate dehydrogenase (EC 1.4.1.3) were investigated for a possible role in the regulation of asparaginase (EC 3.5.1.1) in a *Chlamydomonas* species isolated from a marine environment. Cells grown under nitrogen limitation (0.1 millimolar NH₄⁺, NO₃⁻, or L-asparagine) possessed 6 times the asparaginase activity and approximately one-half the protein of cells grown at high nitrogen levels (1.5 to 2.5 millimolar). Biosynthetic glutamine synthetase activity was 1.5 to 1.8 times greater in nitrogen-limited cells than cells grown at high levels of the three nitrogen sources.

Conversely, glutamate dehydrogenase (both NADH- and NADPH-dependent activities) was greatest in cells grown at high levels of asparagine or ammonium, while nitrate-grown cells possessed little activity at all concentrations employed. For all three nitrogen sources, glutamate dehydrogenase activity was correlated with the residual ammonium concentration of the media after growth (r = 0.88 and 0.94 for NADH- and NADPH-dependent activities, respectively).

These results suggest that glutamate dehydrogenase is regulated in response to ambient ammonium levels via a mechanism distinct from asparaginase or glutamine synthetase. Glutamine synthetase and asparaginase, apparently repressed by high levels of all three nitrogen sources, are perhaps regulated by a common mechanism responding to intracellular nitrogen depletion, as evidenced by low cellular protein content.

The enzyme L-asparaginase (EC 3.5.1.1) has been investigated extensively in bacteria, fungi, and certain mammals because of its importance as an antineoplastic agent (for reviews see 7, 33). L-Asparagine has been described as a major nitrogen transport and storage compound in legumes (3) and L-asparaginase may play a major role in the mobilization of the amide nitrogen for utilization in amino acid and protein synthesis in higher plants (see 19 for review). Recently, a potassium-dependent L-asparaginase has been described in tissues of several higher plants (30) indicating that the distribution of asparaginase among higher plants may be more widespread than previously thought.

Although L-asparaginase regulation has been studied extensively in bacteria (4, 11, 12, 15) and fungi (9), the factors controlling its synthesis in eukaryotic microalgae are not known. Nitrogen deprivation has been shown to cause an increase in L-asparaginase levels in *Saccharomyces cerevisiae* (9, 22), *Aspergillus nidulans* (8), and *Klebsiella aerogenes* (25). Conditions which led to derepression of glutamine synthetase (i.e., nitrogen deprivation) also led to derepression of L-asparaginase in *K. aerogenes* and mutants constitutive for glutamine synthetase were also constitutive for asparaginase (25).

Recently, we described the first algal L-asparaginase in a euryhaline *Chlamydomonas* species (21). This organism could only utilize asparagine, glutamine, ammonia, or nitrate as sole nitrogen source for growth. Studies employing L-[¹⁴C]asparagine suggested that whole cells possessed little capacity to metabolize L-asparaginase, since [¹⁴C]asparagine was deamidated at the cell surface without intracellular accumulation of radioactivity, growth presumably occurring on the liberated amide (20). The L-asparaginase behaved like an intraperiplasmic enzyme, since whole cells possessed equivalent activity as broken cells, the enzyme not being produced extracellularly (21). The enzyme has been subsequently purified to near-homogeneity and characterized (20, and J. H. Paul, in preparation). Since as we had previously shown that the greatest L-asparaginase activity occurred in cells grown under nitrogen limitation (21), we investigated the nitrogen assimilatory enzymes glutamine synthetase and glutamate dehydrogenase for possible co-regulation with L-asparaginase.

**MATERIALS AND METHODS**

**Plant Material.** The organism employed in all experiments has been identified as *Chlamydomonas* species by Dr. John Green, Plymouth Marine Laboratory, Plymouth, United Kingdom, and has been previously described (21).

**Growth of Organism.** All cultures were axenic and aseptic precautions were taken in their handling. Stock cultures were maintained on an artificial seawater medium (ASP II medium; 23) as previously described (21). Cell counts were performed with a haemocytometer.

For the determination of ammonium and aspartate production in the media of cells grown on L-asparaginase, a 20-ml culture containing 3 to 4 × 10⁶ cells ml⁻¹ was washed in ASP II lacking a nitrogen source (ASP II-N) and resuspended in 200 ml ASP II in which the nitrate had been replaced by 10 mM L-asparagine. Five-ml aliquots were aseptically transferred to 40 sterile 25-ml screw cap culture flasks. Flasks were incubated at 27°C at 3,500 lux and harvested after various time intervals. Ammonium and aspartate were determined in the supernatant liquid by the methods described below.

To determine the effect of methionine sulfoximine on growth, quadruplicate 5-ml cultures were grown in the presence of 0, 1, 5,
or 20 mM dl-methionine dl-sulfoximine (Sigma) in ASP II media. The procedure was repeated with 0.1 mM NH₄Cl replacing nitrate as the nitrogen source. Cultures were counted after 72 h.

Cells for asparaginase, glutamine synthetase, and glutamate dehydrogenase assays were grown as follows. A 200-ml culture (3–4 x 10⁶ cells ml⁻¹) was harvested aseptically and cells were washed twice with 100 ml ASP II-N. The pellet was resuspended in 80 ml ASP II-N and 25-ml aliquots were added to three separate 475-ml volumes of ASP II-N. Appropriate volumes of 1 M NH₄Cl, 1.19 M NaNO₃, or 0.25 M l-asparagine were added to each culture to make a final concentration of 0.1, 0.5, 1.5, or 2.5 mM of the three nitrogen sources. Cultures were grown for 60 h as described previously (21). Ten-ml aliquots were taken for asparaginase assays immediately prior to harvesting.

**Preparation of Cell-Free Extracts.** Cell density was determined prior to harvesting. Harvesting and preparation of cell-free extracts were done at 4°C.

**Asparaginase.** Replicate 10-ml aliquots were taken from glutamine synthetase cultures prior to harvesting (see below). Cells were harvested at 3,200 g, washed once with 0.15 M NaCl, 10 mM EDTA, and resuspended in 1 ml 0.05 M sodium borate buffer containing 0.1 mM EDTA, pH 8.3. Since whole cells possessed equal activity as broken cells (21), it was not necessary to prepare cell-free extracts to assay this enzyme.

**Glutamine Synthetase.** The remaining 480-ml culture was harvested by centrifugation at 4,100g for 10 min, the pellet washed once in 0.15 M NaCl containing 10 mM EDTA, and resuspended in 1 ml 0.1 M imidazole-HCl buffer (pH 7.15). Cells were broken by passage twice through a French pressure cell (Amino) at 1.41 x 10⁷ kg m⁻². The cell extract was brought to 2 ml with 0.1 M imidazole-HCl (pH 7.15) and mercaptoethanol added for a final concentration of 25 mM. Cell fragments were removed by centrifugation at 4,600g for 10 min.

**Glutamate Dehydrogenase.** Cultures were harvested as above, and the cell pellet resuspended in 2.5 ml of 50 mM Tris-HCl (pH 8.0), containing 0.5 mM DTT. Cells were broken as above, and extracts centrifuged at 4,600g for 10 min to remove cellular debris. The supernatant was then centrifuged at 100,000 g for 1 h in a Beckman L3-50 preparative ultracentrifuge to clarify extracts. The tight yellow extracts were dialyzed against 2 L 50 mM Tris-HCl (pH 8.0) containing 0.5 mM DTT for at least 4 h.

**Enzyme Assays.**

**Asparaginase.** Asparaginase assays were performed as previously described (21).

**Glutamine Synthetase.** Biosynthetic glutamine synthetase was determined by an adaptation of the method of Rowe et al. (26). Reaction mixtures contained 0.3 ml 100 mM imidazole-HCl (pH 7.15); 0.1 ml 0.5 M Na glutamate (pH 7.0); 0.1 ml 100 mM ATP, disodium salt; 0.020 ml 1 M MgCl₂; 0.1 ml 1 mM hydroxylamine-HCl, freshly prepared, pH 7.0; and 0.18 ml H₂O. The reaction was initiated by the addition of 0.2 ml cell-free extract as prepared above. Controls were incubation mixtures in which the ATP, glutamate, or enzyme was replaced by distilled H₂O. After 15 min at 37°C, 0.5-ml samples were taken and added to 0.75 ml FeCl₃ solution (0.37 M FeCl₃, 0.67 N HCl, 0.2 N TCA). Precipitated protein was removed by centrifugation at 3,200g and filtration through a 0.45 μm membrane filter (Millipore HAWP). Absorbance was measured at 535 nm and compared to γ-glutamyl hydroxamate standards (Sigma). A unit is defined as the amount of enzyme required to form 1 μmol γ-glutamyl hydroxamate min⁻¹ at 37°C. γ-Glutamyltransferase activity was also determined in several cell-free extracts via the method of Bender et al. (5) except that acetyl trimethyl ammonium bromide was omitted from reaction mixtures and the concentration of MnCl₂ in the concentrated reaction mixture increased from 0.27 to 0.7 mM. Reaction mixtures were made to 60 mM Mg²⁺ by the addition of 33 μl of 1 M MgCl₂.

To investigate short-term regulation of glutamine synthetase in response to ammonium concentration a 1-L culture was grown under nitrogen limitation (100 μM NH₄Cl) and divided into two 500 ml lots prior to harvesting. To one lot, 12.5 ml 2 M (NH₄)₂SO₄ was added (98 mM NH₄⁺ final concentration) and incubated for 15 min. Each lot was harvested and cell-free extracts were prepared as above.

**Glutamate Dehydrogenase.** The procedure for the assay of glutamate dehydrogenase activity was adapted from Kates and Jones (13). Reaction mixtures contained 0.6 ml 50 mM Tris-HCl (pH 9.0), 0.1 ml 1 M NH₄Cl, 2 μl 10 mM NADH, 78 μl distilled H₂O, and 0.2 ml of the enzyme extract as prepared above. The reaction was initiated by the addition of 20 μl 0.2 M α-ketoglutarate (pH 7.0), which was replaced by 20 μl water in the control. The rate of the oxidation of NADH was determined by monitoring A at 340 nm. A change in A of 6.22 x 10⁻³ min⁻¹ cm⁻¹ was assumed to correspond to the oxidation of 1 μmol NADH min⁻¹, which is defined as a unit. NADPH-dependent activity was assayed as above except that 0.1 ml 10 mM CaCl₂ and 2 μl 10 mM NADPH replaced the water and NADH in reaction mixtures, respectively, as suggested by Miflin (18).

**Aspartate and Ammonium Determinations—** Aspartate and ammonium in the growth medium was determined by anion exchange chromatography and microdiffusion, respectively, as previously described (21).

Protein was determined via the method of Lowry et al. (16).

**RESULTS**

Production of asparagine and ammonium with time in the medium of cells grown on 10 mM l-asparagine appears in Figure 1. Approximately equimolar quantities of aspartate and ammonium were produced, maximal production occurring after cells had reached stationary phase.

Residual medium ammonium levels after growth at various concentrations of l-asparagine, ammonium, or nitrate appear in Table I. Ammonium was detected in the media after growth at 1.5 and 2.5 mM l-asparagine or ammonium while none was present in the medium of nitrate-grown cells.

Protein content of cells (Fig. 2) grown at 0.1 mM ammonium or asparagine was approximately half that of cells grown at higher nitrogen concentrations (1.5 and 2.5 mM), and no difference was found between these two higher concentrations. Protein content of nitrate grown cells was generally less than asparagine- or ammonium-grown cells except at 2.5 mM, but showed the same general response to increasing nitrogen concentration. Since cel-
Table I. Ammonium Levels in Media after Growth

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Nitrogen Concentration (mm) of Growth</th>
<th>μmol ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Minimal detectable concentration was 0.02 μmol ml⁻¹.

![Fig. 2. Protein content of whole cells grown on nitrate (●), ammonium (□), or L-asparagine (▲) as sole nitrogen source. Values are mean of four replicates +/− sd.](image1)

There is a change in protein content was not constant over the experimental parameters, and a change in specific activity could be the result of a change in activity or a change in cellular protein. To eliminate this problem, all enzyme activities are expressed as activity (cell number)⁻¹.

Enzyme Activities. Asparaginase activity was nearly 6-fold greater in cells grown in 0.1 mm than 1.5 or 2.5 mm concentrations of any of the three nitrogen sources employed (Fig. 3A). Cells grown at 0.5 mm possessed an intermediate level of activity, and cells grown at 1.5 and 2.5 mm had equal activity.

Merscaptoethanol was determined to be a superior reducing agent than DTT in preserving glutamine synthetase activity over the 2 h required to assay all extracts. Reaction mixtures containing 0.1 or 1.0 mm DTT possessed 85 and 95%, respectively, of the activity of mixtures containing 25 mm mercaptoethanol. Addition of 0.1 mg ml⁻¹ cetyl trimethyl ammonium bromide to cells during harvesting as suggested by Bender (5) abolished all glutamine synthetase activity (data not shown).

Biosynthetic glutamine synthetase of cells grown at various concentrations of combined nitrogen appears in Figure 3B. Analysis of variance followed by Student-Neuman–Keuls multiple range testing (34) indicated that glutamine synthetase activity of cells grown at 0.1 mm was greater than that of cells grown at 1.5 and 2.5 mm for all three nitrogen sources (NO₃⁻: P < 0.025, n = 11; NH₄⁺: P < 0.005, n = 12; asparagine: P < 0.025, n = 12). There was no difference between the three nitrogen sources at any one concentration (P > 0.5, n = 12). If the data from the three nitrogen sources were pooled at each concentration, the activity of cells grown at 0.1 mm was significantly greater than that of cells grown at 0.5 mm.

Incubation of cells grown under nitrogen limitation with 98 mm ammonium for 15 min resulted in an approximate 20% decrease in biosynthetic and γ-glutamyltransferase activity (Table II). Biosynthetic glutamine synthetase levels were comparable to γ-glutamyltransferase activities in the presence of Mn²⁺ and Mg²⁺ (Table II). γ-Glutamyltransferase activity as a function of nitrogen concentration of growth appears in Table III. The greatest γ-glutamyltransferase activity occurred in nitrogen-limited cells. The transferase activity expressed as a percent of the biosynthetic activity is greatest in nitrogen limited cultures (74–98%, Table III) and decreases with increasing nitrogen concentration.

![Fig. 3. Asparaginase (A), biosynthetic glutamine synthetase (B), or NADH-dependent glutamate dehydrogenase activity (C) of cells grown on nitrate (●), ammonium (□), or L-asparagine (▲) as a function of nitrogen concentration (values are initial media concentrations).](image2)

![Table II. Short Term Regulation of Glutamine Synthetase in Response to Ammonium](image3)

Values are in units 10⁻⁸ cells, each value is the mean of three determinations. Cells were grown under nitrogen limitation, divided in two lots, and one lot incubated for 15 min with NH₄⁺ prior to harvesting.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biosynthetic GS</th>
<th>0.7 mm Mn²⁺</th>
<th>60 mm Mg²⁺</th>
<th>0.7 mm Mn²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen limited</td>
<td>1.36</td>
<td>1.32</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>98 mm NH₄⁺</td>
<td>1.06</td>
<td>1.06</td>
<td>1.02</td>
<td></td>
</tr>
</tbody>
</table>

![Table III. γ-Glutamyltransferase Activity as a Function of Nitrogen Concentration of Growth](image4)

Values are in units 10⁻⁸ cells. Values in parentheses are % of the biosynthetic activity.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Nitrogen Concentration of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mm</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>1.42</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>1.13</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.40</td>
</tr>
</tbody>
</table>

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FIG. 4. Inhibition of biosynthetic glutamine synthetase activity in cell-free extracts of *Chlamydomonas* species by DL-methionine DL-sulfoximine.

Table IV. NADPH-Dependent Glutamate Dehydrogenase Activity of Cells Grown at Various Concentrations of Ammonium, l-Asparagine, or Nitrate

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Nitrogen Concentration of Growth</th>
<th>0.1 mm</th>
<th>0.5 mm</th>
<th>1.5 mm</th>
<th>2.5 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units 10^-6 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4+</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.51</td>
<td>0.61</td>
</tr>
<tr>
<td>l-Asparagine</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.31</td>
<td>0.50</td>
</tr>
<tr>
<td>NO3^-</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.074</td>
<td>0.088</td>
</tr>
</tbody>
</table>

grown cells at 1.5 and 2.5 mm, values being 11.2 and 10.4 times that observed at 0.1 mm, respectively (Fig. 3C).

Asparagine-grown cells showed the greatest NADH-dependent glutamate dehydrogenase at 2.5 mm (8.6 times the activity at 0.1 mm), with decreasing activities in cells grown at 1.5 and 0.5 mm (Fig. 3C). Nitrate-, ammonium-, or asparagine-grown cells had similar NADH-glutamate dehydrogenase activities at 0.1 and 0.5 mm. However, activity was 38 and 27% of the ammonium-grown cells at 1.5 and 2.5 mm nitrate, respectively (Fig. 3C).

NADPH-dependent glutamate dehydrogenase activity was lowest for nitrate-grown cells (Table IV), being only 15% of the activity in asparagine or ammonium-grown cells at 2.5 mm. Ammonium-grown cells possessed nearly twice the activity of asparagine-grown cells at 1.5 mm, but possessed equal activity as asparagine-grown cells at 2.5 mm.

NADH- and NADPH-dependent glutamate dehydrogenase activities showed significant correlation (P < 0.001; t test of simple linear correlation, 34) to the ammonium levels found in the medium after growth had occurred (data from Table I). The best correlation occurred (r = 0.94) for NADPH-dependent activity versus ammonium concentration, while NADH-dependent activity showed a correlation coefficient of 0.88.

**DISCUSSION**

Cells grown at 0.1 mm NaNO₃, NH₄Cl, or l-asparagine possessed the greatest l-asparaginase and biosynthetic glutamine synthetase activity while possessing only half of the protein content of cells grown at 1.5 and 2.5 mm nitrogen. We have previously shown that growth is limited at 0.1 mm concentrations of these three nitrogen sources (21). Apparently, all three nitrogen sources exert a repressive effect on these two enzymes which is maximal at concentrations greater than 1.5 mm. The magnitude of the change in activity of biosynthetic glutamine synthetase is much less than the change in asparaginase, however.

Previous work (20) has shown that l-asparaginase is regulated in this organism through repression of synthesis only. There apparently exists a mechanism for short term regulation of glutamine synthetase in this organism, since ammonium caused a 20% decrease in activity in 15 min. This change is less than the decreases observed in the biosynthetic (about 50%) and y-glutamyltransferase (about 75%) reactions for cells grown at limiting concentrations of nitrogen.

Glutamine synthetase in *Escherichia coli* and other enteric bacteria has been shown to be regulated in vivo by at least four separate mechanisms (28), including covalent attachment of adenine 5'-monophosphate molecules to subunits. *Anabaena cylindrica* cells showed a 50% loss in glutamine synthetase activity in the presence of 10 mM NH₄⁺ that could be reversed *in vivo* by removing ammonium or *in vitro* by the addition of 40 mM mercaptoethanol (27).

Mechanisms of short term regulation of glutamine synthetase are largely unknown in green algae. Akimova et al. (1, 2) have shown that the rapid inactivation of glutamine synthetase in the presence of ammonium in *Chlorella* was due to feedback inhibition by adenine nucleotides. Through the use of ¹³CO₂ and density gradient centrifugation, Tischner and Hutterman (32) recently found both de novo synthesis and activation of glutamine synthetase when cells of *Chlorella sorokiniana* were transferred to nitrogen-deficient media.

The significance of the Mn²⁺/Mg²⁺ y-glutamyltransferase activity in elucidating the short term regulatory mechanisms of glutamine synthetase in eukaryotic microalgae is not known. The work of Rasulov et al. (24) suggests that the quaternary structure of the *Chlorella* glutamine synthetase may be unique in possessing six subunits, and unique regulatory mechanisms are not ruled out for these enzymes.

Glutamate dehydrogenase activity showed a different response than asparaginase or glutamine synthetase to nitrogen concentration for the three nitrogen sources employed. Increasing nitrate concentrations had little effect on NADH- or NADPH-dependent activities, while increasing concentrations of ammonium or asparagine stimulated both activities. Growth on asparagine (but not nitrate) was accompanied by accumulation of ammonium in the media in this organism, and glutamate dehydrogenase activity was correlated to the residual concentration of ammonium in the media at the time of harvesting the cells. Elevated ammonium levels have been shown to stimulate NADH-dependent glutamate dehydrogenase activity in *Chlamydomonas reinhardtii* (10, 13). It is not known whether the NADH and NADPH activities detected represented isoenzymes or different reduced pyridine nucleotide specificities of the same enzyme. The fact that no NADPH activity was detected at low concentrations of all three nitrogen sources suggests the former possibility. These results corroborate the work of Kretovich (14) and Talley (31) who found that high concentrations of ammonium induced an NADPH-dependent glutamate dehydrogenase electrophoretically distinct from an NADH-dependent enzyme in *Chlorella pyrenoidosa*. Shatilov et al. (29) found a constitutive glutamate dehydrogenase active with both NADH and NADPH in a variety of unicellular green algae. Several of these organisms possessed an ammonium-inducible NADPH-specific glutamate dehydrogenase (29) while others did not.

Presumably, microorganisms which possess glutamine synthetase employ the glutamine synthetase-glutamate synthase pathway for the assimilation of ammonium into glutamate rather than glutamate dehydrogenase under conditions of nitrogen deprivation (6). Although methionine sulfoximine has been shown to inhibit growth of *Chlorella vulgaris* (17) and to inhibit glutamine synthetase *in vitro* in this study, this compound did not inhibit growth in the presence of nitrate or low concentrations of ammonium. Either glutamate dehydrogenase can act as the primary nitrogen assimilatory enzyme or methionine sulfoximine did not get into the cells. The capacity for this organism to transport small molecules may be limited (21). Of a wide variety of monosaccharides and organic acids tested, none could support heterotrophic growth or stimulate phototrophic growth, and of the amino
acids, only asparagine and glutamine could support growth as nitrogen sources. Asparagine was deaminated at the cell surface and the aspartate moiety did not accumulate in the cell (21). Therefore, we feel that the latter possibility may explain the inability of methionine sulfoximine to inhibit growth.

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3. Atkins CA, JS Pate, PJ Sharkey 1975 Asparagine metabolism-key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56: 807-812