Separation, Purification, and Characterization of Two Isoforms of Glutamine Synthetase from Chlamydomonas reinhardii

Francisco J. Florencio
Departamento de Bioquímica, Facultad de Biología y C.S.I.C. Universidad de Sevilla, Sevilla, Spain

and José M. Vega
Departamento de Bioquímica, Facultad de Química, Universidad de Sevilla, Sevilla, Spain

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Two isoforms of glutamine synthetase, GS₁ and GS₂, have been separated from Chlamydomonas reinhardii cells grown autotrophically with nitrate. The intracellular level of GS₂ was higher than that of GS₁. In cells under darkness, the GS₁ peak increased markedly, whereas that of GS₂ became negligible.

The two isoenzymes were purified to electrophoretic homogeneity by a method which included: a) DE-52 cellulose chromatography; b) ammonium sulphate fractionation; and c) affinity chromatography on ADP-sepharose. The specific activity was 114 and 63 U/mg for GS₁ and GS₂ respectively, and both enzymes (M_r = 380 000 and 373 000) are oligomeric proteins composed by 8 subunits of similar size (M_r = 48 000 in GS₁, and 46 000 in GS₂). The basic differences between GS₁ and GS₂ are: a) the effect of light on their intracellular level; b) their Km for ammonium (83 and 244 µM, respectively). Both isoenzymes were inhibited in a similar extent by L-alanine, L-glycine and L-arginine.

Introduction

The assimilation of ammonium by the unicellular green alga Chlamydomonas reinhardii is catalyzed exclusively by the glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle [1, 2]. The synthesis of glutamine is catalyzed by glutamine synthetase (L-glutamate-ammonia-ligase, EC. 6.3.2.1) which is an ubiquitous enzyme found in animals, plants and microorganisms [3]. In Chlamydomonas, glutamine synthetase is also involved in the reassimilation of the intracellular ammonium formed during the photorespiration [4].

The presence of two isoenzymes with glutamine synthetase activity has been reported in rice leaves and other higher plants [5, 6]. However, such two activities have not been observed up to date in photosynthetic microorganisms [3]. In Chlamydomonas, glutamine synthetase is also involved in the reassimilation of the intracellular ammonium formed during the photorespiration [4].

Glutamine synthetase has been purified to electrophoretic homogeneity from several photosynthetic organisms, such as nitrogen-fixing bacteria [7], cyanobacteria [8 – 11], green algae [12], and higher plants [13 – 15]. Generally, affinity chromatography on different supporting matrices has been used as the basic purification step, such as blue-sepharose for the enzyme from Rhodopseudomonas palustris [7], R. sphaeroides and Anabaena sp. 7120 [8, 11], amino-hexane-sepharose 4B for that from Anabaena cylindrica and Nostoc sp. [10], and ADP-sepharose 4B for the purification of rice leaves and roots glutamine synthetase [14].

Two types of glutamine synthetases have been established in photosynthetic organisms: the plant-type enzyme (M_r = 330 000 – 480 000), composed by 8 similar-sized subunits (M_r = 45 000 – 48 000) [13, 14, 16, 17], and the prokaryotic-type enzyme (M_r = 590 000 – 670 000) composed by 12 subunits (M_r = 50 000 – 55 000) [7, 10, 11]. On the other hand, the purified glutamine synthetase from Chlorella pyrenoidosa (M_r = 320 000), consists of 6 similar subunits (M_r = 53 000) [12].

This paper reports the separation of two isoenzymes with glutamine synthetase activity in a photosynthetic microorganism. These isoenzymes, referred as GS₁ and GS₂, have been purified using affinity chromatography on ADP-sepharose 4B, and a comparative study of their physico-chemical, kinetic, and regulatory properties is presented.

Reprint request to Dr. José M. Vega.
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Materials and Methods

Chemicals

ADP, AMP, ATP, dithioerythritol (DTE), morpholinopropane sulfonic acid (MOPS), catalase, alcohol dehydrogenase were purchased from Sigma (St. Louis, USA). N,N,N',N'-Tetramethylethylamine (TEMED), ovalbumin, bovine serum albumin, and apoferritin were from Serva (Heidelberg, FRG). Electrophoresis standard proteins, 2',5'-ADP-sepharose and Sephadex G25 were from Pharmacia (Uppsala, Sweden). Bio-gel A-1.5 from Bio-rad Lab. (Richmond, USA). All other chemicals were reagent grade and used as supplied by Merck (Darmstadt, FRG).

Growth of cells and crude extract preparation

Chlamydomonas reinhardii (strain 6145 c, a gift from Dr. R. Sager, Hunter College, New York) was grown as described elsewhere [2]. In order to obtain high mass of cells, the alga was grown in 20 or 40 1 bottles during 3–4 days. The cells were harvested at the logarithmic phase (absorbance at 660 nm = 1.5–2.0) by centrifugation using a continuous system operating at 23 000 × g with a flow rate of 1 l/min. After washing with 50 mM MOPS buffer, pH 7.0, they were frozen at −20 °C until use.

The cells were broken by thawing in MOPS buffer (5 ml/g fr. wt.); the suspension was slowly stirred for 60 min at 0 °C, and then centrifuged at 27 000 × g for 10 min. The supernatant was used as crude extract.

Enzyme activity

The biosynthetic activity of glutamine synthetase was determined spectrophotometrically at 340 nm by measuring the ADP-dependent oxidation of NADH in the reaction mixture described elsewhere [18].

The synthetic activity of glutamine synthetase was determined by measuring the γ-glutamyl-hydroxamate formed, according with the method previously described [19].

Except where indicated, the GS was estimated by its transferase activity in reaction mixtures which included in a final volume of 1 or 2 ml, respectively, the following compounds: MOPS buffer, pH 7.0, 50 μmol; glutamine, 30 μmol; MnCl₂ · 4H₂O, 3 μmol; ADP, 0.4 μmol; hydroxylamine, 60 μmol; NaOH, 60 μmol; Na₂HAsO₄, 20 μmol; and an adequate amount of enzyme solution (in vitro assay), or 1 ml of a suspension of toluenized cells (in situ assay). The reaction was initiated by the addition of arsenate, and after 15 min at 30 °C, it was finished by the addition of 2 ml of the ferric acid reagent [19]. One unit of activity corresponds to the formation of 1 μmol of γ-glutamyl-hydroxamate per min.

When required, the permeabilization of the cells was obtained by the addition of 20 μl of toluene to 1 ml of cells suspension, and stirring vigorously for 1–2 min.

Analytical methods

Protein was determined by the method of Bailey [20], with bovine serum albumin as standard. Cellular protein was extracted, before determination, with 10% TCA solution.

Electrophoresis

Analytical disc gel electrophoresis of purified GS was performed on gels (5 × 85 mm), containing 6.25% of acrylamide according to Jovin et al. [21]. Samples containing 7–30 μg of protein were used, and electrophoresis was carried out at 1 mA/gel during the first 15 min, and then at 3–4 mA/gel during 90–100 min. Proteins were located by staining the gels with 1% coomassie blue in 7% acetic acid. Glutamine synthetase activity was detected in the gels according to Lepo et al. [9]. SDS-electrophoresis was performed according to Weber and Osborn [22], using gels (5 × 75 mm) containing 10% acrylamide.

Determination of Stokes radius and sedimentation coefficient

The Stokes radius was determined as described by Siegel and Monty [23] using a Bio-gel A-1.5 m column (1.6 × 66 cm) equilibrated with 50 mM MOPS buffer (pH 7.0), containing 0.5 mM EDTA; 2.5 mM MgCl₂. 0.5 ml samples of purified enzyme preparation or standard proteins were loaded on the top of the gel and eluted at a flow rate of 23 ml/h. Fractions of 2 ml were collected. Standard proteins used were: ovalbumin, 20 mg; yeast alcohol dehydrogenase, 1 mg; catalase, 1 mg; and apoferritin, 3.5 mg.

The sedimentation coefficient was determined by sucrose-density-gradient centrifugation as described.
previously [24], using 4.6 ml of a 5—20% linear gradient in the above indicated buffer. Samples of 0.1 ml of purified glutamine synthetase or standard proteins were centrifuged at 4 °C for 8 h at 45 000 rpm. The gradients were eluted from the bottom of the tubes, and 3-drop fractions were collected. The standard proteins used were: apoferritin, 50 µg; catalase, 30 µg; yeast alcohol dehydrogenase, 50 µg; and bovine serum albumin, 80 µg.

Results

Separation of two isoenzymes with glutamine synthetase activity by DE-52 cellulose chromatography

100 g (fresh weight) of Chlamydomonas grown with nitrate in the light were broken and the resulting crude extract was applied to a DE-52 cellulose column (2.6 x 20 cm) equilibrated with 50 mM MOPS, pH 7.0, containing 0.5 mM DTE; 0.5 mM EDTA; and 2.5 mM MgCl₂ (standard buffer). The chromatography was run at a flow rate of 70 ml/h, and the glutamine synthetase activity eluted by supplementing the buffer with 0.2 M NaCl. Those fractions containing high level of enzyme activity were pooled, and afterwards diluted with the standard buffer to a final concentration of 50 mM in NaCl. The solution was then passed through a second DE-52 cellulose column (2.5 x 30 cm), at a flow rate of 52 ml/h. After washing with 150 ml of standard buffer, containing 50 mM NaCl, the GS activity was eluted with a linear gradient (50–250 mM) of NaCl in 600 ml of standard buffer. The elution profile is presented in Fig. 1 (lower). Two peaks of GS activity were obtained; the first one (GS₁) eluted at 90 mM NaCl, and the second one (GS₂) at 130 mM NaCl.

Similar elution profile was obtained when the experiment was performed using a crude extract prepared in the presence of 30 µM of the protease inhibitor phenyl-methanesulfonyl-fluoride (PMSF). Furthermore, separate rechromatography of the isoenzymes showed single peaks (results not shown).

Fig. 1 (upper) shows the results obtained with a crude extract prepared from cells grown with nitrate in the light, followed by 14 h in the dark. In this case, the peak of GS₁ increased significantly, while that corresponding to GS₂ was imperceptible.

Purification of GS₁ and GS₂ enzymes from Chlamydomonas reinhardii

The fractions containing high glutamine synthetase activity, GS₁ or GS₂, were separately pooled and the purification of the corresponding isoenzyme pursued at 4 °C.

Solid ammonium sulphate up to 60% saturation was added with continuous stirring to each enzymatic preparation, and the mixtures were kept, during 15 min at 0 °C, with stirring. The suspensions were centrifuged at 27 000 x g during 15 min, the resulting supernatants were discarded, and the pellets dissolved in a minimum volume of standard buffer. The preparations were desalted using a Sephadex G-25 column (2.6 x 25 cm) equilibrated with standard buffer. The obtained GS₁ and GS₂ preparations were applied to a 2',5'-ADP-sepharose...
Physico-chemical properties of the isoenzymes $GS_1$ and $GS_2$ from Chlamydomonas reinhardii

Table II shows the characterization of $GS_1$ and $GS_2$ which appear to be very similar in their molecular parameters, activation energy, optimum pH, and apparent $K_m$ for glutamate and ATP (physiological substrates), hydroxylamine and Mn$^{2+}$ (non physiological substrates). Significant differences were found in the $K_m$ for ammonium (physiological substrate) and glutamine (non physiological substrate). The corresponding $K_m$ values were determined using saturating concentration of all substrates, except for that of interest, which concentrations were varied.

In vivo regulation of glutamine synthetase activity in Chlamydomonas reinhardii

If we take as reference the level of glutamine synthetase in cells grown autotrophically with nitrate, the enzymatic level was doubled after 6 h treatment with glutamate or glutamine, and decreased about 30% when ammonium substituted for nitrate as nitrogen source. In N-starved cells the intracellular GS activity increased 3-fold.

When Chlamydomonas growing autotrophically with nitrate were darkened, the GS activity decreased to 35%, after 2 h of treatment (Figure 4). The original level of GS activity was restored after 30 min of illumination, even in the presence of cycloheximide, indicating an enzyme activation. On the other hand, the GS activity was reactivated by treating the crude extract with 5 mM dithioerythritol.

Effect of thiols on glutamine synthetase activity

Dithioerythritol was an effective protector of the GS activity, and this thiol was present in the buffers used for the enzyme purification.
Table III. Effects of thiols on the glutamine synthetase activity of GS₁ and GS₂ from Chlamydomonas reinhardii.

The glutamine synthetase activity was measured as indicated in Materials and Methods by using purified preparations of GS₁ and GS₂, extensively dialyzed against the standard buffer, without DTE. The enzymatic assays included in the corresponding reaction mixture the indicated compounds, at a final concentration of 5 mM. 100% of activity corresponded to 0.7 and 1.2 U/ml for GS₁ and GS₂, respectively.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glutamine synthetase [%]</th>
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<tbody>
<tr>
<td></td>
<td>GS₁</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Dithioerythritol</td>
<td>290</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>292</td>
</tr>
<tr>
<td>Cysteine</td>
<td>289</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>203</td>
</tr>
<tr>
<td>Cystine</td>
<td>88</td>
</tr>
</tbody>
</table>

Table IV. Effect of amino acids on the activity of glutamine synthetase isoenzymes from Chlamydomonas reinhardii.

The glutamine synthetase was measured as indicated in Materials and Methods, using aliquots of purified preparations of GS₁ and GS₂, but including in the reaction mixture the indicated amino acids, at a final concentration of 5 mM. 100% activity was 1.8 and 3.0 U/ml respectively.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glutamine synthetase [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS₁</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>L-alanine</td>
<td>28</td>
</tr>
<tr>
<td>L-glycine</td>
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<tr>
<td>L-serine</td>
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<tr>
<td>L-tryptophan</td>
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<tr>
<td>L-histidine</td>
<td>100</td>
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<tr>
<td>L-aspartate</td>
<td>83</td>
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<tr>
<td>L-arginine</td>
<td>66</td>
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</tbody>
</table>

The activity of GS₁ and GS₂ are significantly stimulated by thiols, such as DTE, dithiothreitol, cysteine, and β-mercaptoethanol (Table III). On the other hand, 1 mM p-hydroxymercurobenzoate completely inactivated the GS₁ or GS₂, which were protected, or the inactivation reversed, by 5 mM DTE.

Effect of amino acids on GS₁ and GS₂ from Chlamydomonas

Table IV shows the effect of L-amino acids on the purified isoenzymes GS₁ and GS₂. L-Alanine and L-glycine inhibited the activity 70% and 40%, respectively. L-Arginine and L-serine also inhibited, while tryptophan, histidine and aspartic acid did not decrease the glutamine synthetase activity.

Discussion

The reported results demonstrate the existence of two isoenzymes with glutamine synthetase activity in the green alga Chlamydomonas reinhardii. Up to date, the presence of GS isoenzymes has been reported in higher plants, such as rice [5, 14, 16], barley [6], pea [26], germinating peanuts [27], Cucurbita pepo [17], soybean hypocotyls [13, 28], and leaves from the C₄ plant Sorghum vulgare [29]. The separation of such isoenzymes was generally obtained by ion-exchange chromatography. One type of glutamine synthetase was present in Chlorella pyrenoidosa [12], Anabaena cylindrica [10, 30], Nostoc sp. [10], and Rhodopseudomonas palustris [7].

The separation of two isoenzymes with glutamine synthetase activity, and the observation that in Chlamydomonas exist two enzymes with glutamate synthase activity, one specific for ferredoxin as electron donor, and apparently located in the chloroplast; and other with specificity for NAD(P)H and located in the cytosol [31], suggest in this alga, the presence of two GS-GOGAT systems: one, GS₂: ferredoxin-GOGAT, in the chloroplast, which should be involved in the photosynthetic assimila-
tion of ammonium; and the other, GS2: NAD(P)H-GOGAT, in the cytosol, which should be required for the assimilation of ammonium in the dark, and/or the reassimilation of the ammonium produced by photorespiration [4, 16]. Studies about the intracellular location of GS1 and GS2 in *Chlamydomonas* are in progress in order to confirm this hypothesis, which is in good agreement with the following observations in higher plants: a) the GS1 is located in the cytosol, while GS2 is in the chloroplast [6, 16, 17]; b) ferredoxin-nitrite reductase, which catalyzes the formation of ammonium, during the photosynthetic assimilation of nitrate, is located in the chloroplast [32].

The present results also contribute to the knowledge of the physico-chemical, kinetic, and regulatory properties of glutamine synthetase isoforms from *Chlamydomonas reinhardii*. GS1 and GS2 show similar physico-chemical parameters (Table II), and the following differences: a) GS1 shows higher *Km* for ammonium, which is in agreement with the observation of Iyer et al. [14]; b) the *Km* for glutamine showed by GS1 was lower than that of GS2, as previously described in higher plants [13].

The GS isoenzymes from *Chlamydomonas* are octameric enzymes, with molecular parameters similar to those of plant-type glutamine synthetase, and substantially different to those found in the GS from *Chlorella pyrenoidosa* [12].

The glutamine synthetase appears to be an important control point of the nitrogen metabolism in photosynthetic organisms [28, 30, 33, 34]. We have observed in *Chlamydomonas* that the level of GS depends on the nitrogen source. In addition, the GS1 level was high in cells darkened during several hours, while that for GS2 was low under such conditions. On the other hand, either, GS1 and GS2 were significantly present in cells grown autotrophically with nitrate (Fig. 1). Evidence for light induction of chloroplastic GS, during greening of etiolated rice leaves, has been reported [35].

In general, the GS is inactivated by dark and/or ammonium, and reactivated, *in vivo*, by light, or, *in vitro*, by thiols [19, 36–38]. This is in agreement with the results reported here for *Chlamydomonas*, although the behaviour of GS1 and GS2 appears to be different with respect to dark-light transition of the cells. since GS2, but not GS1, decreased with the dark (Fig. 1). *In vitro* studies indicate that GS1 and GS2 from *Chlamydomonas* are inhibited by L-alanine, L-glycine, L-arginine, and L-serine, as observed in the glutamine synthetase from *Chlorella* [34], higher plants [15, 28], and cyanobacteria [30, 37, 39–41]. Recently, it has been claimed that glutamine synthetase had an important role in the control of the nitrate assimilation by *Chlamydomonas* [42]. Future studies concerning the regulation of GS activity in photosynthetic organisms should be focused on the existence of two GS-GOGAT systems involved in the assimilation of ammonium.

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