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

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Z. Naturforsch. **38c**, 531 – 538 (1983); received March 4, 1983

Chlamydomonas reinhardii, Glutamine Synthetase, Isoenzymes, Purification

Two isoforms of glutamine synthetase, GS_1 and GS_2, have been separated from *Chlamydomonas reinhardii* cells grown autotrophically with nitrate. The intracellular level of GS_2 was higher than that of GS_1. In cells under darkness, the GS_1 peak increased markedly, whereas that of GS_2 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 GS_1 and GS_2 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 GS_1, and 46 000 in GS_2). The basic differences between GS_1 and GS_2 are: a) the effect of light on their intracellular level; b) their K_m 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.

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 GS_1 and GS_2, have been purified using affinity chromatography on ADP-sepharose 4B, and a comparative study of their physico-chemical, kinetic, and regulatory properties is presented.

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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′-Tetramethylethylenamine (TEMED), ovalbumin, bovine serum albumin, and apoferritin were from Serva (Heidelberg, FRG). Electrophoresis standard proteins, 2′,5′-ADP-sepharose and Sephadex G 25 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 l 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; $MnCl_2 \cdot 4H_2O$, 3 μmol; ADP, 0.4 μmol; hydroxylamine, 60 μmol; NaOH, 60 μmol; Na_2HAsO_4, 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 DTE; 0.5 mM EDTA; and 2.5 mM $MgCl_2$. 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 × 20 cm) equilibrated with 50 mM MOPS, pH 7.0, containing 0.5 mM DTE; 0.5 mM EDTA; and 2.5 mM $MgCl_2$ (*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 × 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 (GS_1) eluted at 90 mM NaCl, and the second one (GS_2) at 130 mM NaCl.

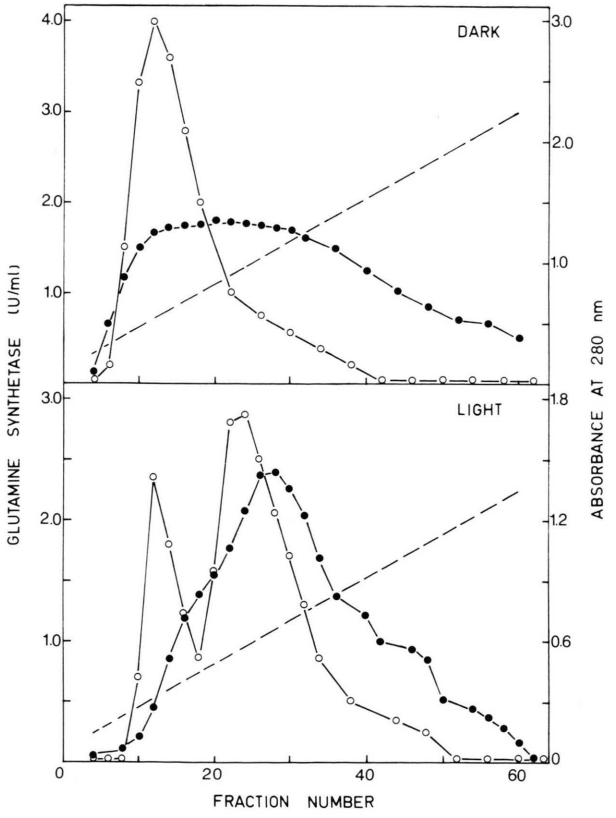

Fig. 1. Elution profile of glutamine synthetase activity on DE-52 cellulose column. Crude extract prepared from cells grown autotrophically with nitrate (*lower*), and placed under darkness for 14 h (*upper*) were treated. The GS activity (○—○) and the absorption at 280 nm (●—●) were measured in aliquots of the 10 ml collected fractions.

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 GS_1 increased significantly, while that corresponding to GS_2 was imperceptible.

Purification of GS_1 and GS_2 enzymes from Chlamydomonas reinhardii

The fractions containing high glutamine synthetase activity, GS_1 or GS_2, 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 × *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 × 25 cm) equilibrated with standard buffer. The obtained GS_1 and GS_2 preparations were applied to a 2′,5′-ADP-sepharose

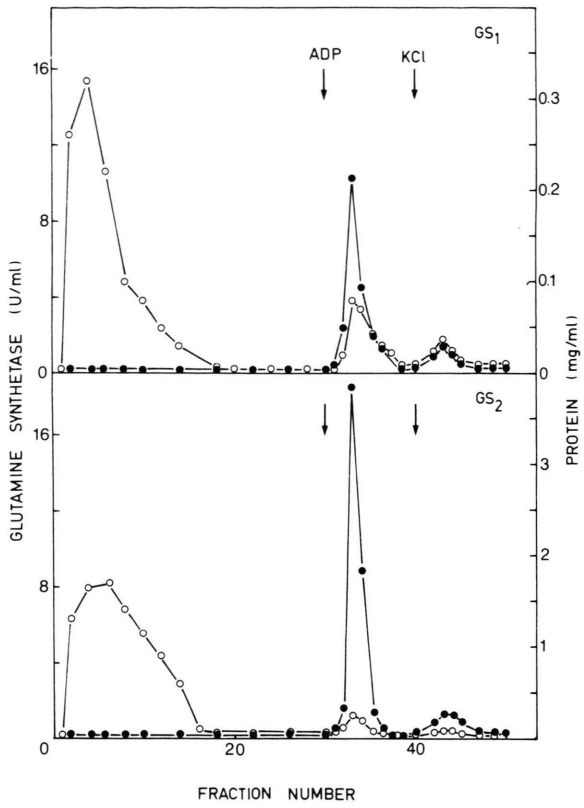

Fig. 2. ADP-Sepharose affinity chromatography of isoenzymes GS_1 and GS_2 from *Chlamydomonas*. Arrows indicate the points of addition to the washing buffer of ADP, 2 mM; or KCl, 1 M. Fractions of 4.5 ml were collected at a flow rate of 9 ml/h, and GS activity (●—●) and protein (○—○) were determined.

column (1 × 12 cm), and the enzymes were eluted with 2 mM ADP. Fig. 2 shows the elution profiles obtained with GS_1 (*upper*) and GS_2 (*lower*).

The purification procedure, summarized in Table I, yielded 0.6 mg of GS_1 and 2.1 mg of GS_2 with the following characteristics: 114 and 63 U/mg specific activity which corresponds to turnover numbers of 43 453 and 23 500 min^{-1}; purification factors of 212 and 110; and yields of 28 and 53% respectively. These preparations exhibited a transferase/biosynthetic activity ratio of about 20, and transferase/synthetic about 5.

The purity of the preparations was tested by electrophoresis on gels containing 6.25% of acrylamide. Similar results were obtained with both preparations: a main protein band (80% of total protein and $R_f = 0.117$) with GS activity, and two minor protein bands lacking activity, with $R_f =$ 0.341 and 0.517 respectively, which indicate that these proteins may represent dissociated forms of the native enzyme. On the other hand, both GS_1 and GS_2 preparations showed a single protein band upon SDS-electrophoresis, which suggests the homogeneity of such preparations and indicates that both isoenzymes posses one type of subunit (results not shown).

Molecular weights

Two procedures were followed for the determination of the molecular weight (M_r) of the native isoenzymes. One of them used filtration through a calibrated column of Bio-gel A-1.5 m (100–200 mesh), and a Stokes radius of 6.3 nm was obtained for GS_1 and GS_2 (Fig. 3A). Moreover, a sedimentation coefficient of 14.2 S was calculated for GS_1 and GS_2 by sucrose-density-gradient centrifugation (Fig. 3B). From these data, a value of 371 000 was determined.

On the other hand, M_r of 390 000 and 375 000 for GS_1 and GS_2 respectively were estimated by electrophoresis as described elsewhere (25) with gels containing 3.50, 5.00, 6.25, and 7.50% of acrylamide. The average values of 380 000 (GS_1) and 373 000 (GS_2) were taken as molecular weights of these isoenzymes.

The M_r for the corresponding subunits were determined by SDS-electrophoresis, and values of 48 000 and 46 000 were obtained.

Table I. Purification of the glutamine synthetase isoforms from *Chlamydomonas reinhardii*. Assays for enzyme activity and protein were conducted as described in Materials and Methods.

Step and Fraction	Vol [ml]	Protein [mg]	Activity [*U*]	Specific activity [*U*/mg]
1. Crude extract	440	464	251	0.54
2. DE-52 cellulose	100	171	520	3.04
GS_1------				
3. DE-52 cellulose	70	14.7	126	8.57
4. $(NH_4)_2SO_4$ (0–60%)	8	9.5	103	10.80
5. ADP-sepharose eluate	6	0.6	69	114.20
GS_2------				
3. DE-cellulose	140	41.2	280	6.92
4. $(NH_4)_2SO_4$ (0–60%)	15	31.0	253	8.16
5. ADP-sepharose eluate	9	2.1	133	63.00

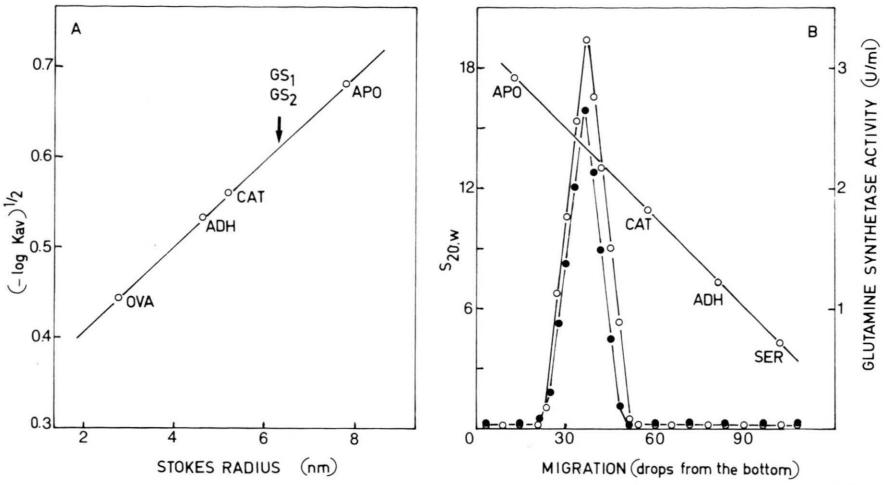

Fig. 3. Determination of Stokes radius and sedimentation coefficient of GS_1 and GS_2 from *Chlamydomonas*. A. Standards of known Stokes radius were: ovalbumin (OVA), 2.75 nm; yeast alcohol dehydrogenase (ADH), 4.60 nm; catalase (CAT), 5.20 nm; apoferritin (APO), 7.80 nm. The arrow locates GS_1 and GS_2. B. Standards for sedimentation coefficient determination were: bovine serum albumin (SER), 4.3 S; yeast alcohol dehydrogenase, 7.4 S; catalase, 11.2 S; apoferritin, 17.6 S. The distribution of the GS_1 and GS_2 activities after sucrose-density-gradient are shown. Details of the procedures are given in Materials and Methods.

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.

Table II. Physico-chemical characterization of the isoenzymes glutamine synthetase from *Chlamydomonas reinhardii*.

	GS_1	GS_2
Stokes radius [nm]	6.3	6.3
Sedimentation coefficient (S)	14.2	14.2
Number of subunits	8	8
Specific activity [*U*/mg]	114.2	63.0
Optimum pH		
transferase activity	6.0	5.5
biosynthetic activity	7.0	7.0
K_m NH_4^+ [mM]	0.0833	0.244
K_m NH_2OH [mM]	2.1	2.0
K_m ATP [mM]	0.317	0.294
K_m glutamine [mM]	2.5	10.0
K_m Mn^{2+} [mM]	0.210	0.200
Activation energy (Kcal/mol)	12.3	11.8

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 GS_1 and GS_2 from *Chlamydomonas reinhardii*. The glutamine synthetase activity was measured as indicated in Materials and Methods by using purified preparations of GS_1 and GS_2, extensively dyalized 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 GS_1 and GS_2, respectively.

Addition	Glutamine synthetase [%]	
	GS_1	GS_2
None	100	100
Dithioerythritol	290	284
Dithiothreitol	292	295
Cysteine	289	294
β-Mercaptoethanol	203	200
Cystine	88	90

Table IV. Effect of amino acids on the activity of glutamine synthetase isoforms from *Chlamydomonas reinhardii*. The glutamine synthetase was measured as indicated in Materials and Methods, using aliquots of purified preparations of GS_1 and GS_2, 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.

Addition	Glutamine synthetase [%]	
	GS_1	GS_2
None	100	100
L-alanine	28	34
L-glycine	57	63
L-serine	74	80
L-tryptophan	99	95
L-histidine	100	96
L-aspartate	83	93
L-arginine	66	75

The activity of GS_1 and GS_2 are significantly stimulated by thiols, such as DTE, dithiothreitol, cysteine, and β-mercaptoethanol (Table III). On the other hand, 1 mM *p*-hydroxymercuribenzoate completely inactivated the GS_1 or GS_2, which were protected, or the inactivation reversed, by 5 mM DTE.

Effect of amino acids on GS_1 and GS_2 from Chlamydomonas

Table IV shows the effect of L-amino acids on the purified isoenzymes GS_1 and GS_2. 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.

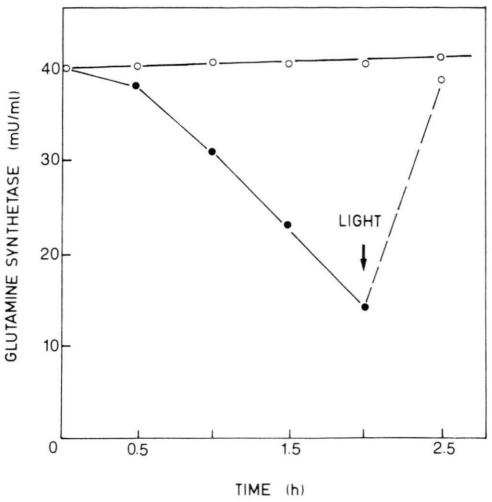

Fig. 4. Effect of dark-light transition on the intracellular level of glutamine synthetase in *Chlamydomonas*. Cells growing with nitrate were harvested at the logarithmic phase and resuspended in 50 mM MOPS buffer (pH 7.0) containing 7 mM $NaHCO_3$, and 2 mM KNO_3. 50 ml of the cell suspension in 250 ml flask were placed in a bath at 25 °C, with continuous stirring and illumination (100 W/m^2) (○—○), or under darkness (●—●). At the indicated times, the GS activity was determined *in situ* using aliquots of the corresponding culture.

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 C_4 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, GS_2: ferredoxin-GOGAT, in the chloroplast, which should be involved in the photosynthetic assimila-

tion of ammonium; and the other, GS_1: 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 GS_1 and GS_2 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 GS_1 is located in the cytosol, while GS_2 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*. GS_1 and GS_2 show similar physico-chemical parameters (Table II), and the following differences: a) GS_1 shows higher K_m than GS_2 for ammonium, which is in agreement with the observation of Iyer *et al.* [14]; b) the K_m for glutamine showed by GS_1 was lower than that of GS_2, 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 of the nitrogen source. In addition, the GS_1 level was high in cells darkened during several hours, while that for GS_2 was low under such conditions. On the other hand, either, GS_1 and GS_2 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 GS_1 and GS_2 appears to be different with respect to dark-light transition of the cells, since GS_2, but not GS_1, decreased with the dark (Fig. 1). *In vitro* studies indicate that GS_1 and GS_2 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.

Acknowledgements

This work was supported by Research Grant, n° 0252, from *Comisión Asesora de Investigación Científica y Técnica* (Spain). One of us (F.J.F.) thanks to the *Instituto Nacional de Asistencia y Promoción del Estudiante* for financial support.

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