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Occurrence of Isoenzymes of Glutamine Synthetase in the Alga *Chlorella kessleri*[1]

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ABSTRACT

Two forms (GS_1 and GS_2) of glutamine synthetase have been isolated, separated by ion exchange chromatography, and partly characterized from cells of the green alga *Chlorella kessleri*. Both forms are present in cells grown autotrophically or heterotrophically on various nitrogen sources, but under all nutritional conditions GS_1 was found to be the major isoenzyme present (60–80%). The activity of both isoenzymes was greatest in cells grown under nitrogen-limiting conditions. Both isoenzymes have molecular weights in the range 340 to 350,000 daltons. GS_1 was found to have a greater thermostability than GS_2: GS_1 was stable at 30°C while GS_2 lost 95% of its activity in 30 minutes. GS_1 was much less sensitive to thiol reactive reagents than GS_2.

Recent work has shown that glutamine synthetase (EC 6.3.1.2), the key enzyme of ammonia metabolism, occurs in multiple molecular forms in many higher plants (1, 7, 12, 15, 16, 18–20, 23, 27). Ion exchange chromatography and subcellular localization studies have shown the presence of two isoenzymes in photosynthetically active tissue: GS_1 is localized in the cytoplasm whereas GS_2 is found in chloroplasts (10, 11, 17). These isoenzymes differ in several respects, including their pH optima, heat stability, and K_m for glutamate (1, 9, 16, 18, 19).

Although glutamine synthetase has been much studied in microorganisms, there is little evidence for the occurrence of isoenzymic forms; in only two species, *Bacillus caldolyticus* (26) and *Rhizobium japonicum* (6), have distinct isoenzymes been demonstrated. There appear to be no reports of glutamine synthetase isoenzymes in algae, although the enzyme has been purified from a thermophilic strain of *Chlorella pyrenoidosa* and characterized in some detail (21). In the present paper, we report the presence of two distinct forms of glutamine synthetase in *Chlorella kessleri* and that their properties resemble those of GS_1 and GS_2 isoenzymes present in some higher plants.

MATERIALS AND METHODS

Organism and Growth Conditions. *Chlorella kessleri* was obtained from the culture center of Algae and Protozoa at Cambridge (No. 211/11g) and maintained on agar slopes. Cells were grown as batch cultures for 3 to 4 d either autotrophically in aerated Dretschel bottles at 25°C in the light (4) or heterotrophically in penicillin flasks on a rotary shaker at 25°C in the dark (5), as appropriate. The basic medium contained 7.26 g KH_2PO_4, 2.32 g K_2HPO_4, 0.41 g $MgSO_4 \cdot 7H_2O$, 1 ml A4 trace elements (2), and 2.4 ml of a stock iron solution (13). Nitrogen sources were at an initial concentration of 280 mg N/l and where required 1% glucose was also added. Nitrogen-starved cultures were obtained by transferring cells grown in medium containing ammonium to a nitrogen-free medium overnight.

Enzyme Extraction and Ion Exchange Chromatography. Cultures were harvested by centrifugation and the cells were resuspended in 50 ml of 50 mM Tris-HCl buffer pH 8.0, containing 1 mM reduced glutathione, 6 mM DTT, 1 mM mercaptoethanol, 1 mM EDTA, 10 mM magnesium sulfate, and 5 mM glutamate, and extracted in an Aminco French pressure cell. The broken cells usually received a second treatment before being centrifuged at 12,000*g* for 20 min at 5°C. The supernatant was applied to a DEAE-Sephacel (Pharmacia Ltd) column (10 × 1 cm), equilibrated with resuspension buffer. Elution was carried out with a 0 to 0.4 M KCl linear gradient made up in resuspension buffer. The gradient (120 ml total volume) was run over a 16 h period and 2-ml fractions were collected.

Gel Filtration. Gel filtration was carried out on a column (45 × 3.3 cm) of Sepharose CL-6B (Pharmacia Ltd.) equilibrated with resuspension buffer. The pooled fractions corresponding to the two peaks of glutamine synthetase obtained from ion exchange chromatography were concentrated by ultrafiltration and applied to the Sepharose column and eluted at a flow rate of 24 ml h^{-1}.

Enzyme Assays. The synthetase and transferase activities were determined as described by Guiz *et al.* (7) and Rhodes *et al.* (22), respectively. Protein was assayed by the method of Bradford (3).

RESULTS

Separation of GS_1 and GS_2 by Ion Exchange Chromatography. Extracts from cells of *Chlorella* grown under a range of nutritional conditions were found to exhibit two peaks of glutamine synthetase activity when subjected to ion exchange chromatography (Fig. 1). The first peak eluted at 70 mM KCl and the second at 230 mM KCl, and by analogy with the corresponding chromatographic forms in higher plants they are designated GS_1 and GS_2 (see Refs. 9 and 16). GS_1 was always the major form present, comprising 60 to 80% of the total activity. It is evident from the results in Figure 1 and those summarized in Table I that the specific activity and relative amounts of the two isoenzymes were influenced by both nitrogen availability and light. The specific activities of both GS_1 and GS_2 were higher in nitrate-compared with ammonium-grown cells, and nitrate-starved cells always exhibited higher specific activities of GS_1 and GS_2 than did nitrate-grown cells. The difference between nitrate- and ammonium-grown cells is more pronounced when they are grown in the dark; under such conditions, nitrate-grown cells resemble those starved of nitrogen.

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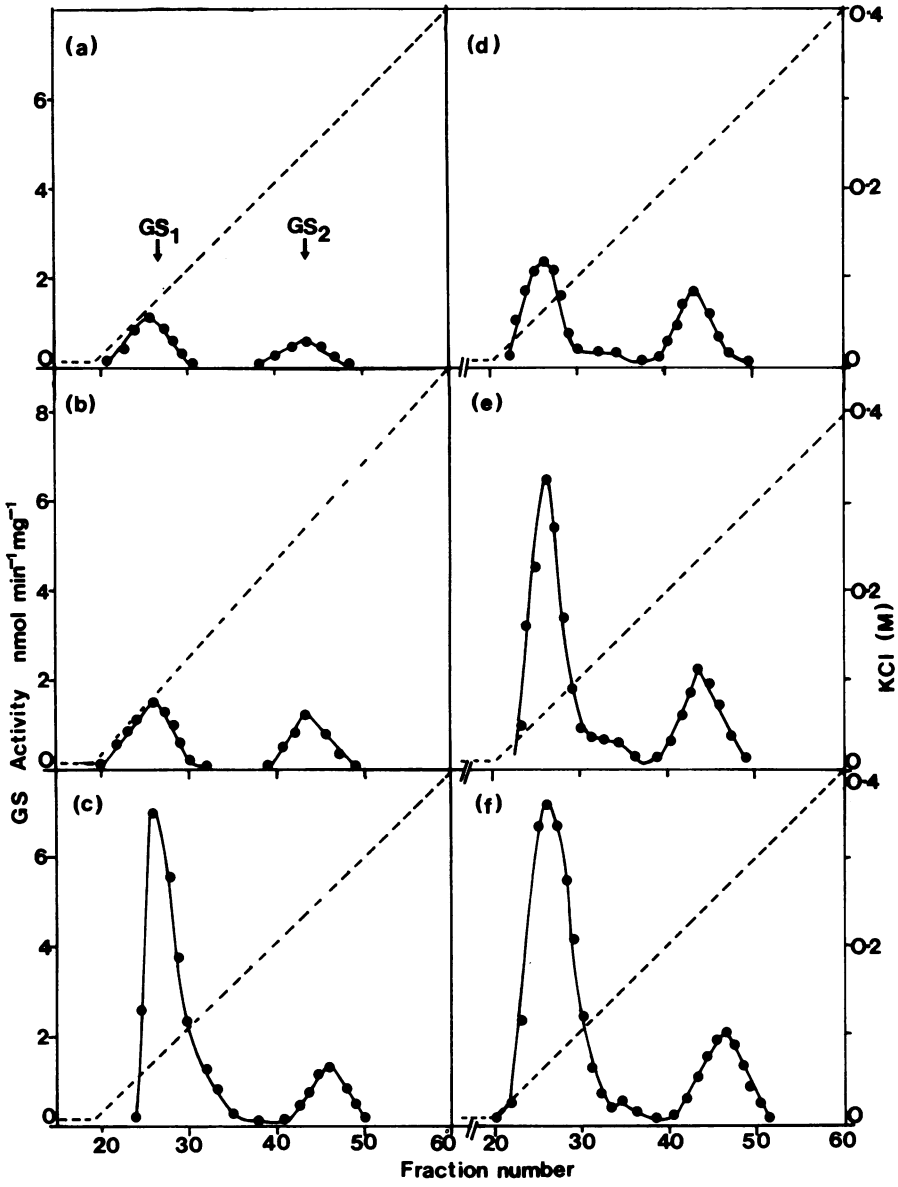

FIG. 1. DEAE-Sephacel elution profiles of glutamine synthetase from *C. kessleri* grown under different conditions. Glutamine synthetase activity was determined by the synthetase assay. (a), Ammonium-grown cells in the light; (b), nitrate-grown cells in the light; (c), nitrogen-starved cells in the light; (d), ammonium-grown cells in the dark; (e), nitrate-grown cells in the dark; (f), nitrogen-starved cells in the dark.

Table I. *Comparison of Specific Activities of Glutamine Synthetases from Chlorella kessleri Grown under Different Conditions*

Specific activity obtained by summing the activities of the fractions from the respective GS_1 and GS_2 peaks in Figure 1.

Growth Conditions	Specific Activity		
	GS_1	GS_2	Total
	nmol min^{-1} mg^{-1} protein		
Ammonium, light	5.9	3.4	9.3
Nitrate, light	8.5	5.3	13.8
Nitrogen starved, light	31	8.3	39.3
Ammonium, dark	13.3	8.4	21.7
Nitrate, dark	28.3	11.9	40.2
Nitrogen starved, dark	42.8	11.9	54.7

In general, the specific activities of GS_1 and GS_2 in dark-grown cells on a particular nitrogen regime are greater than those in corresponding cells grown in the light.

Enzyme Stability. A major difference observed between the GS_1 and GS_2 isoenzymes of higher plants is their heat stability: GS_2 always appears to be less heat stable than GS_1 (1, 9, 16, 18, 19). This difference in heat stability is also shown by the *Chlorella* isoenzymes (Fig. 2). GS_2 was found to inactivate more rapidly than GS_1 at all temperatures examined; at 30°C GS_1 was quite stable but GS_2 lost 95% of its activity in 30 min.

Influence of Thiol-Reactive Reagents. A further characteristic which distinguishes the GS_1 and GS_2 isoenzymes from higher plants is their sensitivity towards thiol-reactive reagents such as *N*-ethylmaleimide and 5-5-dithiobis(2-nitrobenzoic acid). It is evident from the results shown in Figure 3 that GS_2 of *Chlorella* was more sensitive to both NEM[3] and DTNB. A concentration of 0.5 mM NEM gave complete inactivation of GS_2 but only 60% inactivation of GS_1. Similarly, 0.1 mM DTNB brought about 80% inactivation of GS_2, but less than 10% inactivation of GS_1. GS_2 in higher plants is also more susceptible to inactivation by DTNB and NEM (1, 16, 18, 19).

[3] Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5-dithiobis(nitrobenzoic acid).

DISCUSSION

Cells of *C. kessleri*, like photosynthetic tissue of many higher plants, have two isoenzymes of glutamine synthetase. These exhibit differences in their heat stability and sensitivity toward thiol-reactive reagents. *Chlorella* GS_2 was found to be less heat stable and more readily inactivated by NEM and DTNB than GS_1. It is tempting to assume, given the similarity in chromatographic behavior, heat stability, and sensitivity to NEM and DTNB between the *Chlorella* isoenzymes and those of higher plants that GS_1 and GS_2 of *Chlorella* correspond to cytoplasmic and chloroplastic forms of glutamine synthetase.

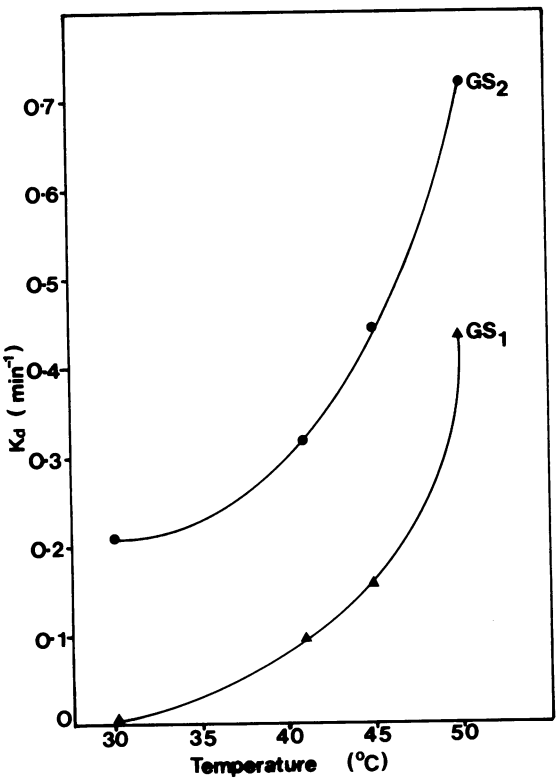

FIG. 2. Thermal inactivation of GS_1 and GS_2. Partially purified GS_1 and GS_2 were incubated at different temperatures and aliquots taken at various time intervals to determine the residual activity. Decay constants (K_d) were calculated from semilog plots of residual activity against time.

Both *Chlorella* isoenzymes are influenced by light and nitrogen availability, although both these factors appear to exert a greater influence on GS_1. The activity of GS_2 in cells subject to different nitrogen regimes was always greater when the cells were grown in the dark. This contrasts with results obtained on higher plants; in both barley (16) and rice (9), GS_2 was found to be present at low levels in etiolated seedlings and to increase rapidly following exposure to light. Tischner and Hutterman (25) working with *Chlorella sorokiniana* cells grown in synchronous cultures reported a light activation of total glutamine synthetase activity in response to the light-dark transitions. In the present study, comparisons were made between cells grown nonsynchronously in continuous illumination or continuous darkness with a carbon source supplied (as glucose).

Another difference in regulatory behavior between the *Chlorella* and higher plant isoenzymes is the effect of nitrogen availability on their activity. Although the isoenzymes of barley show little change in activity in response to nitrogen source or concentration (17), the specific activity of *Chlorella* GS_1 varies 7-fold and that of GS_2 4-fold. Maximum activities of GS_1 and GS_2 were found in nitrogen-starved cells grown in the dark whereas the lowest activities were present in light-grown cells assimilating ammonia. Hipkin and Syrett (8) similarly demonstrated increased glutamine synthetase activity in *Ankistrodesmus braunii* during nitrogen starvation.

Under all growth conditions employed, GS_1 was found to be the major component in *C. kessleri*. In light-grown cells supplied with nitrate or ammonia, GS_1 accounted for 50 to 60% of the total activity while under conditions of nitrogen starvation, GS_1 comprised 70 to 80% of the total activity. Keys *et al.* (14) have suggested that the cytoplasmic isoenzyme (GS_1) in higher plants functions in the re-assimilation of ammonia released in the photorespiratory nitrogen cycle while the chloroplastic isoenzyme (GS_2) functions in primary ammonia assimilation. The depression of *Chlorella* GS_1 in dark grown cells would appear to rule out an exclusive role in the photorespiratory nitrogen cycle.

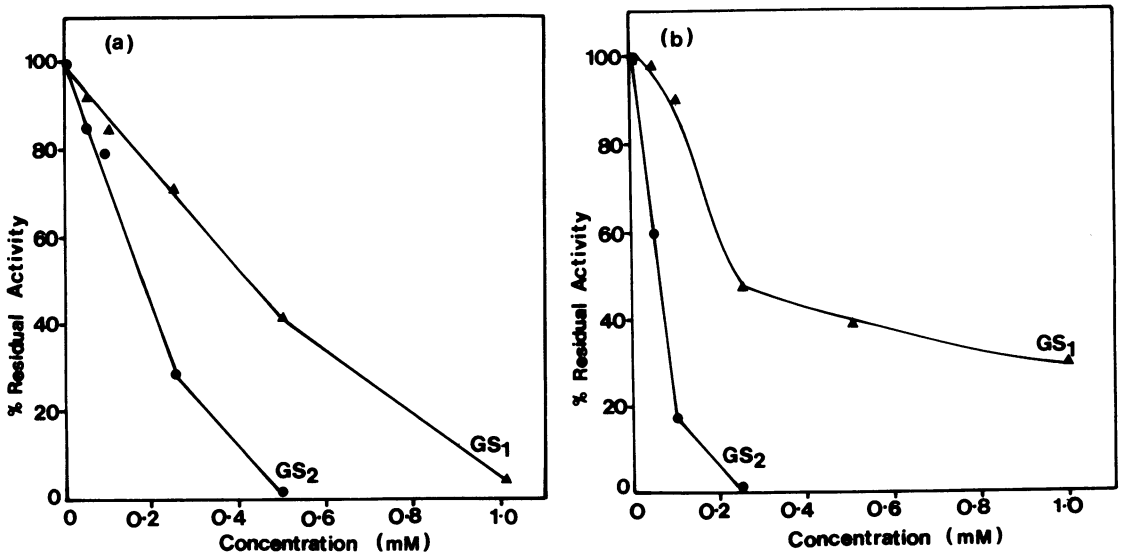

FIG. 3. Effect of thiol-reactive reagents on GS_1 and GS_2. (a), NEM; (b), DTNB. Partially purified GS_1 and GS_2 were incubated with varying concentrations of NEM and DTNB at 30°C for 30 min and aliquots were removed to determine residual activity.

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