Characterization of Glutamine Synthetase Isoforms from Chlorella

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ABSTRACT

Ion-exchange chromatography of extracts derived from Chlorella sorokiniana mutant strain (oxygen resistant) yielded two separate activity peaks of glutamine synthetase (GS). GSs and GSII were purified 220- and 187-fold and have molecular weights of approximately 398,000 and 360,000, respectively. Both enzymes are composed of eight identical subunits with a subunit molecular weight of 47,000 for GSs and 43,000 for GSII. The amino acid composition, catalytic, and immunological properties for both enzymes are similar.

In algae and plant leaves, two molecular forms of GS1 (L-glutamate:ammonia ligase [adenosine 5'-diphosphate forming], EC 6.3.1.2) have been found (2, 5, 15). The enzymes are present in different intracellular compartments. GSs is present in the cytosol and GSII is located in the chloroplasts in plant leaves (15). In algae, the intracellular compartmentation of both enzymes remains to be determined. A detailed comparison of purified GSs and GSII is of interest considering the important roles both enzymes play in photosynthetic organisms. Not only is GS involved in primary ammonia assimilation but it also is responsible for the detoxification of NH3 produced during photorespiration via the glycine-serine pathway of glycolate metabolism (11). It is presently being debated whether GSs, GSII, or both enzymes are involved in the latter process (2, 15). In this paper, we report the purification and characterization of GSs and GSII from a mutant of C. sorokiniana which has an increased capacity for the metabolism of glycolate via the glycine-serine pathway. In addition, this organism exhibits an efficient GS-mediated assimilation of NH3 produced during photorespiration (1, 2).

MATERIALS AND METHODS

Materials. DEAE-Sephaloc and Octyl-Sepharose were from Pharmacia Fine Chemicals. ATP (grade II) disodium salt came from Sigma, as did all other biochemicals.

Organism and Growth Conditions. A Chlorella sorokiniana mutant strain resistant to high oxygen tension (strain OR) was grown autotrophically under light-saturating conditions with nitrate as sole nitrogen source (1). A gas atmosphere of 98% O2—2% CO2 was supplied to the culture. Medium composition has been described (24).

Purification of Glutamine Synthetase. Thirty liters of culture yielding 40 g of cells (wet weight) were harvested rapidly with the aid of a Pellicon Cassette System (Millipore) and concentrated to 1 L in 25 mM Tris/Cl buffer containing 5 mM MgCl2, 10 mM Na-glutamate, 10 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.6 (isolation buffer). Cells were washed in this buffer and disrupted in a French pressure cell (4°C, at 20,000 p.s.i.) for four consecutive cycles. The debris was removed by ultracentrifugation at 60,000 x g for 60 min (4°C) in a Beckman 70 Ti rotor. The 100 ml supernatant was applied to a 30 x 2.5 cm DEAE-Sephaloc column equilibrated in isolation buffer (4°C). Proteins were eluted with a linear gradient of 0 to 400 mM NaCl (total volume, 400 ml) in isolation buffer. Fractions of 5 ml were collected at a flow rate of 20 ml-h−1. Active fractions from both peaks were pooled and (NH4)2SO4 added to 25% (w/v). Solutions were incubated for 30 min at 0°C prior to application to an Octyl-Sepharose column equilibrated in isolation buffer containing 25% (v/v) (NH4)2SO4 (4°C). Proteins were eluted with a linear gradient of decreasing (NH4)2SO4 (25–0% w/v) and increasing ethylene glycol concentrations (0–50% v/v) in isolation buffer (4°C). Active fractions were pooled, concentrated by ultrafiltration, and dialyzed overnight in isolation buffer at 4°C. The dialyzed protein solutions were layered on a 35-ml linear gradient of 0.4 to 0.9 M sucrose in isolation buffer. The sucrose gradients were subjected to ultracentrifugation for 40 h at 83,000 x g (4°C); 1-ml fractions were collected from the bottom of the tube.

Glutamine Synthetase. GS was assayed, using the synthetic reaction, and is based on the formation of γ-glutamylhydroxamate. The assay mixture consisted of 25 mM Tris/Cl buffer, 45 mM MgSO4, 100 mM Na-glutamate, 18 mM ATP, and 6 mM hydroxylamine (13). Glutamate-dependent Pi release (the biosynthetic assay) was also followed (13). Assays were performed at 40°C.

Protein was quantitated by the Coomassie blue binding method (3).

Preparation of Antisera. Purified preparations of GSs and GSII (0.2 mg protein/ml) were emulsified with an equal volume of Freund’s adjuvant; 0.3 ml of this mixture was then injected subcutaneously into a New Zealand white rabbit at the left and right sides of the dorsal area and, 8 d later, intramuscularly into each thigh.

Blood was taken 1 week later. Immunoglobulins were obtained by (NH4)2SO4 (50% w/v) precipitation of the serum at 0°C. After centrifugation for 30 min at 60,000 g (4°C), the pellet was resuspended in isolation buffer to one-half the serum volume. This preparation was dialyzed overnight at 4°C against isolation buffer and subsequently stored in small quantities at −20°C. Control sera were obtained prior to injection of the antigens.

SDS-Polyacrylamide Slab Gel Electrophoresis. Denatured
samples were loaded onto a 3% (w/v) stacking gel and a 12.5% (w/v) running gel using established procedures (12).

**Disc Gel Electrophoresis.** To determine the MW of GS, and GSII, both enzymes were subjected to electrophoresis in tube gels (5 x 85 mm) containing 3.5; 5.0; 6.25; and 7.5% of acrylamide. Mobilities of proteins of known MW were plotted against the percentage gel concentration. The slope of such a plot for each standard protein was used to determine the MW of GS, and GSII (8).

**Amino Acid Composition.** Prior to amino acid analysis, the enzymes were dialyzed for 96 h against four changes of 4000 volumes of distilled H2O. The enzymes were lyophilized and subjected to hydrolysis in 6 N HCl at 110°C. Hydrolysates were analyzed with a Beckman 121 Automatic Analyzer. Cysteine and cystine were determined as cysteinic acid after acid hydrolysis in the presence of 0.21 M DMSO. Alanine served as an internal standard (21). Tryptophan was measured spectrophotometrically (6).

**RESULTS**

**Purification of GS, and GSII.** DEAE-Sepharose chromatography of Chlorella extracts yielded two separate activity peaks. The enzyme eluting at the lower NaCl concentration is termed GS, whereas GSII eluted at a higher salt concentration (2). Reapplication of either GS, or GSII to a second DEAE-Sepharose column resulted in only one activity peak. This clearly indicates that the separation of GS, and GSII is not an isolation artifact.

GS, was purified 224-fold to homogeneity (Table I; Fig. 1). GSII was purified 178-fold but contained minor contaminants as shown by SDS-PAGE (Table I; Fig. 1). Subunit MW of 47,000 and 43,000 were determined for GS, and GSII, respectively, after comparison of the mobilities of the stained dissociated enzymes to the mobility of MW standards (Fig. 1).

Electrophoretic determination of the native MW of the two enzymes yielded a value of 398,000 for GSI and 360,000 for GSII. This is consistent, in each case, with a protein composed of eight identical subunits, as shown with previously isolated eucaryotic GS (2, 15, 18). In sucrose gradients, it is also readily apparent that GS, sediments at a position in the gradient suggestive of a MW greater than GSII. Both isoforms were fairly stable and did not show loss of activity at 40°C for periods up to 1 h. GSII is less stable than GS, during storage for days at 4°C; activity loss at 4°C is about 10%/24 h for GSII and negligible for GS.

**Composition.** The amino acid compositions of C. sorokiniana GS, and GSII are very similar (Table II). Both enzymes have an identical polarity index of 40 to 41%. To assess possible structural homologies for both enzymes, $S \Delta Q$ values were calculated using the formula:

\[
S \Delta Q = \sum (X_i - X_k)^2
\]

where $i$ and $k$ represent the proteins being compared, and $X_i$ is the mol % of amino acid $j$ in protein $i$ (14). For GS, and GSII, an $S \Delta Q$ value of 8.0 was calculated which indicates close homology. The calculated $S \Delta Q$ of GSII from Chlorella with cystolic GS isolated from soybean root nodules (17) is about 16, although data are not available on the tryptophan and cysteine content of this enzyme. Since, so far as we are aware, the amino acid composition of GS isoforms from other plants and algae is not known, no comparison can be made at this time.

**Catalytic Properties.** Both enzymes show similar pH optima (Fig. 2), and affinity constants for NH$_4^+$, ATP, and glutamate.
Table II. Amino Acid Composition of the C. sorokiniana GS Isoforms

<table>
<thead>
<tr>
<th>Amino Acid Residue</th>
<th>GS&lt;sub&gt;1&lt;/sub&gt;</th>
<th>GS&lt;sub&gt;II&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol%</td>
<td>mol%</td>
</tr>
<tr>
<td>Lys</td>
<td>4.8</td>
<td>5.5</td>
</tr>
<tr>
<td>His</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Arg</td>
<td>4.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Cys</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Asx</td>
<td>8.3</td>
<td>8.7</td>
</tr>
<tr>
<td>Thr</td>
<td>4.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Ser</td>
<td>4.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Glx</td>
<td>11.0</td>
<td>11.8</td>
</tr>
<tr>
<td>Pro</td>
<td>8.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Gly</td>
<td>11.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Ala</td>
<td>7.6</td>
<td>8.5</td>
</tr>
<tr>
<td>Val</td>
<td>6.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Met</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Ile</td>
<td>3.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Leu</td>
<td>5.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Phe</td>
<td>2.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Trp</td>
<td>5.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Polarity index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.1</td>
<td>40.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mean values of 48 and 72 h of hydrolysis were used. <sup>b</sup>The polarity index was calculated from the sum of mol % of Asx, Thr, Ser, Glx, His, Lys, and Arg.

Fig. 2. Effect of pH on activity of purified GS isoforms. (●, O), GS<sub>1</sub>; (▲, △), GS<sub>II</sub>.

Table III. Some Properties of GS Isoforms Purified from C. sorokiniana

<table>
<thead>
<tr>
<th>Properties</th>
<th>GS&lt;sub&gt;1&lt;/sub&gt;</th>
<th>GS&lt;sub&gt;II&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native MW</td>
<td>398,000</td>
<td>360,000</td>
</tr>
<tr>
<td>Subunit MW</td>
<td>47,000</td>
<td>43,000</td>
</tr>
<tr>
<td>pH optimum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4-8.0</td>
<td>7.4-8.0</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; ATP (μM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>300</td>
<td>400</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt; (μM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>Glutamic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>variable</td>
<td>variable</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined in the synthetic assay. <sup>b</sup>Determined in the biosynthetic assay.

Fig. 3. The effect of varying glutamine concentrations on the activities of purified GS<sub>1</sub> and GS<sub>II</sub> (●), GS<sub>II</sub> (O), GS<sub>II</sub>. The final concentration of the amino acids in the reaction mixture was 10 mM. Preincubation of the enzymes with the amino acids at 40°C did not affect activity.

Table IV. Effects of Amino Acids on the Activity of Purified GS Isoforms from C. sorokiniana

<table>
<thead>
<tr>
<th>Added Amino Acid</th>
<th>Per Cent of Original Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>L-Ala</td>
<td>100</td>
</tr>
<tr>
<td>L-Gly</td>
<td>100</td>
</tr>
<tr>
<td>L-Gln</td>
<td>88</td>
</tr>
<tr>
<td>L-Ser</td>
<td>92</td>
</tr>
<tr>
<td>L-His</td>
<td>83</td>
</tr>
<tr>
<td>L-Ala + L-Gly + L-Ser + L-</td>
<td>100</td>
</tr>
<tr>
<td>Glu + L-His</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The synthetic assay was employed in all cases.

react with both GS<sub>1</sub> and GS<sub>II</sub>. Antibodies raised against GS<sub>1</sub> only react with GS<sub>1</sub> and not with GS<sub>II</sub> (results not shown).

DISCUSSION

All results obtained indicate that GS<sub>1</sub> and GS<sub>II</sub> are very closely related isoenzymes. The only major difference between both enzymes is the difference in the MW of the subunits, with GS<sub>1</sub> slightly larger than GS<sub>II</sub>. In higher plants, GS<sub>1</sub> is located in the cytosol whereas GS<sub>II</sub> is a chloroplast enzyme (15). Since both enzymes have such similar properties in Chlorella, it seems reasonable to assume that GS<sub>II</sub> is a product of posttranslational processing of GS<sub>1</sub>. Current research is directed at resolving this question. Since antibodies raised against GS<sub>1</sub> cross-react with GS<sub>1</sub> enzyme but not vice versa, we speculate that the antigenic determinants of GS<sub>1</sub> are located on the leader sequence. We cannot, however exclude the possibility that there are separate genes encoding the different size subunits of GS<sub>1</sub> and GS<sub>II</sub>. Certainly, a well described example of posttranslational processing of a chloroplast localized protein is the small subunit of ribulose 1,5-bisP carboxylase, which is synthesized as a 20,000 MW precursor on free cytoplasmic ribosomes. This precursor is then processed upon gaining entry into the chloroplast with the production of a mature subunit of about 13,000 MW (4, 9). Recently, it was suggested that chicken cytosolic GS and mitochondrial GS are encoded for by the same gene and that their ultimate subcellular destination is posttranslationally determined (18).

Photosynthetic oxidation is considered by some to be a wasteful process.
limiting plant productivity (23). However, at least in Chlorella, due to the activity of ribulose 1,5-bisP oxygenase, an important redistribution of fixed carbon may be accomplished and is dependent on the nitrogen status of the cell (2).

In nature, several strategies have evolved to overcome the process of photorespiration. For example, the C4-pathway in plants acts as a mechanism to concentrate CO2 near the active center of ribulose bisP carboxylase/oxygenase and this also serves to decrease the activity of ribulose bisP oxygenase (7). In Chlamydomonas, HCO3- transport and carbonic anhydrase activity are derepressed under CO2 limiting conditions, resulting in a dramatic increase in the intracellular CO2 concentration (20). Despite an extensive search, no oxygenase-deficient ribulose bisP carboxylase has thus far been found (19). The OR actually increases the rate of photorespiration since it metabolizes more of the glycolate produced than wild-type cells due to higher levels of enzymes of the glycine-serine pathway. Consequently, it excretes less glycolate than wild-type cells (1). The mutant also exhibits a more efficient removal of ammonia that is produced during photorespiration, correlating with the relatively high levels of GSII in this organism as compared with wild-type cells (2). Since GS1 and GSII show very similar characteristics, it is concluded that the location of GSII in the chloroplast rather than any alteration of its catalytic properties renders it more efficient in detoxifying NH3 than GS1, which is present in the cytosol (15).

In chloroplasts, reduced thioredoxins are present which markedly activate GS in Chlorella (22). As previously discussed (2), it seems more logical that GS1 might play a major role in the reasimilation of photorespiratory produced ammonia. However, after screening C3, C3-C4 intermediate, and C4 plants, it was found that GSII is most abundant (and in some cases the only isoform) in C3 plants, which show relatively high rates of photorespiration (10, 16). These findings strongly suggest that GSII rather than GS1 is involved in this process. GS1 may thus be responsible for glutamine synthesis in the dark, which is supported by the observation that dark incubation of Chlamydomonas cells results in the disappearance of GSII and a concomitant increase in the level of GS1 (5). We are currently engaged in defining the physiological role of the two glutamine synthetase isoforms in Chlorella.

LITERATURE CITED