Glutamine Synthetase in Rice
A COMPARATIVE STUDY OF THE ENZYMES FROM ROOTS AND LEAVES

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ABSTRACT

Chromatographic, kinetic, and regulatory properties of glutamine synthetase in rice were investigated. By DEAE-Sephacel column chromatography, two forms (glutamine synthetase 1 and glutamine synthetase 2) were identified in leaves and one form (glutamine synthetase R) was identified in roots. Purification on hydroxyapatite and gel electrophoresis showed that glutamine synthetase R was distinct from the leaf enzymes. The three isoforms were purified to similar specific activities and their properties were studied. Heat stability, pH optimum about 8, $K_m$ for L-glutamate of 2 millimolar, and inhibition by glucosamine 6-phosphate were the main characteristics of glutamine synthetase 2. Heat stability, pH optimum about 7.5, $K_m$ for L-glutamate of 2 millimolar, and no effect of glucosamine 6-phosphate differentiated glutamine synthetase 1 from glutamine synthetase 2. Glutamine synthetase R was also an labile protein but its kinetic and regulatory properties were quite similar to those of glutamine synthetase 1. These results clearly demonstrate the existence of three isoforms of glutamine synthetase in rice, two of which are located in the leaves and the third in the roots.

GS2 (L-glutamate:ammonia ligase (ADP), EC 6.3.1.2), a key enzyme in ammonia assimilation, has been studied in many prokaryotic and eucaryotic organisms. Purification, subunit structure, and kinetic and physicochemical properties have been thoroughly investigated in various bacteria (1, 23, 27), cyanobacteria (21), algae (20), and fungi (19). Despite its fundamental role in N metabolism, GS has been studied in detail only in a few plant organs, such as pea leaves (17), soya bean root nodules (13), and rice roots (10). Depending on their origins, these enzymes display specific kinetic properties and, in each case, only one enzyme was investigated but, recently, isoforms of glutamine synthetase have been found in soybean (24), barley (12), and rice (6). In both rice leaves and barley leaves, two enzymes designated as GS1 and GS2 have been characterized. GS1 is predominant in etiolated leaves and GS2 was the major form in green leaves. GS2 located in chloroplasts (7, 12) was also found to be present in etioplasts (7). GS2, absent from the plastids appeared to be a cytosolic enzyme (7, 12). Only one glutamine synthetase was detected in rice and barley roots (10, 12). This enzyme is located mainly in the cytosol (16), although the presence of a low amount in plastids has also been suggested (14). Here, the chromatographic, kinetic, and regulatory properties of the glutamine synthetase isoforms present in rice roots and leaves have been investigated. It appears that the root form named GS2 is different from GS1 and GS2 present in leaves. The metabolic role of these three isoforms is discussed in relation to their intracellular location and their properties.

MATERIALS AND METHODS

MATERIALS

$n$-Hexane and CCl$_4$ were analytical grade. Ammonium sulfate grade I, hydroxylamine, Tris, and Coomassie brilliant blue R were purchased from Sigma and ATP and L-glutamate were from Boehringer (Mannheim). DEAE-Sephacel, Sephacryl S-300, polyacrylamide gel gradient PAA 4/30, and high mol wt proteins calibration kit were purchased from Pharmacia (Uppsala) and hydroxyapatite Bio-Gel HT was from Bio-Rad Laboratories (Richmond, Calif.).

PLANT CULTURE

Oryza sativa L. (var. Delta) was grown for 2 weeks on a modified Lockard solution (6), in a controlled environment chamber at 29°C during light exposure and 25°C in the dark. The daylength was 14 h and the light intensity was about 20,000 lux. RH was 60% during the day and 80% during the night. Etiolated plants were grown in a dark chamber at 29°C for 10 days on the same growth medium but at 80% RH.

EXTRACTION AND PURIFICATION OF ENZYMES

All operations were carried out at 4°C.

Step 1/2: Extraction and Ammonium Sulfate Precipitation of GS1 and GS2. Samples (200 g) fresh etiolated leaves or fresh roots were ground in 2 liters 100 mM Tris-HCl buffer (pH 7.6) containing 1 mM MgCl$_2$, 1 mM EDTA, and 10 mM 2-mercaptoethanol in a Waring Blender for 4 min. The brei was filtered through two layers of cheesecloth and centrifuged at 20,000g for 30 min. The soluble proteins were fractionated by ammonium sulfate precipitation, between 40 and 60% of saturation, by adding progressively solid ammonium sulfate and gently mixing the solution for 20 min. They were collected by centrifugation at 17,000g for 5 min in a Beckman J 21 centrifuge and redissolved in 50 ml 10 mM Tris-HCl buffer containing 1 mM MgCl$_2$ and 1 mM EDTA. This preparation was dialyzed against the same buffer for 12 h.

Step 1/2: Isolation of Chloroplasts in Nonaqueous Medium. The chloroplastic location of GS2 has already been established (7, 12); then nonaqueous medium was used for the isolation of rice chloroplasts (7). Forty g lyophilized green leaves were ground in 800 ml of an organic solvent mixture (density = 1.172) containing 10 volumes CCl$_4$ and 8 volumes $n$-hexane in a Waring Blender running at maximum speed three times for 5 s. The homogenate was filtered through two layers of cheesecloth and centrifuged for
zymograms. gradient destaining apparatus. Mol wt estimation was performed by using hydroxamate glutamyl to according (232,000), achieved and mol wt. phoresis experimental O'Neal mine synthetase NH2OH or by based on in the below. fractions ml pre pared for 5 s, and centrifuged 15 min at 20,000g.

Step 2: DEAE-Sephacel Column Chromatography of GS1, GS2, and GS3. Protein extracts prepared as described in step 1 were layered on the top of a DEAE-Sephacel column (20 x 2 cm) previously equilibrated in a 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM MgCl2. A linear gradient of 0 to 0.4 M NaCl dissolved in 200 ml equilibrating buffer was used to elute the proteins. Four-ml fractions were collected and the flow rate was adjusted to 20 ml h^{-1}.

Step 3: Gel Filtration for GS1, GS2, and GS3. After DEAE-Sephacel chromatography, the fractions exhibiting activities higher than 0.25 unit ml^{-1} were pooled and precipitated by ammonium sulfate at 80% saturation as described in step 1. The preparation was centrifuged for 5 min at 17,000g and the pellet of proteins was dissolved in 2 ml 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM MgCl2 and 2 mM sucrose. This was layered on a Sephacryl S-300 column (100 x 2.5 cm) previously equilibrated with a 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM MgCl2. Elution was done with the same buffer at a flow rate of 10 ml h^{-1}. Two-ml fractions were collected.

Step 4: Hydroxyapatite Column Chromatography for GS1, GS2, and GS3. Fractions exhibiting activities higher than 0.25 unit ml^{-1} were pooled and layered on an hydroxyapatite column (10 x 1 cm) previously equilibrated with 10 mM K-phosphate (pH 7). Elution was performed by using a linear gradient of phosphate buffer (pH 7) from 0.1 to 0.3 M in a total volume of 100 ml. Two-ml fractions were collected, but only those having an activity of 0.25 unit ml^{-1} were pooled and treated with ammonium sulfate (80% of saturation). This was centrifuged for 5 min at 17,000g, dissolved in 0.1 ml 10 mM Tris-HCl buffer and desalted through a Sephadex G-25 column (5 x 0.5 cm) previously equilibrated with the same buffer. At this stage, the purity of the preparation was checked by polyacrylamide gel electrophoresis as described below.

DETERMINATION OF ENZYME ACTIVITY AND PROTEIN MEASUREMENTS

Proteins were determined by the Scopes method (22). Glutamine synthetase was assayed by using the biosynthetic reaction based on \gamma-glutamyl hydroxamate formation in the presence of NH2OH or by a coupled reaction with NH4^{+} as substrate. The experimental procedures were identical to those described by O'Neal and Joy (17) except that diethylenetriamine pentaacetic acid was omitted. One unit of the activity represents 1 \mu mol \gamma-glutamyl hydroxamate formed min^{-1}.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Disc-gel electrophoresis was performed on 5% acrylamide gels according to Davis (4). For the procedure, the gels were incubated 15 min in the standard reaction medium to obtain GSzymograms. Bands containing enzyme activity were visualized adding Fiske and SubbaRow reagent (5) or 0.2 M CaCl2. Electrophoresis in presence of SDS was conducted according to Weber and Osborn (26) by using 10% acrylamide gels to study subunit mol wt. Protein bands were stained 4 h in Coomassie brilliant blue R and destained overnight in 7% CH3COOH solution in a Pluger destaining apparatus. Mol wt estimation was performed by using polyacrylamide gradient gel PAA 4/30. Gel calibration was achieved by using thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000) as standards.
GLUTAMINE SYNTHETASE IN RICE

To the major protein band. Minor contaminations were observed, but their contributions represented less than 10% of the total protein. Extra bands disappeared if in each step only the peak fraction was pooled.

MOL WT

The mol wt of GS1, GS2, and GS3 was about 330,000 as estimated by polyacrylamide gradient gel electrophoresis. Only one type of subunit was identified by SDS-polyacrylamide gel electrophoresis, the mol wt of which was approximately 45,000 for each of the three isoforms.

KINETIC AND REGULATORY PROPERTIES OF GS1, GS2, AND GS3

Kinetic and regulatory properties of GS1, GS2, and GS3 were studied with purified enzymes.

Optimum pH. In the presence of Mg2+, GS1 and GS2 exhibited similar pH optima (7.5) but, towards the acidic pH, the activity of GS1 did not decline as steeply as that of GS2 (Fig. 3). Mn2+ shifted the pH optimum for the two isoforms to 5.5. GS2 exhibited a clearly different response to pH. With Mn2+ and Mg2+, pH optima were always higher, being 6.2 and 7.9, respectively.

Thermal Stability. Heat denaturation of GS1, GS2, and GS3 are shown in Figure 4. GS2 and GS3 were very heat-labile at 45°C; after 15 min, 80% of the original activities disappeared for both enzymes and, after 60 min, almost complete denaturation occurred. GS1 was quite stable; no denaturation was observed during the first 30 min.

Km Values for Substrates. Km for L-glutamate. ATP, and NH2OH were determined by Lineweaver and Burk plots (Table II). Assays based on γ-glutamyl hydroxamate formation or on the coupled spectrophotometric method gave similar results. When NH2OH was used, substrate Km values were identical for the three enzymes. For the two other substrates, GS1 and GS3 exhibited

![Graph 1](image1.png)

**Fig. 2.** Elution patterns of rice GS3 (▲), GS1 (●●), and GS2 (○○) from an hydroxyapatite column. The samples were prepared as described under "Materials and Methods" after DEAE-Sephacel chromatography. Proteins, 14 mg containing 16 units GS activity, 68 mg containing 34 units GS activity, and 4 mg containing 8 units GS activity were applied to the column (10 x 1 cm). Elution of proteins was performed by progressively mixing 50 ml 100 mM K-phosphate (pH 7) with 50 ml 300 mM K-phosphate (pH 7). Two-ml fractions were collected and 200-μl aliquots were assayed for biosynthetic potency with hydroxylamine. Maximum activities for GS3, GS1, and GS2 were 2, 4, and 1, respectively.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etiolated leaf crude extract</td>
<td>1,456</td>
<td>145.6</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>(NH4)2SO4 (40-60%),</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>64.3</td>
<td>26</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>0.4</td>
<td>9.3</td>
<td>23.2</td>
<td>232</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.090</td>
<td>3</td>
<td>33.3</td>
<td>333</td>
</tr>
</tbody>
</table>

| GS2                        |               |             |                   |              |
| Nonaqueous isolated chloroplasts | 208         | 33.6       | 0.16              | 1            |
| DEAE-Sephacel              | 33.6          | 3           | 0.23              | 1.4          |
| Sephacryl S-300            | 0.7           | 2.8         | 4                 | 25           |
| Hydroxyapatite             | 0.060         | 1.86        | 31                | 193          |

| GS3                        |               |             |                   |              |
| Root crude extract         | 301           | 137.5       | 0.45              | 1            |
| (NH4)2SO4 (40-60%),        |               |             |                   |              |
| DEAE-Sephacel              | 13.7          | 16          | 1.17              | 2.6          |
| Sephacryl S-300            | 0.140         | 2.6         | 18.5              | 41.1         |
| Hydroxyapatite             | 0.028         | 1           | 35.7              | 79.3         |

![Graph 2](image2.png)

**Fig. 3.** Optimum pH for GS1 (●●), GS2 (○○), and GS3 (▲▲) in the presence of 20 mM Mg2+ or 20 mM Mn2+. The reaction mixture contained 80 mM L-glutamate, 8 mM ATP, and 6 mM NH2OH.

Assays were performed in 0.2 m acetate buffer between pH 4 and 7 and in 0.2 m Tris-HCl between pH 7 and 9. Glutamine synthetase activity was determined from the reaction with hydroxylamine. Maximum activities of GS1, GS2, and GS3 were about 0.05 unit with Mg2+ or Mn2+.

![Graph 3](image3.png)

**Fig. 4.** Thermal inactivation curve of GS1 (●●), GS2 (○○), and GS3 (▲▲). Samples containing 0.05 unit glutamine synthetase activity were incubated for 30 min at 45°C in 50 mM Tris-HCl. After incubation, the solutions were kept on ice, and the activity was measured under standard assay conditions for reaction with hydroxylamine. Maximum activities for GS1, GS2, and GS3 were about 0.05 unit.

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Table II. Michaelis Constants for Substrates of GS1, GS2, and GS3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GS1</th>
<th>GS2</th>
<th>GS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>ATP</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>NH2OH</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table III. Action of Effectors on Activity of GS1, GS2, and GS3

<table>
<thead>
<tr>
<th>Effecter (5 mM)</th>
<th>GS1</th>
<th>GS2</th>
<th>GS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>64</td>
<td>66</td>
<td>76</td>
</tr>
<tr>
<td>NADH</td>
<td>90</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>CTP</td>
<td>62</td>
<td>74</td>
<td>93</td>
</tr>
<tr>
<td>GTP</td>
<td>50</td>
<td>66</td>
<td>80</td>
</tr>
<tr>
<td>GlcNH2-P</td>
<td>98</td>
<td>10</td>
<td>64</td>
</tr>
</tbody>
</table>

identical $K_m$ values for L-glutamate (2 mm) and for ATP (1 mm). The $K_m$ values of GS2 for ATP (2 mm) and for L-glutamate (20 mm) were much higher than those found for GS1 and GS3.

When the $Mg^{2+}$ concentration was varied, a sigmoidal response was observed for each of the three isoforms (Fig. 5). However, the shape of the curve for each isoform was unique. $Mg^{2+}$ concentration for maximum activity depended on the enzyme studied; it was 15 mm for GS1 and 10 mm for GS2 and GS3. However, $Mg^{2+}$ inhibited GS3 at concentrations higher than 10 mm. For GS2, a clear cooperative effect was observed, with a rapid increase in activity between 5 and 15 mm $Mg^{2+}$.

**Regulation.** Various effectors reported to inhibit glutamine synthetase were assayed at a 5 mm concentration. Asn, Trp, and Arg had a small effect, but only on GS2. GS1 was the most sensitive to Asn, Trp, or Arg, and GS2 was most sensitive to NADH; GS3 was almost unaffected (Table III). The most clear-cut result was the very high efficiency with which GlcNH2-P inhibited GS2 almost completely but had no effect on GS1 (Table III). L-Methionine-DL-sulfoximine, a very well known inhibitor of glutamine synthetase, had a marked effect on each enzyme.

**DISCUSSION**

In a recent publication, Wallsgrove et al. (25) showed that 40% of the GS in the pea leaf cells was in the chloroplasts; the rest was in the cytosol. The cytosolic GS external to the mitochondria is thought to play a central role in the photosynthetic N cycle (11). The results presented here suggest that GS could play this central role in ammonia assimilation in the chloroplast in the dark; in fact, it might account for the light-independent formation of glutamine described by Ito et al. (9). It has also been shown that there are two isoforms of GS in the leaf (6) and that one of the isoforms develops in the light. Mann et al. have shown that, in the barley leaves, one isoform of GS is in the cytosol and the other is in the chloroplasts (12). In another study, it was also found that GS2 was in the cytosol and GS1 was in the chloroplasts (7). GS has also been purified from peas leaves (17, 18). Many of the properties described for that enzyme are similar to those for GS2 described here. In pea leaves, the affinity for L-glutamate was directly proportional to the ATP concentration and ranged from 3.5 to 12.4 mm. In experiments described here, the low affinity for L-glutamate ($K_m = 20$ mm) was determined at 5 mm ATP and 20 mm $Mg^{2+}$ and is in agreement with the results reported by O’Neal and Joy (18). The Michaelis constants for other substrates, ATP, and NH2OH, are similar to those of glutamine synthetases of many other plants and, in fact, are very close in the three GS of rice. There is also abundant evidence that the chloroplast GS is only active in the light (2, 15). Thus, it is concluded that the level of ATP limited the rate of GS activity in the chloroplast. However, a comparison of known changes within the chloroplast in the light with the optimum conditions for GS2 suggest two other possibilities for the regulation of this enzyme. For example, during illumination the pH in the stroma is 7.9, whereas, in the dark, it is 7.0 (28). This shift in pH could account for a 50% decrease in GS activity in the dark. $Mg^{2+}$ could also be a potential regulator of GS2 activity in the chloroplast. A sigmoidal saturation curve between 5 and 15 mm $Mg^{2+}$ was obtained for GS2; thus, the cation enrichment found in the chloroplast during illumination (3) could be an important factor leading to increased GS activity. A modification in enzyme activity during illumination has been reported for many of the chloroplast enzymes involved in carbon metabolism. The modifications can involve the formation or activation of the enzymes (8).

**Comparison** of GS2 to GS1 is quite heat stable and its pH optimum (about 7.5) is lower. The $K_m$ for L-glutamate was 10-fold lower, suggesting that rates for this isoform are probably not limited by concentration of glutamate. GS1 is also much less sensitive to feedback inhibition by amino acids or GlcNH2-P.

In rice roots, as in barley roots, only one form of glutamine synthetase is detected (10, 12). In each case, it is eluted from DEAE-Sephacel with the same ionic strength as GS2. Mann et al. (12) concluded that in barley the root enzyme and GS2 were probably the same protein. However, in rice it has been demonstrated that GS1 and the root enzyme have quite different chromatographic properties. In addition, the root enzyme is much more heat-labile than GS1. Other properties, such as pH optima, $K_m$ values for substrates, and inhibition by end products, are very similar for GS2 and GS1 and also for another root enzyme described by Kanamori and Matsumoto (10). GS2 is located in the cytosol (16) as is GS1. Although located in the same cellular compartment, the physiological functions of the two enzymes are, in fact, different. GS2 is probably involved in the primary ammonia assimilation in roots and GS1 function is very likely to be implicated in the recycling of ammonia during photosynthesis but also in glutamine synthesis in the dark.
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