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Glutamine Synthetase in Rice

A COMPARATIVE STUDY OF THE ENZYMES FROM ROOTS AND LEAVES[1]

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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 lability, pH optimum about 8, K_m for L-glutamate of 20 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 a 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.

GS[2] (L-glutamate:ammonia ligase (ADP), EC 6.3.1.2), a key enzyme in ammonia assimilation, has been studied in many procaryotic 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 GS_1 and GS_2 have been characterized. GS_1 was predominant in etiolated leaves and GS_2 was the major form in green leaves. GS_2 located in chloroplasts (7, 12) was also found to be present in etioplasts (7). GS_1 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 GS_R is different from GS_1 and GS_2 present in leaves. The metabolic role of these three isoforms is discussed in relation to their intracellular location and their properties.

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[2] Abbreviations: GS: glutamine synthetase; $GlcNH_2$6-P: glucosamine 6-phosphate; GS_1 and GS_2: leaf glutamine synthetase; GS_R: root glutamine synthetase.

MATERIALS AND METHODS

MATERIALS

n-Hexane and CCl_4 were analytical grade. Ammonium sulfate grade I, hydroxylamine, Tris, and Coomassie brillant 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/A: Extraction and Ammonium Sulfate Precipitation of GS_1 and GS_R. 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 Blendor for 4 min. The brei was filtered through two layers of cheesecloth and centrifuged at 20,000*g* 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 17,000*g* 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/B: Isolation of Chloroplasts in Nonaqueous Medium. The chloroplastic location of GS_2 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 Blendor running at maximum speed three times for 5 s. The homogenate was filtered through two layers of cheesecloth and centrifuged for

5 min at 4,000g. The supernatant was discarded and the residual solvent of the pellet was thoroughly eliminated under vacuum in a desiccator kept cold in an ice bath. The dry pellet then was resuspended in 50 ml 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$ and 10 mM DTT, homogeneized with a "Polytron mixer" for 5 s, and centrifuged 15 min at 20,000g.

Step 2: DEAE-Sephacel Column Chromatography of GS_1, GS_2, and GS_R. Protein extracts prepared as described in step 1 were layered on the top of a DEAE-Sephacel column (20 × 2 cm) previously equilibrated in a 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$. 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 GS_1, GS_2, and GS_R. 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 $MgCl_2$ and 2 M sucrose. This was layered on a Sephacryl S-300 column (100 × 2.5 cm) previously equilibrated with a 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$. 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 GS_1, GS_2, and GS_R. Fractions exhibiting activities higher than 0.25 unit ml^{-1} were pooled and layered on an hydroxyapatite column (10 × 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 × 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 γ-glutamyl hydroxamate formation in the presence of NH_2OH or by a coupled reaction with NH_4^+ 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 μmol γ-glutamyl hydroxamate formed min^{-1}.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Disc-gel electrophoresis was performed on 5% acrylamide gels according to Davis (4). After the procedure, the gels were incubated 15 min in the standard reaction medium to obtain GS zymograms. Bands containing enzyme activity were visualized adding Fiske and SubbaRow reagent (5) or 0.2 M $CaCl_2$. 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 brillant blue R and destained overnight in 7% CH_3COOH 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.

RESULTS

CHROMATOGRAPHIC AND ELECTROPHORETIC PROPERTIES OF GS_1, GS_2, AND GS_R

Since GS_1 was the predominant form in etiolated leaves (6), etiolated material was used for the purification of GS_1. The results in Figure 1 show that GS_1 was the predominant form. For the GS_2 isoform, a nonaqueous extraction procedure for the isolation of chloroplasts was developed. This fraction contained mainly GS_2 (Fig. 1). Root extracts had only one GS peak which eluted at 0.15 M NaCl (Fig. 1). This is in good agreement with published results (10, 12). In this investigation, the root and leaf enzymes were also purified on hydroxyapatite (Fig. 2). GS_R was eluted with 0.14 M phosphate buffer (Fig. 2), whereas GS_1 and GS_2 came off at 0.18 and 0.22 M, respectively (Fig. 2). To establish these different elution patterns, further hydroxyapatite column chromatography was carried out using a mixture of extracts from roots and etiolated leaves. Again, GS_1 and GS_R were eluted with ionic strengths of 0.14 and 0.18 as above. Results obtained with 5% polyacrylamide gel electrophoresis also indicated distinct GS bands for extracts obtained from roots (R_F = 0.23) and etiolated leaves (R_F = 0.34 and 0.49).

These specific chromatographic and electrophoretic properties of the three enzymes suggest that GS_1, GS_2, and GS_R are different proteins.

PURIFICATION OF GS_1, GS_2, AND GS_R

The purification of each of the enzymes is summarized in Table I. The best purification was obtained for GS_1, but the final specific activity obtained was almost the same for each isoform. The purity of these proteins was estimated by electrophoresis on polyacrylamide gels. The band exhibiting the enzyme activity corresponded

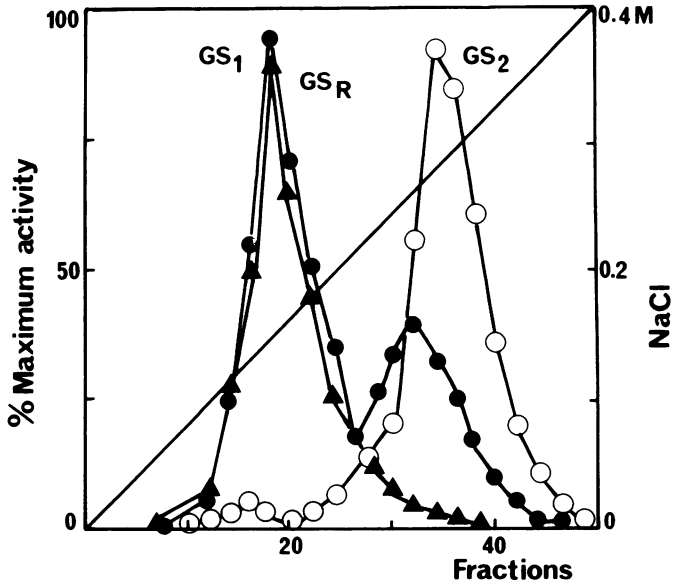

FIG. 1. Elution patterns of glutamine synthetase of a rice root extract (▲——▲), etiolated leaf extract (●——●), and chloroplast extract (○——○) from a DEAE-Sephacel column. The samples were prepared as described under "Materials and Methods." Proteins, 260 mg containing 137 units GS activity, 1300 mg containing 145 units GS activity, and 208 mg containing 34 units of GS activity were applied to the column (20 × 2 cm). Elution of proteins was performed by progressively mixing 100 ml 10 mM Tris-HCl (pH 7.6), containing 1 mM $MgCl_2$ with 100 ml of the same 0.4 M buffer in NaCl. Four-ml fractions were collected and 200-μl aliquots were assayed for biosynthetic potency with hydroxylamine. Maximum activities for GS_R, GS_1, and GS_2 were 4, 8, and 2 units, respectively.

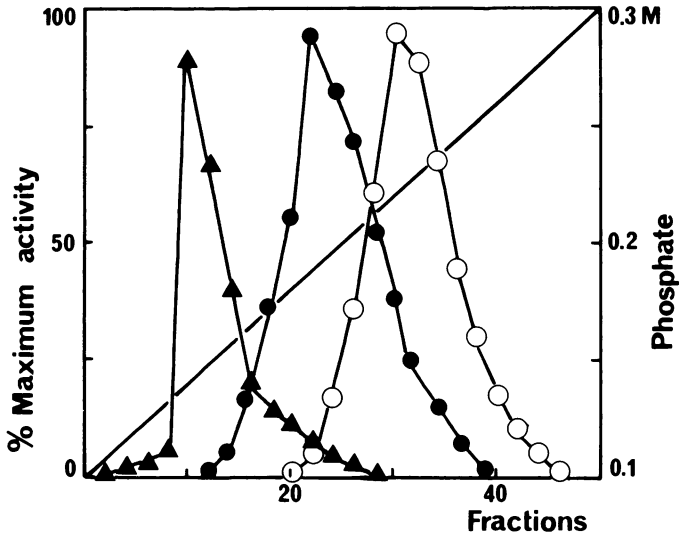

FIG. 2. Elution patterns of rice GS_R (▲——▲), GS_1 (●——●), and GS_2 (○——○) from an hydroxyapatite column. The samples were prepared as described under "Materials and Methods" after DEAE-Sephacel chromatography. Proteins, 14 mg containing 16 units GS activity, 68 mg containing 34 units GS activity, and 4 mg containing 8 units GS activity were applied to the column (10 × 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 GS_R, GS_1, and GS_2 were 2, 4, and 1 units, respectively.

Table I. *Purification of GS_1, GS_2, and GS_R from Rice Plants*

Purification Step	Total Protein	Total Units	Specific Activity	Purification
	mg	*μmol/min*	*μmol/min·mg protein*	*-fold*
GS_1				
Etiolated leaf crude extract	1,456	145.6	0.1	1
$(NH_4)_2SO_4$ (40–60%), DEAE-Sephacel	64.3	26	0.4	4
Sephacryl S-300	0.4	9.3	23.2	232
Hydroxyapatite	0.090	3	33.3	333
GS_2				
Nonaqueos isolated chloroplasts	208	33.6	0.16	1
DEAE-Sephacel	33.6	8	0.23	1.4
Sephacryl S-300	0.7	2.8	4	25
Hydroxyapatite	0.060	1.86	31	193
GS_R				
Root crude extract	301	137.5	0.45	1
$(NH_4)_2SO_4$ (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

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 GS_1, GS_2, and GS_R 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 GS_1, GS_2, AND GS_R

Kinetic and regulatory properties of GS_1, GS_2, and GS_R were studied with purified enzymes.

Optimum pH. In the presence of Mg^{2+}, GS_1 and GS_R exhibited similar pH optimums (7.5) but, towards the acidic pH, the activity of GS_R did not decline as steeply as that of GS_1 (Fig. 3). Mn^{2+} shifted the pH optimum for the two isoforms to 5.5. GS_2 exhibited a clearly different response to pH. With Mn^{2+} and Mg^{2+}, pH optima were always higher, being 6.2 and 7.9, respectively.

Thermal Stability. Heat denaturation of GS_1, GS_2, and GS_R are shown in Figure 4. GS_2 and GS_R 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. GS_1 was quite stable; no denaturation was observed during the first 30 min.

***K_m* Values for Substrates.** K_m for L-glutamate, ATP, and NH_2OH 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 NH_2OH was used, substrate K_m values were identical for the three enzymes. For the two other substrates, GS_1 and GS_R exhibited

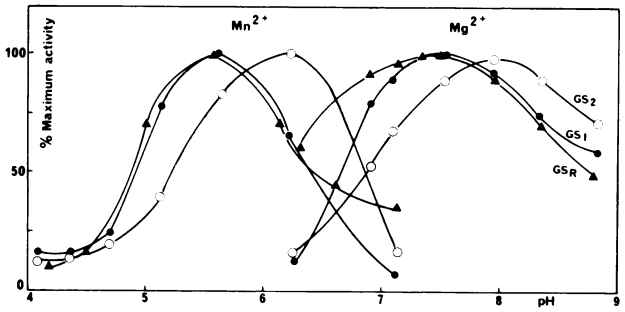

FIG. 3. Optimum pH for GS_1 (●——●), GS_2 (○——○), and GS_R (▲——▲) in the presence of 20 mM Mg^{2+} or 20 mM Mn^{2+}. The reaction mixture contained 80 mM L-glutamate, 8 mM ATP, and 6 mM NH_2OH. Assays were performed in 0.2 M acetate buffer between pH 4 and 7 and in 0.2 M Tris-HCl between pH 7 and 9. Glutamine synthetase activity was determined from the reaction with hydroxylamine. Maximum activities of GS_1, GS_2, and GS_R were about 0.05 unit with Mg^{2+} or Mn^{2+}.

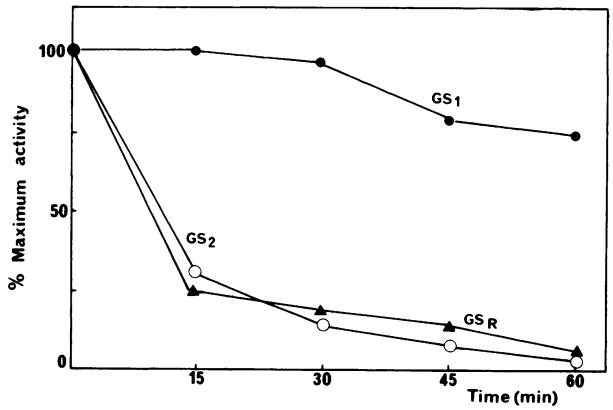

FIG. 4. Thermal inactivation curve of GS_1 (●——●), GS_2 (○——○), and GS_R (▲——▲). Samples containing 0.05 unit glutamine synthetase activity were incubated for 30 min at 45 C in 50 mM Tris-HCl. After incubation, the solutions were kept on ice, and the activity was measured under standard assay conditions for reaction with hydroxylamine. Maximum activities for GS_1, GS_2, and GS_R were about 0.05 unit.

Table II. *Michaelis Constants for Substrates of GS_1, GS_2, and GS_R*

Substrate	Enzyme		
	GS_1	GS_2	GS_R
		mM	
L-Glutamate	2	20	2
ATP	1	2	1
NH_2OH	1	1	1

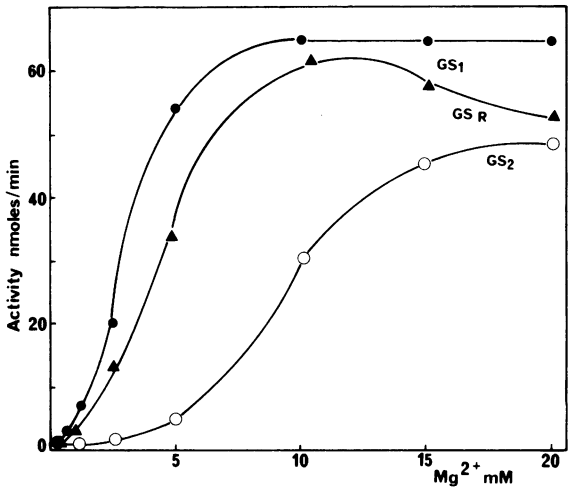

FIG. 5. Effect of Mg^{2+} concentration on the activity of GS_1 (●——●), GS_2 (○——○), and GS_R (▲——▲). The reaction mixture contained 80 mM L-glutamate, 8 mM ATP, 6 mM NH_2OH, and various concentrations of Mg^{2+} (20–0.625 mM). Samples containing 0.05 unit enzyme were added to the reaction mixture and glutamine synthetase activity was measured from the reaction with hydroxylamine.

Table III. *Action of Effectors on Activity of GS_1, GS_2, and GS_R*

Effector (5 mM)	Enzyme		
	GS_1	GS_2	GS_R
		% control activity	
AMP	64	66	76
NADH	90	54	100
CTP	62	74	93
GTP	50	66	80
$GlcNH_2$6-P	98	10	64

identical K_m values for L-glutamate (2 mM) and for ATP (1 mM). The K_m values of GS_2 for ATP (2 mM) and for L-glutamate (20 mM) were much higher than those found for GS_1 and GS_R.

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 GS_2 and 10 mM for GS_1 and GS_R. However, Mg^{2+} inhibited GS_R at concentrations higher than 10 mM. For GS_2, 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 GS_2. GS_1 was the most sensitive to AMP, CTP, or GTP, and GS_2 was most sensitive to NADH; GS_R was almost unaffected (Table III). The most clear-cut result was the very high efficiency with which $GlcNH_2$6-P inhibited GS_2 almost completely but had no effect on GS_1 (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 photorespiratory N cycle (11). The results presented here suggest that GS_1 could play this central role in ammonia assimilation in the cytosol 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 GS_1 was in the cytosol and GS_2 was in the chloroplasts (7). GS has also been purified from pea leaves (17, 18). Many of the properties described for that enzyme are similar to those for GS_2 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 8 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 NH_2OH, 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 GS_2 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 GS_2 activity in the chloroplast. A sigmoidal saturation curve between 5 and 15 mM in Mg^{2+} was obtained for GS_2; thus, the cation enrichment found in the chloroplast during illumination (3) could be an important factor leading to increased GS_2 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 and or activation of the enzymes (8).

Compared to GS_2, GS_1 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. GS_1 is also much less sensitive to feedback inhibition by amino acids or $GlcNH_2$6-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 GS_1. Mann *et al.* (12) concluded that in barley the root enzyme and GS_1 were probably the same protein. However, in rice it has here been demonstrated that GS_1 and the root enzyme have quite different chromatographic properties. In addition, the root enzyme is much more heat-labile than GS_1. Other properties, such as pH optima, K_m values for substrates, and inhibition by end products, are very similar for GS_R and GS_1 and also for another root enzyme described by Kanamori and Matsumoto (10). GS_R is located in the cytosol (16) as is GS_1. Although located in the same cellular compartment, the physiological functions of the two enzymes are, in fact, different. GS_R is probably involved in the primary ammonia assimilation in roots and GS_1 function is very likely to be implicated in the recycling of ammonia during photorespiration but also in glutamine synthesis in the dark.

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