Occurrence of ferredoxin-dependent glutamate synthase in plant cell fraction of soybean root nodules *(Glycine max)*

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Ferredoxin-dependent glutamate synthase (EC 1.4.7.1) and NADH-dependent glutamate synthase (EC 1.4.1.14) have been identified in the plant cells of soybean nodules. Ferredoxin-dependent glutamate synthase is 2-fold more active than NADH-dependent enzyme in vitro. Ferredoxin-dependent glutamate synthase cross-reacts with IgG against ferredoxin-dependent glutamate synthase of rice green leaves, whereas NADH-dependent glutamate synthase does not recognize the IgG, indicating that there are two distinct enzyme proteins. Ferredoxin-dependent glutamate synthase is composed of polypeptide chain(s) of 165 kDa and has a high affinity to spinach leaf ferredoxin as an electron carrier.

Glutamate synthase *Isoform* *Ferredoxin* *Pyridine nucleotide*
Glycine max *Immunoglobulin G*

1. INTRODUCTION

Glutamate synthase (glutamine : 2-oxoglutarate aminotransferase, GOGAT) catalyzes the second step of ammonia nitrogen assimilation via the amido group of glutamine to the α-amino group of glutamate. The enzyme has been shown to be present in various cell organisms [1–3], and 3 forms of glutamate synthase have been characterized by their electron donor specificity for NADPH (EC 1.4.1.13), NADH (EC 1.4.1.14), and reduced ferredoxin (EC 1.4.7.1). In higher plants, both ferredoxin-dependent and NAD(P)H-dependent glutamate synthases have been shown to be widely distributed in different tissues [4–11]. In nodules, an NADH-dependent glutamate synthase has been detected in several species of plants [12–17]. We report here the presence of ferredoxin-dependent glutamate synthase in the plant cell fraction of soybean root nodules, and some physiological functions of the enzyme are discussed in relation to glutamate cycling during assimilation of ammonia derived from dinitrogen fixation.

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2. MATERIALS AND METHODS

2.1. Plant material

Soybean (*Glycine max* L. Weber) seeds were germinated and inoculated in vermiculite with *Rhizobium japonicum* strain G 49 obtained from Laboratoire de Microbiologie des Sols, Dijon. Nodules were grown under 16 h light ($250\,\mu E \cdot m^{-2} \cdot s^{-1}$) per day at 27°C and 70% humidity, and harvested 16 days after soaking.

2.2. Enzyme extraction

Nodules were suspended and extracted in 10 vols (w/v) of 100 mM Tris–HCl (pH 8.0) containing 0.5 M sucrose, 1 mM NAD, 14 mM β-mercaptoethanol, and 20% (g/g fresh wt) polyclar AT. The homogenate was filtered through cheese-cloth, and centrifuged at $100\,000 \times g$ for 15 min. The resulting supernatant was used for enzyme assays. The pellet containing bacteroids was subjected to

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sonication (Annemasse SA, France) for 1 min at 40 W to determine enzyme activities.

2.3. Immunochemical analysis

Antibodies against ferredoxin-dependent glutamate synthase from green leaves of rice were prepared as in [11]. A double diffusion test was performed on 2% (w/v) agar plate according to [18]. Immunotitration analysis was carried out by incubating the enzyme sample with antibodies for 12 h at 4°C. After centrifugation at 10000 × *g* for 10 min, enzyme activity was measured in the supernatant fraction.

2.4. Electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as in [19]. Immunoprecipitated proteins were dissociated by incubating at 100°C for 10 min in the presence of 1% SDS and 1% β-mercaptoethanol. Using 5% polyacrylamide gels, a constant electric current of 4 mA/tube was applied for 5 h at room temperature. After electrophoresis, gels were stained with Coomassie brillant blue R, and destained in 7.5% (v/v) acetic acid and 5% (v/v) methanol solution. Molecular masses of polypeptides were estimated by calibration with cross-linked bovin serum albumin: 66, 132, 198, 264 kDa.

2.5. Enzyme assay

Ferredoxin-, methyl viologen-, or pyridine nucleotide-dependent glutamate activities were assayed as in [11]. Glutaminase (EC 3.5.1.2) was measured according to [20]. In these assays, glutamate formation was determined using high-pressure liquid chromatography as in [21]. One unit of enzyme activity represents one μmol glutamate formed·min^{-1}.

2.6. Protein determination

Protein content was determined as in [22].

3. RESULTS

Table 1 shows glutamate formation with cell-free extracts of plant cell fraction of soybean nodules. In the presence of glutamine and α-ketoglutarate, glutamate synthase activity corresponding to 0.490 unit·g^{-1} fresh wt was detected with chemically reduced spinach leaf ferredoxin as an electron carrier. When glutamine was substituted for ammonia, no significant glutamate formation was observed. Dithionite-reduced methyl viologen gave about 75% of the enzyme activity compared to the ferredoxin assay. As reported in other nodules, NADH-dependent glutamate synthase activity was present, and it represented about half relative to the ferredoxin-dependent enzyme activity. No significant glutamate was detected with NADPH as an electron donor. Glutaminase activity was absent from the plant cell fraction (table 1), and enzyme activity (420 nmol glutamate formed·min^{-1}·g^{-1} fresh wt) was restricted to the bacteroid fraction. Distribution of glutaminase, a marker enzyme of bacteroids, indicated no significant bacteroid contamination in the plant cell fraction, and the glutamate synthase activities determined in our assay conditions could not derive from bacteroids.

Table 1

Glutamate synthase activity dependent on different substrates and electron donors with cell-free extracts of plant cell fraction of soybean nodules

	nmol glutamate formed·min^{-1}·g^{-1} fresh wt
Complete with ferredoxin	490
Glutamine + NH_4Cl	13
α-Ketoglutarate–ferredoxin*	6
Ferredoxin	11
Ferredoxin + methyl viologen	407
Ferredoxin + NADH	223
Ferredoxin + NADPH	23

Glutamate synthase was assayed in a reaction mixture consisting of 2.25 μmol phosphate buffer (Na_2HPO_4–KH_2PO_4) (pH 7.3), 0.5 μmol glutamine, 0.5 μmol α-ketoglutarate and enzyme sample. The reaction mixture included 2 nmol ferredoxin or 33 nmol methyl viologen which was reduced with 0.9 μmol sodium dithionite in 1.9 μmol $NaHCO_3$, or 2.2 μmol NAD(P)H as reductant

* Concentration of glutamine was 3 μmol

Fig.1 shows that glutamate formation increased by the concomitant increase of ferredoxin concentration. Lineweaver–Burk plots gave an apparent Michaelis constant (K_m) of 1.9 μM. The K_m value of glutamate synthase for methyl viologen was estimated to be 133.3 μM in our assay conditions (not shown).

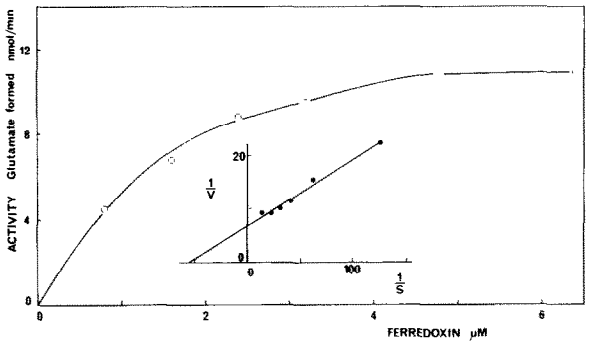

Fig.1. Glutamate synthase activity in plant cell fraction of soybean nodules dependent on different concentrations of spinach leaf ferredoxin, and corresponding Lineweaver–Burk plot.

When concentrated cell-free crude extracts were subjected to the Ouchterlony double immunodiffusion test, one immunoprecipitin line was observed with IgG anti-ferredoxin-dependent glutamate synthase of rice green leaves (fig.2). This suggests that in plant cells of soybean nodules, glutamate synthase is present which has related antigenic determinants to those of ferredoxin-dependent glutamate synthase in rice green leaves.

Glutamate synthase activity dependent on different reductants was assayed after incubation with the IgG anti-ferredoxin-dependent glutamate

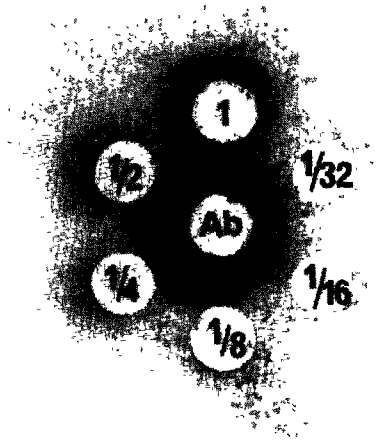

Fig.2. Ouchterlony double immunodiffusion test. The center well contained 10 μl IgG against ferredoxin-dependent glutamate of rice green leaves. The outer wells contained 10 μl of serially diluted (1 to 1/32) cell-free extract of plant fraction of soybean nodules.

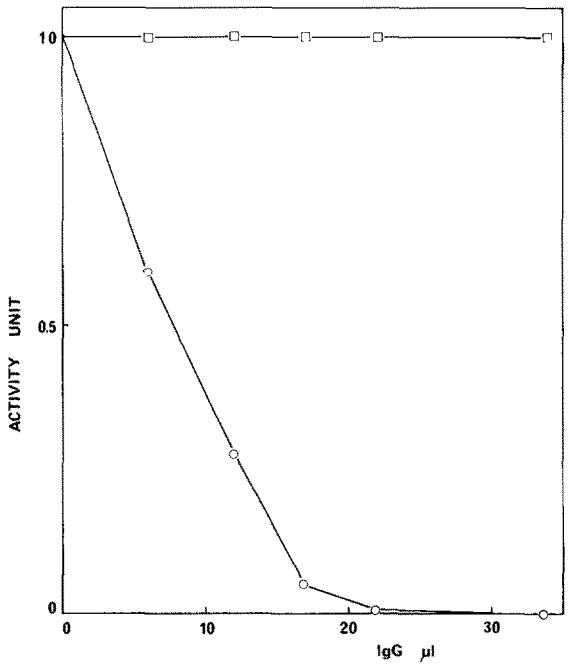

Fig.3. Immunotitration curves of glutamate synthase in plant cell fraction of soybean nodules with IgG against ferredoxin-dependent glutamate synthase of rice green leaves: assay with ferredoxin (○), and NADH (□).

synthase of rice green leaves (fig.3). Ferredoxin-dependent glutamate synthase was completely immunoprecipitated by increasing the amount of IgG. Methyl viologen-linked glutamate synthase activity was also precipitated by IgG. About 8 μl IgG was needed to precipitate one half of one unit of ferredoxin-dependent glutamate synthase. On the other hand, IgG did not cross-react with NADH-dependent glutamate synthase. Nonimmune serum had no effect on ferredoxin-, methyl viologen-, and NADH-dependent glutamate synthase activities.

Immunoprecipitated ferredoxin-dependent glutamate synthase obtained above by the immunotitration analysis was subjected to SDS–PAGE after dissociation in the presence of SDS and β-mercaptoethanol. Dissociated proteins in the gel gave only one polypeptide band of ferredoxin-dependent glutamate synthase (glutamate synthase), and heavy (H) and light (L) polypeptide chains of IgG (fig.4). The molecular mass of ferredoxin-dependent glutamate synthase polypeptide was estimated, by calibration with marker proteins, to be 165 kDa.

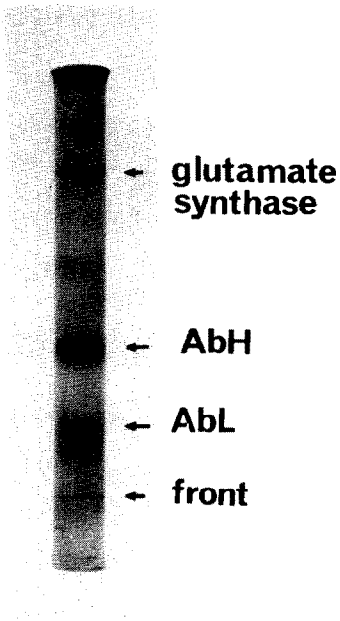

Fig.4. SDS–PAGE of ferredoxin-dependent glutamate synthase in plant cells of soybean nodules. Ferredoxin-dependent glutamate synthase was immunoprecipitated, dissociated, and subjected to electrophoresis as described in section 2.

4. DISCUSSION

In plant cell fraction of soybean root nodules, ferredoxin-dependent glutamate synthase (EC 1.4.7.1) was present together with NADH-dependent glutamate synthase (EC 1.4.1.14). No significant activity of NADPH-dependent glutamate synthase (EC 1.4.1.13) was detected. Ferredoxin-dependent glutamate synthase in cell-free crude extract represents 0.490 unit·g^{-1} fresh wt. The ferredoxin-dependent glutamate synthase is about 2-fold more active than NADH-dependent glutamate synthase in vitro. In other nodules examined, the NADH-dependent glutamate synthase level varies according to developmental stages [12] and to plant species [17,23]. It should be noted that NADH-dependent glutamate synthase activity in these nodules is comparable or higher (25–100 nmol NADH oxidized $min^{-1} \cdot mg^{-1}$ protein) than in soybean nodules, although the developmental stage is different according to the nodules used [12,16,17]. It remains to be determined whether the higher activity is related to the presence of only NADH-dependent glutamate synthase in these cells.

Ferredoxin-dependent glutamate synthase in plant cells of soybean nodules cross-reacts with IgG against ferredoxin-dependent glutamate synthase of rice green leaves. On the other hand, the IgG does not recognize NADH-dependent glutamate synthase, showing that ferredoxin-dependent glutamate synthase is a protein distinct from NADH-dependent glutamate synthase in plant cells of soybean nodules. Immunoprecipitated ferredoxin-dependent glutamate synthase has a molecular structure composed of polypeptide chain(s) of 165 kDa. Ferredoxin-dependent glutamate synthase in spinach green leaves is a single polypeptide of 180 kDa [24], and the enzyme in rice leaves consists of two identical polypeptides with molecular mass of 125 kDa [11,25]. In the plant cell fraction of lupin nodules, NADH-dependent glutamate synthase is a single polypeptide of 235 kDa [23]. It seems therefore that glutamate synthase activity is carried out by different protein(s) according to plant species. Previous studies demonstrated NADH-dependent glutamate synthase in plant cell fraction of nodules [12,23]. The enzyme is shown to be located in the plastid of plant cells, and absent from bacteroids [14,15,17]. During nodulation of root legumes, the activity of NADH-dependent glutamate synthase increases concomitantly with nitrogenase activity [12], and the enzyme was considered to be responsible for glutamate cycling during assimilation of ammonia derived from dinitrogen assimilation [13,26]. Ferredoxin-dependent glutamate synthase is in fact more active than NADH-dependent glutamate synthase in vitro, and this form of glutamate synthase could be involved in ammonia assimilation during dinitrogen assimilation in the presence of glutamine synthetase. In roots of higher plants, ferredoxin-dependent glutamate synthase is also a distinct protein molecule from NAD(P)H-dependent glutamate synthase [11], and these glutamate synthases have a strict electron donor specificity [27]. In contrast to the root ferredoxin-dependent glutamate synthase which has an apparent K_m value of 20 μM for spinach leaf ferredoxin [27], ferredoxin-dependent glutamate synthase in plant cells of soybean nodules has a higher affinity of ferredoxin (K_m 1.9 μM). The enzyme therefore has an electron donor affinity comparable to that of ferredoxin-dependent glutamate synthase in green

leaves of higher plants (K_m 1.7–5.5 μM) [25,28,29]. The presence of ferredoxin-dependent and NADH-dependent glutamate synthase isoforms in the plant cells of nodules requires further study in relation to the mechanism of regulation of their expression during nodulation of root legumes and of electron transfer to ferredoxin-dependent glutamate synthase in these cells.

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