Glutamate Synthase Isoforms in Rice

IMMUNOLOGICAL STUDIES OF ENZYMES IN GREEN LEAF, ETIOLATED LEAF, AND ROOT TISSUES

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ABSTRACT

Rabbit antiserum was raised against ferredoxin-dependent glutamate synthase (EC 1.4.7.1) purified from green leaves of *Oryza sativa* L. cv Delta. Ferredoxin-dependent glutamate synthase, detected in green leaf, etiolated leaf, and root tissues cross-reacted completely with the anti-ferredoxin glutamate synthase immunoglobulin G. In contrast, the immunoglobulin G did not cross-react with NADH-dependent (EC 1.4.1.14) and NADPH-dependent (EC 1.4.1.13) glutamate synthases found in nonphotosynthetic etiolated leaf and root tissues. In addition, ferredoxin-dependent glutamate synthase was separated and distinguished by its affinity to ferredoxin from NAD(P)H-dependent glutamate synthase on ferredoxin-Sepharose affinity chromatography. Based on the immunological studies, it is suggested that ferredoxin-dependent glutamate synthases in green leaf and etiolated leaf tissues are closely related proteins; in contrast, ferredoxin-dependent glutamate synthase in root tissue is a distinct protein from the leaf enzymes.

It is now well accepted that glutamate synthase (glutamine:α-ketoglutarate aminotransferase; GOGAT[2]) catalyzes the second step of nitrogen assimilation into glutamate via glutamine in the presence of glutamine synthetase (EC 6.3.1.2) in bacteria (18, 32), algae (10, 14), nitrogen-fixing organisms (3, 24), and higher plants (16).

In higher plants, glutamate synthase was first identified in cultured cells (5) and roots (6) in which the enzyme was found to be specific for reduced pyridine nucleotides: NADH (EC 1.4.1.14) or NADPH (EC 1.4.1.13) as electron donors. Later, Fd-dependent glutamate synthase (EC 1.4.1.7) was characterized in higher plants (9, 23) and algae (10, 24). In nonphotosynthetic tissues of higher plants, further studies have shown the presence of a Fd-dependent glutamate synthase activity together with NAD(P)H-dependent glutamate synthase in soybean cotyledons (28) and pea roots (17). In contrast, glutamate synthase in developing shoots of halophytes (27) and rice roots (1) was specific for Fd and was inactive with NAD(P)H. Recently, two forms of glutamate synthase have been identified in pea cotyledons (12), etiolated pea shoots (13), *Chlamydomonas* (4) in which Fd and NADH were physiological reductants.

We describe here the presence of Fd-dependent and pyridine nucleotide-dependent glutamate synthases in rice green leaf, etiolated leaf, and root tissues, and immunological properties of the enzymes are studied using antibodies raised against Fd-dependent glutamate synthase from rice green leaves.

[1] Recipient of a scholarship from the French Foreign Ministry

[2] Abbreviations: GOGAT, glutamate synthase; IgG, immunoglobulin G.

MATERIALS AND METHODS

Plant Materials. *Oryza sativa* L. cv Delta was germinated and grown for 14 d on a 0.1 strength Lockard solution (11). In a growth chamber, day and night temperatures were regulated to be 29°C and 25°C, respectively, with a 14-h photoperiod. Etiolated plants were grown on the same medium in darkness at 29°C for 10 d. Root tissue was obtained from seedlings germinated on agar as already described (30).

Standard Enzyme Extraction. All procedures were carried out at 4°C. Leaf and root tissues were homogenated in a Waring Blendor with 10 volumes of 25 mM phosphate buffer ($Na_2HPO_4 \cdot KH_2PO_4$), pH 7.5 (standard buffer), containing 14 mM β-mercaptoethanol and 1 mM EDTA. After filtration through four layers of cheesecloth, the filtrate was centrifuged at 11,000*g* for 30 min. The 30% to 70% saturated ammonium sulfate fraction was taken. The precipitate was dissolved in the standard buffer containing 14 mM β-mercaptoethanol and dialyzed for 12 h against the same buffer. The glutamate synthase thus obtained was used for enzyme assays and immunological studies.

Purification of Fd-Glutamate Synthase from Rice Green Leaf Tissue. Fd-dependent glutamate synthase in rice green leaf tissue was purified to homogeneity by the method described previously (29).

Immunization and Preparation of Rabbit Anti-Fd-Glutamate Synthase Antibodies. Homogeneous Fd-glutamate synthase (350 μg) emulsified with the complete Freund's adjuvant was administrated subcutaneously to a rabbit (Fauve de Bourgogne). The booster injection was performed 4 weeks later by the same amount of the antigen through an ear vein. One week after the second administration, the serum was collected and the λ-globulin fraction was purified by 0% to 33% saturated ammonium sulfate fractionation. The IgG was dissolved in 150 mM borate buffer, pH 8.1, containing 0.8% NaCl and was dialyzed against the same buffer. Nonimmune serum was taken from the rabbit before immunization.

Immunotitration Analysis. Immunotitration was performed by incubating antibodies with enzyme samples for 15 min at room temperature and then for 12 h at 4°C. The antigen-antibody complex was collected by centrifugation at 10,000*g* for 10 min. Glutamate synthase activity was assayed in the supernatant fraction, and the immunoprecipitate was washed three times with 25 mM sodium phosphate buffer, pH 7.5, prior to SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis. Immunoprecipitated proteins were dissociated by incubating at 100°C for 10 min in the presence of 1% SDS and 1% β-mercaptoethanol and subjected to electrophoresis on 5% polyacrylamide gel according to the method of Weber and Osborn (34). After electrophoresis, the gels

were stained for 30 min in Coomassie Blue R, and destained in 7.5% (v/v) acetic acid and 5% (v/v) methanol solution. The mol wt of the polypeptide chains were estimated by calibrating with standard mol wt proteins: cross-linked albumin bovin (264,000 D, 198,000 D, 132,000 D, 66,000 D).

Ouchterlony Double Immunodiffusion. Double immunodiffusion analysis was carried out according to the method of Ouchterlony (20) on 2% (w/v) agar plates as we reported previously (8). The plates were incubated at 4°C for 6 to 12 h and immunoprecipitin bands were stained with Coomassie Brillant Blue R.

Affinity Column Chromatography. Glutamate synthase preparation obtained by standard extraction procedure was applied to Fd-Sepharose 4B column, equilibrated with the standard buffer containing 14 mM β-mercaptoethanol. After washing with the same buffer, glutamate synthase was eluted with linear NaCl gradient from 0 (50 ml) to 400 mM (50 ml) at a flow rate of 6.5 ml/h as described previously (29).

Enzyme Assay and Protein Determination. Glutamate synthase activities dependent on Fd, methyl viologen, or NAD(P)H were assayed either by determining glutamate formation or by following the decrease in A at 340 nm spectrophotometrically as described previously (29, 30). One unit of enzyme activity represents 1 μmol of glutamate formed/min at 30°C. Proteins were determined by the method of Scopes (25).

RESULTS

Specificity of Rabbit Anti-Fd-Glutamate Synthase IgG. The specific cross-reactivity of the IgG fraction was tested by Ouchterlony double immunodiffusion analysis (Fig. 1). Serial dilution of the anti-Fd-glutamate synthase IgG formed a single precipitin line with the enzyme from green leaf tissue. No contaminating band was detected. The IgG fraction of nonimmune serum gave no immunoprecipitin band with the enzyme from green leaf tissue, suggesting strongly that the IgG obtained is monospecific to Fd-glutamate synthase from rice green leaf tissue. The anti-Fd-glutamate synthase IgG was therefore used for further studies on glutamate synthase from leaf and root tissues.

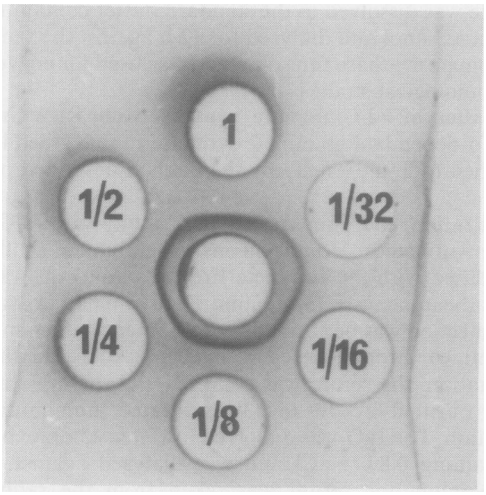

FIG. 1. Ouchterlony double immunodiffusion. The center well contained 10 μl of the standard enzyme extract, and the outer well contained serial dilution (1 to 1/32) of the anti-Fd-glutamate synthase IgG.

Table I. *Comparison of Glutamate Synthase Activity in Rice Green Leaf, Etiolated Leaf, and Root Tissues*

Reductant	Green Leaf	Etiolated Leaf	Root
	nmol glutamate formed/min·g fresh wt		
Fd	2308.7	339.5	88.0
Methyl viologen	918.9	126.0	22.0
NADH	0	90.3	16.7
NADPH	0	87.6	17.1

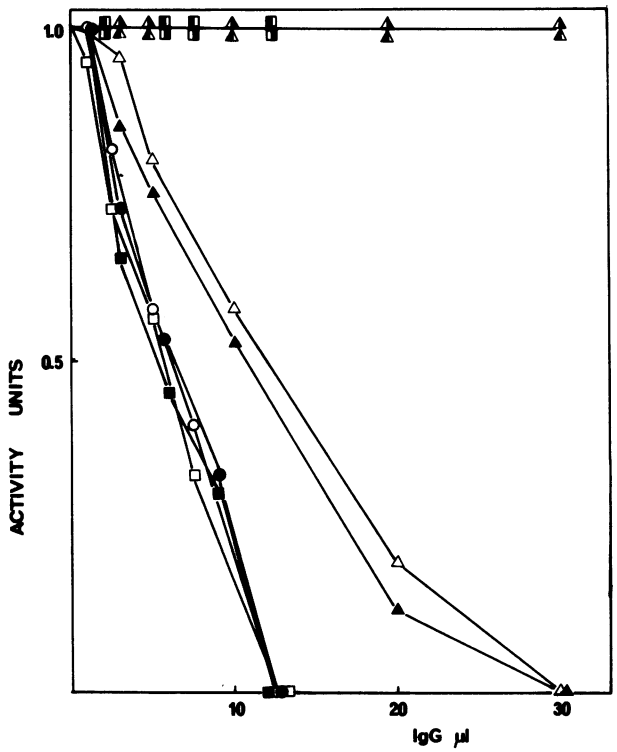

FIG. 2. Immunotitration curves of glutamate synthases from rice green leaf, etiolated leaf, and root tissues. The anti-Fd-glutamate synthase IgG was incubated with a constant amount of glutamate synthase. The enzyme activity was assayed as described in "Materials and Methods." Green leaf tissue: Fd assay (○), methyl viologen assay (●); etiolated leaf tissue: Fd assay (□), methyl viologen assay (■), NADH assay (◨), NADPH assay (◧); root tissue: Fd assay (△), methyl viologen assay (▲), NADH assay (◭) NADPH assay (◬).

Reactivity of Different Forms of Glutamate Synthase towards the Anti-Fd-Glutamate Synthase IgG. Table I shows the glutamate synthase activities in green leaf, etiolated leaf, and root tissues. Fd-dependent glutamate synthase activity in green leaf tissue was about 7-fold higher than that in etiolated leaf tissue. In root tissue, Fd-dependent activity was also detected, and in this tissue as well as in etiolated leaf tissue, Fd-dependent glutamate synthase activities were 4- to 5-fold higher than those assayed with NADH or NADPH. No glutamate formation was detected either with NADH or with NADPH in the enzyme extract from green leaf tissue.

Behavior of glutamate synthases towards the anti-Fd-glutamate synthase IgG was studied by quantitative immunotitration analysis. Figure 2 shows the immunotitration curves of glutamate synthases from different plant tissues. With the enzyme samples from green leaf, etiolated leaf, and root tissues, Fd-dependent glutamate synthase activity in the supernatant decreased with the concomitant increasing amount of the IgG. When the activity in the supernatant was found to be zero, about 5% of the initial enzyme activity was associated with the immunoprecipitate. It can therefore be concluded that all the enzyme was precipitated. In green leaf and etiolated leaf tissues, one half of one unit enzyme was precipitated with 5.8 μl of the IgG, but 12 μl of the IgG were necessary for the enzyme from root tissue. Therefore, the root-Fd-enzyme was less susceptible to precipitation with the IgG than the leaf enzymes. The titration curves when the enzyme activity was assayed with methyl viologen showed a similar pattern. Nonim-

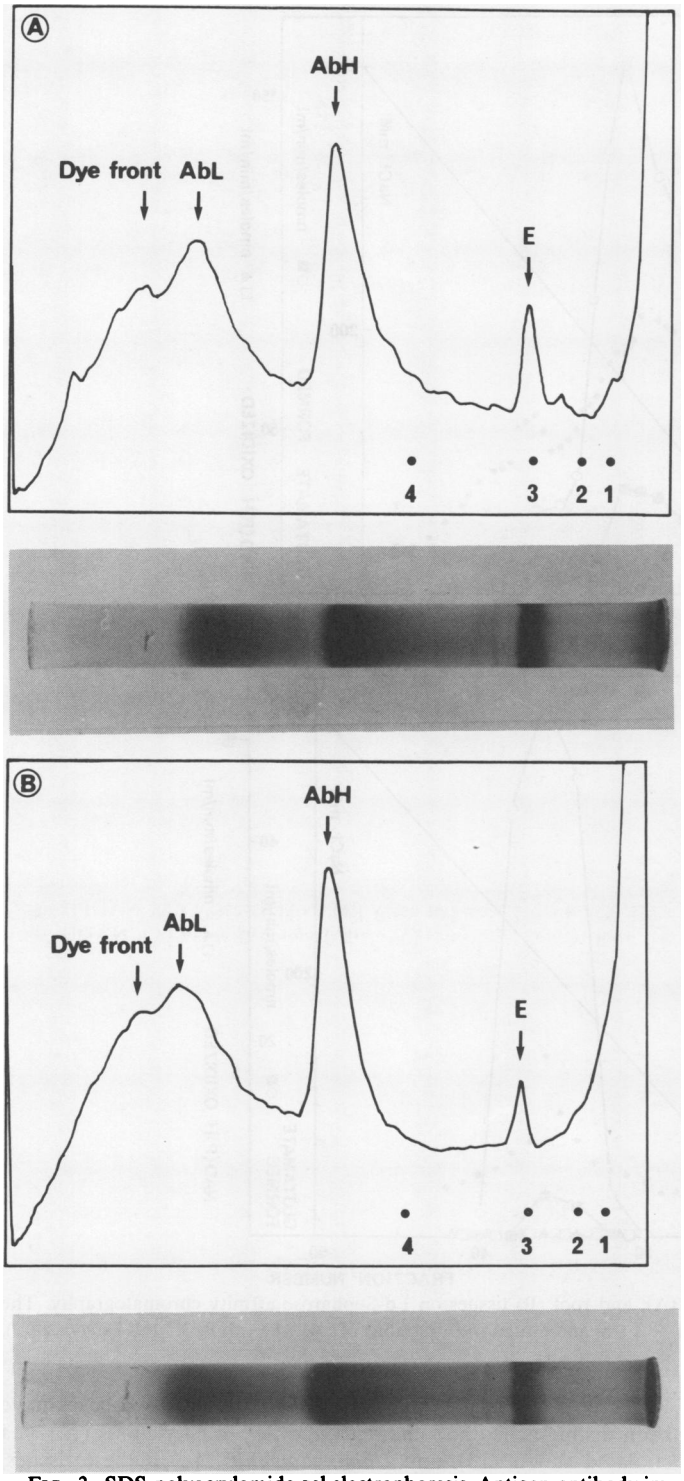

FIG. 3. SDS-polyacrylamide gel electrophoresis. Antigen-antibody immunoprecipitates obtained from rice green leaf (A) and etiolated leaf (B) tissues were dissociated and subjected to electrophoresis on 5% SDS polyacrylamide gel. A constant electric current of 5 mamp/tube was applied for 5 h at room temperature. The gels were scanned spectrophotometrically and the *A* at 280 nm determined before staining with Coomassie Blue R. The marker proteins represent cross-linked serum albumin bovin; 264,000 D (1), 198,000 D (2), 132,000 D (3), and 66,000 D (4).

mune IgG had no effect on both Fd- and methyl viologen-dependent activities. These results indicate that there are different antigenic sites on the Fd-glutamate synthase from rice tissues. In contrast, the IgG did not recognize the NAD(P)H-dependent activities in etiolated leaf and root tissues since the initial activity was detected in the supernatant from all the fractions assayed with different quantities of the IgG used for Fd and methyl viologen assays.

SDS-Electrophoresis of Immunoprecipitated Proteins. The antigen-antibody immunoprecipitates obtained from green leaf and etiolated leaf tissues were subjected to SDS-polyacrylamide gel electrophoresis. Dissociated immunoprecipitates obtained from green leaf tissue gave only one enzyme polypeptide band (E), and heavy (AbH) and light (AbL) chains of the IgG (Fig. 3A). By calibration with the standard mol wt proteins, the mol wt of the polypeptide chain was estimated to be 125,000 D which is in agreement with the subunit mol wt (115,000 D) determined by another method (29). The dissociated immunoprecipitate obtained from etiolated leaf tissue showed also the single glutamate synthase polypeptide which corresponds to the mol wt of 125,000 D (Fig. 3B).

Affinity Chromatography of Extracts from Etiolated Leaf and Root Tissues on Fd-Sepharose Column. Standard enzyme extracts obtained from etiolated leaf (Fig. 4A) and root (Fig. 4B) tissues were applied to Fd-Sepharose affinity column. Figure 4A shows that NAD(P)H-glutamate synthase activities passed through the column by washing with the buffer, and no activity was recovered in the NaCl gradient fractions. On the other hand, the enzyme activities dependent on reduced Fd were retained on the column and eluted by NaCl gradient with the activity peak at 160 mM, although one part of the enzyme activity was washed out by the buffer. The result has been repeated including the second chromatography of the Fd-glutamate synthase fractions which were retained on the column by the first chromatography. Although it could be due to the overloading of the column, the reason for the non-fixation of the enzyme is not yet clearly understood. The methyl viologen-linked enzyme activity peak coincided with that of Fd-glutamate synthase activities. A similar chromatographic pattern was observed from the root extract (Fig. 4B). Therefore, by the affinity of glutamate synthase to Fd, pyridine nucleotide-dependent glutamate synthase and Fd-dependent glutamate synthase activities were separated which suggests that these enzyme activities were due to distinct enzymes in both etiolated leaf and root tissues.

Immunochemical Relationship between Fd-Glutamate Synthase in Green Leaf, Etiolated Leaf, and Root Tissues. Fd-Glutamate synthase in etiolated leaf and root tissues, separated from pyridine nucleotide-glutamate synthase on affinity chromatography column was subjected to Ouchterlony double immunodiffusion analysis in order to compare the immunological relationship of Fd-glutamate synthase in green tissue to Fd-glutamate synthase in etiolated leaf tissue (Fig. 5A) and Fd-glutamate synthase in root tissue (Fig. 5B). We observed (Fig. 5A) that the immunoprecipitin lines formed an arc with complete fusion. In contrast, Fd-glutamate synthase in root tissue showed a spur in the precipitin lines (Fig. 5B). The results indicate that Fd-glutamate synthase molecules in green leaf and etiolated leaf tissues have identical immunological deterinants which are related but not identical to those of Fd-glutamate synthase in root tissue.

DISCUSSION

The results presented here indicate that Fd-dependent glutamate synthase is a distinct protein from pyridine nucleotide-dependent glutamate synthase in rice etiolated leaf and root tissues. An important feature of the Fd-dependent glutamate synthase is that the enzyme molecule in etiolated leaf tissue has both identical immunological determinants and a similar mol wt of the polypeptide chain to those of Fd-glutamate synthase in green leaf tissue. On the immunological bases it is likely that the Fd-dependent enzyme activities in these tissues are carried out by closely related protein molecules. Furthermore, it is to be noted that Fd-gluta-

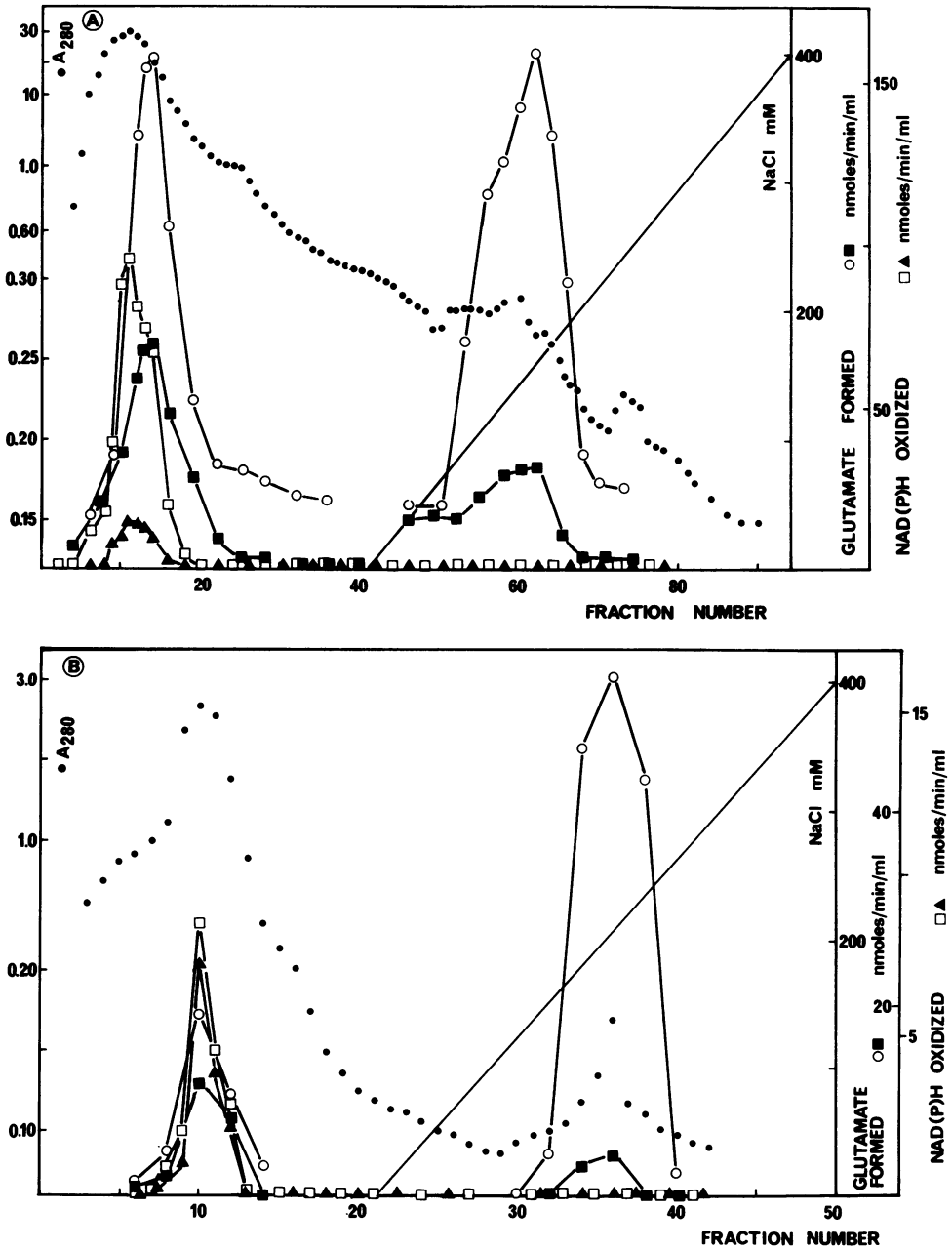

FIG. 4. Elution patterns of glutamate synthases from rice etiolated leaf (A), and root (B) tissues on Fd-Sepharose affinity chromatography. The standard enzyme extract was applied to the column: A, 1.2 × 10.5 cm; B, 2.1 × 3 cm; and eluted into fractions of: A, 1.85 ml; B, 3.3 ml. Fd assay (○), methyl viologen assay (■), NADH assay (□), and NADPH assay (▲).

mate synthase in root tissue also cross-reacts with the IgG anti-Fd-glutamate synthase purified from green leaf tissue. The Fd-glutamate synthase in root tissue has related but not identical antigenic components to those of Fd-glutamate synthase in green leaf and etiolated leaf tissues, showing that the enzyme in root tissue is a distinct protein from Fd-glutamate synthase in leaf tissues.

The immunotitration curves show that pyridine nucleotide-dependent glutamate synthases in etiolated leaf and root tissues are not recognized by the IgG. These results indicate that pyridine nucleotide-dependent glutamate synthase has a quite different structural conformation from that of Fd-glutamate synthase, which is consistent with the observation that pyridine nucleotide-dependent glutamate synthases in etiolated leaf and root tissues lack an affinity to Fd on Fd-Sepharose affinity chromatography.

Recently, Fd- and NADH-dependent glutamate synthases have been distinguished and characterized in pea cotyledons (12), pea etiolated shoots (13), and *Chlamydomonas* (4). In these tissues, however, NADPH-dependent glutamate synthase activity is not found. In contrast, both NADH and NADPH are efficient electron donors for enzymes in rice etiolated leaf and root tissues at a pyridine nucleotide concentration of 0.22 mM. The lack of pyridine nucleotide specificity has been found for enzymes in cultured cells (5), roots (6, 17, 19, 27), and endosperms (19, 26). In these tissues, the ratio of NADPH- to NADH-dependent activities ranges between 0.5 and 1.0. Beevers and Storey (2) have observed that NADH is the preferred electron donor for enzyme in pea cotyledons, and have speculated that NADPH-dependent activity is due to the enzymic conversion of NADPH to NADH. Since NADH- and NADPH-dependent glutamate synthases in rice etiolated leaf

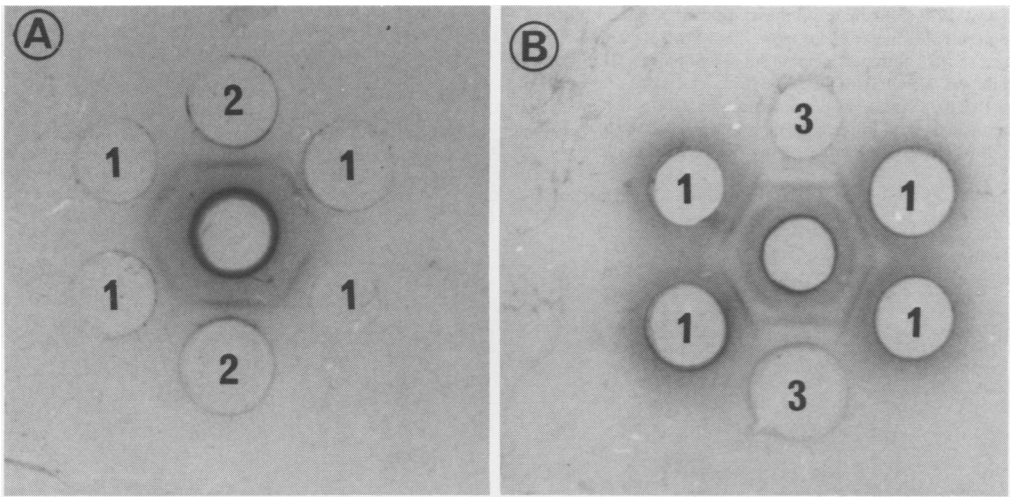

FIG. 5. Ouchterlony double immunodiffusion. The center well contained the anti-Fd-glutamate synthase IgG. Cross-reaction was performed by a combination of: A, Fd-glutamate synthases from rice green leaf (1) and etiolated leaf (2) tissues; B, Fd-glutamate synthases from green leaf (1) and root (3) tissues.

and root tissues lack the immunological cross-reactivity with the IgG and the affinity to Fd, further evidence is necessary to clarify the pyridine nucleotide specificity of glutamate synthase.

For a detemination of Fd-glutamate synthase activity, methyl viologen is used as a reducing dye (22, 31, 33). In pea etiolated shoots (13) and *Chlamydomonas* (4), methyl viologen-linked enzyme activity is associated with the NADH-glutamate synthase rather than with Fd-glutamate synthase. As it is clear from the results with the immunotitration analysis and affinity chromatography on Fd-Sepharose column, Fd- and methyl viologen-dependent activities are associated with the same enzyme protein in rice. The reasons for this discrepancy are not yet well understood.

The significance of Fd-dependent glutamate synthase in non-photosynthetic tissues is not clear, since the presence and the physiological role of Fd in these tissues are not yet clarified, and reduced pyridine nucleotides are generally considered to be electron donors. An important characteristic of Fd-glutamate synthase in rice etiolated leaf and root tissues is that the enzyme activity is 4- to 5-fold higher than the NAD(P)H-glutamate synthase activities *in vitro*, suggesting that Fd-glutamate synthase could possibly undertake ammonium assimilation in dark together with pyridine nucleotides-glutamate synthase in these tissues under some catalytic conditions. The presence of both Fd and Fd-NADP reductase (EC 1.6.7.1) has been studied in etiolated leaves of bean (7, 15) and barley (21) during greening of etiolated leaf tissues in relation to the development of components in electron transport chain. Assuming that the functional stage of electron transport components in etiolated bean and barley leaf tissues is similar to that in rice etiolated leaf tissue, a possible presence of a natural physiological electron carrier to Fd-dependent glutamate synthase in this tissue could not be ruled out. Up to now, the presence of Fd or of functionally or structurally similar proteins, as well as of Fd-NADP reductase has not been reported in root tissue. The present evidence of Fd-dependent glutamate synthase protein particularly in root tissue requires the characterization and identification of the mechanism which could supply the reducing power to the enzyme in this tissue, and further analytical studies are needed to elucidate the physiological function of the root Fd-dependent glutamate synthase.

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