Detection of the Messenger RNA Encoding for the Ferredoxin-Dependent Glutamate Synthase in Maize Leaf

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ABSTRACT

Ferredoxin-dependent glutamate synthase (EC 1.4.7.1), glutamate oxoglutarate aminotransferase (glutamate synthase) (GOGAT) messenger RNA was extracted from maize (Zea mays L.) leaves and partially purified through oligo(dT)-cellulose chromatography and ultracentrifugation in a sucrose gradient. mRNA was translated in vitro using a reticulocyte system. The glutamate synthase subunit was characterized by immunoprecipitation with antibodies raised against the rice (Oryza sativa L.) ferredoxin-glutamate synthase. The in vitro synthesized protein and the 145 kilodaltons genuine maize leaf subunit of GOGAT were found to comigrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments.

Glutamate synthase is a key enzyme of nitrogen metabolism. It was first discovered in bacteria (17) and was subsequently reported to be present in other procaryotic cells, in algae, in higher plants, and in fungi (10). It is now quite clear that in most cases this enzyme catalyzes the second step of ammonia assimilation via amide group of glutamine into amino group of glutamate in plant cells. In higher plants the enzyme seems to be restricted to the chloroplasts (6, 8).

Chromatographic and immunochemical investigations discriminated a pyridine nucleotide (NAD or NADP)-dependent GOGAT1 (EC 1.4.1.13 and EC 1.4.1.14) and a ferredoxin-dependent GOGAT (EC 1.4.7.1) which appear to be unrelated proteins.

We have previously reported the occurrence of a Fd-GOGAT in rice leaves, which is restricted to the chloroplast and whose activity is increased 4 to 20 times during greening; the enzyme is a homodimer composed of 125,000 D subunits whereas GOGAT from spinach leaf has a mol wt of 180,000 (16). Moreover it was shown that maize contains a Fd-dependent GOGAT in both leaf and root (13). The root ferredoxin-enzyme exhibits immunological and kinetic properties different from the leaf ferredoxin-enzymes (15). The electron donor associated with the root enzyme is a nonheme-iron protein which exhibits immunological reactivity with Fd antibodies. Current studies on developmental aspects of nitrogen assimilation in different tissues of higher plants have elucidated the distinct expression of enzyme activity in relation to the isofoms of glutamate synthase in greening etiolated leaves and developing nodules; in both cases the activity of Fd-dependent enzyme is increased (14, 15) whereas that of NAD(P)-dependent enzyme is decreased (10, 14, 15). These contrasting developmental patterns led us to examine how this particular enzymic system is regulated at translational and transcriptional levels.

As a first step to this study we report here the extraction and detection of a messenger RNA coding for the Fd-GOGAT in maize leaf. The mRNA was partially purified and the translation products were characterized immunologically using the antibody directed against rice enzyme.

MATERIALS AND METHODS

Plant Culture. Seeds of maize (Zea mays L.) hybrid INRA 508 were obtained from FRASEMA (Française des Semences de Mais). Seeds were sown in sand and provided daily with a nutrient solution (3). They were grown for 7 d in controlled chambers (16 h at 180 μE m–2 s–1 and 25°C day, 20°C night, in an 80% moisture atmosphere).

Preparation of Antibodies. An antiserum was raised against purified Fd-GOGAT from rice (Oryza sativa L. cv Delta). Antibodies were shown to be specific for the enzyme and to cross-react with the maize leaf enzyme (15).

Molecular Weight Determination of the GOGAT Subunit. Enzyme extraction, purification, immunoprecipitation, immunocomplex dissociation, and SDS gel electrophoresis were performed as described in Suzuki et al. (15).

Purification of Poly (A*) RNA. Maize leaves (25 g) were frozen in liquid N2 and ground in a mortar. The fine powder was mixed with 100 ml of 50 mm Tris-HCl buffer (pH 8.0) containing 10 mm EDTA, 5 mm guanidinium thiocyanate, 2% (w/v) N-lauroyl sarcosine, and 5% (v/v) 2-mercaptoethanol. The mixture was incubated for 15 min at 37°C and centrifuged at 20,000 g for 30 min. The pellet was treated as above in 50 ml of the same medium. Supernatants were pooled, adjusted to 0.1 mg ml–1 CsCl and layered on 8 ml cushions of 5.7 M CsCl, 50 mm EDTA (pH 8.0) in 30 ml polylamol centrifuge tubes. The tubes were centrifuged for 24 h at 25,000 rpm in a SW 27 rotor at 15°C. The RNA pellet was resuspended in 10 mm Tris-HCl (pH 7.5) containing 1 mm EDTA and 0.1% (v/v) SDS, and heated for 10 min at 65°C to ensure complete dissolution; RNA was reprecipitated after addition of 0.1 volume 3 M sodium acetate (pH 6.0) and 2.2 volumes of ethanol.

Poly(A*)-RNA was twice purified on an oligo(dT)-cellulose (Sigma) column as described by Bantle et al. (1) and finally ethanol precipitated.

Typical yields were 20 mg RNA, and 0.5 mg poly(A*)-RNA per 25 g fresh weight.

1 Abbreviation: GOGAT, glutamate oxoglutarate aminotransferase (glutamate synthase).
RNA Fractionation. Linear, 5 to 20% sucrose gradients were prepared in 12 ml polyallomer tubes as described by Luthe (7). Hundred μg poly(A * )-RNA were redissolved in 100 μl of buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) heated for 90 s at 70°C, layered on the top of gradient, then centrifuged for 16.5 h in a SW 41 rotor at 29,000 rpm, and 4°C. Fractions (0.6 ml each) were collected from the bottom of the tubes and stored frozen at −20°C until use.

Cell-Free Translation. The in vitro assay for mRNA fractions was performed in a nuclease-treated reticulocyte lysate system obtained from Amersham.

Translation experiments were started by adding 4 μl aliquot of each gradient fraction to 7 μl of lysate containing 10 μCi of [35S] methionine (900 Ci/mmol, Amersham, England); incubation was performed at 30°C for 1 h. Translation products were analyzed by electrophoresis on a 7.5% acrylamide gel, and revealed by fluorography after 72 h exposure. (2) Control without RNA; (4–16), fraction number; (B), no mRNA added.

Immunoprecipitation. Protein A-Sepharose Cl 4 B (Pharmacia) was equilibrated in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 2% (w/v) Triton X 100 or 0.5% (w/v) Tween, 150 mM NaCl, 0.2% NaN3 (TNE buffer) and 25 μl gels were poured in 1 ml pipetman tips. Fifty μl of specific antiserum were passed twice through the column. The translation mixture was diluted to 500 μl in TNE buffer containing 10 mM unlabeled methionine and passed twice through the column. The column was rinsed with 5 ml TNE buffer made 1 mM in NaCl and then with 50 mM Tris-HCl buffer (pH 7.5). The tip of the column was cut off and

Fig. 1. Analysis of GOGAT immunoprecipitate by SDS-gel electrophoresis. The immunoprecipitate (50 μg of protein) was dissolved in electrophoresis buffer in the presence of 1% (w/v) SDS and 10% (v/v) 2-mercaptoethanol as described by Weber and Osborn (18). Electrophoresis was performed at 40 V, 150 mamp, in 7% (w/v) acrylamide gel in presence of 0.1% (w/v) SDS. Track 1, mol wt markers; track 2, proteins from the immunoprecipitate, GOGAT subunit, ABH, and ABL, respectively, heavy and light chain of the antibodies.

Fig. 2. In vitro translation of poly (A *) RNA fractionated on sucrose gradients. Aliquot of 2.5 μl of each fraction was incubated in 5 μl reticulocyte lysate as described in “Materials and Methods.” The translation products were analyzed by electrophoresis on a 7.5% acrylamide gel, and revealed by fluorography after 72 h exposure. (2) Control without RNA; (4–16), fraction number; (B), no mRNA added.
the protein A-Sepharose collected in an Eppendorf tube with 25 µl of 10 mm Tris-HCl buffer (pH 7.5) containing 1 ml EDTA, 2% (w/v) SDS, and 5% (w/v) 2-mercaptoethanol. Desorbed proteins were electrophoresed on a SDS polyacrylamide gel and detected by fluorography as above.

RESULTS AND DISCUSSION

In a previous work, the Fd-GOGAT from rice leaf was purified to homogeneity and used as an antigen to raise a specific immune serum (15). Subsequently it was established that the IgG were multivalent since they recognized GOGAT found in other plant tissues (14) or organs (15), whatever the metabolic type of the plant (C₃ or C₄).

An (NH₄)₂SO₄-purified extract of GOGAT from maize leaf was incubated with the immune serum, and the collected pellet was dissociated as described (15). By SDS gel electrophoresis of the dissociated immunocomplex, the mol wt of the maize leaf enzyme was found to be approximately 145,000 (Fig. 1, lane 2). Matoh et al. (9) reported a mol wt close to 160,000 for the native enzyme extracted from another maize cultivar. These results strongly suggest that the maize leaf enzyme is a monomer. The high mol wt of the protein chain implies that the size of the mRNA should also be unusually high. As a consequence, in order to enrich our preparations in mRNA coding for GOGAT, total poly (A⁺) RNA was fractionated on sucrose gradients. These gradients were collected starting from the bottom of the tubes and the resulting twenty fractions (0.6 ml each) were assayed for translational capacity in a cell free reticulocyte lysate. The mRNAs were distributed in the gradient fractions according to their size. In control gradients run in parallel on which ribosomal RNAs were sedimented, 18S rRNA (approximately 1800 nucleotides) was detected in fraction 13 and 28S rRNA (approximately 3600 nucleotides) was detected in fraction 10. In the range of the high mol wt, strong bands were detected in [³⁵S]methionine labeled translation products (Fig. 2). The main band of mol wt about 105,000 is thought to be pyruvate-Pi-dikinase (4).

When GOGAT antibodies were added to the translation products synthesized from mRNA of fraction 8, a single radioactive peptide was selected as shown by gel fluorography (Fig. 3B, lane 2). The labeled peptide comigrated with an authentic GOGAT peptide under denaturing conditions as revealed by Coomassie blue staining (Fig. 3A, lane 1), thus allowing us to identify the in vitro synthesized protein as GOGAT. As expected from Figure 2, Fd-GOGAT mRNA is found ahead of the bulk of total poly (A⁺)-RNA. Compared with migrations of 18S- and 28S-rRNA sequences (result not shown), Fd-GOGAT mRNA migrates as a 4000 nucleotides molecule, as expected from the enzyme mol wt (coding sequence approximately 3300 nucleotides long). In fraction 8 the purification yield for GOGAT mRNA was estimated to be about 30 times.

As shown by Sommerville and Ogren (12), the GOGAT protein is encoded by the nuclear genome and, as expected, the corresponding mRNA is polyadenylated and can be purified through oligo(dT)-cellulose. On the other hand, the enzyme Fd-GOGAT is restricted to the chloroplasts (10). According to the current view, the peptide should be synthesized as a precursor in the cytoplasm before entering the chloroplast (11). In our experiment, a slight difference between the mol wt of the genuine and the in vitro synthesized peptides was detected. We intend to determine whether or not the GOGAT peptide is synthesized in the cytoplasm as a precursor with a leader sequence.

The result obtained here provides us a test to study the level of GOGAT mRNA in plants under different physiological conditions, and will make possible the preparation of a cDNA probe.

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LITERATURE CITED