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Different Characteristics of the Two Glutamate Synthases in the Green Leaves of *Lycopersicon esculentum*[1]

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ABSTRACT

The two glutamate synthases, NAD(P)H- and ferredoxin-dependent, from the green leaves of tomato plants (*Lycopersicon esculentum* L. cv Hellfrucht frühstamm) differed in their chemical properties and catalytic behavior. Gel filtration of NAD(P)H enzyme gave an apparent molecular size of 158 kilodalton, whereas the ferredoxin enzyme molecular size was 141 kilodalton. Arrhenius plots of the activities of the two enzymes showed that the NAD(P)H enzyme had two activation energies; 109.6 and 70.5 kilojoule per mole; the transition temperature was 22°C. The ferredoxin enzyme however, had only one activation energy; 56.1 kilojoule per mole. The respective catalytic activity pH optima for the NAD(P)H-dependent and the ferredoxin dependent enzymes were around 7.3 and 7.8. In experiments to evaluate the effects of modulators aspartate enhanced the NAD(P)H-linked activity, with a K_a value of 0.25 millimolar, but strongly inhibited that of the ferredoxin-dependent glutamate synthase with a K_i of 0.1 millimolar. 3-Phosphoserine was another inhibitor of the ferredoxin dependent enzyme with a K_i value of 4.9 millimolar. 3-Phosphoglyceric acid was a potent inhibitor of the ferredoxin-dependent form, but hardly affected the NAD(P)H-dependent enzyme. The results are discussed and interpreted to propose different specific functions that these activities may have within the leaf tissue cell.

Glutamate synthase activity catalyzes the transfer of amide nitrogen from glutamine to 2-oxoglutarate. This process requires an input of reducing power. This activity, thus occupies a key position along the ammonia assimilation and reassimilation pathways in higher plants.

Two enzyme forms have been distinguished; each depends on a different electron donor. One receives its reducing power from reduced pyridine nucleotide (EC 1.4.1.14), the other from reduced Fd (EC 1.4.7.1) (19). Their presence is reported in green tissues of higher plants, barley, and pea shoots (30) and cultured tobacco cells (26). It is well established that the two activities correspond to two different enzymes with different molecular sizes and individual specificities for their electron donors (17).

Furthermore, they are reported to have different antigenicities, since antibodies against Fd dependent glutamate synthase from green rice leaves did not react with NAD(P)H-dependent enzyme from roots and etiolated leaves (27). Although the properties of these isoforms have been known for some time, the reason for their simultaneous presence in the same tissue has not yet been clarified. Two forms of glutamate synthase have been separated from tomato green leaves (1). The present communication reports some differences in their structural and regulatory properties and discusses the significance of their proposed different physiological roles.

MATERIALS AND METHODS

Tomato plants (*Lycopersicon esculentum*, L. cv Hellfrucht frühstamm) were cultured under natural sunlight in a glasshouse. The plants were supplied with a 2.5 μM NH_4NO_3, 5 mM KNO_3, 1.3 mM $(NH_4)_2HPO_4$, 2.8 mM $MgSO_4$ aqueous solution at 2 to 3 d intervals. Leaf samples for enzyme extraction were from 6 to 7 week-old plants.

Enzyme Assays. The extraction and purification of the enzymes was as described elsewhere (1). The final enzyme preparations were electrophoretically and immunologically homogeneous for the Fd-dependent enzyme, but for NAD(P)H-dependent one additional protein band appeared after PAGE of the extract. The NAD(P)H-dependent glutamate synthase was measured by absorbance decrease at 340 nm at 35°C and the reaction mixture contained 2 mM L-glutamine, 1 mM 2-oxoglutarate, 1 mM EDTA, 25 mM Hepes (pH 7.5), and 0.1 mM NADPH or NADH. The Fd-dependent glutamate synthase activity was assayed in a reaction mixture of 1 ml containing 22.5 mM phosphate buffer (pH 7.3), 5 mM L-glutamine, 5 mM 2-oxoglutarate, and 0.02 mM Fd, 9 μmol sodium dithionite dissolved in 0.05 ml of 190 mM $NaHCO_3$. The Fd was obtained from spinach leaves according to Keresztes-Nagy and Margoliash (10). Reaction mixtures were treated at the end of the incubation period with 20 mM DNS-chloride[2] for 1 h at room temperature as previously described (13). The amino acids were separated by HPLC with a Spherisorb ODS-2 5 μ column (150 × 4.6 mm i.d., Tracer Analítica S.A., Spain), thus allowing the determination of the glutamate formed in the enzymic assay by external standard method. Solvents for elution were methanol (A) and 0.008% (w/v) triethylamine in 0.6% acetic acid aqueous solution (B). Isocratic elution was used (A:B, 45:55) which gave excellent amino acid resolution in most cases; when aspartate was present in the assay medium the elution gradient was varied from 30:70 (A:B) to 50:50 in 50 min. The flow rate was increased from 1 to 2 ml/min to improve resolution. A pre-column (7 μm disposable New Guard RP-18, 15 × 3.2 mm i.d., Brownlee Labs.) was always used. The peaks were integrated from valley to valley by a SP4100 computing integrator (Spectra Physics). All the data showed correspond to mean values of triplicated measurements. Both assays for NAD(P)H- and Fd-dependent glutamate synthases were linear to the enzyme concentrations throughout the incubation period assayed.

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[2] Abbreviations: DNS-, dansyl-; GOGAT, glutamate synthase; GS, glutamine synthetase; PGA, 3-phosphoglyceric acid.

Protein Determination. The method of Lowry *et al.* (12) was used after precipitation with 10% TCA.

Immunochemical Methods. Antibodies against the purified Fd-GOGAT were prepared as described elsewhere (3), and the double immunodiffusion procedure, as described by Ouchterlony and Nilsson (20), was used in the cross-reaction experiments.

RESULTS

In the green leaves of tomato plants there are two proteins which catalyze the transfer of amide nitrogen from glutamine to 2-oxoglutarate, but they differ in their reducing power donors. One enzyme can use either NADH or NADPH as its electron donor, the affinity for NADH being greater (K_m = 1.7 μM, Avila *et al.* [1]) than for NADPH, K_m 6.0 μM. The other enzyme receives the electrons from reduced Fd. It should be noted that in the GOGAT assays, methyl viologen, chemically reduced with dithionite, is often substituted for reduced Fd.

Figure 1 shows the influence of pH on enzyme activities; the optimum pH for NAD(P)H-GOGAT was around 7.3, whereas the Fd-dependent glutamate synthase pH optimum was around 7.8 when the electron donor was ferredoxin.

The separated proteins showed clear differences in their molecular sizes, with a mol wt of 158 kD of the NAD(P)H-dependent enzyme (Fig. 2). This value is less than those previously reported for this enzyme obtained from other sources like pea shoots (14), lupin nodules (2), and *Chlamydomonas reinhardii* (6). The Fd-dependent enzyme has a mol wt of 141 kD, which is lower than the value previously reported for this enzyme from rice leaves (25) and close to the enzymes from field bean (28), corn leaves (15), pea cotyledons (17), and spinach leaves (8). The mol wt for this form was also determined by SDS-electrophoresis giving a value of 153 kD. The differences between these forms was further tested by double immunodiffusion using specific antibodies raised against the Fd-GOGAT which did not react with the NAD(P)H dependent enzyme (results not shown). Previous studies of Suzuki *et al.* (27) showed that nicotinamide dependent GOGAT from roots and etiolated leaves did not recognize the antibodies raised against the Fd-dependent enzyme from green leaves.

The values obtained for the activation energies from the Arrhenius plots were different for the two enzymes. Furthermore,

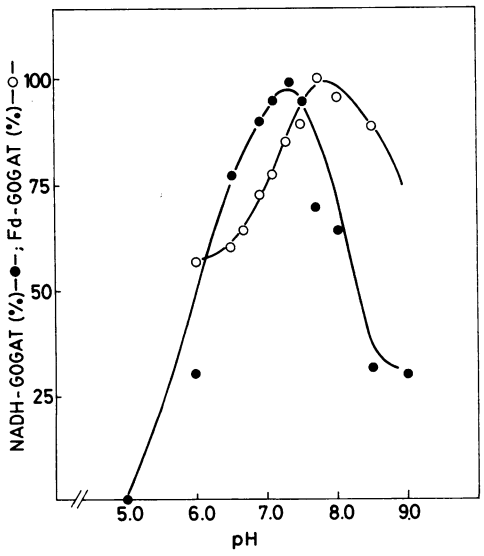

FIG. 1. Effect of pH on NAD(P)H-GOGAT and Fd-GOGAT activities. Buffers used were 0.1 M acetate (pH 5.0), 0.1 M potassium phosphate (pH 6–7.8), and 0.1 M Tris/HCl (pH 8–8.5). Mean values are represented (SE always lower than 0.3 for NAD[P]H- and 0.08 for Fd-GOGAT).

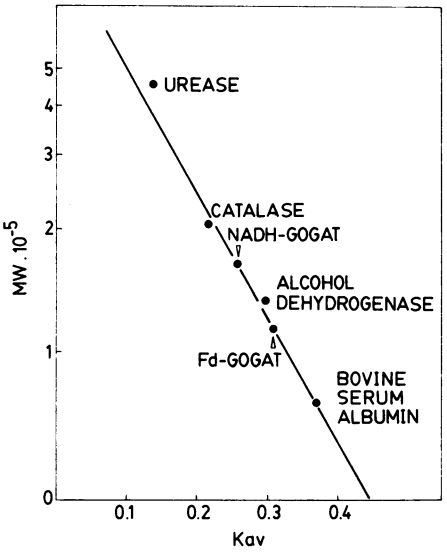

FIG. 2. Linear relationship between the partition coefficient (Kav = Ve − Vo/Vt − Vo) of the proteins eluted through a Sephacryl S-300 column (64 × 1.6 cm) and the molecular size of the BSA (66 kD), alcohol dehydrogenase (150 kD), catalase (240 kD), and urease (489 kD).

Table I. *Effects of Amino Acids and PGA on GOGAT Activities from Green Tomato Leaves*

	NAD (P) H-dependent		Fd-dependent	
	1 mM	5 mM	1 mM	5 mM
		%		
Control	100	100	100	100
Gly	100	108	70	45
Ser	93	102	54	41
Ala	93	115	69	52
Leu	108	103	87	46
Met	96	95	57	37
Thr	93	98	82	65
Lys	95	98	76	63
Orn	99	88	67	49
Arg	99	95	90	37
His	85	92	98	104
Val	93	88	96	91
Pro	95	96	106	72
PGA	110	115	19	18

Table II. *Effect of Asp and P-Ser on Glutamate Synthase Activities from Tomato Leaves*

Effector	NAD (P) H-GOGAT	Fd-GOGAT
Aspartate	Nonessential activator K_a = 0.25 mM	Competitive inhibitor[a] K_i = 0.1 mM
P-Serine	None	Competitive inhibitor[b] K_i = 4.9 mM

[a] Assayed against 2-oxoglutarate. [b] Assayed against glutamine.

the experimental values for the NAD(P)H-GOGAT fitted better to two straight lines than to one, and the transition occurs at 22°C. This gave two activation energies, 109.6 kJ mol^{-1} and 70.5 kJ mol^{-1} above and below this temperature, respectively. Coefficients for linear correlation were −0.995 and −0.978 for the two lines. For the Fd-GOGAT assay the value of the coefficient

was −0.992. The calculated Q_{10} values were 4.7 below, and 2.5 above the transition temperature for the NAD(P)H-GOGAT; and 2.0 for the Fd-GOGAT over the entire range tested. Thus, the NAD(P)H-dependent activity was greatly affected by low temperatures which did not affect the Fd-dependent activity. Some experiments to elucidate whether a conformational or kinetic change is involved have to be done.

The effects of some physiological compounds on GOGAT activities were also tested. The amino acids, the end products of nitrogen metabolism, when added to the assay medium were ineffective against NAD(P)H-GOGAT, but some of them inhibited the Fd-GOGAT as shown in Table I. Among these should be stressed the effect of some small amino acids like Gly, Ser, Ala, and Leu, some of them photorespiratory intermediates, and other essential amino acids like Thr, Lys, and Met, end products of metabolic pathways starting from Asp. Also highly basic amino acids, like Arg and Orn, are clear inhibitors. Also, the 3-P-Glycerate when added to the assay medium with the purified enzyme was a strong inhibitor of Fd-GOGAT but did not affect the NAD(P)H-GOGAT (Table I).

The greater effects were, however, for aspartate and phosphoserine (Table II). The first strongly increased the NAD(P)H-dependent activity (K_a 0.25 mM, see Ref. 21). This amino acid is a strong competitive inhibitor against 2-oxoglutarate for the Fd-dependent GOGAT (K_i 0.1 mM). Phosphoserine, whereas irrelevant for NAD(P)H-GOGAT, was a competitive inhibitor against glutamine for the Fd-dependent enzyme (K_i 4.9 mM).

DISCUSSION

There is no apparent explanation for the presence of two glutamate synthase isoforms in the same green tissue of tomato leaf, since there is only one glutamine synthetase (3, 5), probably located inside the chloroplast (18). Consequently, it is reasonable to suppose that one of GOGAT forms might be localized within this organelle with its function closely linked to that of GS in the glutamate synthase cycle, to catalyze the primary ammonia assimilation and the reassimilation of photorespiratory evolved ammonium ions; this form has been proven to be the Fd-dependent in *Arabidopsis* (23) and barley (9). In tomato leaves a chloroplastic localization of Fd GOGAT has been found (JR Botella, unpublished data). On the other hand, the NAD(P)H-dependent form could be an important enzyme at very early stage of development, as stated for peas (16). The optimum pH values of these enzymes obtained from tomato leaves lends additional support to this proposed role assignment because this value is close to 8 for the Fd-dependent enzyme, and somewhat lower for the NAD(P)H-dependent one.

The proven opposed influence of aspartate on these enzyme activities strongly suggests this physiological function assignment. Aspartate enhances NAD(P)H-dependent activity by acting as indicator that nitrogen is being imported into the leaf. Moreover, asparagine has been reported a major nitrogen transport compound in some plants (11), and a high asparaginase activity is detected at early stages of pea leaf development (22) when the nitrogen sink effect in the leaf is high.

On the other hand, aspartate delivered to the chloroplast could be used within this compartment as precursor of several essential amino acids (4), or to shuttle reducing equivalents to the cytosol (7). The biosynthesis of essential amino acids in the chloroplast is important in early leaf development, whereas the rate of shuttle reducing equivalents would be high in actively photosynthesizing leaf tissue. Thus, aspartate accumulation inside the chloroplast would be a signal for nitrogen assimilation within this organelle to cease by its progressive inhibition of Fd-GOGAT. The effects of amino acids other than aspartate on Fd-GOGAT have also to be considered under this point of view. Furthermore, phosphoserine, which also inhibits tomato leaf glutamine synthetase (5), could be another argument to link Fd-GOGAT to glutamine synthetase in the glutamate synthase cycle. The role of this phosphorylated amino acid needs further research after knowing that the K_i values for both enzymes are in the mM range (2.4 mM for GS and 4.9 mM for Fd-GOGAT).

Finally, the PGA effect on this Fd-GOGAT enzyme could be another possible link of this activity inside the chloroplast to light events (29). In spinach and pea protoplast it has been found that PGA rises inside the chloroplast in the dark period (24).

The above findings and their discussion agree with the results reported for greening barley and pea leaves (30), where NAD(P)H-GOGAT remains steady during the greening process and there is an increase in Fd-GOGAT; in fact, the Fd-GOGAT activity level in the green leaves of tomato plants is much higher than that of NAD(P)H-GOGAT (1). The results here reported on GOGAT isoforms may represent another step towards the definition of their physiological roles. Meanwhile NAD(P)H-GOGAT would be important at very early stage of leaf development, whereas Fd-GOGAT would be linked to GS and responsible for primary and secondary ammonium assimilation in mature green leaves. NAD(P)H-GOGAT localization needs to be established since previous reports on the literature indicate its association with plastid fractions (16).

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