The activity of NADH-, NADPH- and Fd-dependent glutamate synthase in the plastids and cytosol of *Pisum arvense* L. root cells

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Abstract

Three forms of glutamate synthase (NADH-GOGAT, NADPH-GOGAT and Fd-(ferredoxin) GOGAT) were found in the plastids and cytosol of *Pisum arvense* root cells. The activities of the enzymes of both fractions decreased with increasing age of the plants, with the exception of plastid NADPH-GOGAT which exhibited markedly stable activity. NADH-GOGAT dominated in the cytosol of root cells of several day-old plants but after 14 days of cultivation, the activities of all of the GOGAT forms equalized. Plastid NADH-GOGAT and Fd-GOGAT showed similar activities in the root cells of 3-5 day-old plants, with Fd-GOGAT becoming the dominant enzyme form after 14 days. The entire activity of NADH-GOGAT and Fd-GOGAT was confined to the plastid stroma. The plastid membrane fraction contained 37% of the NADPH-GOGAT activity. Isolated plastids synthesized glutamate from 2-ketoglutarate and glutamine, and glucose-6-phosphate and 6-phosphogluconate clearly stimulated this process. It is supposed that the synthesis of glutamate in *Pisum arvense* root plastids may be dependent on the intensity of the carbohydrates conversion in the pentose phosphate pathway.

Key words: plastids, cytosol, roots, glutamate synthase

INTRODUCTION

Glutamate synthase, an enzyme participating in the alternative glutamate synthesis pathway, occurs in the tissues of higher plants in the following forms: NADH-GOGAT (EC 1.4.1.14), NADPH-GOGAT (EC 1.4.1.13) and

Fd-GOGAT (EC 1.4.7.1). Studies on GOGAT isolated from pea hypocotyls have shown that the NADH- and Fd-dependent enzymes had different molecular weights and were characterized by strict specificity towards electron donors (Matoh et al. 1979).

Root cells of various species of plants have been shown to contain Fd-GOGAT and NAD(P)H-GOGAT (Mifflin and Lea 1975, Oaks et al. 1979, Matoh et al. 1980a, Matoh and Takahashi 1982, Suzuki et al. 1983), and it has also been determined that part of the activity found in root cells is located in plastids (Emes and Fowler 1979a, Suzuki et al. 1981, Kłobus et al. 1983, Kubik-Dobosz and Kłobus 1985).

Root plastids contain enzymes participating in the metabolism of inorganic nitrogen and in the pentose phosphate pathway (Emes and Fowler 1978, Dennis and Miernyk 1982). Studies by Dalling et al. (1972), Mifflin (1974), Emes and Fowler (1979b), Dry et al. (1981) and Kłobus (1984) have shown that the rate of NO_2 reduction in root plastids depends on the amount of NADPH produced in the reaction catalysed by glucose-6-phosphate dehydrogenase. Because the activity of glutamate synthase in plant roots is also dependent on the level of reduced pyridine nucleotides, it is possible that the biosynthesis of glutamate in roots is regulated by the intensity of the processes occurring in the pentose phosphate pathway.

This paper presents the results of a study on the changes in the activities of Fd-GOGAT, NADH-GOGAT and NADPH-GOGAT in plastids and cytosol from root cells during two weeks of growth of *Pisum arvense* seedlings, and shows the effect of some of the pentose phosphate pathway metabolites on the biosynthesis of glutamate in isolated plastids.

MATERIALS AND METHODS

Pisum arvense L. var. "Niezpaniecka" seeds were germinated in darkness at 27°C, then placed in a medium of pH 6.5 containing (in mM): 5 $\text{Ca}(\text{NO}_3)_2$, 5 KNO_3 , 1 MgSO_4 , 1 KH_2PO_4 and (in μM): 15 Fe-citrate, 34 H_3BO_3 , 10 MnSO_4 , 1 CuSO_4 , 1 ZnSO_4 , 0.1 CoSO_4 , 0.5 Na_2MoO_4 . The plants were grown under a 17 hr day (15.5 W m^{-2} , 25°C) and 7 hr night (19-20°C). Plastids were isolated according to a slightly modified method of Emes and England (1986). The roots were homogenized in a porcelain mortar at a 1:1 ratio with 50 mM KH_2PO_4 -KOH (pH 7.5) buffer, 330 mM sorbitol, 1 mM EDTA, 1 mM MgCl_2 and 0.1% bovine serum albumin (buffer A). The homogenate was filtered through six layers of gauze and centrifuged for 2 min at $200 \times g$. The supernatant was divided into two parts. One part was centrifuged for 15 min at $21\,000 \times g$, and the cytosol fraction was obtained. The remaining part was

layered over a 10% solution of Percoll in 50 mM KH_2PO_4 -KOH (pH 7.5) buffer, 330 mM sorbitol, 0.1% bovine serum albumin (buffer B) and centrifuged for 6 min at $3800 \times g$. The plastid pellet was suspended in buffer A. Triton X-100 was added to the suspension to a final concentration of 0.5% in order to separate plastid membranes and stroma; after 15 min at 4°C , the suspension was centrifuged for 15 min at $21\,000 \times g$. In all of the experiments using isolated plastids, the appropriate substrates dissolved in buffer B were added to 0.5 cm^3 of plastid suspension. The final volume of the mixture equalled 3 cm^3 . The samples were incubated for 30 min at 30°C , after which the reaction was terminated in a boiling water bath (1 min). After centrifuging off the precipitated protein ($10\text{ min } 10\,000 \times g$), the glutamate content in the supernatant was determined by the method of Matoh et al. (1979, 1980a).

When the activities of NADH-GOGAT, NADPH-GOGAT and Fd-GOGAT were assayed, the plastids or cytosol were obtained as described above, except that the plastid pellet was treated with 0.5% Triton X-100 in 50 mM KH_2PO_4 -KOH buffer (pH 7.5) containing 10 mM dithiothreitol. The enzymic activity was measured according to the Matoh and Takahashi method (1982). The final 3 cm^3 volume contained 50 mM KH_2PO_4 -KOH buffer (pH 7.5), 10 mM 2-ketoglutarate, 10 mM glutamine, 0.5 cm^3 enzyme and appropriately 0.6 mM NAD(P)H or 0.23 mg ferredoxin (plus 12 mM $\text{Na}_2\text{S}_2\text{O}_4$ and 13.5 mM NaHCO_3). After 20 min of incubation at 30°C the reaction was stopped in a boiling water bath (1 min) and the glutamate content was determined as described above. The activity of the enzymes is expressed as nmoles of glutamate per g fresh weight per hour.

The methods of Shin (1971) and Buchanan and Arnon (1971) were used to purify ferredoxin from spinach leaves.

The presented results are the means from triplicate experiments.

RESULTS

Plastids isolated from *Pisum arvense* roots were capable of synthesizing glutamate (Table 1). The highest amount of glutamate was determined following incubation of plastids with glutamine and 2-ketoglutarate. It was found that glutamate synthase catalyzing the biosynthesis of glutamate with NADH, NADPH or ferredoxin as the electron donors was present in the plastids (Table 2). Triton X-100 was used to disintegrate the isolated plastids into membranes and stroma, and the activities of all of the forms of glutamate synthase were studied. The results indicated that almost all of the activity of NADH-GOGAT and Fd-GOGAT was limited to the stroma of the plastids (Table 2). Only a small part of the activity of these two enzymes was found in

the membrane fraction, in comparison with 37% of the activity of NADPH-GOGAT.

The activity of glutamate synthase in plastids from root cells changed during growth of plants (Table 3). The highest NADH-GOGAT and Fd-GOGAT activities were found in plastids on the fifth day of growth. On

Table 1

The effect of 2-ketoglutarate and glutamine on the synthesis of glutamate in isolated *Pisum arvense* root plastids

Substrate	Nmoles glutamate $\times$ cm ⁻³ fraction $\times$ hr ⁻¹
2-Ketoglutarate (10 mM)	23.2
Glutamine (10 mM)	197.8
Glutamine (10 mM) + 2-keto-glutarate (10 mM)	302.6

Table 2

The distribution of NADH-GOGAT, NADPH-GOGAT and Fd-GOGAT activities expressed as nmoles glutamate $\times$ g⁻¹ fr.w. $\times$ hr⁻¹ in the membrane and stroma fractions of *Pisum arvense* root plastids

Fraction	NADH-GOGAT	NADPH-GOGAT	Fd-GOGAT
Plastids:			
intact	40.3	13.8	66.0
membranes	9.1 (5)	24.0 (37)	4.6 (1)
stroma	165.8 (95)	53.1 (63)	304.2 (99)

The results in brackets represent changes in the activities of particular enzymes expressed as percents.

the following days, the activity of both enzymes radically dropped. In comparison to NADH-GOGAT and Fd-GOGAT, the activity of NADPH-GOGAT in the root plastids of the youngest plants was low. However, during the course of the experiments, the level of NADPH-GOGAT activity was found to be very stable, which led to distinct differentiation of the mutual proportions of the activity of the particular forms of GOGAT depending on the age of the plants (Table 3). The ratio of NADH-GOGAT and Fd-GOGAT activities to that of NADPH-GOGAT in root plastids decreased from 12.4 and 13.2 to 3.2 and 7.8, respectively, during the development of the plants. It was also observed that after 14 days the ferredoxin-dependent enzyme became the dominant form of glutamate synthase in these organelles.

Table 3

Changes in the activities of NADH-GOGAT, NADPH-GOGAT and Fd-GOGAT in *Pisum arvense* root plastids during development

Day of growth	Nmoles glutamate $\times$ g ⁻¹ fr.w. $\times$ hr ⁻¹			Activity ratios		
	NADH-GOGAT	NADPH-GOGAT	Fd-GOGAT	$\frac{\text{NADH-GOGAT}}{\text{NADPH-GOGAT}}$	$\frac{\text{Fd-GOGAT}}{\text{NADPH-GOGAT}}$	$\frac{\text{Fd-GOGAT}}{\text{NADH-GOGAT}}$
3	296.6 (100)	23.9 (100)	315.8 (100)	12.4	13.2	1.1
5	489.4 (165)	25.3 (106)	404.2 (128)	19.3	15.9	0.8
7	216.5 (73)	22.5 (94)	337.9 (107)	9.6	15.0	1.5
12	97.9 (33)	41.3 (173)	189.5 (60)	2.3	4.6	1.9
14	74.1 (25)	22.7 (95)	176.8 (56)	3.2	7.8	2.3

The results in brackets represent changes in the activities of particular enzymes expressed as percents.

The activity of glutamate synthase in the cytosol was many times higher than in the plastids (Table 4). It was also characterized by a lower differentiation of the activities of the various enzyme forms. After 3 days of growth the activity of NADH-GOGAT in the cytosol was 2.4 and 1.5 times that of NADPH-GOGAT and Fd-GOGAT, respectively, while after 14 days, the

Table 4

Changes in the activities of NADH-GOGAT, NADPH-GOGAT and Fd-GOGAT in the cytosol fraction of *Pisum arvense* root cells during development

Day of growth	Nmoles glutamate $\times$ g ⁻¹ fr. w. $\times$ hr ⁻¹			Activity ratios		
	NADH-GOGAT	NADPH-GOGAT	Fd-GOGAT	$\frac{\text{NADH-GOGAT}}{\text{NADPH-GOGAT}}$	$\frac{\text{Fd-GOGAT}}{\text{NADPH-GOGAT}}$	$\frac{\text{Fd-GOGAT}}{\text{NADH-GOGAT}}$
3	4834.2 (100)	2007.0 (100)	3141.4 (100)	2.4	1.6	0.6
5	6492.1 (134)	2007.0 (100)	2059.3 (65)	3.2	1.0	0.3
7	4310.6 (89)	1780.1 (89)	2862.1 (91)	2.4	1.6	0.7
12	1466.0 (30)	820.2 (41)	1727.7 (55)	1.8	2.1	1.2
14	1326.3 (27)	1029.7 (51)	1518.3 (48)	1.3	1.5	1.1

The results in brackets represent changes in the activities of particular enzymes expressed as percents.

activities of all three forms of GOGAT attained similar values, with that of Fd-GOGAT being slightly higher.

Table 5 presents results showing the changes in glutamate synthesis intensity in isolated plastids treated with solutions of hexose phosphates. It was found that glucose-6-phosphate and 6-phosphogluconate and, to a lesser degree, fructose-6-phosphate, increased the amount of synthesized glutamate.

Table 5

The effect of hexose phosphates on the biosynthesis of glutamate in isolated *Pisum arvense* root plastids

Substrate	Nmoles glutamate $\times$ cm ⁻³ fraction $\times$ hr ⁻¹
Control	120.6 (100)
Fructose-1,6-diphosphate	98.0 (81)
Fructose-6-phosphate	180.9 (150)
Glucose-6-phosphate	256.3 (212)
6-Phosphogluconate	233.7 (194)

The incubation medium contained 2-ketoglutarate (2.5 mM), glutamine (2.5 mM) and the appropriate hexose phosphate at a concentration of 2.5 mM, dissolved in buffer B. The values in brackets represent the changes in the amount of determined glutamate in relation to the control, expressed as percentages.

DISCUSSION

Studies conducted earlier (Kłobus et al. 1983, Kubik-Dobosz and Kłobus 1985) have shown that *Pisum arvense* root plastids contain several nitrogen metabolism enzymes, namely nitrite reductase, glutamate synthetase and NADH-glutamate synthase. From the results presented in this report it can be seen that three molecular forms of GOGAT are responsible for the synthesis of glutamate in *Pisum arvense* root plastids. Each of them requires a different cofactor as the electron donor (NADH, NADPH or reduced ferredoxin), with NADH-GOGAT and Fd-GOGAT occurring mainly in the stroma of the plastids, while NADPH-GOGAT seems to be highly associated with the plastid membranes.

Soluble forms of NADH-, NADPH- and Fd-GOGAT were also found in the cytosol fraction of *Pisum arvense* roots. The activity of NADH-GOGAT in the early phase of seedling development was greater than that of the remaining GOGAT forms, which is in agreement with the results of Matoh and Takahashi (1982) obtained on pea roots. The cited authors observed a drastic rise in the activity of root Fd-GOGAT accompanying the further stages of pea plant development. These increases were responsible for the activity of Fd-GOGAT becoming 30 times greater than that of NADH-GOGAT after 17 days. Similar differences in the activities of both enzyme forms in the cytosol of *Pisum arvense* root cells were not observed in this study, although here too, after 14 days the activity of Fd-GOGAT exceeded that of NADH-GOGAT. The relative proportions of the activities of three forms of GOGAT in the plastids of *Pisum arvense* root cells during different phases of development presented themselves differently. The initially modest participation of NADPH-GOGAT in the production of glutamate in these organelles increased during the successive days of growth as the result of

the change in the ratios of NADH- and Fd-GOGAT to NADPH-GOGAT activities. Although some authors still do not clearly distinguish NADH-GOGAT from NADPH-GOGAT (see the review by Oaks and Hirel 1985), as early as in 1976 Dougall and Bloch showed that both these enzymes act independently during the growth of carrot cultures. Even though NADPH-GOGAT has not yet been obtained in pure form, indirect evidence of the existence of enzyme forms dependent on NADH or NADPH was supplied by the work by Matoh et al. (1980b). These authors purified NADH-GOGAT from etiolated pea shoots and found that NADPH was not an effective electron donor for this enzyme. The different nature of the changes in enzymatic activity in *Pisum arvense* root plastids found for NADH-GOGAT and NADPH-GOGAT suggests that two independent forms of GOGAT associated with pyridine nucleotides exist in these organelles.

The main source of NADPH in root plastids is the conversion of glucose-6-phosphate in the pentose phosphate pathway. Stepan-Sarkisian and Fowler (1978) have proved that the pentose phosphate pathway in some areas of the root satisfied the demand for NADPH by the enzymes assimilating nitrogen localized in pea root plastids. In rice roots as well, was the system producing NADPH with the participation of glucose-6-phosphate dehydrogenase found to effectively produce glutamate in amounts comparable with exogenous NADPH (Suzuki et al. 1983). All of the enzymes of the pentose phosphate pathway have been found in root plastids (Emes and Fowler 1979b), and as shown by the study by Emes and Fowler (1983), the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in pea root plastids increased in response to the addition of NO_3^- . Glucose-6-phosphate stimulated the synthesis of amino acids from nitrites and 2-ketoglutarate in the proplastids of tobacco cell cultures (Washitani and Sato 1977). In our experiments, glucose-6-phosphate and 6-phosphogluconate added to the medium stimulated the synthesis of glutamate in isolated *Pisum arvense* root plastids, which suggests at least a partial integration of glutamate synthesis in these organelles with the intensity of the oxidation-reduction processes in the pentose phosphate pathway.

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Aktywność syntazy glutaminianowej zależnej od NADH, NADPH lub ferredoksyny w plastydach i cytosolu komórek korzeni Pisum arvense L.

Streszczenie

W plastydach i cytosolu komórek korzeni *Pisum arvense* znaleziono trzy formy syntazy glutaminianowej (NADH-GOGAT, NADPH-GOGAT i Fd(ferredoksyna)-GOGAT). Aktywność enzymów w obu frakcjach malała z wiekiem roślin, z wyjątkiem plastydowej NADPH-GOGAT wykazującej dużą stabilność aktywności. W cytosolu korzeni kilkudniowych roślin dominowała NADH-GOGAT, jednakże po 14 dniach uprawy obserwowano zrównanie poziomu aktywności wszystkich form GOGAT. Plastydowe NADH-GOGAT i Fd-GOGAT wykazywały zbliżoną aktywność w korzeniach 3-5 dniowych roślin, natomiast po 14 dniach Fd-GOGAT stanowiła przeważającą formę enzymu w tych organellach. W stromie plastydów była zgromadzona cała aktywność NADH-GOGAT i Fd-GOGAT. Z frakcją błon plastydów było związane 37% aktywności NADPH-GOGAT. Izolowane plastydy syntezowały glutaminian z 2-ketoglutaranu i glutaminy, a glukozo-6-fosforan i 6-fosfoglukonian wyraźnie stymulowały ten proces. Przypuszcza się, że synteza glutaminianu w plastydach korzeni *Pisum arvense* może zależeć od intensywności przemian w szlaku pentozofosforanowym.