ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 55, nr 4: 611-619
1986

The role of malate in the synthesis of glutamate in *Pisum arvense* roots

GENOWEFA KUBIK-DOBOSZ

Department of Plant Physiology, Institute of Botany, Wrocław University, Kanonia 6/8, 50-328 Wrocław, Poland

(Received: December 9, 1985; Acepted: January 22, 1986)

Abstract

The in vivo and in vitro activities of NADH-dependent glutamate synthase in excised *Pisum arvense* roots increased several-fold under the influence of malate while pyruvate, oxaloacetate, citrate and succinate inhibited this enzyme. The plastids isolated from *Pisum arvense* roots, when incubated with glutamine and α-ketoglutarate, released glutamate into the medium. Malate clearly stimulated this process. Albizziin (25 mM) completely reduced the presence of glutamate in the incubation mixture. These results indicate that reduced pyridine nucleotides arising in P. *arvense* root plastids during oxidation of malic acid may constitute the indispensable source of electrons for glutamic acid synthesis.

Key words: roots, NADH-glutamate synthase, malate

INTRODUCTION

Glutamate synthase dependent on pyridine nucleotides (NADH-GOGAT--E.C. 1.4.1.14) takes part in the synthesis of glutamate in plant roots (Miflin and Lea 1977). This enzyme is located in root-cell plastids (Emes and Fowler 1979, Suzuki et al. 1981, Kubik-Dobosz and Kłobus 1985). The activity of NADH-GOGAT in roots is especially high in the early stages of plant development (Matoh and Takahashi 1982, Kłobus et al. 1983).

Root plastids have a double membrane with a still relatively unknown permeability for particular metabolites (Falk and Stocking 1976). Because of the structure of the membranes surrounding these organelles and their

little-differentiated inner structure, a similarity between them and etiolated plastids from green tissues may be seen to exist. Studies on isolated chloroplasts have shown that plastid membranes are impermeable to pyridine nucleotides which is the reason why specific compounds, including malic acid, take part in their transport across this membrane (Heber 1974, Walker 1976). The work of Matoh and Takahashi (1982) and our previous study (Kubik-Dobosz and Kłobus 1985) have suggested that malate can take part in supplying reducing equivalents for the reaction catalysed by NADH-GOGAT.

This paper presents the results of studies done on excised *Pisum arvense* roots and on isolated plastids, aimed at explaining the role of malate in the process of glutamate biosynthesis in the roots of these plants.

MATERIALS AND METHODS

The experiments were done on the roots of *Pisum arvense* L. var. 'Nieznaniecka' grown in Hoagland's medium for 7 days. The conditions of culture were as given in the paper by Kłobus et al. (1983).

The activity of glutamate synthase (in vivo) was determined essentially as described by Singh et al. (1983), with modifications. *P. arvense* roots (400 mg) were cut into fragments 1 cm in length and placed in 25 cm^3 flasks containing 1.5 cm^3 100 mM Hepes-NaOH buffer (pH 7.5) and 0.075 cm^3 toluene which increases the permeability of lipoprotein membranes. After 80 min. of preincubation, the reaction medium containing α-ketoglutarate (2 mM), glutamine (10 mM), $NADH_2$ (0.1 mM), ATP (2.5 mM) and either pyruvate, oxaloacetate, citrate, succinate or malate at a concentration of 2 mM, was added. The final volume was 3 cm^3. The control samples did not contain glutamine. $NADH_2$ was not added to some of the samples. After incubation for 60 min. at 30° C, the reaction medium was separated from the roots and heated for 1 min. in a boiling water bath. The cooled solution was centrifuged for 10 min. at 10000 × g. The glutamate was determined in the supernatant by the method of Matoh et al. (1979, 1980). Glutamate was separated from the remaining components on a column of Dowex 1 × 8 (200–400 mesh, acetate) by applying 2 cm^3 of the supernatant to it. The column was first washed with distilled water, then with 1 M CH_3COOH. The glutamate concentration was determined by the ninhydrin method (Garrels et al. 1972) as modified by Matoh et al. (1980). The staining solution contained 0.4 g ninhydrin, 80 cm^3 95% ethanol, 1 g $CdCl_2$, 10 cm^3 CH_3COOH and 20 cm^3 H_2O. After mixing 2 cm^3 of this solution with 1 cm^3

of the eluate, the samples were heated for 10 min. at 80°C and then cooled 10 min. in an ice bath. Absorbance was determined at 506 nm. The activity of glutamate synthase was expressed in nmoles glutamate per min. per g of fresh weight.

For the determination of glutamate synthase activity in vitro *P. arvense* roots were homogenized in 67 mM phosphate buffer (pH 7.8) containing 10 mM KCl, 2 mM $MgCl_2$, 5 mM EDTA, 10 mM dithiothreitol and 1% bovine serum albumin. The ratio of the extraction buffer to the weight of the tissues was 1:1. The homogenate was centrifuged for 15 min. at 16000 × g. The activity of glutamate synthase was determined in the supernatant according to Emes and Fowler (1979) after incubation of the enzyme extract with α-ketoglutarate, $NADH_2$ and glutamine. Glutamine was not added to the control samples. The decrease in the absorbance was followed at 340 nm. The activity of glutamate synthase is presented in nmoles $NADH_2$ per min. per g fresh weight.

Plastids from *P. arvense* roots were isolated according to the method described in a previous paper (Kubik-Dobosz and Kłobus 1985). The homogenate was filtered through a double layer of Miracloth and applied to a discontinuous sucrose density gradient made from 60%, 50% and 30% (w/w) sucrose. The plastid pellet was suspended in 2 cm^3 of 50 mM Hepes-NaOH buffer (pH 7.0) containing 200 mM sucrose and 300 mM mannitol. The plastid suspension (0.5 cm^3) was incubated for 60 min. at 30°C with the appropriate substrate (2.5 mM α-ketoglutarate, 2.5 mM glutamine, 1.25 mM ATP, 2.5 mM malate, 25 mM albizziin) dissolved in 50 mM Hepes-NaOH buffer (pH 7.0), 200 mM sucrose, 300 mM mannitol and 0.3% bovine serum albumin. The final volume of the reaction medium was 3 cm^3. Next, the plastids were removed by centrifugation at 15000 × g for 10 min. The samples were deproteinized with 0.25 volume of 2 M $HClO_4$, and after centrifuging off the protein, were neutralized with 1 M K_2CO_3. The solution above the precipitate of potassium perchlorate was used to determine the content of glutamate by the enzymic method of Bernt and Bergmeyer (1970) and oxaloacetate by the method of Hohorst and Reim (1970) measuring the reduction of NAD^+ in the presence of exogenous glutamate or malate dehydrogenases.

RESULTS

The roots of *Pisum arvense* seedlings which were peincubated in a toluene solution and then treated with α-ketoglutarate, $NADH_2$ and ATP, released significant amounts of glutamate into the incubation medium

Table 1

The effect of organic acids on the in vivo activity of glutamate synthase in the roots of *Pisum arvense* and on the content of glutamate in the incubation medium

Substrates	Glutamate content, nmoles	Glutamate synthase activity, nmoles glutamate × g^{-1} fresh weight × $min.^{-1}$			
		$-NADH_2$	(%)	$+NADH_2$	(%)
α-Ketoglutarate + ATP	969.4 ± 37.7	—		—	
α-Ketoglutarate + ATP + glutamine	1550.2 ± 46.5	12.7	(100)	24.2	(100)
α-Ketoglutarate + ATP + glutamine + pyruvate	1275.9 ± 57.4	6.1	(48)	13.2	(54)
α-Ketoglutarate + ATP + glutamine + citrate	1426.8 ± 51.3	3.4	(27)	21.6	(89)
α-Ketoglutarate + ATP + glutamine + oxaloacetate	1315.8 ± 67.2	5.5	(43)	11.2	(46)
α-Ketoglutarate + ATP + glutamine + succinate	1496.5 ± 40.1	8.6	(67)	13.8	(57)
α-Ketoglutarate + ATP + glutamine + malate	1620.4 ± 49.7	65.2	(512)	91.0	(376)

Pieces of *P. arvense* roots were incubated for 60 min. at 30 C in a medium containing 2 mM α-ketoglutarate, 2.5 mM ATP, 10 mM glutamine with or without the addition of 0.1 mM $NADH_2$ and 2 mM of the tested organic acids. Glutamate was determined colorimetrically (see Material and Methods). The activity of glutamate synthase (in vivo) in the samples containing the organic acids was calculated in respect to the control samples without glutamine. The results are the means of three repetitions. Differences between the mean values of two different combinations were accepted as significant if they were over twice the value of SE.

Table 2

The effect of malate on the in vitro activity of glutamate synthase in *Pisum arvense* roots

Substrate	Glutamate synthase activity	
	nmoles $NADH_2 \times min^{-1} \times g^{-1}$ fresh weigh	%
Control	7.7±0.21	100
Malate (10 mM)	14.2±0.43	184
Malate (30 mM)	12.9±0.41	167

Excised roots were incubated in 67 mM phosphate buffer (pH 7.5) and malate for 6 hrs. at room temperature. The activity of glutamate synthase in the roots was determined according to the method in vitro. The results are the mean of three repetitions. Differences between the mean values of two different combinations were accepted as significant if they were over twice the value of SE.

Table 3

The effect of malate and albizziin on the content of oxaloacetate and glutamate in the medium after incubation with plastids isolated from the roots of *Pisum arvense*

Substrates	Oxaloacetate, nmoles	Glutamate, nmoles
α-Ketoglutarate + ATP + glutamine	0	29.5±1.3
α-Ketoglutarate + ATP + glutamine + albizziin	0	0
α-Ketoglutarate + ATP + glutamine + malate	18.3±0.9	78.9±5.2
α-Ketoglutarate + ATP + glutamine + malate + albizziin	4.5±0.3	0

0.5 cm^3 of the plastid suspension were incubated for 60 min at 30 C in the presence of 2.5 mM α-ketoglutarate, 1.25 mM ATP, 2.5 mM glutamine, 2.5 mM malate and 25 mM albizziin. Oxaloacetate and glutamate were determined in the incubation medium enzymatically (see Material and Methods). The results are the mean values of three repetitions. Differences between the mean values of two different combinations were accepted as significant if they were over twice the value of SE.

(Table 1). After 10 mM glutamine had been added, a clearly evident rise in the glutamate content of the solution was seen, which indicates that at least part of the assayed amino acid was the product of the reaction catalysed by glutamate synthase. Because glutamate dehydrogenase also occurs in *P. arvense* roots (Kłobus et al. 1983), the in vivo activity of glutamate synthase was calculated in respect to the samples not containing glutamine, thus eliminating glutamate dehydrogenase from the production of glutamate in these tissues.

The addition of pyruvate, citrate, oxaloacetate or succinate to the incubation medium caused an insignificant decrease in the concentrations of glutamate determined in the solution bathing the roots (Table 1). At the same time, a distinct fall in the activity of glutamate synthase (in vivo) was seen in the samples treated with these acids. However, in the *P. arvense* roots treated with malate, the activity of the studied

enzyme increased by about 4-fold. A similar effect of the exogenous organic acids on the activity of glutamate synthase was observed in samples not containing $NADH_2$. Only malate clearly increased the activity of the enzyme while the remaining acids had an inhibitory effect.

The results complied in Table 2 present the changes in the activity of glutamate synthase measured in vitro in *P. arvense* roots treated with different concentrations of malate. It was found that the activity of the studied enzyme increased under the influence of malate, although the concentration of this acid did not have an effect on the degree of stimulation.

The *P. arvense* root plastids isolated in a sucrose density gradient and then incubated in a solution of α-ketoglutarate and glutamine in the presence of ATP, released glutamate into the reaction medium (Table 3). The addition of 2.5 mM malate to the incubation medium caused an over two-fold increase in the glutamate concentration in the medium. In addition, the presence of oxaloacetate was found in this solution, a fact not observed in the control samples. An analogous of glutamine, albizziin (L-2-amino-3-ureido-propionic acid), an inhibitor of glutamate synthase (Lea and Fowden 1975, Oaks et al. 1979) completely inhibited the release of glutamate both in the presence and in the absence of malate in the incubation medium.

DISCUSSION

On the basis of the conducted experiments on excised *P. arvense* roots, a significant stimulation of NADH-GOGAT activity was demonstrated when malate was present in the incubation medium. The observed effect of malate is probably connected with its metabolism, because other organic acids, metabolites of the tricarboxylic acid cycle, even caused a decrease in the glutamate synthase activity in these roots. The inhibitory effect of oxaloacetate or succinate on the activity of the studied enzyme was thus similar to the in vitro effect presented by Boland and Benny (1977) showing that NADH-GOGAT isolated from lupine papillae is inhibited competetively by oxaloacetate.

Matoh and Takahashi (1982) found that the addition of malate to a mixture containing isolated chloroplasts caused the amount of ^{14}C--glutamate produced to be doubled, which indicated that the rate of glutamine metabolism in these organelles increased. This process was inhibited by azaserine (a GOGAT inhibitor). Our experiments done on *P. arvense* root plastids showed that after albizziin was used as a a GOGAT inhibitor, glutamate was not found in the reaction medium.

P. arvense root plastids contain a NADH-dependent glutamate synthase and also nitrite reductase (Kłobus et al. 1983, Kubik-Dobosz and Kłobus 1985). As results from the studies by Kłobus (1984), the reduced nucleotides taking part in the reduction of nitrites in *P. arvense* root plastids can be formed in the process of oxidation of glucose-6-phosphate or malate. The transfer of the amide group from glutamine to α-keto-glutarate catalysed by NADH-glutamate synthase is a reduction process and requires the input of electrons. As is known, plastid membranes are impermeable to pyridine nucleotides (Heber 1974), which necessitates the involvement of dicarboxylic acids in their transport. From the studies of Nobel and Wang (1970) and Poincelot (1975) it is seen that dicarboxylic acids easily permeate the chloroplast membrane. The external membrane is no obstacle in their transport because it is unspecifically permeable to low molecular weight compounds (Heldt and Rapley 1970, Heldt and Sauer 1971). The specific transport of these acids across the inner membrane is dependent on the presence of the appropriate carrier (Walker 1976). The transport of dicarboxylic acids across the membrane of root plastids has not yet been satisfactorily explained. In the experiments presented in this paper, the appearance of oxaloacetate in the reaction medium was noted when the latter contained malate and root plastids isolated from *P. arvense* roots. Because its presence was not found in the mixture incubated without the addition of malate, it may be supposed that oxaloacetic acid is a product of malate metabolism inside *P. arvense* root plastids. A molecular form of malate dehydrogenase with a different isolectric point from that of the mitochondrial or cytosolic forms of this enzyme has been found (Kubik-Dobosz 1986) inside these plastids. The malate dehydrogenase contained in the plastids had a distinct affinity for NAD^+ (Kubik-Dobosz and Kłobus 1985). It would seem, then, that analogically to the oxaloacetate-malate shuttle functioning in the chloroplasts of higher plants, which sends reduced pyridine nucleotides outside of the chloroplast (Heber 1974) a similar system operating in the opposite direction is functioning between the plastids and cytosol of *P. arvense* root cells. This enables the necessary reduced pyridine nucleotides to be transferred to the inside of the plastids. Of course, such a system requires the participation of cytosolic and plastid malate dehydrogenases. The oxidation of malate linked with the reduction of NAD^+ going on inside root plastids may supply them with reducing equivalents. The results obtained in this study thereby allow the conclusion to be drawn that reduced pyridine nucleotides, supplying the necessary reducing power for the biosynthesis of glutamate, are transported from the cytosol to the inside of *P. arvense* root plastids. Malic acid would play the role of their transport intermediate.

Acknowledgment

This study was financed through problem MR II/7.3.06.

REFERENCES

Bernt E., Bergmeyer H. U., 1970. L-Glutamat Bestimmung mit Glutamat-Dehydrogenase und NAD. In: Methoden der enzymatischen Analyse. H. U. Bergmeyer (ed.), Akademie--Verlag, Berlin, Bd. III, pp. 1659–1663.

Boland M. J., Benny A. G., 1977. Enzymes of nitrogen metabolism in legume nodules: purification and properties of NADH-dependent glutamate synthase from lupin nodules. Eur. J. Biochem. 79: 355.

Emes M. J., Fowler M. W., 1979. The intracellular location of the enzymes of nitrate assimilation in the apices of seedling pea roots. Planta 144: 249–253.

Falk R. H., Stocking C. R., 1976. Transport in plants. III. Intracellular interactions and transport processes. In: Encyclopedia of Plant Physiology. Stocking C. R. and Heber U. (eds.), Springer-Verlag, New York, vol. 3, pp. 3–50.

Garrels J. J., Elgin S. C. R., Benner J., 1972. A histone protease of rat liver chromatin. Biochem. Biophys. Res. Commun. 46: 545–551.

Heber U., 1974. Metabolite exchange between chloroplasts and cytoplasm. Ann. Rev. Plant Physiol. 25: 393–421.

Heldt H. W., Rapley L., 1970. Specific transport of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetonephosphate, and of dicarboxylates across the inner membrane of spinach chloroplasts. FEBS Lett. 10: 143–148.

Heldt H. W., Sauer F., 1971. The inner membrane of the chloroplast envelope as the site of specific metabolite transport. Biochim. Biophys. Acta 234: 83–91.

Hohorst H-J., Reim M., 1970. Oxalacetat. UV-Spektrophotometrische Bestimmung. In: Methoden der enzymatischen Analyse. H. U. Bergmeyer (ed.), Akademie-Verlag, Berlin, Bd. III, pp. 1560–1564.

Kłobus G., 1984. Sources of reducing equivalents for nitrite reductase in *Pisum arvense* roots. Acta Soc. Bot. Pol. 53: 507–513.

Kłobus G., Kubik-Dobosz G., Buczek J., 1983. The intracellular location of nitrogen metabolism enzymes in *Pisum arvense* L. roots. Acta Soc. Bot. Pol. 52: 215–222.

Kubik-Dobosz G., 1986. The intracellular localization of malate dehydrogenase isoenzymes in *Pisum arvense* L. roots. Acta Soc. Bot. Pol. 55: 621–627.

Kubik-Dobosz G., Kłobus G., 1985. The localization of nitrate reductase, glutamate synthase and malate metabolism enzymes in *Pisum arvense* L. roots. Acta Soc. Bot. Pol. 54: 85–93.

Lea P. J., Fowden L., 1975. Asparagine metabolism in higher plants. Biochem. Physiol. Pflanzen 168: 3–14.

Matoh T., Ida S., Takahashi E., 1980. A rapid and sensitive assay for ferredoxin glutamate synthase. Bull. Res. Inst. Food Sci. Kyoto Univ. 43: 1–6.

Matoh T., Suzuki F., Ida S., 1979. Corn leaf glutamate synthase: Purification and properties of the enzyme. Plant Cell Physiol. 20: 1329–1340.

Matoh T., Takahashi E., 1982. Changes in the activities of ferredoxin- and NADH--glutamate synthase during seedling development of peas. Planta 154: 289–294.

Miflin B. J., Lea P. J., 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28: 299–329.

Nobel P. S., Wang C., 1970. Amino acid permebility of pea chloroplasts as measured by osmotically determined reflection coefficients. Biochim. Biophys. Acta 211: 79–87.

Oaks A., Jones K., Misra S., 1979. A comparsion of glutamate synthase obtained from maize endosperm and roots. Plant Physiol. 63: 793–795.

Poincelot R. P., 1975. Transport of metabolites across isolated envelope membranes of spinach chloroplasts. Plant Physiol. 55: 849–852.

Singh V. K., Mathur M., Mukerji D., Mathur S. N., 1983. A colorimetric method for assay of glutamate synthase in leaf tissue of *Vigna mungo*. Can. J. Bot. 61: 290–294.

Suzuki A., Gadal P., Oaks A., 1981. Intracellular distribution of enzymes associated with nitrogen assimilation in roots. Planta 151: 457–461.

Walker D. A., 1976. Transport in plants. III. Intracellular interactions and transport processes. In: Encyclopedia of Plant Physiology. Stocking C. R. and Heber U. (eds.), Springer-Verlag, New York, pp. 85–136.

Funkcja jabłczanu przy syntezie glutaminianu w korzeniach Pisum arvense

Streszczenie

Aktywność in vivo i in vitro zależnej od NADH syntazy glutaminianowej w odciętych korzeniach *Pisum arvense* zwiększała się kilkakrotnie pod wpływem kwasu jabłkowego, natomiast pirogranian, szczawiooctan, cytrynian i bursztynian hamowały aktywność enzymu. Plastydy, wyizolowane z korzeni *Pisum arvense*, inkubowane z glutaminą i 2-ketoglutaranem wydzielały do medium inkubacyjnego kwasu glutaminowy. Jabłczan wyraźnie stymulował ten proces. Albizzina (25 mM) redukowała całkowicie obecność glutaminianu w roztworze inkubacyjnym. Uzyskane wyniki wskazują na to, że zredukowane nukleotydy pirydynowe, powstające w plastydach korzeni *Pisum arvense* podczas utleniania kwasu jabłkowego, mogą stanowić niezbędne źródło elektronów do biosyntezy kwasu glutaminowego.