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Ammonia assimilation in *Vitis vinifera* L.: II. Leaf and root glutamine synthetase

by

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Die Ammonium-Assimilation bei *Vitis vinifera* L.: II. Die Glutaminsynthetase der Blätter und Wurzeln

Zusammenfassung. — Bei der Rebsorte Chenin blanc (*Vitis vinifera* L.) lag nach differenzierter Zentrifugation sowohl in den löslichen wie in den strukturierten Fraktionen Glutaminsynthetase- (GS-)Aktivität vor. Die prozentuale Verteilung der GS-Aktivität auf Blatt- und Wurzelextrakte betrug jeweils 39,5 und 49,2 % im 10 000-*g*-Sediment, 38,6 und 41,1 % im 23 500-*g*-Sediment sowie 21,9 und 9,7 % im 23 500-*g*-Überstand. In den Blättern war die GS-Aktivität stets höher als in den Wurzeln. Untersuchungen der Enzymkinetik erbrachten bei dem 10 000-*g*-Sediment keine signifikanten Unterschiede zwischen der GS-Aktivität der Blätter und der Wurzeln. Die K_m-Werte für L-Glutamat, ATP und Hydroxylamin betrugen 3,2 ± 0,7 mM, 0,8 ± 0,2 mM und 0,8 ± 0,2 mM. Die Bildung von γ-Glutamylhydroxamat verlief in den ersten 35 min linear. *In vitro* waren die optimalen Reaktionsbedingungen ein pH von 7,70—8,10, eine Inkubationstemperatur von 37 °C und eine Enzymmenge, die dem Gehalt von 75—105 mg Frischgewebe entsprach. L-Arginin, L-Ornithin und Carbamylphosphat in Konzentrationen von 5 mM hemmten die Enzymwirkung um 13,6 bzw. 12 %.

Introduction

Recent studies on ammonia assimilation in higher plants have indicated that, in addition to the reaction catalyzed by glutamate dehydrogenase, another route for ammonia entry into nitrogenous compounds exists; namely, the ATP-driven addition of ammonia to glutamate and transfer of the amide group of glutamine to α-ketoglutarate (LEA and MIFLIN 1974). These two reactions are catalyzed by the enzymes glutamine synthetase (L-glutamate : ammonia ligase, ADP, EC 6.3.1.2.; GS) and glutamate synthase (L-glutamate : $NADP^+$ oxidoreductase, EC 1.4.1.13., or L-glutamate : ferredoxin oxidoreductase, EC 1.4.7.1.; GOGAT), respectively.

GS has been extracted, partially purified and studied in several plants, such as *Vicia faba* (HAYSTEAD 1973), *Cucurbita moschata* (LIGNOWSKI *et al.* 1971), *Pisum sativum* (VARNER and WEBSTER 1955, O'NEAL and JOY 1974, 1975, GIVAN 1976), *Oryza sativa* (KANAMORI and MATSUMOTO 1972, GUIZ *et al.* 1979, HIREL and GADAL 1980) and germinating peanuts (WINTER *et al.* 1982).

In a previous communication (ROUBELAKIS-ANGELAKIS and KLIEWER 1983) the presence of glutamate dehydrogenase was demonstrated in roots and leaves of grapevine along with some of its kinetic properties. In this report, information on the extraction, intracellular localization and some properties of GS from leaf and root tissue of *Vitis vinifera* L. cv. Chenin blanc is presented. Numerous attempts to obtain active GOGAT preparations from grapevine roots have not yet been successful.

Materials and methods

GS was extracted from leaf and root tissue of *Vitis vinifera* L. cv. Chenin blanc. The plant material, sampling technique and sample treatment used were the same as described previously (ROUBELAKIS-ANGELAKIS and KLIEWER 1983).

Enzyme extraction procedure and assay conditions: 5—10 g of fresh tissue was used for enzyme extraction. The extraction procedure and the steps of cell fractionation by differential centrifugation were as already described (ROUBELAKIS-ANGELAKIS and KLIEWER 1983). The grinding medium consisted of 100 mM Tris-HCl, pH 7.8, 6 mM L-cysteine-HCl and 4 % (w/v) Polyethylene Glycol 4000. The homogenizing medium consisted of 100 mM Tris-HCl, pH 7.8, 10 mM L-cysteine-HCl, 4 mM $MgCl_2$ and 1 % (v/v) Triton X-100. Determination of enzymic activity was based on the reaction of L-glutamate, hydroxylamine and ATP to form γ-glutamyl hydroxamate in the presence of a divalent ion. The reaction mixture consisted of 83 mM Tris-HCl, pH 7.8, 20 mM L-glutamate, 5 mM hydroxylamine, 20 mM $MgSO_4$, 3.6 mM ATP, enzyme extract equivalent to about 100 mg of fresh tissue and deionized water to a total volume of 3 ml. All reactants were made up in 50 mM Tris-HCl, pH 7.8 buffer and were neutralized, if necessary. Complete reaction mixtures lacking ATP were allowed to equilibrate in a water bath at 37 °C for 5 min; then ATP was added and the reaction allowed to proceed for time intervals up to 1 h. Controls lacking hydroxylamine were included. The reaction was terminated by adding 1 ml of freshly prepared ferric chloride reagent, consisting of equal volumes of 24 % (w/v) TCA, 10 % (w/v) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, and 6 M HCl (LIPMAN and TUTTLE 1945, LIGNOWSKI *et al.* 1971). Precipitated protein was removed by centrifugation and absorbance was read at 520 nm. Standards of γ-glutamyl hydroxamate with all the reaction mixture components except ATP were run simultaneously.

Enzyme activity was calculated from the net initial velocity and expressed as units per g fresh tissue. 1 unit was defined as the amount of enzyme required to mediate the formation of γ-glutamyl hydroxamate equivalent to an increase of 0.1 A_{520} unit per h. Under the described experimental conditions this was equivalent to about 42 µM of product.

Results and discussion

GS activity was present in extracts and partially purified preparations from grape-vine leaf and root tissues. Enzyme activity was dependent on the presence of glutamate, hydroxylamine, ATP and a divalent ion, preferably magnesium. For active enzyme preparations, polyethylene glycol was required in the extracting medium.

In vitro GS activity in grapevine leaves was greater than in roots; the respective values were 35.4 ± 7.3 and 22.1 ± 3.4 units/g fresh tissue. In most of the plants that have been studied, GS in aerial parts exceeded root enzyme activity (STEWART *et al.* 1980). The magnitude of root and leaf GS activity and their contribution to total ammonia assimilation via the GS/GOGAT pathway varies with the plant species.

The distribution of GS among the subcellular fractions of leaf and root extracts obtained by differential centrifugation is presented in Table 1. In both tissues, a similar pattern of GS distribution among the fractions was observed. In leaf extracts, approximately 40 % of enzyme activity was associated with the 10,000 *g* pellet fraction, 40 % with the 23,500 *g* pellet fraction and 20 % with the 23,500 *g* soluble fraction. In root extracts, the corresponding respective distribution was 50, 40 and 10 % (Table 1). Preliminary work indicated that the 10,000 *g* pellet fraction contained mainly plastids

Table 1

Subcellular distribution of glutamine synthetase activity in leaf and root tissues from *Vitis vinifera* L. cv. Chenin blanc[1])

Subcelluläre Verteilung der Glutaminsynthetase-Aktivität in Blatt- und Wurzelgewebe der Rebsorte Chenin blanc (*Vitis vinifera* L.)

Subcellular fraction[2])	Glutamine synthetase activity[3]), %	
	Leaf	Root
P_1	18.5 ± 4.8	4.2 ± 2.1
P_2	20.1 ± 3.6	36.9 ± 7.9
P_3	36.4 ± 7.4	31.1 ± 6.8
P_4	3.1 ± 2.1	18.1 ± 4.9
S	21.9 ± 6.4	9.7 ± 4.9

[1]) The values are the means of 12 and 5 extractions for leaf and root tissue, respectively.
[2]) Subcellular fractions were separated by differential centrifugation as it appears in the flow diagram of Part I; see Bibliography, no. 16.
[3]) Assay conditions were as described in the text.

whereas the 23,500 *g* one contained mostly mitochondria (ROUBELAKIS-ANGELAKIS, unpublished data). MIFLIN (1974) working with *Spinacia oleracea*, O'NEAL and JOY (1973) with *Pisum sativum* and HAYSTEAD (1973) with *Vicia faba* leaves found most of GS activity associated with the chloroplastid fraction. On the other hand, WALLSGROVE *et al.* (1979) found 60 % of pea leaf GS activity associated with the chloroplastid fraction and 40 % with the cytosolic one. In addition, JACKSON *et al.* (1979) reported GS activity in the mitochondrial fraction of leaf extracts from pea seedlings; it was not possible, however, to conclude whether the activity originated from mitochondria or from plastid enzyme contamination.

In the present investigation it is highly unlikely that GS activity in the mitochondrial fraction resulted from contamination from plastids, since nearly equal percentages of total enzyme activity (40 %) was found in the two fractions. If GS contamination from the plastid fraction had occurred, then the relative activity of the mitochondrial fraction would likely be significantly less than the activity of plastid fraction. Recently, multiple forms of GS were found in rice (GUIZ *et al.* 1979, HIREL and GADAL 1980), barley leaves (MANN *et al.* 1979) and soybean hypocotyl (STASIEWICZ and DUNHAM 1979) which were of cytoplasmic and chloroplastid origin, whereas in etiolated tissues and roots only the cytoplasmic form existed (MANN *et al.* 1980).

Kinetic properties of plastid (10,000 *g* pellet) GS from grapevine leaf and root tissues showed no significant differences. These results support findings for soybean hypocotyl GSs (STASIEWICZ and DUNHAM 1979) as well as barley (MANN *et al.* 1979), rice (GUIZ *et al.* 1979, HIREL and GADAL 1980) and peanut GS isoenzymes (WINTER *et al.* 1982), which showed similar apparent K_m values for ammonium and ATP.

GS activity was dependent on glutamate concentration (Fig. 1). Both, grape leaf and root enzyme showed standard Michaelis-Menten kinetics for L-glutamate (Fig. 1, inset) with an average K_m value of 3.2 ± 0.7 mM. At glutamate concentrations greater than about 20 mM there was a partial inhibition of enzyme activity; however, addition of high concentration of ATP and Mg^{2+} to the reaction mixture could fully reverse the inhibition. K_m values for glutamate reported for some annual plants enzyme varied

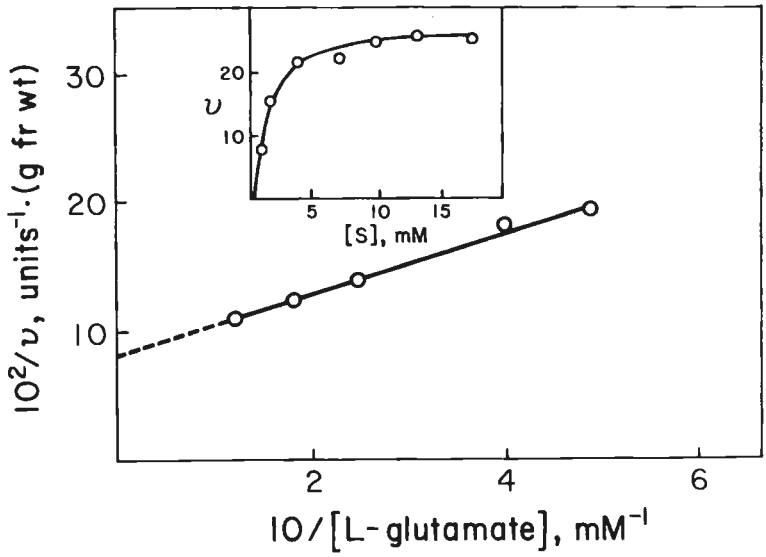

Fig. 1: Dependence of glutamine synthetase activity on L-glutamate concentration: Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions and components were as described in the text.

Abhängigkeit der Glutaminsynthetase-Aktivität von der L-Glutamat-Konzentration: Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen und -komponenten s. Text.

from 1 to 13.3 mM (STEWART *et al.* 1980). Most of these GSs exhibited normal Michaelis-Menten kinetics whereas chloroplastid GS from rice leaves (GUIZ *et al.* 1979) and barley leaves (MANN *et al.* 1980) showed negative cooperative binding to glutamate.

Grapevine GS showed an absolute dependence on a divalent ion for *in vitro* activity. Among the tested divalent ions, Mg^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} were the most effective. The relative activities for the same concentration of these ions were 100, 54 and 22 %, respectively.

The response of GS to increasing concentrations of ATP depended upon the concentration of Mg^{2+}. Only when the Mg^{2+} concentration in the reaction mixture exceeded 4-fold or more the concentration of ATP normal Michaelis-Menten kinetics were observed (Fig. 2, inset). The average K_m value calculated from the double reciprocal plots (Fig. 2) was 0.75 ± 0.2 mM, which is within the range reported for other plants enzyme (see review by STEWART *et al.* 1980).

The response of GS to hydroxylamine is generally considered to be analogous to ammonia (VARNER and WEBSTER 1955). Grapevine GS activity at increasing concentrations of hydroxylamine appear in Fig. 3, inset. Concentrations of hydroxylamine greater than 25 mM caused inhibition of enzyme activity. The K_m value for hydroxylamine in grapevine extracts was 0.8 ± 0.2 mM, which is greater than the corresponding values reported for rice root (KANAMORI and MATSUMOTO 1972) and pumpkin germinating seed enzyme (LIGNOWSKI *et al.* 1971) but lower than for barley root GS (STEWART *et al.* 1980).

Optimum conditions for *in vitro* GS activity were pH 7.70—8.10, when Mg^{2+} fulfilled the cation requirements of the enzyme; enzyme amount equivalent to 75—105 mg

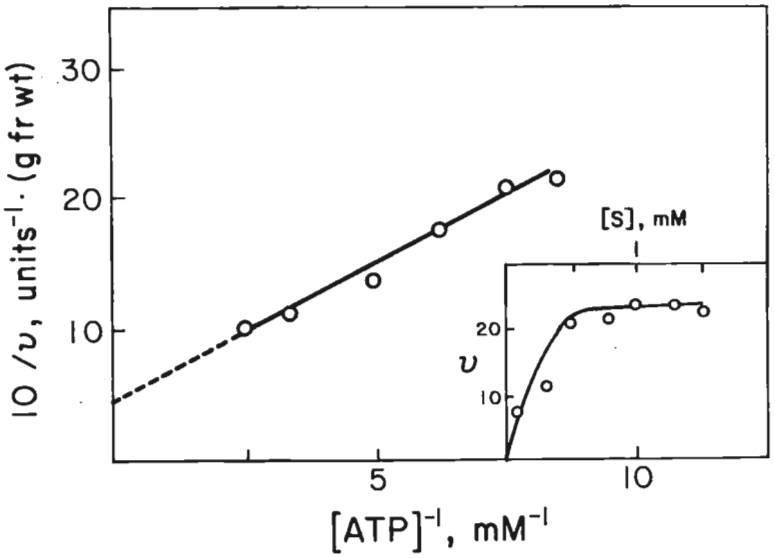

Fig. 2: Dependence of glutamine synthetase activity on ATP concentration: Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions and components were as described in the text.

Abhängigkeit der Glutaminsynthetase-Aktivität von der ATP-Konzentration: Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen und -komponenten s. Text.

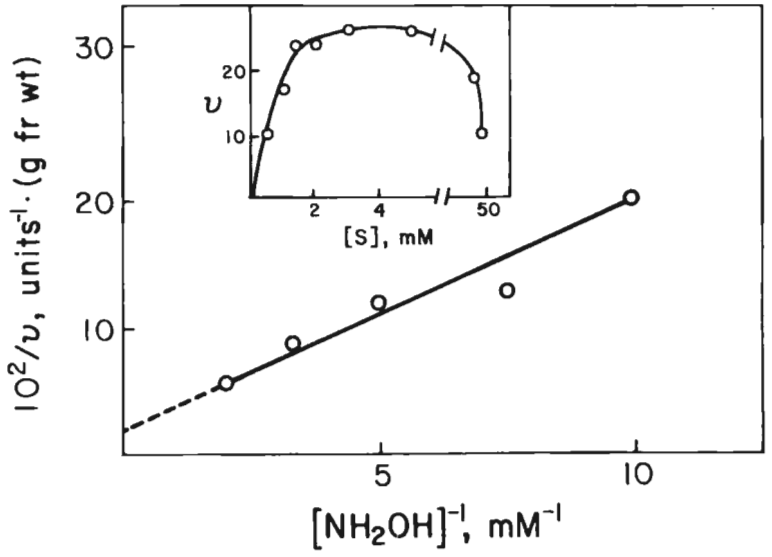

Fig. 3: Dependence of glutamine synthetase activity on hydroxylamine concentration: Lineweaver-Burk and Michaelis-Menten (inset) plots. Other reaction conditions and components were as described in the text.

Abhängigkeit der Glutaminsynthetase-Aktivität von der Hydroxylamin-Konzentration: Enzymkinetik nach Lineweaver-Burk und Michaelis-Menten (Einsatz). Übrige Reaktionsbedingungen und -komponenten s. Text.

Table 2

Effect of L-arginine, L-ornithine and carbamyl-phosphate on glutamine synthetase activity from grapevine leaf tissue

Einfluß von L-Arginin, L-Ornithin und Carbamylphosphat auf die Glutaminsynthetase-Aktivität von Rebenblattgewebe

Tested compound (5 mM)	Glutamine synthetase activity[1]), %
L-Arginine	77.0
L-Ornithine	94.0
Carbamyl phosphate	88.0
Control	100.0

[1]) Reaction conditions were as described in the text.

of fresh tissue; and incubation temperature 37 °C. Linearity between product formation and incubation period was observed for 35 min under the described experimental conditions.

Studies with *Escherichia coli* have shown that GS is a strategic target for cellular control and a cumulative feedback control by each metabolite derived from glutamine exists (STADTMAN 1973). This regulation involves inhibition and/or repression of GS. The regulatory characteristics of bacterial GS suggest that factors other than ammonium availability may regulate its assimilation through the GS/GOGAT pathway. O'NEAL and JOY (1975) found that several metabolites, such as histidine, ornithine, arginine, and carbamyl phosphate inhibited pea GS. In the present study, L-arginine, L-ornithine and carbamyl phosphate at 5 mM concentration caused inhibition of 13, 6 and 12 % of grapevine GS activity, respectively (Table 2). The nature of inhibition was not studied for these compounds. However, according to the above authors ornithine was a competitive inhibitor with respect to glutamate in pea enzyme.

Summary

Glutamine synthetase (GS) activity in *Vitis vinifera* L. cv. Chenin blanc leaf and root tissues was present in the supernatant and particulate fractions. The percentage distribution of GS activity in leaf and root extracts were respectively 39.5 and 49.2 % in the 10,000 *g* pellet, 38.6 and 41.1 % in the 23,500 *g* pellet, and 21.9 and 9.7 % in the 23,500 *g* supernatant fractions. Leaf GS activity was always greater than root enzyme activity. Kinetic studies revealed no significant differences between leaf and root GS from the 10,000 *g* pellet fraction. The K_m values for L-glutamate, ATP and hydroxylamine were respectively 3.2 ± 0.7 mM, 0.8 ± 0.2 mM, and 0.8 ± 0.2 mM. Formation of γ-glutamyl hydroxamate was linear for the first 35 min. Optimum *in vitro* reaction conditions were pH 7.70—8.10, incubation temperature 37 °C, and amount of enzyme equivalent to 75—105 mg of fresh tissue. L-arginine, L-ornithine and carbamyl phosphate at a concentration of 5 mM caused inhibition of 13.6 and 12 %, respectively.

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