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γ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization*

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Abstract. γ-Glutamylcysteine synthetase activity (EC 6.3.2.2) was analysed in Sephacryl S-200 eluents of extracts from cell suspension cultures of *Nicotiana tabacum* L. cv. Samsun by determination of γ-glutamylcysteine as its monobromobimane derivative. The enzyme has a relative molecular mass (M_r) of 60000 and exhibits maximal activity at pH 8 (50% at pH 7.0 and pH 9.0) and an absolute requirement for Mg^{2+}. With 0.2 mM Cd^{2+} or Zn^{2+}, enzyme activity was reduced by 35% and 19%, respectively. Treatment with 5 mM dithioerythritol led to a heavy loss of activity and to dissociation into subunits (M_r 34000). Buthionine sulfoximine and L-methionine-sulfoximine, known as potent inhibitors of γ-glutamylcysteine synthetase from mammalian cells, were found to be effective inhibitors of the plant enzyme too. The apparent K_m values for L-glutamate, L-cysteine, and α-aminobutyrate were, respectively, 10.4 mM, 0.19 mM, and 6.36 mM. The enzyme was completely inhibited by glutathione ($K_i = 0.42$ mM). The data indicate that the rate of glutathione synthesis in vivo may be influenced substantially by the concentration of cysteine and glutamate and may be further regulated by feedback inhibition of γ-glutamylcysteine synthetase by glutathione itself.

γ-Glutamylcysteine synthetase is, like glutathione synthetase, localized in chloroplasts as well as in the cytoplasm. Chloroplasts from *Pisum sativum* L. isolated on a Percoll gradient contained about 72% of the γ-glutamylcysteine synthetase activity in leaf cells and 48% of the total glutathione synthetase activity. In chloroplasts of *Spinacia oleracea* L. about 61% of the total γ-glutamylcysteine synthetase activity of the cells were found and 58% of the total glutathione synthetase activity. These results indicate that glutathione synthesis can take place in at least two compartments of the plant cell.

Key words: Cell suspension culture – γ-Glutamylcysteine synthetase – Glutathione synthesis – *Nicotiana* (Glutathione synthesis)

Introduction

Biosynthesis of glutathione (GSH) takes place in two steps. In the first step the dipeptide γ-glutamylcysteine (γ-GC) is produced from L-glutamate and L-cysteine in an ATP-dependent reaction catalyzed by γ-glutamylcysteine synthetase (EC 6.3.2.2). In the second step, glycine is added to the C-terminal site of the dipeptide to yield glutathione; this reaction is catalyzed by glutathione synthetase (EC 6.3.2.3) and depends also on ATP:

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \rightarrow \gamma\text{-glutamylcysteine} + \text{ADP} + P_i \quad (1)$$

$$\gamma\text{-glutamylcysteine} + \text{glycine} + \text{ATP} \rightarrow \gamma\text{-glutamylcysteinylglycine} + \text{ADP} + P_i \quad (2)$$

Both enzymes have been purified and characterized (Seelig and Meister 1985; Meister 1985) and the two reactions have been intensively studied in animals and microorganisms (Meister and Anderson 1983).

Despite the importance of glutathione in higher plants (Rennenberg 1982), studies on glutathione synthesis in plants so far are fragmentary. Only recently has GSH synthetase been found and characterized in several species (Law and Halliwell 1986; Macnicol 1987; Hell and Bergmann 1988; Klapheck et al. 1988). The catalytic properties described in these papers indicate a strong similarity of the plant enzyme to GSH synthetase from animal sources and suggest that GSH synthesis in plants proceeds via the same pathway as it does in animals. Corresponding data on the γ-GC synthetase from higher

* Dedicated to Professor A. Pirson on the occasion of his 80th birthday

Abbreviations: DTE = dithioerythritol; γ-GC = γ-glutamylcysteine; GSH = glutathione; Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

plants are missing. There is only one paper by Webster and Varner (1954) on the mechanism of γ-GC synthesis in wheat germ, which has never been confirmed, and a preliminary report indicating the presence of γ-GC synthetase in cultured tomato cells (Steffens and Williams 1987). In animal cells, γ-GC synthesis is considered to be the rate-limiting step in GSH synthesis. γ-glutamylcysteine synthetase has been shown to be inhibited by GSH at concentrations which are similar to those found in vivo, indicating a physiologically significant feedback inhibition of GSH synthesis (Jackson 1969; Richman and Meister 1975). On the other hand, γ-GC synthesis may be regulated by the availability of cysteine in the cells. The γ-GC synthetases from different species exhibit K_m values for cysteine that are near intracellular concentrations of cysteine so that changes in cellular cysteine level can influence the rate of γ-GC formation and, by this, GSH synthesis (Meister and Anderson 1983).

For an understanding of GSH metabolism in higher plants it is important to know whether γ-GC synthetases of plants show the same properties as the enzymes isolated from animal cells. Therefore, we have employed the technique used for the determination of GSH synthetase and homoglutathione synthetase in higher plants (Hell and Bergmann 1988; Klapheck et al. 1988) to measure and characterize γ-GC synthetase from suspension cultures of *Nicotiana tabacum*. In the present paper we report on the catalytic properties of γ-GC synthetase isolated from cells of tobacco suspension cultures, and compare synthesis rates and subcellular localization of γ-GC synthetase and GSH synthetase in leaves of *Pisum sativum* and *Spinacia oleracea*. The experiments show that the principal catalytic properties of γ-GC synthetase from tobacco cells are similar to the characteristics reported for γ-GC synthetase from animal cells, and that γ-GC synthetase as well as GSH synthetase in leaves are present in chloroplasts and in the cytoplasm.

Material and methods

Plant material. The tobacco suspension cultures (*Nicotiana tabacum* L. cv. Samsun) used in the present experiments originate from a single cell isolated by Bergmann (1960). Photoheterotrophic suspensions were grown in a modified Murashige and Skoog medium as described earlier (Hell and Bergmann 1988). Heterotrophic tobacco cells were cultivated under the same conditions, but on a rotary shaker (100 rpm) in the dark. Enzyme activity was determined in exponentially growing cells 8 d after inoculation into fresh medium.

Seedlings of *Pisum sativum* L. cv. Kleine Rheinländerin were grown in shallow trays of soil and compost (1:3, v/v) in a growth cabinet at 20° C (light period) and 18° C (dark period) and 70%–80% humidity. A period of 16 h light daily was supplied by Sylvania cool-white tubes (F-58W-GRO; Sylvania, Danvers, Mass., USA) and Osram cool-white tubes (L 65/22R; Osram, München, FRG) providing 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Leaves of 9–11-d-old plants were used for experiments. Seedlings of *Triticum aestivum* cv. Star were grown under the same conditions in Vermiculite supplied with Hoagland solution and primary leaves of 12-d-old seedlings were used for enzyme extraction. *Nicotiana tabacum* L. cv. Samsun and *Spinacia oleracea* L. cv. Hiverna were grown in soil in a greenhouse under natural conditions but with additional illumination supplied by fluorescent tubes on overcast days. Leaves of 4–6-week-old tobacco plants and of 4–5-month-old spinach plants were used in the experiments.

Enzyme extraction. Cells from three tobacco cultures were mixed and separated from the medium by filtration and subsequent washing with distilled water. The leaves of the different plants were washed, the midrib was removed and the leaves were cut into small pieces. The plant material was frozen in liquid N_2 and ground in a mortar containing liquid N_2 and quartz sand. An aliquot of 10 g of the powdered, frozen material was mixed with 20 ml buffer (100 mM Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol)-HCl, pH 7.5, 5 mM EDTA (Ethylenediaminetetraacetic acid)) and 1 g polyvinylpolypyrrolidone (Sigma, München, FRG) and the resulting slurry was homogenized again in a mortar at 4° C. The homogenate was centrifuged (40000 · *g*, 15 min), the pellet discarded, and the supernatant centrifuged again. The clear supernatant of the second centrifugation step was either used as crude enzyme extract or subjected to ammonium-sulfate precipitation. The crude enzyme extract from tobacco cells (1 ml) was dialysed against 200 ml of buffer (50 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH, pH 7.8, 1 mM EDTA) for 6 h with buffer change after the first 3 h. The supernatant obtained by centrifugation of the dialysed enzyme solution (10000 · *g*, 3 min) was used for the determination of the enzyme activities. The crude extract from plant leaves was purified by gel filtration on Sephadex G 25 (PD-10; Pharmacia, Freiburg, FRG), using 50 mM Hepes-NaOH buffer, pH 7.8, 1 mM EDTA, for elution.

Enzyme purification. Ammonium-sulfate precipitates (35%–60% saturation) of crude enzyme extracts were dissolved in 6.5 ml Hepes buffer (50 mM Hepes-NaOH, pH 7.8, 1 mM EDTA) and unsolved protein removed by centrifugation. The clear supernatant (6 ml) was applied to a Sephacryl S-200 column (16 mm diameter, 970 mm long), equilibrated with Hepes buffer, and eluated with the same buffer at a flow rate of 56 $ml \cdot h^{-1}$. Fractions of 2.8 ml were collected and immediately tested for γ-GC synthetase activity. The fractions containing activity were pooled and stored over night at 4° C before use.

Estimation of molecular weight. A column (16 mm diameter, 970 mm long) of Ultrogel AcA 44 (IBF Biotechnics, Villeneuve la Gaunne, France) was equilibrated with 50 mM K-phosphate buffer, pH 7.0, and calibrated with myoglobin (17800), chymotrypsinogen (25000), ovalbumin (45000), and bovine serum albumin (67000) (all Serva, Heidelberg, FRG).

Determination of γ-GC synthetase activity. γ-GC synthetase activity was assayed by high-performance liquid chromatography (HPLC) quantification of synthesized γ-GC as its monobromobimanederivative. In order to prevent losses of cysteine, the enzyme assay was carried out under strictly anaerobic conditions, which were achieved by incubation under argon and addition of an O_2-consuming system (Papen et al. 1986). The standard reaction mixture (final volume 1 ml) contained 1 mM cysteine, 20 mM glutamic acid, 4 mM ATP, an ATP-regenerating system (8 mM phosphocreatine, 10 U creatine kinase; Sigma, 1 U = 1 $\mu mol \cdot min^{-1}$), 50 mM $MgCl_2$, 10 mM NADH (Serva), an O_2-consuming system [1 U glucose oxidase (Sigma), 200 U catalase (Sigma), 25 mM glucose], 0.1 M Hepes-NaOH, pH 8.0, and enzyme extract (final concentration 1–3 mg protein $\cdot ml^{-1}$ assay). The assay mixtures were given into small Fernbach-flasks (5–8 ml volume), which were tightly closed by rubber septa (Aldrich, Steinheim, FRG). The assay mixtures in the flasks were evacuated and flushed with oxygen-free argon for several times. Enzyme reaction was started by injection of cysteine-glucose solution (50 µl) which had been prepared by dissolving cysteine under anaerobic conditions in oxygen-free glucose solution. Incubation was carried out at 30° C for 30–60 min. At 0, 15, 30, and 60 min, samples of 10 µl were taken with a syringe

(Hamilton, Darmstadt, FRG) and the reaction was stopped by transferring these samples into a derivatization solution, containing 0.1 ml of 0.1 M Tris-HCl, pH 8.0, and 5 µl of 10 mM monobromobimane (Calbiochem, La Jolla, Calif., USA) dissolved in acetonitrile. After 15 min, 5% (v/v) acetic acid was added to a total volume of 1 ml.

Separation of the thiol derivatives was achieved on an ODS Hypersil column (4.6 mm diameter, 250 mm long; 5 µm corn size) with a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan). Aliquots of 10–20 µl were injected and eluted with a linear gradient of 0.1 M K-acetate, pH 5.5, and methanol (10% to 20% methanol in 12 min, flow rate 1 ml·min^{-1}). The monobromobimane derivatives were detected fluorometrically (excitation at 380 nm, emission at 480 nm, wavelength accuracy 5 nm).

The activity of γ-GC synthetase with α-aminobutyrate as substrate was assayed by HPLC quantification of synthesized γ-glutamyl-α-aminobutyrate as its orthophthalaldehyde (OPA) derivative (Lenda and Svenneby 1980). The standard reaction mixture (final volume 0.1 ml) contained 20 mM α-aminobutyrate, 20 mM L-glutamic acid, 4 mM ATP, an ATP-regenerating system [8 mM phosphocreatine, 10 U creatine kinase (Sigma)], 50 mM $MgCl_2$, and 0.1 M Hepes-NaOH, pH 8.0. The reaction was started by adding enzyme extract or α-aminobutyrate to the reaction mixture. Incubation was carried out at 30° C. The reaction was stopped by addition of 0.1 ml 6% (v/v) trichloroacetic acid, and denatured proteins were removed by centrifugation (10000·*g*, 5 min).

The derivatization of γ-glutamyl-α-aminobutyrate with *o*-phthalaldehyde was carried out as pre-column derivatization with a Waters-WISP 710 autosampler as described previously (Hell and Bergmann 1988).

The same protocol as for γ-glutamyl-aminobutyrate determination was used for the control of glutamic acid and the detection of glutamine synthesis and γ-aminobutyrate formation in the enzyme assays.

The identity of γ-glutamyl-α-aminobutyrate and γ-GC synthesized in the enzyme assays was proved by derivatization and co-chromatography of reference substances (Kohjin, Tokyo, Japan).

Determination of GSH synthetase activity. Glutathione synthetase activity was assayed by HPLC quantification of synthesized GSH as its monobromobimane derivative as described before (Hell and Bergmann 1988), except that incubation of the enzyme assays was performed at 30° C.

Isolation of chloroplasts. Purified chloroplasts were obtained by Percoll centrifugation according to Joy and Mills (1987). Leaves (50 g) of *Pisum sativum* or *Spinacia oleracea* were cut into small pieces and mixed with 200 ml of ice-cold isolation medium (0.33 M sorbitol, 50 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, and 2 mM EDTA). The mixture was homogenized three times for 1 s in a Kannangara-blender. The brei was filtered through four layers of cheesecloth and a nylon net (30 µm pore size) and the chloroplasts were separated from the filtrate by centrifugation (2500·*g*, 45 s, swing-out rotor). The chloroplasts were resuspended in the isolation medium and 7.5 ml of this suspension were layered on top of a step gradient consisting of 10 ml 40% Percoll layered on 3 ml 80% Percoll, both containing isolation buffer. After centrifugation at 2500·*g* for 5 min (swing-out rotor) the intact chloroplasts were removed from the 40%:80% Percoll interface using a syringe. Intactness of the chloroplasts was determined by the ferricyanide test according to Lilley et al. (1975).

In order to release the chloroplastic enzymes, the chloroplasts were pelleted by centrifugation (2500·*g*, 5 min) and the pellets were suspended in extraction buffer (50 mM Hepes-NaOH, pH 7.8, 1 mM EDTA, 0.1% (v/v) Triton X-100). The clear supernatant obtained by centrifugation (2500·*g*, 5 min) was applied to a PD-10 column. Enzymes were eluted with 50 mM Hepes-NaOH buffer, pH 7.8, 1 mM EDTA, and the eluents used for enzyme assays. Chlorophyll was extracted with 80% acetone and measured according to Lichtenthaler and Wellburn (1983).

Marker enzymes. Cytochrome-*c* oxidase (EC 1.9.3.1) was assayed according to Hodges and Leonard (1974), $NADP^+$-phosphoglyceraldehyde dehydrogenase (EC 5.3.1.1) according to Jackson et al. (1979), α-mannosidase (EC 3.2.1.24) according to Boller and Kende (1979), pyruvate kinase (EC 2.7.1.40) and catalase (EC 1.11.1.6) according to Bergmeyer (1974).

Results

Enzyme determination and purification. The activity of γ-GC synthetase was assayed by quantification of γ-GC and other thiols in the reaction mixture with monobromobimane followed by reversed-phase HPLC and fluorescence detection (Newton et al. 1981). Besides high sensitivity – quantification of 2 pmol in 0.25 ml assay is possible – this method allows the simultaneous determination of γ-GC, cysteine, and GSH in the assays, which proved to be critical for the determination of γ-GC synthetase activity. Using crude extracts of tobacco cells, high activities of GSH synthetase were found, but no activity of γ-GC synthetase. Determination of γ-GC synthetase was possible only after removal of low-molecular weight substances by dialysis or ammonium-sulfate precipitation and after deoxygenation of the enzyme assays. Under these conditions synthesis of γ-GC and the simultaneous decrease of cysteine were linear with time for at least 60 min (Fig. 1). Taking into account the small losses of cysteine in the controls, cysteine consumption and γ-GC formation agreed satisfactorily and could both be used as a measure for enzyme activity. No GSH was observed in the assays, which is in accordance with the correspondance of γ-GC formation and cysteine consumption. The formation of γ-GC was positively correlated with protein between 0.6 and 1.65 mg protein in the assay mixture; below 0.6 mg protein, enzyme activi-

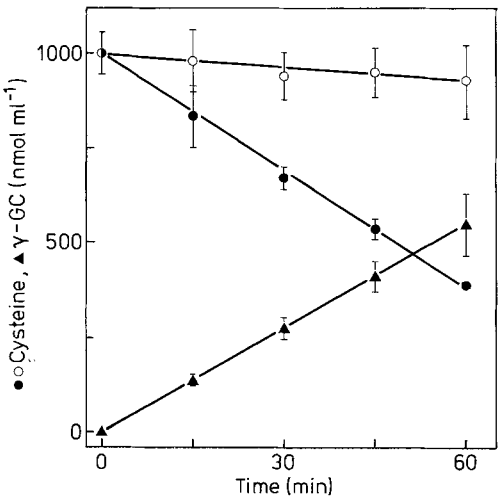

Fig. 1. γ-Glutamylcysteine synthesis by extracts of tobacco cells. Sephacryl S-200 eluents from cell extracts of heterotrophic suspension cultures were assayed under anaerobic conditions with 1.65 mg protein·ml^{-1} assay as described in *Material and methods.* (●) Cysteine, (▲) γ-GC, (○) cysteine in controls without protein. The values represent means of four replicates each. The SDs are omitted when smaller than symbols

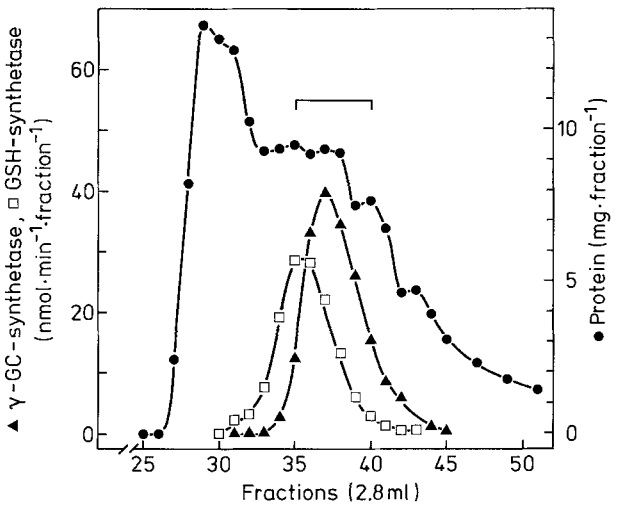

Fig. 2. Elution profile of γ-glutamylcysteine synthetase (▲) and glutathione synthetase (□) activity from ammonium-sulfate precipitates (35%–60% saturation) of extracts of heterotrophic tobacco suspension cultures. Separation was carried out by gel-chromatography (Sephacryl S-200) as described in *Material and methods*. Fractions marked by a bar, containing 80% of total γ-GC synthetase activity, were pooled and used for characterization of the enzyme

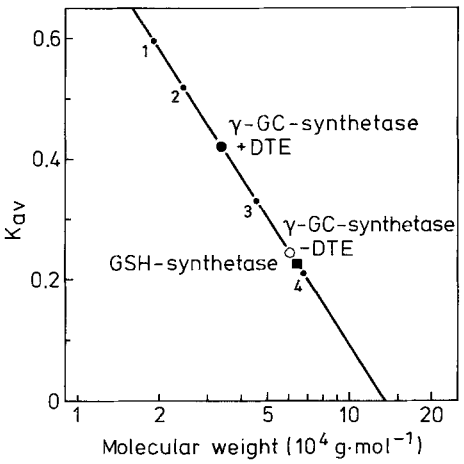

Fig. 3. Determination of M_r of glutathione synthetase (■) and γ-glutamylcysteine synthetase with (●) and without (○) DTE by means of filtration through an Ultrogel AcA 44 column (1.6 cm diameter, 97 cm long). Markers: *1*, myoglobin (18 kDa); *2*, chymotrypsinogen (25 kDa); *3*, ovalbumin (45 kD); *4*, bovine serum albumin (67 kDa)

ties were not linear. γ-Glutamylcysteine was not detectable in assays with boiled enzyme extract or without glutamate, cysteine, ATP, or enzyme extract.

Ammonium-sulfate fractionation of a tobacco cell extract resulted in precipitation of 96% of the γ-GC-synthesizing enzyme between 35 and 60% saturation and 2.4-fold purification of the enzyme in this fraction, compared to the fraction obtained by 90% saturation. Specific activities up to 2.5 nmol·(mg protein)$^{-1}$·min^{-1} were measured in the 35–60% fraction. By gel filtration on Sephacryl S-200 a tenfold concentration of the protein was achieved, but nearly 50% of the total enzyme activity applied to the gel were lost; so the specific activity in the Sephacryl fraction went up only to 5.4 nmol·(mg protein)$^{-1}$·min^{-1}. This purification step did not succeed in separation of γ-GC synthetase from GSH synthetase (Fig. 2). Attempts at further purification by affinity chromatography on agarose aminohexyl-N6-ATP, successfully used to purify GSH synthetase from spinach leaves (Law and Halliwell 1986), led to rapid loss of enzyme activity. Therefore Sephacryl S-200 eluents were used for the evaluation of the catalytic properties of γ-GC synthetase. The eluents lost more than half of their activity in 48 h if kept at 4° C, but could be stored at −20° C for at least two weeks without further losses of activity. In crude extracts and ammonium-sulfate precipitates the enzyme showed a marked instability; freezing (−20° C) and thawing of extracts or precipitates resulted in losses between 70% and 80% of enzyme activity.

Effect of dithioerythritol on enzyme activity and on molecular weight of γ-GC synthetase. Addition of reducing agents to the homogenization medium or to the assay mixtures led to severe inhibition of γ-GC synthetase. Incubation of enzyme extracts from tobacco cells in the presence of dithioerythritol (DTE) or mercaptoethanol (5 mM each) reduced enzyme activity by 80% and 46%, respectively. Dithioerythritol also inibited γ-GC synthetase activity from pea and spinach leaves by 74% and 69.5%, respectively.

Chromatography of the γ-GC synthetase from tobacco cells on Ultrogel AcA 44 revealed a molecular weight of the enzyme near 60000 (Fig. 3). Addition of DTE to the enzyme solution applied to the column and to the elution buffer led to the dissociation of γ-GC synthetase into subunits of M_r about 34000. This dissociation was accompanied by a loss of enzyme activity, compared to the chromatography of the same enzyme extract without DTE. After removal of DTE from the eluates by ultracentrifugation (Amicon, Centricon 30, 4 h, Amicon, Danvers, Mass., USA), γ-GC synthetase activity increased 1.62-fold. This increase probably results from a partial reassociation of subunits, as has been observed with the γ-GC synthetase from rat kidney (Seelig et al. 1984).

For the GSH synthetase of tobacco cells a molecular weight of 64000 was found. Neither the elution pattern nor the enzyme activity were altered by the addition of DTE.

Catalytic properties. γ-Glutamylcysteine synthetase was active over a fairly broad pH range (pH 6.5–9.5). The highest activity was found at pH 8.0 in Hepes-NaOH buffer, with 50% of maximal activity at pH 7.0 and pH 9.0. Nearly equal activities were observed in Tris-HCl, 2-(N-cyclohexylamino)ethanesulfonic acid (CHES)-NaOH, and Hepes-NaOH buffer.

Table 1. Effect of Cd^{2+}, Zn^{2+} and Cu^{2+} on γ-glutamylcysteine synthetase activity. Ammonium-sulfate precipitates (35–60% saturation) of tobacco cell extracts were dialysed against 50 mM Hepes buffer, pH 7.0, 0.1 mM EDTA and assayed under standard conditions with and without metal ions. The assay mixtures were incubated for 30 min with the ions before the reaction was started by adding cysteine. Time linearity of the reactions was checked by taking samples after 0, 30 and 60 min of incubation. A γ-GC synthetase activity of 100% corresponds to 2.9 nmol·(mg protein)$^{-1}$·min^{-1}. The values given in columns 2 and 3 represent the amounts of γ-GC and of cysteine found in the assay mixtures after 60 min of incubation

	Activity of γ-GC synthetase (%)	Content of γ-GC in the assay (nmol·ml^{-1})	Content of cysteine in the assay (nmol·ml^{-1})
Control	100	305	322
$CdCl_2$ 0.2 mM	64.4	174	428
$ZnCl_2$ 0.2 mM	81	248	375
$CuCl_2$ 0.2 mM	7	19	24

Enzyme activity depended on the presence of Mg^{2+} in the assay. Without Mg^{2+} no γ-GC formation was observed. Optimal activity was observed with 30–50 mM Mg^{2+} with a slight decrease at higher concentrations. The enzyme activity did not depend on K^+ as has been reported for γ-GC synthetase from wheatgerm (Webster and Varner 1954), and it was not stimulated by K^+ as has been observed with GSH synthetase (Law and Halliwell 1986; Hell and Bergmann 1988).

The activity of γ-GC synthetase was reduced by heavy-metal ions that are known to induce the formation of heavy-metal-binding phytochelatins in plants, which are synthesized at the expense of GSH (Grill et al. 1989). Since oxidation of thiols is increased in the presence of metal ions, the enzyme assays were checked carefully for possible disappearance of cysteine or the synthesized γ-GC. As shown in Table 1, enzyme activity was reduced to 64.4% of the activity of the control in the presence of 0.2 mM Cd^{2+} and to 81% in the presence of 0.2 mM Zn^{2+}. In contrast to Cu^{2+}, addition of Cd^{2+} and Zn^{2+} did not increase the oxidation rate, since the sum of cysteine and γ-GC in both assays was as high as in the control. Therefore, the reduced synthesis rate of γ-GC has to be attributed to inhibition of the enzyme itself. This inhibition may explain why in some plants, which respond to low doses of Cd^{2+} with phytochelatin synthesis, a much smaller accumulation of phytochelatins is observed at higher Cd^{2+} concentrations (Scheller et al. 1987).

The apparent K_m values for L-glutamate and L-cysteine were determined by analysing the initial velocity of the reaction with different concentrations of one of these substrates at saturating concentrations of the other one and of ATP. The γ-GC synthetase exhibited saturation kinetics with respect to both substrates. Using Lineweaver-Burk plots an apparent K_m of 10.4 ± 0.6 mM for glutamate ($V_{max} = 5.21 \pm 1.27$ nmol·(mg protein)$^{-1}$·min^{-1}) and an apparent K_m of 0.19 ± 0.05 mM for cysteine ($V_{max} = 3.61 \pm 1.38$ nmol·(mg protein)$^{-1}$·min^{-1}) were observed in three experiments with different enzyme preparations. Since the γ-GC synthetase used was not purified to homogeneity the assays were carefully analysed for the presence of γ-aminobutyrate and glutamine, which could have been formed in side reactions by L-glutamate decarboxylase (EC 4.1.1.15) and by glutamine synthetase (EC 6.3.1.2). Both substances were not detectable after orthophthalaldehyde derivatization of the assays and HPLC analysis. The oxidation rate of cysteine, determined in control assays without enzyme, was always below 5% and therefore did not influence the determination of the K_m value for cysteine.

Besides cysteine, α-aminobutyrate was used as substrate by the tobacco γ-GC synthetase. An apparent K_m of 6.36 ± 0.69 mM for α-aminobutyrate was found, based on three separate experiments with different enzyme preparations. Only at high concentrations of α-aminobutyrate (20 mM) was the turnover rate equal to that found with 1 mM cysteine: 2.75 ± 0.88 nmol·(mg protein)$^{-1}$·min^{-1} for α-aminobutyrate compared to 2.93 ± 0.86 nmol·(mg protein)$^{-1}$·min^{-1} for cysteine in measurements with the same enzyme extracts. This high concentration together with the high saturation concentration of glutamate (20 mM) complicate pre-column OPA derivatization and abolish the advantages of α-aminobutyrate over the readily oxidable cysteine.

The γ-GC synthetases from tobacco and pea were inhibited by buthionine sulfoximine and, to a smaller degree, by L-methionine sulfoximine, which are both effective inhibitors of γ-GC synthetase in mammalian cells (Richman et al. 1973; Griffith and Meister 1979). Incubation of the tobacco enzyme with 1 mM buthionine sulfoximine (30 min) resulted in an 80% decrease of activity, whereas incubation with 10 mM L-methionine sulfoximine (30 min) caused a 25.4% decrease in enzyme activity. In experiments with γ-GC synthetase from pea leaves, 100% inhibition was found with 1 mM buthionine sulfoximine and 35% inhibition with 1 mM L-methionine sulfoximine. The same degree of inhibition was observed using γ-glutamyl-aminobutyrate as substrate for the enzyme.

The γ-GC synthetase from animal sources is inhibited by GSH in vitro and it appears that such inhibition constitutes a physiologically significant feedback mechanism for the control of GSH synthesis (Richman and Meister 1975). When the tobacco enzyme was assayed under standard conditions in the presence of added 1 mM and 5 mM GSH its activity was reduced by 20% and 95%, respectively. Addition of homoglutathione led to a smaller but, at higher concentrations, also significant inhibition of γ-GC synthesis (Fig. 4).

The inhibition by GSH is competitive with respect to L-glutamate as has been demonstrated by the experiments shown in Fig. 5. The data clearly reflect a competitive inhibition of γ-GC synthesis by GSH at concentrations found in plant cells. The apparent K_i value for GSH, calculated from the data of two experiments, was 0.42 ± 0.12 mM.

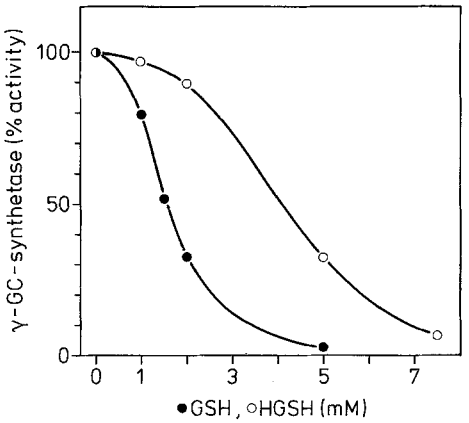

Fig. 4. Effect of glutathione (●) and homoglutathione (HGSH) (○) on γ-glutamylcysteine synthetase activity. Sephacryl S-200 eluents from cell extracts of heterotrophic tobacco suspension cultures were assayed under standard conditions with 0.8–1.2 mg protein · ml^{-1} assay and 10 mM glutamic acid. The GSH- and HGSH concentrations were varied as shown. The assays were incubated with GSH and HGSH at the concentrations indicated for 30 min at 30° C before the reaction was started with cysteine. Data shown are mean values of two experiments with two replicates each

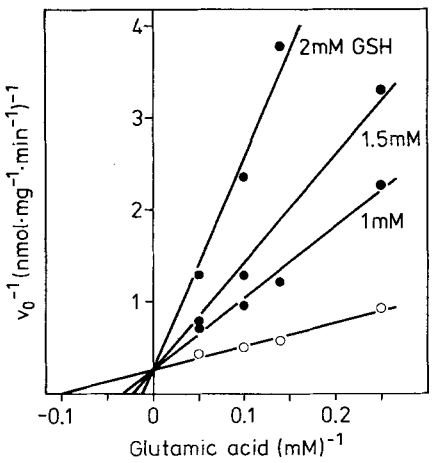

Fig. 5. Effect of glutamate on inhibition of γ-glutamylcysteine synthetase by glutathione. Sephacryl S-200 eluents from cell extracts of heterotrophic tobacco suspension cultures were assayed as described in *Material and methods*, but with variant concentrations of glutamic acid for 30 min at 30° C before the reaction was started by adding cysteine. The values represent means of two experiments with two replicates each

γ-Glutamylcysteine synthetase activity and GSH synthetase activity in tobacco suspension cultures and in plant leaves. Activities of γ-GC synthetase in extracts from tobacco cells purified either by dialysis alone or by precipitation at 90% ammonium sulfate and subsequent dialysis were always lower than GSH synthetase activities. In heterotrophically grown cells a specific activity of 0.96 ± 0.14 nmol · (mg protein)$^{-1}$ · min^{-1} was obtained for γ-GC synthetase compared to 2.03 ± 0.15 nmol · (mg protein)$^{-1}$ · min^{-1} for GSH synthetase. The specific activities for γ-GC synthetase and GSH synthetase of chloroplast-containing, photoheterotrophically grown cells were found to be 0.97 ± 0.17 and 1.66 ± 0.14 nmol · (mg protein)$^{-1}$ · min^{-1}, respectively. Homogenisation of the cells with 0.5% Triton did not change the quantities of the enzymes extracted (data not shown).

Table 2. Rate of synthesis of γ-glutamylcysteine and glutathione in leaf extracts of *Pisum sativum*, *Spinacia oleracea*, and *Triticum aestivum*. Crude enzyme extracts were assayed after gel filtration on Sephadex G-25 under standard conditions. Values represent means of five independent experiments ±SD

Plant	Rate of synthesis (nmol · (mg protein)$^{-1}$ · min^{-1})	
	γ-GC	GSH
Pisum sativum	0.601 (0.083)	0.214 (0.050)
Spinacia oleracea	0.454 (0.110)	0.444 (0.064)
Triticum aestivum	0.499 (0.078)	0.530 (0.060)

The higher activity of GSH synthetase compared to γ-GC synthetase indicates that the synthesis of γ-GC is probably the limiting step in GSH synthesis in the tobacco cells. It has not been possible to support this assumption by comparing the corresponding enzyme activities in leaves of tobacco plants because both enzymes could not be quantified correctly in the leaf extracts, which contained large amounts of phenolic compounds. However, experiments with three other species revealed that the activity levels of the two enzymes vary in different plants. As can be seen from the data in Table 2, the specific activities of γ-GC synthetase and GSH synthetase in leaf extracts of *Spinacia oleracea* and *Triticum aestivum* are of equal size, whereas in leaf extracts of *Pisum sativum* the specific activity of γ-GC synthetase was found to be nearly three times higher than the activity of GSH synthetase, which probably signifies that in pea leaves the activity of GSH synthetase is the limiting factor in GSH synthesis.

Subcellular localization. Studies on the localization of GSH synthetase in peas (Klapheck et al. 1987) and tobacco (Hell and Bergmann 1988), and of homoglutathione synthetase of french bean (Klapheck et al. 1988) have shown that these enzymes are present in the cytoplasm and in the chloroplasts. Therefore the distribution of γ-GC synthetase between chloroplast and cytoplasm was investigated and compared with the intracellular distribution of GSH synthetase. Since the variability in size, shape and density make the isolation of chloroplasts from photoheterotrophically cultured tobacco cells very difficult, chloroplasts were isolated from leaves of *Pisum sativum* and *Spinacia oleracea* by Percoll centrifugation according to Joy and Mills (1987). The activities of γ-GC synthetase, GSH synthetase and marker enzymes in the chloroplast fraction were compared with the activities found in the leaf extracts. The chloroplasts isolated by this procedure showed an intactness of between 83 and 92%, according to the ferricyanide test, and low contamination by enzymes of other cellular compartments. Using glyceraldehyde-3-phosphate dehydrogenase

Table 3. Localization of γ-glutamylcysteine synthetase and glutathione synthetase in chloroplasts from *Pisum sativum* (I) and *Spinacia oleracea* (II). The activity of marker enzymes, of γ-GC synthetase and of GSH synthetase in leaf extracts is compared with the activity in chloroplasts, isolated on a Percoll gradient. Activities are related to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. A GAPDH activity (ml enzyme·extract)$^{-1}$·min^{-1} of 1000 corresponds to 4.136 μmol (I) and 2.128 μmol (II) for the leaf extract, and 2.593 μmol (I) and 1.408 μmol (II) for the chloroplast fraction. The values represent means of two (I) and three (II) independent experiments. n.d. = not detectable

Enzyme	Relative activities		% of activity found in the chloroplast fraction
	Leaf extract	Chloroplast	
I.			
GAPDH	1000	1000	
α-Mannosidase	11.9	n.d.	<1
Pyruvate Kinase	106.2	n.d.	<1
Catalase	590.4	11.3	1.9 (0.6)
Cyt.-*c* oxidase	287.8	10.9	5.2 (4.8)
γ-GC synthetase	1.62	1.17	72.0 (9.5)
GSH synthetase	0.66	0.31	47.8 (4.6)
II.			
GAPDH	1000	1000	
α-Mannosidase	21.8	0.48	2.0 (0.9)
Pyruvate Kinase	354.3	10.3	3.0 (1.0)
Catalase	337.4	7.6	2.2 (0.5)
Cyt.-*c* oxidase	276.9	7.4	5.8 (4.2)
γ-GC synthetase	1.14	0.696	61.2 (14.0)
GSH synthetase	1.22	0.662	57.7 (13.5)

(GAPDH) activity as the point of reference, only 5.2% of cytochrome-*c* oxidase, 1.9% of catalase and less than 1% of α-mannosidase and pyruvate kinase, but 72% of γ-GC synthetase and 47.6% of GSH synthetase were found in the chloroplast fractions, compared with leaf extracts of *Pisum sativum* (Table 3). In experiments with *Spinacia oleracea* similar results were obtained. Between 2 and 5.8% of the cytoplasmic marker enzymes were detected in the chloroplast fraction, but 61.2% of the γ-GC synthetase activity and 57.7% of the GSH synthetase activity of the leaf extracts were found in the chloroplasts. These experiments confirm previous results on the localization of GSH synthetase in *Pisum* (Klapheck et al. 1987) and clearly demonstrate, that γ-GC synthetase is present in the chloroplasts as well as in the cytoplasm. Moreover, the experiments show an interesting feature of the intracellular distribution of both enzymes. In chloroplasts of *Pisum* nearly 3.7-fold more γ-GC synthetase than GSH synthetase was found, whereas the ratio of both enzymes in the cytoplasm was approximately one. In contrast, equal amounts of γ-GC synthetase and GSH synthetase were detected in the chloroplasts and the cytoplasm of *Spinacia* leaf cells.

Discussion

Synthesis of γ-GC in extracts of tobacco cell suspensions and in leaf extracts of *Pisum sativum*, *Spinacia oleracea*, and *Triticum aestivum* has been shown to proceed as for γ-GC synthesis in mammals and microorganisms: the dipeptide is produced from L-glutamate and L-cysteine in an ATP-dependent reaction. The reaction is catalysed by a γ-GC synthetase, described in the present paper, that shows close similarities to γ-GC synthetase from mammals (Seelig and Meister 1985), and those of yeast (Dennda and Kula 1986), and of *Escherichia coli* (Apontoweil and Berends 1975). The inhibition of the enzyme by methionine sulfoximine and buthionine sulfoximine indicates that the formation of γ-GC in plants proceeds by the same reaction mechanism as described for the mammalian enzyme, the formation of enzyme-bound γ-glutamylphosphate (Orlowski and Meister 1971) and not, as proposed by Webster and Varner (1954), by the formation of a phosphorylated enzyme which reacts with glutamate to form a γ-glutamyl-enzyme intermediate.

The molecular weight of the tobacco enzyme (60000) differs from the molecular weights of γ-GC synthetase from mammals and yeast, which range from 104000–123000 (Seelig et al. 1984; Dennda and Kula 1986). Like γ-GC synthetase from rat kidney (Sekura and Meister 1977), the tobacco enzyme dissociates during incubation with low concentrations of DTE into smaller subunits and looses most of its activity. It also reacts with a heavy loss of activity upon incubation with low doses of mercaptoethanol. This reaction against the two reagents, which are often used to protect thiol groups in plant extracts, is probably one of the reasons why attempts to detect the enzyme in plants have failed so far. In this connection it is interesting that in *Candida boidinii* DTE can be used to stabilize the enzyme during extraction (Dennda and Kula 1986). The low molecular weight of γ-GC synthetase should allow its separation by gel filtration from glutamine synthetase and glutamate decarboxylase which can interfere with the determination of γ-GC synthetase activity, and which show molecular weights of 350000–400000 (Steward et al. 1980) and 256000–310000 (Inatomi and Slaughter 1975), respectively. This possibility has not been particularly investigated in the present work, but the absence of glutamine and γ-aminobutyrate synthesis in enzyme assays with Sephacryl S-200 filtrates indicates such a separation. On the other hand, a separation of γ-GC synthetase and GSH synthetase will be difficult as the molecular weights of both enzymes are very similar. Therefore, the possibility of GSH formation in enzyme assays of γ-GC synthetase activity, in which glycine can be generated by proteolytic release, has to be examined.

Isolation of intact chloroplasts by Percoll centrifugation showed that about 72% of the γ-GC synthetase activity of pea leaves and 61% of the γ-GC synthetase activity of leaves from spinach were associated with the chloroplasts. A similar distribution has been found for GSH synthetase of which 47% was localized in pea chloroplasts and 58% in chloroplasts of spinach. Most probably the rest of γ-GC synthetase and GSH synthetase is localized in the cytoplasm, as has already been shown for GSH synthetase in *Pisum* (Klapheck et al. 1987).

The localization of γ-GC synthetase in our experiments is corroborated by recent results of Lancaster et al. (1989), who found γ-GC synthetase in isolated chloroplasts from mesophyll cells of *Allium cepa*. Thus GSH can be synthesized in at least two compartments of the plant cell as has been previously suggested (Klapheck et al. 1987; Hell and Bergmann 1988).

With regard to the localization of γ-GC synthetase and GSH synthetase in chloroplasts the effect of pH as well as the effect of Mg^{2+} concentration on the activity of γ-GC synthetase are of special interest. When chloroplasts are illuminated the stroma pH is shifted from pH 7.0 to pH 8.0 and at the same time the stromal Mg^{2+} concentration increases to about 2 mM (Heldt 1979). The alkalinization of the stroma and the increase in Mg^{2+} concentration are in a range where they can effectively influence the rate of γ-GC synthesis and the rate of GSH synthesis in the chloroplasts, as GSH synthetase also shows 50% of its maximal activity at pH 7.0 and a broad pH optimum between pH 8.0 and pH 9.0 (Hell and Bergmann 1988). Such an increase of the rate of GSH synthesis during illumination could be involved in the light-dependent diurnal fluctuations of GSH concentration observed recently in leaves (Koike and Peterson 1988; Schupp and Rennenberg 1989). In those experiments, low levels of GSH were found in the dark periods and high levels in periods which corresponded to the changes in photosynthetic active radiation, and could be caused by an enhanced GSH synthesis.

The substrate affinity of the tobacco enzyme for L-glutamate is quite low (K_m (apparent) = 10 mM) compared with the K_m values for glutamate observed with γ-GC synthetase from mammalian cells, yeast, and *E. coli*, which are in the range of 1.1–1.8 mM, whereas the substrate affinity for L-cysteine (K_m(apparent) = 0.19 mM) is in fair agreement with the substrate affinities of γ-GC synthetase from animal cells and microorganisms (Seelig and Meister 1985; Dennda and Kula 1986; Apontoweil and Berends 1975). On the other hand, the apparent K_m value for cysteine found in our experiments is more than one order of magnitude smaller than the K_m value of 4.5 mM reported by Webster and Varner (1954) for the enzyme of wheat germ. Since these authors did not take precautions to protect cysteine against oxidation in their experiments, this high K_m value for cysteine has no kinetic significance.

A comparison of the K_m values found in the present experiments with the intracellular amino-acid concentrations shows the physiological significance of substrate affinities. From the data on the amino-acid content of chloroplasts a glutamate concentration in the stroma of between 4 mM and 6.5 mM, and a cysteine concentration around 0.3 mM can be calculated (Aach and Heber 1967; Kirk and Leech 1972; Chapman and Leech 1979; Mills and Joy 1980). These concentrations indicate that the γ-GC synthesis in chloroplasts may be controlled by the availability of substrates.

A comparison of the K_m values with the amino-acid concentrations in the cytosol is more difficult. All available data on the contents of soluble amino acids in plant tissues based on a fresh-weight or dry-weight basis give only a very rough outline of the conditions in the cytosol since neither the distribution of single amino acids between vacuole, cytoplasm and plastids nor the volume of the cytosolic compartment are known. However, on the supposition that the amino-acid concentrations in the cytosol do not differ markedly from the concentrations of amino acids in the chloroplasts, it seems reasonable to assume that the rate of GSH synthesis in the cytoplasm is also dependent on the availability of the substrates. Naturally, it would be very important for the understanding of the function of GSH as transport metabolite to know more about the intracellular distribution of cysteine, whose concentration in the chloroplasts could be appreciably higher than in the cytoplasm.

Besides indicating the control of GSH synthesis by the availability of substrates, the findings reported here also show that in plants GSH can regulate its own synthesis by feedback inhibition of γ-GC synthetase, as has been observed with rat-kidney enzyme (Richman and Meister 1975). As the present experiments show, γ-GC synthetase from tobacco cells is inhibited in vitro by concentrations which approximate GSH concentrations found in chloroplasts of different species. These concentrations range from 1 mM to 6 mM (Foyer and Halliwell 1976; Law et al. 1983; Smith et al. 1985; Bielawski and Joy 1986; Gillham and Dodge 1986; Klapheck et al. 1987), and could effectively inhibit γ-GC synthesis in vivo. Assuming a glutamate concentration of 4 mM in the stroma, which corresponds to concentrations found in chloroplasts (see above), GSH at a concentration of 2 mM would reduce the rate of γ-GC synthesis to a seventh part of the rate without GSH, and GSH concentrations of 4 mM or above would result in an almost complete inhibition of γ-GC synthesis. A feedback inhibition of γ-GC synthetase in vivo is in good correspondance with the low levels of γ-GC normally observed in plant cells. Very often the level of γ-GC is below the level of detection, or the γ-GC concentration is 100-fold lower than the concentration of GSH (S. Klapheck, Botanisches Institut, Universität Köln, FRG, personal communication).

A feedback inhibition of GSH synthesis will be of great advantage for plant cells because it will allow cells which suddenly loose their GSH under conditions of chemical stress to replenish GSH immediately. However, the possibility should not be overlooked that biosynthesis of GSH in higher plants may also be controlled by changes in the amounts of the enzymes, which would enable plants to tolerate higher demands for GSH in longer-lasting stress situations. The increase of GSH synthetase activity after induction of phytochelatin synthesis in pea roots reported by Schmutz and Brunold (1989), and the recently observed increase in GSH content and extractable GSH synthetase activity in the endosperm of germinating *Ricinus* seeds (Klapheck and Zimmer 1989) point in this direction, as does an light-dependent increase in the GSH content in *Euglena gracilis* that is inhibited by cycloheximide (Shigeoka et al. 1987). On the basis of the present data on γ-GC synthe-

tase and the previously published data on GSH synthetase this possibility can now be explored.

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