Degradation of Glutathione in Plant Cells: Evidence against the Participation of a γ-Glutamyltranspeptidase

Reinhard Steinkamp and Heinz Rennenberg

Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41, Bundesrepublik Deutschland

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When γ-glutamyltranspeptidase activity in tobacco cells was measured using the artificial substrate γ-glutamyl-*p*-nitroanilide, liberation of *p*-nitroaniline was not reduced, but stimulated by addition of glutathione. Therefore, glutathione was not acting as a donator, but as an acceptor of γ-glutamyl moieties in the assay mixture, suggesting that γ-glutamyltranspeptidase is not participating in degradation of glutathione. Feeding experiments with [^{35}S-*cys*]glutathione supported this conclusion. When tobacco cells were supplied with this peptide as sole sulfur source, glutathione and γ-glutamylcysteine were the only labelled compounds found inside the cells. The low rate of uptake of glutathione apparently prevented the accumulation of measurable amounts of radioactivity in the cysteine pool. A γ-glutamylcyclotransferase, responsible for the conversion of γ-glutamylcysteine to 5-oxo-proline and cysteine was found in ammonium sulfate precipitates of tobacco cell homogenates. The enzyme showed high activities with γ-glutamylmethionine and γ-glutamylcysteine, but not with other γ-glutamyldipeptides or glutathione. From these and previously published experiments [(Rennenberg *et al.*, Z. Naturforsch. **35 c**, 708–711 (1980)], it is concluded that glutathione is degraded in tobacco cells *via* the following pathway: γ-glu-cys-gly → γ-glu-cys → 5-oxo-proline → glu.

Introduction

In animal cells, glutathione (γ-L-glu-L-cys-gly) is degraded *via* a series of reactions which are part of the γ-glutamyl-cycle (*cf.* [1]). The initial step of glutathione degradation in this pathway is the transfer of the γ-glutamyl-moiety of the peptide to an amino acid acceptor catalyzed by a γ-glutamyltranspeptidase. Whereas hydrolysis of the remaining dipeptide is obtained by a dipeptidase, the γ-glutamyl-moiety undergoes cyclization to 5-oxo-proline. The latter is hydrolyzed to glutamic acid in an ATP-dependent reaction (Fig. 1, pathway I). Analysis of degradation products of glutathione conjugates with pesticides in animal cells was found to be consistant with this pathway [2].

In contrast to animal cells, where cysteinylglycine and cysteine-derivatives were the degradation products of glutathione conjugates with pesticides, in plant cells the γ-glutamylcysteine- and the cysteine-derivatives were found (*cf.* [3]). From these observations, it has been suggested [3] that the initial step in the degradation of glutathione conjugates is catalyzed by a carboxypeptidase, and that the resulting γ-glutamylcysteine conjugate is converted to the cysteine conjugate by the action of a γ-glutamyltranspeptidase (Fig. 1, pathway II). However, in experiments with cultured tobacco cells, fed [^{14}C-glu]-glutathione, evidence was obtained that 5-oxo-proline is generated during degradation of "free" glutathione [4]. Therefore, it has been assumed that "free" glutathione might be degraded *via* the pathway shown in Fig. 1, III [5]. After hydrolysis of the C-terminal glycine, 5-oxo-proline may be released from γ-glutamylcysteine by a γ-glutamylcyclotransferase. The 5-oxo-proline generated this way may be converted to glutamic acid by a 5-oxo-prolinase.

If glutathione is indeed degraded *via* this pathway, two major assumptions have to be verified: 1. γ-Glutamyltranspeptidase, an enzyme that has repeatedly been demonstrated in higher plants [8, 14–18], is not participating in degradation of glutathione. 2. Higher plants contain a γ-glutamylcyclotransferase capable of releasing 5-oxo-proline from γ-glutamylcysteine. In the present report, evidence supporting both assumptions is presented.

Reprint requests to Dr. H. Rennenberg.
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Material and Methods

Plant material

The tobacco cells (*Nicotiana tabacum* L. var. Samsun) used in the present investigation originate

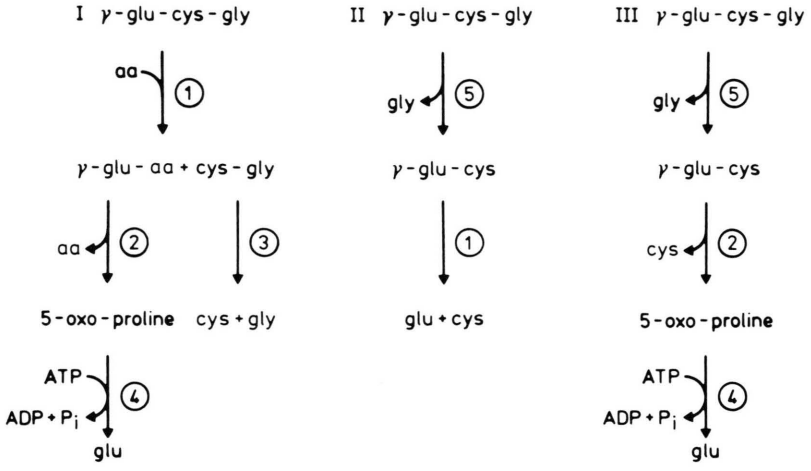

Fig. 1. Possible paths of glutathione degradation. 1: γ-Glutamyltranspeptidase, 2: γ-Glutamylcyclotransferase, 3: dipeptidase, 4: 5-oxo-prolinase, 5: carboxypeptidase.

from a callus culture isolated by Bergmann [6]. Cells were grown in a modified, liquid Murashige and Skoog medium [7] at 25 °C and 60–70% air humidity under continuous illumination of 48–56 μE m^{-2} s^{-1} (HPS L 65W/150 ultra white; Osram L Fluora 35W/77R; Phillips TL 40W/25). Suspension (20 ml) was inoculated every 10th day of cultivation into 300 ml culture medium under sterile conditions. When tobacco cells were grown with glutathione as the only sulfur source, GSH was added as filter sterilized solution with a pH of 5.6–5.8 to sulfur free medium (1.7 mM final conc.). Sulfur free medium was prepared by replacing the sulfate-containing salts in the Murashige and Skoog medium by the corresponding chlorides.

Enzyme assays

Preparation of tobacco cells and enzyme assay for the determination of γ-glutamyltranspeptidase activity were as previously described [8]. For the determination of γ-glutamylcyclotransferase activity, tobacco cells were homogenized [8] and then subjected to ammonium-sulfate precipitation. The protein fraction at 40–70% saturation was resuspended in bidest. H_2O, 50 mM mercaptoethanol and then used for the enzyme assay. The assay mixture contained the following substances in a total volume of 1 ml: 100 mM Tris/HCl, pH 8.5; 4 mM γ-glutamyldipeptide; 0.2 ml enzyme preparation. γ-Glutamyldipeptides used as substrates in the assay mixture were synthesized according to the methods reported by Orlowski and Meister [9]. After 30 min incubation at 30 °C, the reaction was stopped by heat denaturation (5 min) of the protein. The 5-oxo-proline content of the samples was determined by gaschromatography as described by Wilk and Orlowski [10] using a Perkin-Elmer Sigma 3B gaschromatograph with a Sigma 15 Data Station.

Incubation of tobacco cells with [^{35}S-cys]glutathione

[^{35}S-cys]glutathione was isolated from the medium of photoheterotrophic tobacco cells supplied with [^{35}S]sulfate as previously described [11]. As addition of sodium ions to the medium reduced the growth of the tobacco cells (Rennenberg, unpublished results) it was removed from [^{35}S]Na_2SO_4 (NEN, Dreieich) by ion exchange chromatography on Dowex 50 WX4 (100–200 mesh). [^{35}S-cys]glutathione (37.75 mCi $mmol^{-1}$; identical with 0.66 μmol ml^{-1}) was added to 2 g wet weight tobacco cells preincubated for 24 h in a sulfur free medium in 25 ml Erlenmeyer flasks under continuous shaking (30 rpm) in the light. The incubation was stopped by removing the medium. Cells were washed with 40 ml glutathione-containing medium and then homogenized in a Potter Elvejehm homogenizer. After heat denaturation of the protein, the homogenates were centrifuged, the supernatant lyophilized and the samples stored in 2 ml 0.1 M acetic acid.

Identification and quantitation of [^{35}S-cys]glutathione metabolites

Metabolites of [^{35}S-cys]glutathione were determined after reduction, carboxymethylation and microdansylation by TLC-technique and quantified

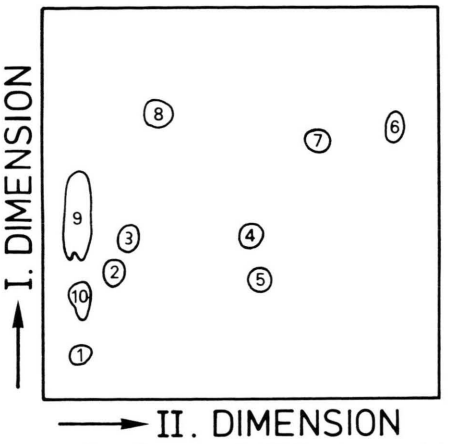

Fig. 2. Two-dimensional TLC of potential glutathione metabolites. Tobacco cells were supplied with [^{35}S-cys]-glutathione, homogenized and subjected to microdansylation after reduction and carboxymethylation. Aliquots of these samples were analyzed by two-dimensional TLC and radioactivity was determined by liquid scintillation counting. 1: start; 2: γ-glu-cys; 3: GSH; 4: cys-gly; 5: cys; 6: front; 7: gln; 8: background; 9 and 10: dansylic acid.

by liquid scintillation counting. From a 100 µl aliquot of each sample (5.9–6 µmol α-amino-N; [12]) acetic acid was removed *in vacuo* and then 100 µl of 300 mM $NaHCO_3$, pH 8.2, plus 50 µl 10 mM DTE were added to reduce disulfides in the sample. After 60 min incubation at room temperature, 20 µl of 100 mM $NaCOOCH_2J$ were added for carboxymethylation of the thiol groups. Excess $NaCOOCH_2J$ was removed after 30 min by addition of 20 µl of 100 mM mercaptoethanol. After 30 min incubation at 25 °C, samples were concentrated *in vacuo* to dryness and 300 µl bidest. H_2O plus 300 µl of 40 mM dansylchloride in acetone were added. Dansylation was stopped after 2 h incubation at 37 °C by addition of 1 drop acetic acid. Excess dansylchloride was removed by addition of 100 µl of 60 mM diethylamine. After 15 min incubation at 25 °C the samples were concentrated *in vacuo* and 1 ml of 0.1 N HCl was added. The samples were purified over Porapak Q, 100–120 mesh (Waters, Ass., Milford) according to the method of Macnicol [13], eluted with 6 ml 80% acetone, concentrated to dryness and solubilized in 100 µl of 95% ethanol, containing 1% triethylamine (w/w). An aliquot of 1 µl of these samples was subjected to two-dimensional thinlayer chromatography on pre-coated micropolyamide foils (7.5 × 7.5 cm, A 1700, Schleicher and Schüll, Dassel) using 1.5% formic acid (1st dimension) and benzene/acetic acid (3 : 1; v/v; 2nd dimension) as solvent systems. Foils were chromatographed 1 times in the first and 3 times in the second dimension. Localization on the foils was obtained by co-chromatography of reference solutions subjected to the same procedure using a UV-lamp for the detection of the dansylated compounds at 254 nm (Fig. 2). For liquid scintillation counting the UV-positive spots were cut off, transferred into polyethylene vials and then eluted with 1 ml ethanol/triethylamine/bidest. H_2O (95 : 1 : 4; w/w/w). After 3 h, 3 ml Toluol Scintillation Fluid (Packard, Zürich) was added and radioactivity was determined with a LS 7500 (Beckmann Irvine) spectrometer.

Results and Discussion

γ-Glutamyltranspeptidase activity catalyzing hydrolysis of γ-glutamyldipeptides (Eqn. (1)) and the transfer of γ-glutamyl-moieties to amino acid acceptors (Eqns (2) + (3)) has repeatedly been demonstrated in higher plant cells [8, 14–18]. Using the artificial substrate γ-glutamyl-*p*-nitroanilide, the

γ-glutamyl amino acid → glutamate + amino acid (1)

γ-glutamyl amino acid + met → γ-glutamyl-met + amino acid (2)

2 γ-glutamyl amino acid → γ-glutamyl-glutamyl-amino acid + amino acid (3)

specific activity of this enzyme in cultured tobacco cells is only slightly enhanced upon addition of the γ-glutamyl acceptor methionine (Table I) or other amino acids (not shown). This observation indicates that γ-glutamyltranspeptidase predominantly catalyzes hydrolysis of γ-glutamyl-*p*-nitroanilide (Eqn. (1)) or the autotransfer of γ-glutamyl-moieties to this substrate (Eqn. (3)). If glutathione is a suitable substrate of tobacco γ-glutamyltranspeptidase, it would compete with γ-glutamyl-*p*-nitroanilide in this assay mixture thereby reducing the amount of *p*-nitroaniline liberated from this compound. As shown in Table I, glutathione did not reduce, but stimulated the liberation of *p*-nitroaniline. Such a stimulation may be explained by a reaction in which glutathione acts as a γ-glutamyl acceptor (Eqn. (2)), while hydro-

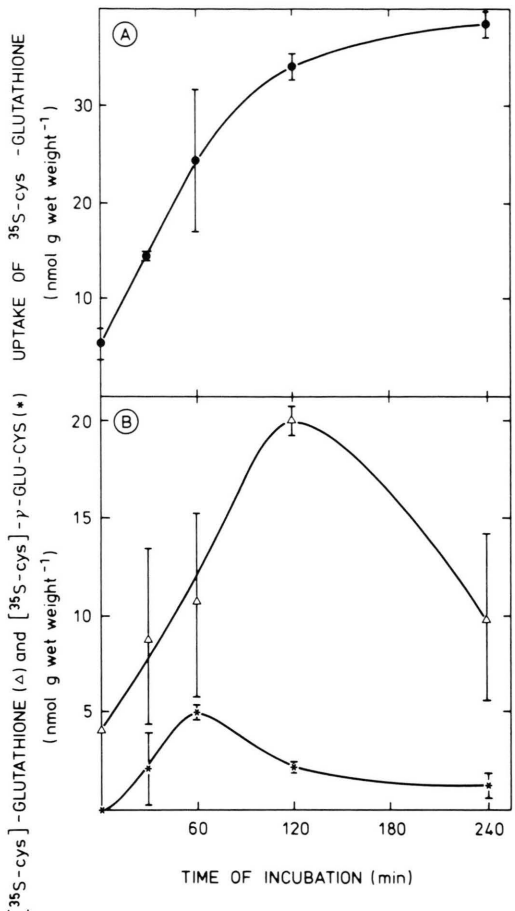

Fig. 3. Uptake and metabolism of [^{35}S-cys]glutathione. Tobacco cells were fed [^{35}S-cys]glutathione. After the periods of time indicated the medium was removed and the cells were washed and homogenized. Aliquots of each sample were reduced, carboxymethylated and dansylated. Analysis of the radioactively labelled compounds was performed by two-dimensional TLC. Glutathione and γ-glutamylcysteine were quantified by liquid scintillation counting. Radioactivity after 0 min of incubation reflects unspecific binding of [^{35}S-cys]glutathione to the cell walls. Three independent experiments were performed with results similar to the data shown.

Table I. Effect of GSH and L-methionine (met) on γ-glutamyltranspeptidase activity of tobacco cells fed sulfate as sulfur source. γ-Glutamyltranspeptidase activity was determined in ammonium sulfate precipitates of tobacco cell homogenates using γ-glutamyl-*p*-nitroanilide as substrate. The liberation of *p*-nitroaniline from γ-glutamyl-*p*-nitroanilide was measured photometrically at 405 nm. Data given are means of three independent experiments with 3 replicates each.

GSH	Methionine	Specific activity ± SE	
		nmol × min^{-1} × mg protein^{-1}	% [a]
0	0	1.79 ± 0.06	100 ± 3.6
10	0	2.42 ± 0.15	135.4 ± 8.5
0	10	2.02 ± 0.09	112.9 ± 5.1
10	10	2.64 ± 0.13	147.5 ± 7.3

[a] γ-Glutamyltranspeptidase activity in reaction mixtures without GSH or met is set 100%.

Table II. Substrate specificity of γ-Glutamylcyclotransferase from cultured tobacco cells. γ-Glutamylcyclotransferase activity was determined in ammonium sulfate precipitates of tobacco cell homogenates. Assays contained 4 mM substrate, 20 mM DTE and 0.2 ml enzyme preparation. After 30 min incubation at 30 °C, generation of 5-oxo-proline was measured by gaschromatography. Data shown are means of 3 independent experiments with 3 replicates each.

Substrate	Specific activity ± SE	
	nmol min^{-1} mg protein^{-1}	% [a]
γ-glutamyl-methionine	6.36 ± 0.98	100 ± 15.4
γ-glutamyl-cysteine	1.46 ± 0.13	22.9 ± 2.0
γ-glutamyl-glutamate	0.70 ± 0.06	11.0 ± 0.9
γ-glutamyl-α-aminobutyrate	0.65 ± 0.15	10.2 ± 2.4
γ-glutamyl-alanine	0.63 ± 0.01	9.9 ± 0.1
γ-glutamyl-cysteinyl-glycine	0.21 ± 0.01	3.3 ± 0.2

[a] γ-Glutamylcyclotransferase activity with γ-glutamyl-methionine as substrate is set 100%.

lysis (Eqn. (1)) proceeds unaffected. Therefore, it appears as if glutathione is not a donator, but an acceptor of γ-glutamyl-moieties in the γ-glutamyltranspeptidase reaction. From these experiments, it can be concluded that a γ-glutamyltranspeptidase is not participating in degradation of glutathione in tobacco cells; γ-glutamyltranspeptidase is more likely to catalyze the degradation of γ-glutamyldipeptides in tobacco cells, a function previously suggested by other investigators [15, 16].

The conclusion that γ-glutamyltranspeptidase is not participating in degradation of glutathione in tobacco cells is supported by feeding experiments with [^{35}S-cys]glutathione. When tobacco cells were supplied with this peptide as sole sulfur source, it is initially taken up at a high rate. With increasing time of exposure, the rate of uptake declined (Fig. 3A). Inside the tobacco cells only two radioactively labelled compounds were found, *i.e.* glutathione and γ-glutamylcysteine. The radioactivity in

the glutathione pool of the cells increased for 2 h, the radioactivity in the γ-glutamylcysteine pool for 1 h. Thereafter, the radioactivity in both pools declined (Fig. 3 B). This observation may be explained by the decrease in the rate of uptake of glutathione (Fig. 3 A). Apparently, the uptake of glutathione after 1 to 2 h of incubation is insufficient to fullfill the tobacco cells' needs for reduced sulfur. This conclusion is supported by the observation that radioactive cysteine was not found in the cysteine pool, although the protein of the cells was radioactively labelled. It is further supported by previous experiments showing that tobacco cells supplied with glutathione as sole sulfur source exhibit sulfur deficiency symptoms with respect to 5-oxo-prolinase activity [19] and accumulation of arginine (Klapheck, unpublished results). Therefore, the present data show that hydrolysis of the C-terminal glycine-moiety is the initial step in glutathione degradation in tobacco cells.

Previously published feeding experiments with [^{14}C-glu]glutathione revealed that 5-oxo-proline is an intermediate in glutathione degradation in tobacco cells [4]. However, a γ-glutamylcyclotransferase, responsible for the generation of 5-oxo-proline from γ-glutamylpeptides, has not been reported in higher plant cells. Using ammonium sulfate precipitates at 40–70% saturation, we were able to demonstrate the presence of γ-glutamyl-cyclotransferase activity in tobacco cells. As shown in Table II, the enzyme exhibits considerable activities with sulfur containing γ-glutamyl-dipeptides, but not with sulfur free γ-glutamyl-peptides or glutathione. This substrate specificity indicates a function of the enzyme in the liberation of sulfur amino acids from sulfur containing γ-glutamyldipeptides.

The present observations suggest that glutathione is degraded in plant cells without participation of a γ-glutamyltranspeptidase *via* the pathway shown in Fig. 1, III. Further experiments will show whether a glutathione specific carboxypeptidase is also present in the tobacco cells.

Acknowledgements

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[1] A. Meister, Current Topics in Cellular Regulation, **Vol. 18,** p. 21, Academic Press, New York 1981.
[2] R. H. Shimabukuro, G. L. Lamoureux, and D. S. Frear, Chemistry and Action of Herbicide Antidotes, p. 133, Academic Press, New York 1978.
[3] G. L. Lamoureux and D. G. Rusness, Sulfur in Pesticide Action and Metabolism, ACS Symposium, Series **Vol. 158,** p. 132, Americal Chemical Society, 1981.
[4] H. Rennenberg, R. Steinkamp, and A. Polle, Z. Naturforsch. **35 c,** 708–711 (1980).
[5] H. Rennenberg, Phytochemistry **21,** 2771–2781 (1982).
[6] L. Bergmann, J. Gen. Physiol. **43,** 841–851 (1960).
[7] H. Logemann and L. Bergmann, Planta **121,** 283–292 (1974).
[8] R. Steinkamp and H. Rennenberg, Physiol. Plant. **61,** 251–256 (1984).
[9] M. Orlowski and A. Meister, J. Biol. Chem. **246,** 7095–7105 (1971).
[10] S. Wilk and M. Orlowski, Anal. Biochem. **69,** 100–113 (1975).
[11] H. Rennenberg, Z. Pflanzenphysiol. **105,** 31–40 (1981).
[12] W. R. Gray, Methods in Enzymology, **Vol. 25 b,** 121–128 (1972).
[13] P. K. Macnicol, Anal. Biochem. **85,** 71–78 (1978).
[14] M. Y. Goore and J. F. Thompson, Biochim. Biophys. Acta **132,** 15–26 (1967).
[15] T. Kasai, A. Ohmiya, and S. Sakamura, Phytochemistry **21,** 1233–1239 (1982).
[16] Y. Kawasaki, T. Ogawa, and K. Sasaoka, Biochim. Biophys. Acta **716,** 194–200 (1982).
[17] E. A. Kean and E. R. Hare, Phytochemistry **19,** 199–203 (1980).
[18] J. F. Thompson, D. H. Turner, and R. R. Gering, Phytochemistry **3,** 33–46 (1964).
[19] H. Rennenberg, A. Polle, and I. Grundel, Physiol. Plant. **59,** 61–66 (1983).