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Characterization of Glutathione Uptake in Broad Bean Leaf Protoplasts[1]

Aziz Jamaï, Roberto Tommasini, Enrico Martinoia, and Serge Delrot*

Laboratoire de Physiologie et Biochimie Végétales, Unité Associée Centre National de la Recherche Scientifique 574, Université de Poitiers, Bâtiment Botanique, 40, Avenue du Recteur Pineau, 86022 Poitiers Cédex, France (A.J., S.D.); Institute of Plant Sciences, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland (R.T.); and Laboratoire des Echanges Membranaires Végétaux, Université de Poitiers, Bâtiment Botanique, 40 Avenue du Recteur Pineau, 86022 Poitiers Cédex, France (E.M.)

Transport of reduced glutathione (GSH) and oxidized glutathione (GSSG) was studied with broad bean (*Vicia faba* L.) leaf tissues and protoplasts. Protoplasts and leaf discs took up GSSG at a rate about twice the uptake rate of GSH. Detailed studies with protoplasts indicated that GSH and GSSG uptake exhibited the same sensitivity to the external pH and to various chemical reagents. GSH uptake was inhibited by GSSG and glutathione conjugates. GSSG uptake was inhibited by GSH and GS conjugates, and the uptake of metolachlor-GS was inhibited by GSSG. Various amino acids (L-glutamic acid, L-glutamine, L-cysteine, L-glycine, L-methionine) and peptides (glycine-glycine, glycine-glycine-glycine) affected neither the transport of GSH nor GSSG. Uptake kinetics indicate that GSH is taken up by a single saturable transporter, with an apparent K_m of 0.4 mM, whereas GSSG uptake exhibits two saturable phases, with an apparent K_m of 7 μM and 3.7 mM. It is concluded that the plasma membrane of leaf cells contains a specific transport system for glutathione, which takes up GSSG and GS conjugates preferentially over GSH. Proton flux measurements and electrophysiological measurements indicate that GSH and GSSG are taken up with proton symport. However, a detailed analysis of these measurements suggests that the ion movements induced by GSSG differ from those induced by GSH.

In plants, GSH (γ-glutamyl-cysteinyl-Gly) and its analogs (γ-glutamyl-cysteinyl-β-alanine and γ-glutamylcysteineserine) are the most abundant storage and transport forms of the sulfur reduced in the chloroplasts. Phloem transport of GSH and homoglutathione from exporting leaves supply the young leaves, the young roots, and the fruits with reduced sulfur (Bergmann and Rennenberg, 1993). Sulfate uptake is inhibited by GSH in tobacco plants, and GSH may be considered as a signal transported from the shoot to the root to mediate interorgan regulation of sulfur nutrition (Herschbach and Rennenberg, 1991, 1994). In addition to its nutritional role, GSH is also important for the protection of the plant. Glutathione, which is usually found in millimolar concentrations in a wide range of plants, is also involved in the reduction of proteins, in the destruction of H_2O_2 in chloroplasts, in the detoxification of xenobiotics (Rennenberg, 1982; Edwards and Owen, 1986; Martinoia et al., 1993), and in protection against various stresses, such as irradiation (Meister and Anderson, 1983), heat (Nieto-Sotelo and Ho, 1986), oxidative stress due to O_3 or SO_2 (Alscher, 1989), and exposure to heavy metals (Grill et al., 1985). Supply of GSH to suspension-cultured cells of bean stimulates the transcription of various defense genes, including those encoding cell wall Hyp-rich glycoproteins, phenylalanyl ammonia-lyase, and chalcone synthase (Wingate et al., 1988). These data led to the suggestion that GSH may function as a signal of redox perturbations induced by various biological stresses (Wingate et al., 1988; Dhindsa, 1991).

Homoglutathione is mainly found in Leguminosae and its biological roles are similar to those of GSH (Klapheck, 1988; Zopes et al., 1993). The relative proportion between GSH and homoglutathione varies greatly within different plant tribes of legumes, and homoglutathione was not detected at all in *Vicia faba* (Klapheck, 1988).

With respect to its important role as a form of sulfur transport, as an effector of sulfate uptake in roots, and as a putative signal involved in various stress responses, relatively few studies have dealt with long distance transport and membrane transport of glutathione. Analysis of *Ricinus* phloem exudate showed that the bulk of sulfur is transported as inorganic sulfate (70% of total transported sulfur), but GSH accounted for 70% of the organic sulfur translocated in the phloem (Bonas et al., 1982). GSH may be readily exchanged between phloem and xylem in spruce trees (Schneider et al., 1994). Schneider et al. (1992) showed that in cultured tobacco cells uptake of GSH is mediated via a high-affinity system (apparent K_m = 17 μM) and a low-affinity system (apparent K_m = 310 μM). In this material, the uptake of GSH is active and competitively inhibited by Cys. γ-Glutamyl transpeptidase, an enzyme involved in GSH degradation in animal cells, was shown not to be involved in glutathione uptake in tobacco cells (Schneider et al., 1992).

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* Corresponding author; e-mail serge.delrot@cri-univ.poitiers.fr; fax 33–49–45–41–86.

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; GS, glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; PD, transmembrane potential difference.

Although GSH is assumed to be transported in its reduced form in the phloem, thanks to a glutathione reductase, which may maintain the reduced form (Alosi et al., 1988), no such reductase was found in xylem exudates, and it is likely that GSH may be at least partially oxidized in the apoplast because of the presence of H_2O_2. Moreover, freshly prepared leaf samples may possess transport properties that differ from cultured cells previously used in the only study concerning the transport of glutathione at the cellular level (Schneider et al., 1992). Those questions, together with the lack of detailed characterization of glutathione uptake and the important biological roles played by both forms of glutathione, led us to investigate the uptake of GSH and GSSG in broad bean leaf tissue.

MATERIALS AND METHODS

Uptake in Protoplasts

Broad beans (*Vicia faba* L. cv Aguadulce) were grown as described by Jamaï et al. (1994). The fourth and fifth fully expanded bifoliate leaves from 3-week-old plants were used to prepare the protoplasts. After the lower epidermis of the leaves was peeled with fine forceps, leaf pieces (about 12 g fresh weight) were cut and incubated for 90 min at 28°C onto 10 mL of a medium containing 500 mM sorbitol, 1 mM $CaCl_2$, 10 mM Mes/KOH (pH 5.5), 0.5 mM polyvinylpolypyrrolidone, 0.1% BSA, 2.5% cellulase YC, and 0.1% pectolyase YC 23 (Seishin, Tokyo, Japan). At the end of incubation, the protoplast suspension was recovered and centrifuged for 5 min at 1200*g* in a tube containing 1 mL of 100% Percoll (Pharmacia), 500 mM sorbitol, and 20 mM Mes/NaOH, pH 5.6. The supernatant was removed, and the protoplasts that were sedimented at the buffer/Percoll interface were mixed with the Percoll present at the bottom of the tube to obtain a 40% Percoll solution. Ten milliliters of 25% Percoll solution (pH 6.0) and 5 mL of 5% Percoll solution (pH 6.0) were then carefully added to this protoplast suspension, and this discontinuous gradient was centrifuged for 5 min at 1200*g*. Only the protoplasts sedimenting at the 25/5% interface were used for the uptake experiments. The protoplasts were resuspended at a density of 4.5×10^6 protoplasts mL^{-1} in a medium containing 500 mM glycinebetaine, 20 mM Mes/KOH (pH 5.6), 1 mM $CaCl_2$, 2 mM DTT, and 0.1% (w/w) BSA (medium A). Uptake experiments were started in 0.5-mL Eppendorf microfuge tubes by adding one part of protoplast suspension to two parts of incubation medium containing 0.75 mM [^{35}S]GSH or [^{35}S]GSSG (except when stated otherwise). For experiments concerning the uptake of GSSG, DTT was omitted from the incubation medium. At selected times, 100-μL aliquots of the incubation medium were sampled and overlaid on a preformed gradient consisting of 25 μL of 25% Percoll, 500 mM sorbitol, 20 mM Mes/NaOH (pH 6.0), and 200 μL of silicone oil AR 200 (Wacker Chemie, Munich, Germany). Uptake was terminated by sedimenting the protoplasts through these cushions at 7500*g* for 18 s in a M11 Beckman microfuge. The tip of the tubes containing the protoplasts was cut with a sharp razor blade and deposited in a scintillation vial containing 4 mL of Ecolite (ICN). The radioactivity was counted by liquid scintillation counting after correction for background and quenching (Packard Instruments, Les Ulis, France).

[^{35}S]Glutathione was synthesized as described previously (Tommasini et al., 1993). The conjugate of GSH with NEM was synthesized as described by Martinoia et al. (1993).

Uptake in Leaf Discs

Leaf discs (12 mm in diameter) were prepared from mature broad bean leaves, and the lower epidermis was peeled with fine forceps. Peeled discs were used for uptake experiments as described by Jamaï et al. (1994).

Measurements of GSH Content of the Medium

To check the time course of the GSH content in the incubation medium used (10 mL, initially containing 50 μM GSH), aliquots of 100 μL of the medium without tissues or containing 12 peeled leaf discs (270 mg fresh weight) were sampled at selected times. The samples were incubated for 5 min in the presence of 1 mM 1-chloro-2,4-dinitrobenzene and 0.5 unit glutathione *S*-transferase (EC 2.5.1.18) in 200 mM Na_2HPO_4 (pH 6.5). The GSH content was determined at 340 nm against a control value (incubation medium without GSH, incubated under the same conditions with the reagents) (Mannervik and Guthenberg, 1986). GSSG is unable to conjugate with 1-chloro-2,4-dinitrobenzene.

pH Measurements

Peeled leaf fragments (0.5 g fresh weight) were floated onto 20 mL of a medium containing 250 mM mannitol, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$ (initial pH 6.0). The pH of the medium was recorded as already described (Jamaï et al., 1994). At appropriate times, GSH or GSSG was added to the medium, and its effect on the evolution of the pH of the medium containing the tissues was studied.

Electrophysiological Measurements

The lower epidermis of a leaf fragment was removed, and the fragment was incubated, peeled side up, in a medium containing 250 mM mannitol, 20 mM Mes/KOH (pH 5.5), 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$. The PD of mesophyll cells was measured using these leaf fragments according to standard electrophysiological techniques described by M'Batchi et al. (1986).

RESULTS

Uptake of GSH and GSSG

At pH 5.6, uptake of GSH and GSSG into broad bean leaf protoplasts was linear for at least 30 min (Fig. 1). The hexapeptide GSSG was taken up at a rate more than twice the rate of GSH. The absence of DTT in the medium stimulated the uptake of GSH. Similar results were obtained with broad bean leaf discs (data not shown). The data show that GSSG is taken up preferentially over GSH. The effects of DTT suggest that either this compound inhibited the

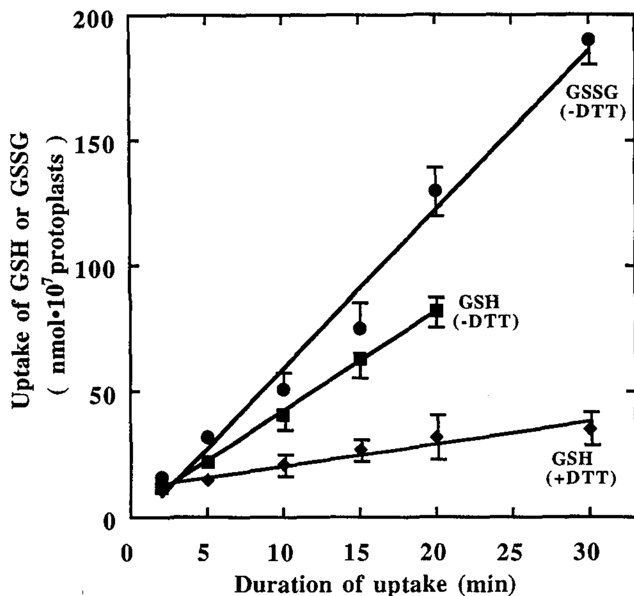

Figure 1. Time course of GSH and GSSG uptake into broad bean leaf protoplasts. ●, Uptake of 0.5 mM GSSG in the absence of DTT; ■, uptake of 0.5 mM GSH in the absence of DTT; ♦, uptake of 0.5 mM GSH in the presence of 2 mM DTT. Data are means ± SE of 20 samples (four experiments). The experiments were run at pH 5.6.

uptake system of GSH or GSH rapidly oxidizes into GSSG in the absence of DTT.

The content of GSH in the incubation medium decreased about 13% within 30 min in the absence of tissue and about 25% in the presence of leaf discs (data not shown). Although we did not measure the appearance of GSSG, it may be suggested that GSH spontaneously oxidizes in the medium and that this oxidation is accelerated by the presence of leaf tissues.

Twelve-gram fresh weight tissues yielded about 25×10^6 purified protoplasts, and 1 cm^2 of peeled leaf tissue weighs about 19.5 mg fresh weight. It is therefore possible to compare the rate of GSH and GSSG uptake in protoplasts and in leaf discs, even though this is an approximate comparison, because of the losses during protoplast preparation and because the uptake conditions differ somewhat (osmolarity of the medium). These calculations indicate that the rate of GSH and GSSG uptake in protoplasts was about 40 and 60%, respectively, of the corresponding rates measured with leaf discs.

pH dependence studies showed that the uptake of GSH and GSSG exhibited the same sensitivity to the pH of the medium (Fig. 2). Uptake of both compounds was optimal at pH 4.0, strongly declined between pH 4.0 and 4.5, and then slowly declined to pH 7.0. At any pH, the uptake of GSSG was about twice the uptake of GS. Later studies were conducted at pH 5.6 because protoplast viability was strongly reduced at a pH lower than 5.0 and because a pH of 5.6 approximately corresponds to the apoplastic pH.

Preliminary measurements gave an estimate of 20 to 30 μM for the GSH concentration of broad bean leaf apoplastic samples (data not shown). This is somewhat higher than other values reported in the literature, e.g. 6 μM in the extracellular washing fluid of spruce needle (Polle et al., 1990). Concentration dependence studies were run with GSH and GSSG concentrations between 0.002 and 15 mM. The uptake of GSH exhibited signs of saturation at approximately 1 mM (Fig. 3A), but this saturable component was superimposed by a linear phase at higher concentrations, as shown by the vertical line in Woolf plots (Fig. 3B). Woolf (Fig. 3B) and Lineweaver-Burk (data not shown) plots yielded an apparent K_m of 0.4 mM and a V_{max} of 2.1 nmol GSH 10^{-7} protoplasts min^{-1} for the saturable component of GSH uptake into broad bean leaf protoplasts. No evidence for an uptake system with a K_m lower than 0.4 mM could be found at low GSH concentrations (Fig. 3A, inset).

Kinetics of GSSG uptake indicate the presence of two apparent carrier-mediated phases superimposed by a diffusion-like component (Fig. 4). The highest affinity component, which was clearly apparent between 0.002 and 0.02 mM GSSG, exhibited a K_m of 7 μM and a V_{max} of 0.13 nmol GSSG 10^{-7} protoplasts min^{-1} (Fig. 4A, inset, and Fig. 4B, horizontal line). A second saturable component was apparent between 0.5 and 8 mM GSSG (Fig. 4A and Fig. 4B, oblique line). Woolf analysis (Fig. 4B) and Lineweaver-Burk plots (not shown) gave an apparent K_m value of 3.7 mM and a V_{max} of 27.1 nmol 10^{-7} protoplasts min^{-1} for this saturable component of GSSG uptake. This component was superimposed by a diffusional component parallel to the vertical axis (Fig. 4B). The data indicate that the uptake of GSH and GSSH into protoplasts is carrier mediated at relatively low concentrations of the peptide in the external medium (<3 mM), whereas diffusion plays a major part at higher concentrations. Whatever the concentration tested,

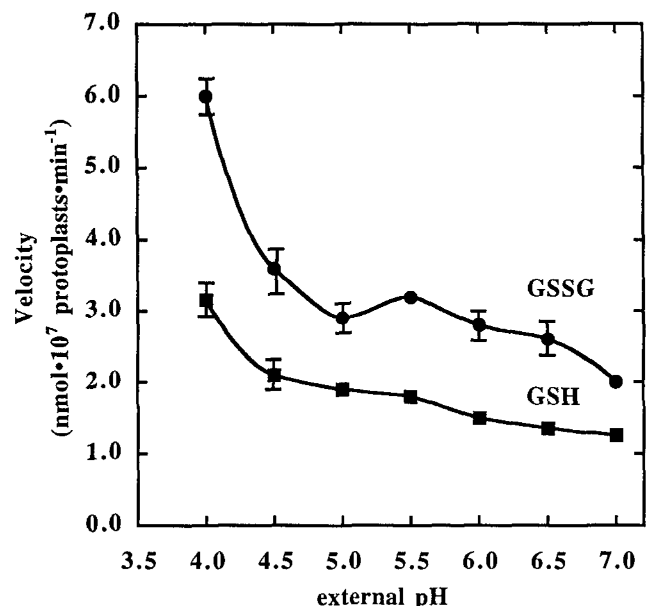

Figure 2. pH dependence of GSH (●) and GSSG (■) uptake from a 0.5 mM solution. The protoplasts were transferred to the incubation medium buffered at various pH values with 10 mM sodium citrate/20 mM disodium phosphate. Data are means ± SE of 15 samples (three experiments). Duration of uptake was 20 min.

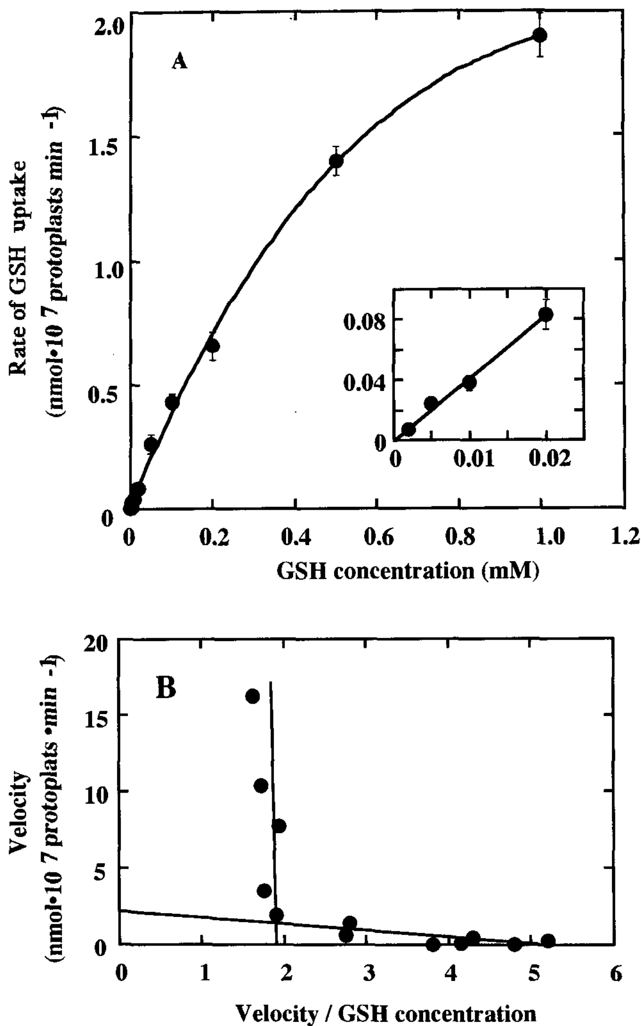

Figure 3. Concentration dependence of GSH uptake into broad bean leaf protoplasts. A, Michaelis-Menten kinetics; inset, low concentration range. B, Woolf plots. The rate of uptake was measured as the difference between the uptake measured after 20 and 2 min of incubation at pH 5.6. Data are means ± SE of 15 samples (three experiments).

GSSG uptake is higher than GSH uptake. To allow for a more direct comparison between GSH and GSSG uptake, and because of the limited sensitivity of proton fluxes and PD measurements, subsequent experiments were conducted using GSH and GSSG concentrations equal to or more than 0.5 mM.

CCCP, which rapidly dissipates the proton gradient in this material (Despeghel and Delrot, 1983), inhibited the uptake of both GSH and GSSG (Table I). Likewise, a 30-min pretreatment with 1 mM NEM (a permeant thiol reagent that may block both the proton-pumping ATPase and the transporters) inhibited subsequent uptake of GSH and GSSG. Inhibitor studies, therefore, indicate that glutathione uptake was energy-dependent.

The various amino acids constituting glutathione (Glu, Cys, Gly) inhibited neither GSH nor GSSG uptake (Table II). Because a transporter mediating proton-peptide transport was recently characterized in mature broad bean leaf, and because this transporter exhibited a broad specificity for "protein" peptides (Jamaï et al., 1994), it was interesting to test whether GSH and GSSG could be taken up by this transport system. The dipeptide Gly-Gly and the tripeptide Gly-Gly-Gly, which are both strongly recognized by the proton-peptide co-transporter mentioned above, inhibited neither the uptake of GSH nor the uptake of GSSG (Table II). Met and Gln did not affect the transport of GSH or GSSG either. It is interesting that the alkyl glutathione GS-NEM strongly inhibited both the uptake of GSH and the uptake of GSSG (Table II). The uptake of 20 μM [^{14}C]metolachlor-glutathione was inhibited 32 and 26%, respectively, by the addition of 0.5 mM GSH or 0.5 mM GSSG (data not shown).

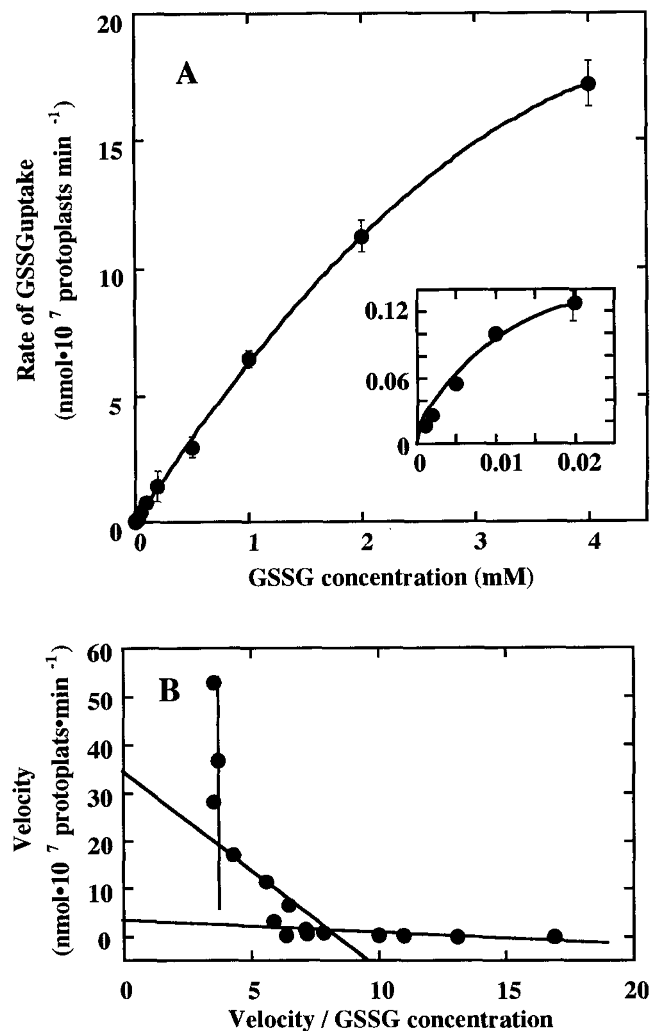

Figure 4. Concentration dependence of GSSG uptake into broad bean leaf protoplasts. A, Michaelis-Menten kinetics; inset, low concentration range. B, Woolf plots. The rate of uptake was measured as the difference between the uptake measured after 20 and 2 min of incubation at pH 5.6. Data are means ± SE of 15 samples (three experiments).

Table I. *Effects of CCCP and NEM on [^{35}S]GSH (0.5 mM) and [^{35}S]GSSG (0.5 mM) uptake by broad bean leaf protoplasts*

CCCP was present during the incubation (20 min). NEM was present during only the pretreatment (30 min), followed by a 5-min centrifugation (rinsing) on a discontinuous Percoll gradient, and incubation (20 min) in the presence of labeled GSH or GSSG. Means ± SE of 20 samples (four experiments) for CCCP and of 10 samples (two experiments) for NEM.

	GSH Uptake		GSSG Uptake	
	nmol 10^{-7} protoplasts min^{-1}	% of control	nmol 10^{-7} protoplasts min^{-1}	% of control
Control	1.75 ± 0.28	100.0	3.80 ± 0.21	100.0
CCCP (0.01 mM)	1.00 ± 0.18	57.1	1.80 ± 0.10	47.4
Control	1.77 ± 0.27	100.0	3.69 ± 0.66	100.0
NEM (1 mM)	1.02 ± 0.30	57.6	2.15 ± 0.41	58.3

GSH- and GSSG-Induced Proton Fluxes

Peeled broad bean leaf fragments acidify the pH of their incubation medium (initial pH 6.0) to pH 4.5, because of the activity of the plasma membrane proton-pumping ATPase. Proton fluxes induced by proton/sugar co-transport are best observed when the pH of the medium has reached 4.7 to 4.8 (Delrot, 1981). Addition of various di- and tripeptides at this pH also allowed investigators to observe peptide-induced proton co-transport (Jamaï et al., 1994). In the same conditions as those used for the previous experiments, addition of GSH (2 mM, final concentration) to the medium induced a decrease of the acidification rate, whereas addition of GSH at 5 mM induced a transient alkalinization of the medium (Fig. 5A). Figure 5B summarizes various experiments during which GSSG was added at various concentrations. Increasing the concentration of the added GSSG decreased and eventually almost stopped the acidification of the medium. Although GSSG is absorbed about twice as much as GSH (Fig. 1), increasing the final concentrations of added GSSG up to 20 mM never resulted in an alkalinization of the medium (Fig. 5B), in contrast to what is observed with GSH (Fig. 5A).

Electrophysiological Measurements

Addition of either GSH or GSSG to the incubation medium induced a transient depolarization of the PD, followed by a transient hyperpolarization (Fig. 6, traces a–c). This evolution of the PD is similar to that observed with peptides containing the normal *N*-peptide bond (Jamaï et al., 1994), but it differs from the reactions caused by the addition of amino acids, which induces a depolarization but no consecutive hyperpolarization (Felle et al., 1979; Golle and Lüttge, 1983; Felle, 1984; Kinraide et al., 1984; Mounoury et al., 1984; Pichelin et al., 1984; Petzold et al., 1989). Addition of GSSG to the incubation medium also induced a transient depolarization of the PD (Fig. 6, traces b–d). This depolarization was faster and slightly stronger than that observed after GSH addition. However, the GSSG-induced depolarization was followed only by a weak hyperpolarization (Fig. 6, trace d) or no hyperpolarization (Fig. 6, trace c), depending on the experiment. When GSSG was added after GSH, GSSG-induced depolarization was followed by a clear hyperpolarization (in Fig. 6, cf. trace b with traces c and d). The addition of GSSG to the medium prior to GSH addition did not affect the PD changes normally induced by GSH (Fig. 6, trace c).

Figure 7 shows the concentration dependence of GSH-induced depolarization and hyperpolarization of the PD. GSH-induced depolarization obeyed a single saturation phase with an apparent K_m of 0.5 mM and a maximal depolarization of +20 mV. Maximal hyperpolarization was about −15 mV, with an apparent K_m of 0.5 mM. These K_m values are in good agreement with those found for the saturable component of GSH uptake.

DISCUSSION

Glutathione is transported for long distances in the plant both in the phloem and in the xylem (Schneider et al., 1994), and it plays many important biological roles, including interorgan regulation of sulfur nutrition and protection against various stresses (Rennenberg and Brunold, 1994). We suggest that glutathione is present in the leaf apoplast, where it plays an important role in maintaining the redox status of the plasma membrane and cell-wall-associated

Table II. *Effects of various compounds (5 mM) on [^{35}S]GSH (0.5 mM) and [^{35}S]GSSG (0.5 mM) uptake by broad bean leaf protoplasts*

Means ± SE of 15 samples (three experiments).

	GSH Uptake		GSSG Uptake	
	nmol 10^{-7} protoplasts min^{-1}	% of control	nmol 10^{-7} protoplasts min^{-1}	% of control
Control	1.70 ± 0.12	100.0	3.40 ± 0.10	100.0
Gly-Gly	1.71 ± 0.20	100.6	3.30 ± 0.23	97.0
L-Glutamine	1.73 ± 0.18	101.8	3.53 ± 0.24	103.8
Gly	1.69 ± 0.14	99.1	3.55 ± 0.06	104.4
L-Cys	1.70 ± 0.19	100.0	3.43 ± 0.16	100.8
Triglycine	1.69 ± 0.13	99.4	3.55 ± 0.11	104.4
L-Met	1.72 ± 0.13	101.2	3.72 ± 0.08	109.4
L-Glu	1.68 ± 0.14	98.8	3.35 ± 0.24	98.5
GS-NEM	0.65 ± 0.04	38.2	1.45 ± 0.3	42.6

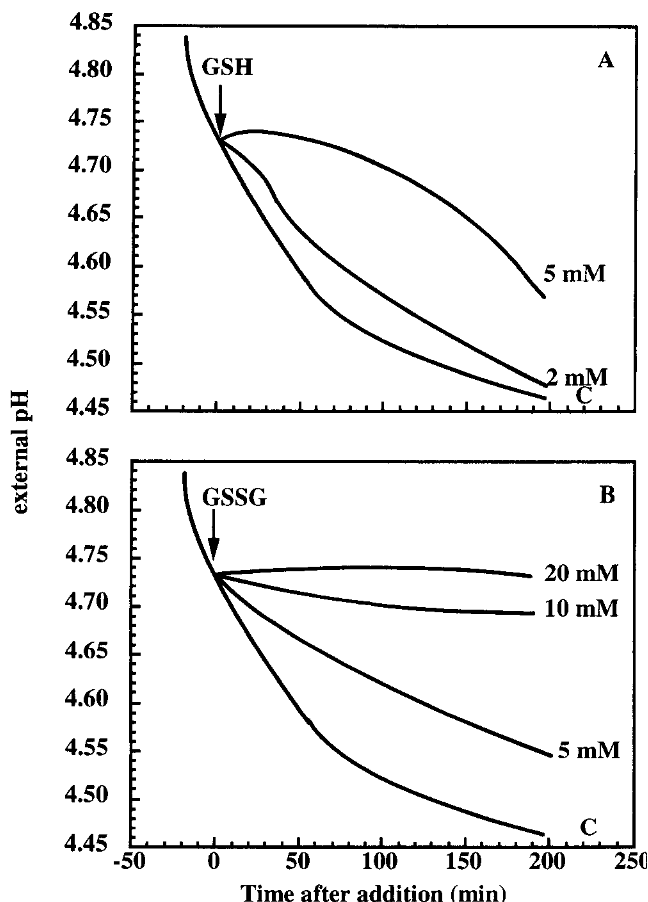

Figure 5. pH changes induced by the addition of various concentrations of GSH (A) and GSSG (B) to a medium containing broad bean leaf tissues. GSH was added from a 250 mM stock solution prepared in the same medium and at the final concentration indicated on the graph. GSSG was added from a 100 mM stock solution. The graphs summarize the results of three experiments for each concentration of GSH and at least four experiments for each concentration of GSSG.

proteins. Although intracellular GSSG accounts for less than 5% of total glutathione content of the cell (Rennenberg and Brunold, 1994), GSH may be oxidized in the apoplast. We compared the uptake of GSH and GSSG to test whether this γ-glutamyl peptide was transported via the peptide transport system already characterized in leaf tissues (Jamaï et al., 1994).

Uptake data indicate that GSSG is absorbed at a rate about twice the rate of GSH absorption, whatever the concentration range tested (Figs. 1, 3, and 4). GSH uptake obeys single saturable kinetics with an apparent K_m of 0.4 mM, whereas GSSG uptake kinetics exhibit two saturable phases with apparent K_ms of 7 μM and 4 mM. At high concentrations uptake of both GSH and GSSG is dominated by a diffusion-like component, but it is likely that this component has no physiological significance in vivo. These data therefore indicate substantial differences in the kinetics of GSH and GSSG uptake. These differences may reflect intrinsically different kinetics of the carrier for GSH and GSSG, but they may also result from a different redox status of the transporter in the presence of GSH and GSSG. Several lines of evidence suggest that a common transporter may mediate GSH and GSSG uptake. At a concentration of 0.5 mM GSH or GSSG, the uptake of both compounds exhibits the same pH sensitivity (Fig. 2) and the same sensitivity to CCCP and NEM (Table I). Furthermore, among all tested compounds, GS-NEM was the strongest inhibitor of uptake both for GSH and GSSG (Table II). GSH and GSSG also compete with each other in uptake experiments (data not shown), but both forms readily interchange with each other; therefore, this is not a strong argument in favor of the idea of a common transporter. Because of these experimental limitations (modification of the redox status of the membrane by the tested substrates, possible interconversion of GSH and GSSG) and the complex kinetics observed, the evidence for a common transporter for GSH and GSSG is therefore indirect. More definitive conclusions await the cloning of these transporters. However, it is clear that the glutathione transport system of the cell preferentially takes up GSSG rather than GSH, although the size of GSSG (molecular weight 612) is about twice the size of GSH (molecular weight 307).

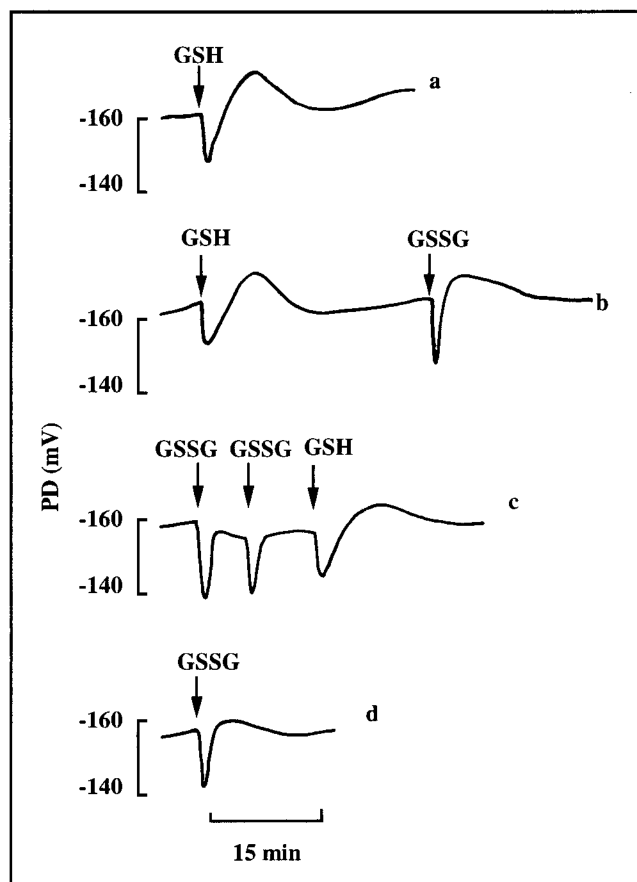

Figure 6. GSH- and GSSG-induced variations of the PD of mesophyll cells. All additions were made at 1 mM (final concentration) from a 100 mM stock solution prepared in a solution identical to the incubation medium. Numbers at the right refer to different traces. Data are chart records representative of at least three similar experiments.

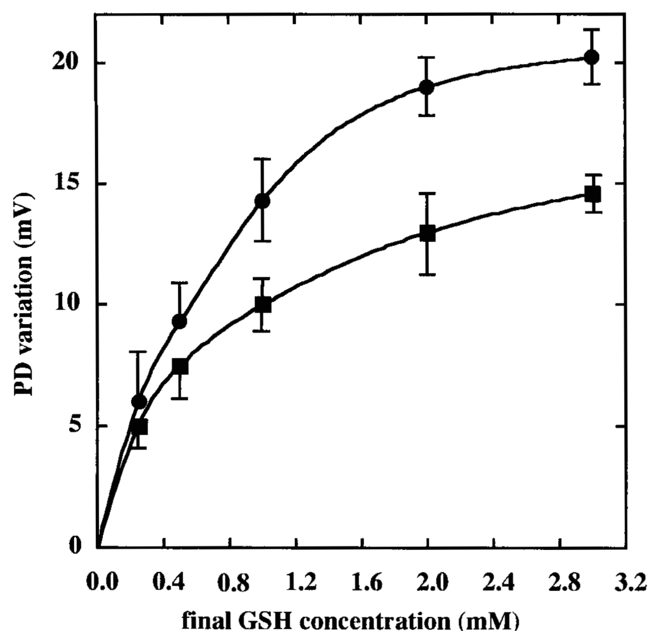

Figure 7. Concentration dependence of GSH-induced depolarization (●) and hyperpolarization (■).

The pH optimum, the kinetics of uptake, and the sensitivity to Cys found in the present study differ somewhat from the only other study concerning uptake of GSH at the cellular level, which was conducted with cultured tobacco cells (Schneider et al., 1992). These differences may be due to the plant material and to the experimental conditions, since we include DTT in our incubation medium to maintain GSH under its reduced form.

The glutathione transport system is not able to recognize amino acids such as L-Glu, L-Cys, and Gly (Table II), which indicates that glutathione uptake is not mediated by one of the several amino acid transporters already characterized (Bush, 1993). Likewise, the glutathione transport system is unable to recognize di- or tripeptides containing a normal peptidic bond (Table II). In addition, uptake of Gly-Gly by the peptide transporter previously characterized in broad bean leaf tissues is maximal at pH 6.0 (Jamaï et al., 1994), whereas GSH and GSSG uptake are maximal at a pH of less than 5.0. This indicates that the glutathione transport system is also different from this peptide transporter. The glutathione transporter(s) must therefore be considered as a specific system of the plasma membrane, adding to the amino acid and peptide uptake systems already described for mesophyll cells (Bush, 1993; Jamaï et al., 1994).

pH measurements and electrophysiological data show that GSH induces concentration-dependent proton influx in the tissues (Fig. 5) and concentration-dependent depolarization of the PD (Fig. 7), which demonstrate that GSH uptake occurs with proton co-transport. The same conclusion may be drawn from similar experiments with GSSG. However, the proton fluxes induced by GSSG are weaker and last longer than those induced by GSH at the same concentration (in Fig. 5, CF. A and B). The weak proton influx induced by GSSG is in contrast with its high rate of uptake and with the fact that it carries two net negative charges instead of one net negative charge for GSH. Also, GSSG uptake induces a PD depolarization that is only slightly stronger than that induced by GSH uptake (Fig. 6). The electrical data suggest that the charges carried by GSSG are neutralized in some way, and the pH measurements suggest that this neutralization does not primarily involve protons. Other cations such as K^+ may be involved in the neutralization process, as has been suggested for acidic amino acids (Kinraide and Etherton, 1980). The hyperpolarization that follows the initial depolarization induced by GSH or GSSH addition (Fig. 6) does not occur after the addition of amino acids (Felle et al., 1979; Golle and Lüttge, 1983; Felle, 1984; Kinraide et al., 1984; Mounoury et al., 1984; Pichelin et al., 1984; Petzold et al., 1989), but it has been reported after the addition of nitrate (McClure et al., 1990) and peptides (Jamaï et al., 1994). McClure et al. (1990) explained this hyperpolarization by the fact that the plasma membrane H^+-ATPase was stimulated as a result of the operation of a nitrate/H^+ symport.

The tissues do not re-acidify their incubation medium after GSSG addition, contrary to what is observed after GSH addition. This may be due to the inhibiting effect of GSSG on the plasma membrane H^+-ATPase (Qian and Murphy, 1993).

The occurrence of a glutathione transporter at the plasma membrane indicates that leaf cells are able to retrieve GSH transported by the phloem and the xylem. The mechanism of glutathione export to the apoplast from the cytoplasm of cells synthesizing this compound remains to be investigated. It may be argued that only the cells connected to the xylem would mediate uptake of xylem-sap glutathione, whereas only a few cells closely connected to the leaf phloem may mediate glutathione export. Thus, working with leaf discs and protoplasts may have the disadvantage that one studies a mixture of cells that are not all involved in glutathione transport. However, it must be remembered that in our system the rate of GSH and GSSG uptake in protoplasts was about 40 and 60%, respectively, of the rates measured in discs. Because the enzymatic digestion of the vein network is never complete, the protoplasts used in our experiments come mainly from the mesophyll tissue. The comparison between protoplasts and leaf discs therefore suggests that glutathione uptake is not restricted to the cells directly connected to conducting tissue. Furthermore, all of the PD measurements have been made with microelectrodes inserted into the bulk of the mesophyll, which also shows that mesophyll cells possess a glutathione transport system.

The glutathione transporter(s) described in the present experiments has three potential roles: (a) retrieval of glutathione transported by the phloem and the xylem, (b) recycling of GSSG oxidized in the apoplast, and (c) detoxification of GS conjugates synthesized in the apoplast. The occurrence of GSSG in the apoplast may be the result of the oxidizing conditions present in the cell wall and from the interaction between GSH and disulfides of membrane enzymes, including membrane transporters. The glutathione transporter would therefore retrieve GSSG for its reduction and recycling in the cytoplasm. In this respect, transport of

GSH/GSSG is similar to the transport of ascorbate/dehydroascorbate recently described in barley mesophyll protoplasts, since dehydroascorbate is preferentially taken up by these cells (Rautenkranz et al., 1994). The occurrence of glutathione conjugates in the apoplast may result either from a spontaneous reaction between GSH and electrophilic compounds, including some xenobiotics, or from an enzyme-mediated reaction if one of the glutathione transferase isozymes is present in the leaf apoplast.

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