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Vacuolar transport of the glutathione conjugate of *trans*-cinnamic acid

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Abstract

Red beet (*Beta vulgaris* L.) tonoplast membrane vesicles and [^{14}C]*trans*-cinnamic acid-glutathione were used to study the vacuolar transport of phenylpropanoid-glutathione conjugates which are formed in peroxidase-mediated reactions. It was determined that the uptake of [^{14}C]*trans*-cinnamic acid-glutathione into the tonoplast membrane vesicles was MgATP dependent and was 10-fold faster than the uptake of non-conjugated [^{14}C]*trans*-cinnamic acid. Uptake of the conjugate in the presence of MgATP was not dependent on a trans-tonoplast H^+-electrochemical gradient, because uptake was not affected by the addition of NH_4Cl (1 mM; 0% inhibition) and was only slightly affected by gramicidin-D (5 μM; 14% inhibition). Uptake of the conjugate was inhibited 92% by the addition of vanadate (1 mM) and 71% by the addition of the model substrate *S*-(2,4-dinitrophenyl) glutathione (500 μM). Uptake did not occur when a nonhydrolyzable analog of ATP was used in place of MgATP. The calculated K_m and V_{max} values for uptake were 142 μM and 5.95 nmol mg^{-1} min^{-1}, respectively. Based on these results, phenylpropanoid-glutathione conjugates formed in peroxidase-mediated reactions appear to be transported into the vacuole by the glutathione *S*-conjugate pump(s) located in the tonoplast membrane. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Beta vulgaris*; Chenopodiaceae; Red beet; Vacuolar transport; Glutathione *S*-conjugate (GS-X) pump; ATP-binding cassette (ABC) transporter; Peroxidase-mediated glutathione conjugation; Phenylpropanoids; *trans*-Cinnamic acid-glutathione

1. Introduction

Detoxification and metabolism of a variety of cytotoxins in cells typically involves four phases (Ishikawa, 1992; Ishikawa, Li, Lu & Rea, 1997; Kreuz, Tommasini & Martinoia, 1996). Phase I is an activation phase where enzymes such as mixed function oxidases and cytochrome P450-dependent monooxygenases introduce or expose reactive functional groups on the compound. In phase II, the activated compound is conjugated to a water-soluble molecule such as glutathione or glucose through the action of glutathione S-transferases (GST) and glucosyltransferases, respectively. These conjugates are then transported out of the cytoplasm in phase III. In phase IV, the transported conjugates are metabolically altered into transport-inactive derivatives.

In regards to phases II and III, the detoxification system involving glutathione (GSH) is probably one of the best-characterized in plant cells. A number of GST enzymes have been identified that will use various herbicides as substrates for GSH conjugation reactions (Frear & Swanson, 1970; Irzyk & Fuerst, 1993; Shimabukuro, Frear, Swanson & Walsh, 1971; Shimabukuro, Swanson & Walsh, 1970). These GSH conjugates are then recognized by the GSH S-conjugate (GS-X) pump(s) located in the tonoplast membrane and moved out of the cytoplasm and into the vacuole for further metabolism and/or storage (Gaillard, Dufaud, Tommasini, Kreuz, Amrhein & Martinoia, 1994; Li,

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Zhao & Rea, 1995; Martinoia, Grill, Tommasini, Kreuz & Amrhein, 1993). The GS-X pump(s) is a multi-specific transporter (i.e. it does not exclusively transport GSH conjugates) that is related to the subclass of multidrug resistance-associated proteins (MRP) of the ATP-binding cassette (ABC) transporter superfamily (Rea, Li, Lu, Drozdowicz & Martinoia, 1998). Because most prior work has focused on GST-mediated reactions involving herbicides or other synthetic compounds, it is these same GSH conjugates that have been used to demonstrate transport by the tonoplast GS-X pump(s). Very little is known about the GSH conjugation and vacuolar sequestration of natural products. In corn, the *Bz2* (*Bronze 2*) gene encodes a GST that catalyzes one of the last steps in cyanidin-3-glucoside (C3G) synthesis (Marrs, Alfenito, Lloyd, & Walbot, 1995). In the presence of BZ2, the purple C3G accumulates in the vacuole, however, in *bz2* mutants, C3G accumulates in the cytoplasm and is oxidized to a brown pigment. It has been speculated that this vacuolar sequestration occurs because BZ2 catalyzes the formation of a glutathione conjugate of C3G (C3G-GS) that is subsequently transported into the vacuole by the tonoplast GS-X pump(s) (Marrs et al., 1995). GSTs and the GS-X pump(s) are also thought to function in the glutathionation and vacuolar sequestration of the phytoalexin medicarpin (Li, Alfenito, Rea, Walbot & Dixon, 1997). The only other natural product that has been shown to be conjugated to GSH is *trans*-cinnamic acid (CA) and some of its derivatives (Diesperger & Sandermann, 1979; Dean, Devarenne, Lee & Orlofsky, 1995; Dean, Gronwald & Anderson, 1991; Dean & Machota, 1993; Edwards & Dixon, 1991; Edwards & Owen, 1987; Edwards & Owen, 1988).

Originally, GSH conjugation to CA was thought to be catalyzed by GSTs, however, it is now known that this reaction occurs as a result of peroxidase activity (Dean & Devarenne, 1997). The GSH conjugation reaction catalyzed by peroxidase enzymes is very different from the reaction catalyzed by GST enzymes. Whereas GST-mediated conjugation involves the formation of a thiolate anion (Armstrong, 1991), peroxidase-mediated conjugation involves the formation of thiyl radicals (Dean & Devarenne, 1997). These thiyl radicals are known to react very strongly with alkenes such as CA and other similar unsaturated phenylpropanoids (Oswald, Griesbaum, Thaler & Hudson, 1962). Though it is known that GSH conjugates formed in GST-mediated reactions are recognized and transported by the tonoplast GS-X pump(s), it has not been shown that GSH conjugates of phenylpropanoids formed in peroxidase-mediated reactions can also be transported by this mechanism. In this report, we demonstrate that GSH conjugates of phenylpropanoids formed in peroxidase-mediated reactions are moved across the tonoplast by a transporter(s) with characteristics identical to those described for the GS-X pump(s) that is known to transport GSH conjugates formed in GST-mediated reactions.

2. Results and discussion

The glutathione conjugate of [^{14}C]CA used in this investigation was synthesized in vitro in the presence of horseradish peroxidase (HRP) and 7-hydroxycoumarin (7-HC). The presence of 7-HC enhanced the rate of conjugate formation several fold, as described previously (Dean & Machota, 1993); the reaction mechanism of peroxidase-mediated conjugation also has been reported previously (Dean & Devarenne, 1997). During in vitro synthesis, up to 80% of the [^{14}C]CA is converted to [^{14}C]CA–GS with a purity greater than 90% as judged by TLC analysis. The ability to synthesize relatively pure [^{14}C]CA–GS in sufficient quantities in vitro provided a unique opportunity to characterize the tonoplast transport of a naturally occurring GSH conjugate.

The time and MgATP dependence of [^{14}C]CA–GS uptake by red beet (*Beta vulgaris*) tonoplast membrane vesicles is shown in Fig. 1. After 60 min, the uptake of CA–GS was 30-fold greater with MgATP present. Since uptake was nearly linear for 20 min, this time point was chosen for all subsequent assays.

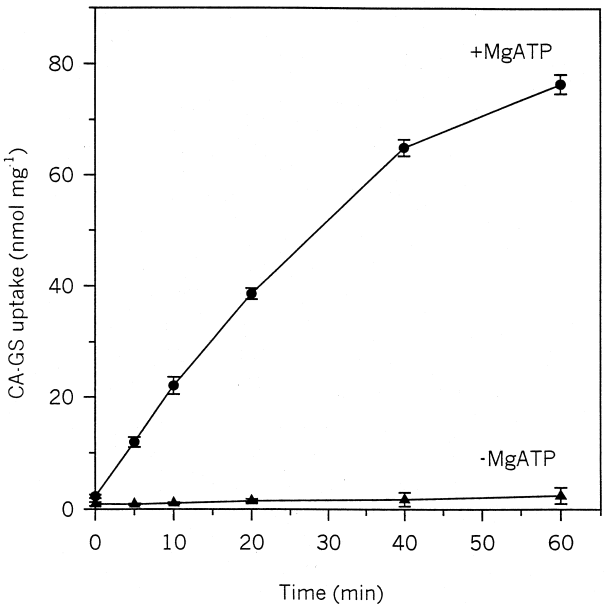

Fig. 1. Time course of [^{14}C]CA–GS uptake by red beet tonoplast vesicles. Uptake was measured using the procedure described by Li et al. (1995) in the presence (+MgATP) or absence (−MgATP) of 3 mM MgATP. The concentration of [^{14}C]CA–GS was 64 μM, and the specific activity was 5 μCi/μmol. Values shown are means ± SD ($n = 3$).

A double reciprocal plot of the uptake of [^{14}C]CA–GS as a function of [^{14}C]CA–GS concentration in the assay medium is shown in Fig. 2. Values for K_m and V_{max} were calculated to be 142 μM and 5.95 nmol mg^{-1} min^{-1}, respectively. Although the K_m value for uptake of CA–GS by red beet tonoplast vesicles is substantially higher than the K_m for uptake of medicarpin-GS (22 μM; Li et al., 1997) and C3G-GS (46 μM; Li et al., 1997) by mung bean (*Vigna radiata*) tonoplast vesicles, the V_{max} is approximately 50% greater. However, it must be noted that these kinetic comparisons describe tonoplast vesicles isolated from different species. Though tonoplast vesicles isolated from mung bean have frequently been used as a model system to measure transport of GSH conjugates, we used tonoplast vesicles prepared from red beet storage root because of the ease of obtaining the plant material and the high capacity for GSH conjugate transport exhibited by these tonoplast vesicles (Li et al., 1995).

Gramicidin-D and NH_4Cl are agents that act to dissipate H^+ gradients formed across membranes (Li et al., 1995). In our assays with tonoplast vesicles, an H^+-electrochemical gradient was established in the presence of MgATP by the vacuolar H^+-ATPase of the tonoplast membrane. The inclusion of gramicidin-D resulted in only a 14% decrease in the uptake of CA–GS compared to controls, and uptake in the presence of NH_4Cl was unaffected (Table 1). Therefore, the bulk of CA–GS uptake into red beet tonoplast vesicles does not require the generation of a transmembrane H^+-electrochemical potential difference.

Vacuolar uptake in the presence of magnesium and 5′-adenylylimidodiphosphate (AMP–PNP), a nonhydrolyzable analog of ATP, was only 3.9% of the uptake observed in the presence of MgATP (Table 1). In the presence of vanadate, uptake of CA–GS was only 7.6% of the transport observed in controls. Therefore, uptake of CA–GS by red beet tonoplast vesicles appears to be dependent on the hydrolysis of ATP and the formation of a phosphorylated transporter intermediate.

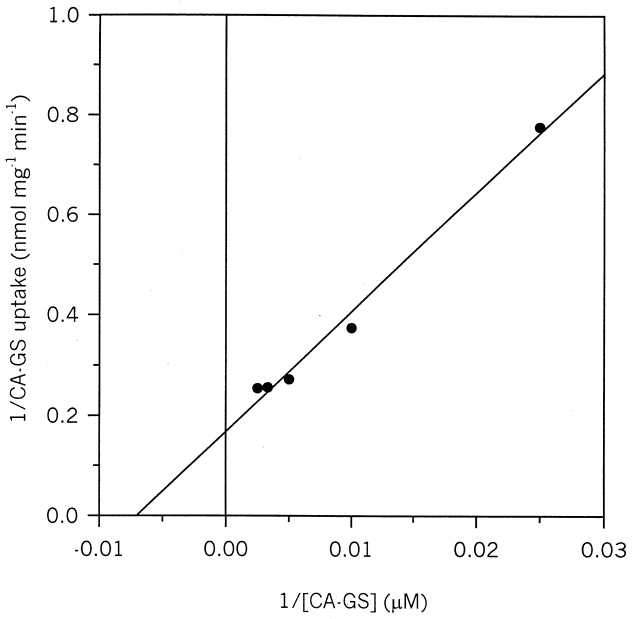

Fig. 2. Double-reciprocal plot of [^{14}C]CA–GS uptake versus [^{14}C]CA–GS concentration. Uptake was measured after 20 min using the procedure described by Li et al. (1995). The specific activity of [^{14}C]CA–GS was 5 μCi/μmol. Values shown are means ± SD ($n = 3$). Values for K_m and V_{max} were calculated to be 142 μM and 5.95 nmol mg^{-1} min^{-1}, respectively.

The MgATP-dependent uptake of CA–GS by tonoplast vesicles was 10-fold greater than the uptake of non-conjugated CA (Table 1). The uptake rates of non-conjugated CA were nearly equal in the presence or absence of MgATP.

Inhibition of CA–GS uptake by the GSH conjugate of 1-chloro-2,4-dinitrobenzene (DNP–GS) is shown in Fig. 3. The rate of CA–GS uptake decreased steadily as the concentration of DNP–GS in the assay medium increased. Inclusion of 500 μM DNP–GSH decreased the rate of uptake approximately 3.5-fold. To verify that inhibition was due to the inclusion of DNP–GS and not due to the individual components of the conjugate, the effects of GSH and 1-chloro-2,4-dinitroben-

Table 1
Uptake of [^{14}C]CA and [^{14}C]CA–GS in the presence or absence of MgATP and the effects of various compounds on the uptake of [^{14}C]CA–GS by red beet tonoplast vesicles

Substrate (64 μM)	Addition to assay medium	MgATP (3 mM)	Uptake[a] (nmol mg^{-1} min^{-1})	% of control[b]
[^{14}C]CA–GS	None	–	0.026 ± 0.02	1.9
[^{14}C]CA–GS	None	+	1.352 ± 0.305	100
[^{14}C]CA–GS	Gramicidin-D (5 μM)	+	1.159 ± 0.078	85.7
[^{14}C]CA–GS	NH_4Cl (1 mM)	+	1.372 ± 0.231	102
[^{14}C]CA–GS	AMP–PNP (3 mM)	–	0.052 ± 0.021	3.9
[^{14}C]CA–GS	Vanadate (1 mM)	+	0.103 ± 0.017	7.6
[^{14}C]CA	None	–	0.114 ± 0.002	8.4
[^{14}C]CA	None	+	0.135 ± 0.021	10

[a] Values shown are mean ± SD ($n = 3$).
[b] Uptake of [^{14}C]CA–GS in the presence of 3mM MgATP without other additions served as the control.

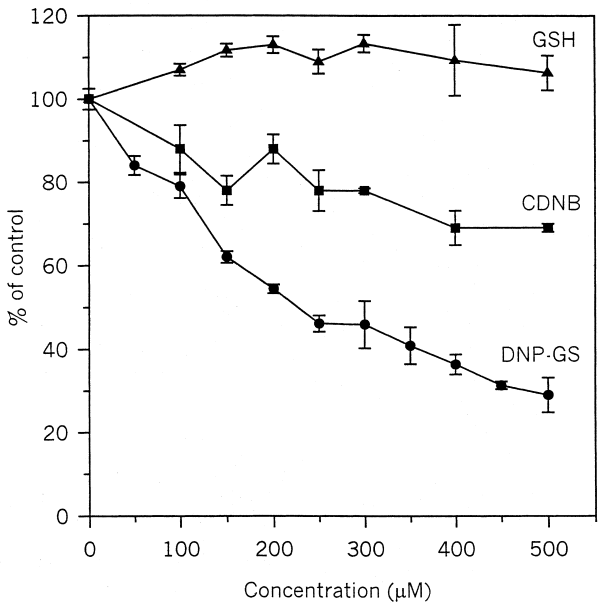

Fig. 3. Effects of DNP–GS, CDNB, and GSH on [^{14}C]CA–GS uptake by red beet tonoplast vesicles. The control value for uptake of [^{14}C]CA–GS (64 μM; sp. act. 5 μCi/μmol) was 0.785 ± 0.029 nmol mg^{-1} min^{-1} and was determined in the presence of MgATP (3 mM) using the procedure described by Li et al. (1995). DNP–GS, CDNB, and GSH were added to the uptake assay medium at the concentrations indicated. Values shown are means $\pm$ SD ($n = 3$).

zene (CDNB) on the rate of uptake were also examined. CA–GS uptake in the presence of 500 μM CDNB or 500 μM GSH was 69 and 106%, respectively, of the uptake in the absence of these compounds. Though the inhibition observed by non-conjugated CDNB was of some consequence, it was not as great as the inhibition observed in the presence of DNP–GS. Because CDNB is a lipophilic electrophile, it is possible that its inhibitory action reflects nonspecific perturbations of either the membrane vesicle or the transporter(s). Conjugation of GSH to CDNB would change the chemical nature of this compound and could alleviate its ability to cause these nonspecific disruptions. We propose that inhibition by DNP–GS is more likely to be due to competition between CA–GS and DNP–GS for the binding site of the GS-X pump(s) rather than by non-specific perturbations. A more detailed kinetic analysis might be able to identify the mechanism of transport inhibition by CDNB and DNP–GS.

In summary, transport of [^{14}C]CA–GS by red beet tonoplast vesicles has the following characteristics: 1) transport requires MgATP; 2) transport is directly energized by MgATP and does not require an H^+-electrochemical potential difference across the membrane; 3) Nonhydrolyzable ATP analogs such as AMP–PNP do not support transport; 4) transport is strongly inhibited by vanadate; 5) transport of CA–GS in the presence of MgATP is significantly greater than the transport of non-conjugated CA; 6) transport is inhibited by DNP–GS, but not by GSH; and 7) CA–GS is not chemically altered during transport (data not shown). Therefore, the CA–GS conjugate synthesized in a peroxidase-mediated reaction appears to be transported across tonoplast membranes by GS-X pumps that have the same characteristics as those described for the transport of GSH conjugates synthesized in GST-mediated reactions (Li et al., 1995; Martinoia et al., 1993).

Medicarpin and CA are the only two naturally occurring plant compounds that have been shown to be conjugated to GSH in vitro. Although GSTs play a role in the vacuolar sequestration of the anthocyanin C3G, it has been difficult to observe the in vitro formation of a C3G–GS conjugate by the *Bz2*- or *An9*- (a functional analog of *Bz2*) encoded GST (Mueller, Silady & Walbot, 1999). Recently, it has been suggested that BZ2 may bind to C3G rather than catalyze enzymatic conjugation (Mueller et al., 1999). If this is the case, then the mechanism of C3G transport into the vacuole by the GS-X pump still needs to be more clearly defined. Though GSH conjugation of medicarpin and transport of the medicarpin–glutathione conjugate have been demonstrated in vitro (Li et al., 1997), the in vivo formation of this conjugate has not been observed. In regards to CA, peroxidase-mediated GSH conjugation has been demonstrated in vitro, the GS–CA conjugate has been reported to be synthesized in vivo by soybean (*Glycine max*) and chickpea (*Cicer arietinum*) cell suspension cultures (Barz & Mackenbrock, 1994; Diesperger & Sandermann, 1979), and in this report we demonstrate that a transporter(s) with characteristics identical to those described for the GS-X pump(s) can transport CA–GS. CA and CA–GS might therefore serve as model substrates for future investigations on GSH conjugation and vacuolar sequestration of natural products.

In addition to CA, peroxidase enzymes are also able to catalyze GSH conjugation to a variety of other phenylpropanoids including *p*-, *m*-, and *o*-coumaric acid, ferulic acid, and coniferyl alcohol (Dean et al., 1995). We have also demonstrated in preliminary experiments that the GSH conjugate of *p*-coumaric acid can be moved across the tonoplast by a transporter(s) with characteristics identical to those described for the GS-X pump(s) (data not shown). Therefore, it is possible to speculate that peroxidase-mediated GSH conjugation and subsequent vacuolar sequestration are important phase II and III detoxification reactions of phenylpropanoids. These reactions might be important in the detoxification of phenylpropanoids encountered exogenously in the form of allelochemicals (Baziramakenga, Leroux & Simard, 1995; Li et al., 1993) or produced endogenously as pathogen-elicited compounds (Loake, Faktor, Lamb & Dixon, 1992).

It is not known if peroxidase-mediated GSH conjugation reactions extend beyond the simple phenylpropanoids. However, it seems likely, since plant cells produce a wide variety of compounds that contain alkenes that could be conjugated to GSH in these types of reactions (Van Sumere, 1989). It is also not known to what extent secondary compounds are conjugated to GSH in GST-mediated reactions. However, it does appear as if GSH conjugates formed by both GST enzymes and peroxidase enzymes are recognized and transported by the tonoplast GS-X pump(s). The presence of both types of GSH conjugating enzymes in plant cells may increase the range of chemical structures that could be conjugated to GSH and transported into the vacuole for sequestration.

3. Experimental

3.1. Preparation of vacuolar membrane vesicles

Red beet (*Beta vulgaris* L.) storage roots were purchased at a local market, and the vacuolar membrane vesicles were purified as described by Li et al. (1995). All vacuolar membrane preparations that were not used immediately were frozen in liquid nitrogen and stored at −70°C.

3.2. Synthesis of [^{14}C]CA and [^{14}C]CA–GS

[^{14}C]CA was synthesized from L-[U-^{14}C]phenylnylalanine as described by Dean et al. (1995). The [^{14}C]CA–GS was synthesized by incubating 1.2 mM [^{14}C]CA (5.0 μCi/μmol), with 108 mM GSH, 0.173 mM 7-HC, and 0.56 ml (23 μg/ml) HRP type VI (Sigma, St. Louis, MO) in a final volume of 1.44 ml. Conjugation proceeded for 4 h at 30°C, and the reaction was stopped by the addition of 0.16 ml of 32.5% (w/v) TCA. Non-conjugated [^{14}C]CA was removed from the aqueous phase by extraction with 3.2 ml CH_2Cl_2. The two phases were separated by centrifugation and the aqueous phase containing the [^{14}C]CA–GS was collected and loaded onto a C_{18} cartridge (Millipore, Bedford, MA). The cartridge was washed with 3 ml H_2O:HOAc (99:1, v/v), and the [^{14}C]CA–GS was eluted from the cartridge with 5 ml of H_2O:CH_3CN:HOAc (49.5:49.5: 1, v/v/v) into 1 ml fractions. Fractions containing the greatest amount of radioactivity were pooled and evaporated to dryness in vacuo. The resulting residue was dissolved in water and purified by TLC on silica gel plates (LK6, Marksson, Hillsboro, OR) developed in *n*-BuOH:HOAc:H_2O (2:1:1, v/v/v). Radioactivity on the TLC plates was visualized with an imaging scanner (Bioscan, Washington, DC). The conjugate was eluted from the TLC plates with water, concentrated to dryness in vacuo, redissolved in water and either used immediately or stored at −20°C.

3.3. Synthesis of DNP–GS

DNP–GS was synthesized enzymatically from GSH and CDNB by a modification of the procedure of Kunst, Sies & Akerboom (1989) and Li et al. (1995). The reaction mixture consisted of 50 μmol of GSH, 50 μmol of CDNB, 5 units of equine GST (Sigma, St. Louis, MO), and 25 mM Tris-MES (pH 6.5) in a final volume of 5 ml. Conjugation was allowed to proceed for 1 h at room temperature after which the reaction was stopped with 0.56 ml of 32.5% (w/v) TCA. Non-conjugated CDNB was removed from the aqueous phase by extraction with 10 ml of ether. After centrifugation to separate the phases, the ether was removed by aspiration. The aqueous fraction was applied to C_{18} cartridges and eluted as described in Section 3.2. The fractions containing the yellow DNP–GS were concentrated to dryness in vacuo and redissolved in 0.2 M Tris (pH 8). The DNP–GS conjugated was purified by TLC as described in Section 3.2. The yellow DNP–GS band was eluted from the TLC plates with 0.2 M Tris (pH 8), evaporated to dryness in vacuo, and redissolved in 0.2 M Tris (pH 8). The concentration of DNP–GS was determined spectrophotometrically at 340 nm using $\epsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$ (Habig, Pabst & Jakoby, 1974).

3.4. Measurement of [^{14}C]CA–GS uptake

Uptake of [^{14}C]CA–GS was measured using the procedure of Li et al. (1995).

3.5. Protein

The protein concentration was estimated by the method of Bradford (1976) using BSA as a protein standard.

Acknowledgements

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