Transport of Oxidized Glutathione into Barley Vacuoles: Evidence for the Involvement of the Glutathione-S-Conjugate ATPase

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Z. Naturforsch. **48c,** 867–871 (1993); received August 23, 1993

Glutathione, Transport, Vacuole

Glutathione in its oxidized form (GSSG) is rapidly taken up into isolated barley vacuoles, while uptake of reduced glutathione (GSH) is only marginal. GSSG transport is strictly ATP-dependent and is a saturable process (K_m 0.4 to 0.6 mM). Uptake is inhibited by vanadate, but not by bafilomycin, a specific inhibitor of the vacuolar H^+-ATPase. The observation that i) the non-hydrolysable ATP analogue adenylylimidodiphosphate does not stimulate, but rather inhibits the ATP-dependent uptake of GSSG and ii) that a four-fold accumulation of GSSG is observed in the presence of bafilomycin, suggests that GSSG is transported into the vacuole by a primary active process similar to that of glutathione S-conjugates (E. Martinoia, E. Grill, R. Tommasini, K. Kreuz, N. Amrhein, Nature **364,** 247–249 (1993)). Uptake of GSSG is competitively inhibited by the glutathione S-conjugate of the herbicide metolachlor (K_i 80 μM), indicating that transport of GSSG is mediated by the glutathione S-conjugate ATPase.

Introduction

Glutathione is among the dominant organic sulfur containing metabolites of higher plants [1] and plays a central role in maintaining the redox potential of the cell. For the functioning of many enzymes a reduced environment is required. The ratio of reduced to oxidized glutathione is high in the cytosol [2, 3]. It has been shown that in the endoplasmic reticulum of animals the redox state of the ER lumen is more oxidized [3]. Correct folding of proteins in the ER occurs only under balanced concentrations of thiols and disulfides [3, 4]. The more oxidized environment is maintained through the activity of the GSH/GSSG transporter which transports GSSG more efficiently than GSH [3]. A more oxidized environment has also been reported for plant vacuoles [2]. In this case the functional significance of the more oxidized redox state is not clear. However, as reported for the ER, the rate of GSSG transport was found to be several fold higher than that of GSH.

Another important function of glutathione is its role in the detoxification of xenobiotics. Potentially toxic substances may be detoxified by conjugation to glutathione in a reaction catalyzed by glutathione S-transferases. The conjugates of xenobiotics are subsequently stored in the vacuole [5]. While the energized uptake of solutes is generally thought to be a secondary active process [6] it has recently been shown that the uptake of the glutathione S-conjugates into vacuoles is a transport process directly energized by ATP [7]. This ATPase exhibits many similarities to the glutathione-S-conjugate ATPase of animal tissues [8–11]. In the light of the discovery of the glutathione S-conjugate ATPase in the vacuolar membrane we wondered, if the uptake of GSSG and GS-conjugates is driven by the same ATPase as it is in liver [8–11], or whether it is mediated by separate transport systems, as described for the low affinity GS-conjugate transport ATPase of erythrocytes [12, 13]. GSSG but not GSH have previously been found to be rapidly taken up by isolated vacuoles in the presence of ATP [2]. However, neither the energetics nor the kinetics of this transport process were investigated.

In this report we provide evidence that, similar to the glutathione-S-conjugate ATPase of the canalicular membrane of liver, transport of GSSG and glutathione-S-conjugates across the vacuolar membrane is mediated by the same transport system.

Abbreviations: AMPPNP, adenylylimidodiphosphate; GSH, glutathione; GSSG, oxidized glutathione; NEM-GS, glutathione S-conjugate of N-ethylmaleimide.

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Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/1100–0867 $ 01.30/0

Materials and Methods

Barley (*Hordeum vulgare* L. *cv.* Baraka) was grown in a growth cabinet with 12 h fluorescent light (45 μmol × m^{-2} s^{-1}) at 22 °C and 12 h dark at 18 °C; relative humidity was 75%. Protoplasts and vacuoles were isolated as described previously [14]. Contamination of the vacuolar preparation with other cell constituents was less than 1% as measured by marker-enzyme activities [14]. Uptake of [^{35}S]-GSSG was measured as described by [7]. Unless indicated otherwise, for each condition and time point, polyethylene microcentrifugation tubes (400 μl capacity) were prepared as follows: 70 μl of basal medium (0.4 M sorbitol, 30 mM K gluconate, 25 mM Hepes KOH pH 7.2) containing 28% (v/v) Percoll, 0.12% bovine serum albumin (BSA), and either 0.5 kBq [^{35}S]-GSH or [^{35}S]-GSSG, as well as 1 kBq ^3H_2O, and further constituents as indicated in the figures and tables were placed in the bottom of the tubes. Uptake was started by the addition of 30 μl of vacuolar suspension. The samples were rapidly overlayered with 200 μl of silicone oil AR 200 (Fluka, Buchs, Switzerland) and 60 μl of water. The incubation was terminated by centrifugation at 10000 × *g* for 15 s. ^3H_2O equilibrates rapidly between the medium and the vacuolar space and can be used to quantify the number of vacuoles. 10^7 vacuoles correspond to a volume of 160 μl and approximately 1 mg chlorophyll in barley mesophyll cells. [^{35}S]-GSSG was synthesized *in vivo* by growing *Saccharomyces cerevisiae* in the presence of [^{35}]-SO_4^{2-}. The yeast (wild type strain Portland) was cultivated at 30 °C in a minimal medium [15] in which the $MgSO_4$ level was reduced to 0.05 mM. Yeast cells of an overnight culture were collected by centrifugation (2000 × *g*, 5 min), washed in sulfate-free minimal medium and resuspended in the medium at a concentration of OD_{600} 15. The cell suspension (0.5 ml) was incubated with 37 MBq labelled sulfate (specific activity 370 MBq/μmol) and 10 mM glycine at 30 °C for 90 min on a gyratory shaker (150 rpm/min). The cells were sedimented (4000 × *g*, 1 min), washed twice with 0.5% glucose, pelleted again and finally resuspended in 50% ethanol (0.1 ml). The cell suspension was frozen in liquid nitrogen and then kept in a 70 °C waterbath for 3 min. After this process had been repeated twice, the clear supernatant (13000 × *g*, 5 min) was diluted to 1 ml with 10 mM acetic acid, and 70 nmol of unlabelled glutathione was added. The extract was chromatographed on an ion exchange column (50 μl Dowex-1) as described [16]. The final yield of the purified glutathione was 98 nmol with a radioactivity of 11.8 MBq (32%). Contamination of the preparation with sulfur-labelled cysteine was less than 1 percent as judged by HPLC analysis and monitoring the β-radiation [17].

Results

As shown by Dietz *et al.* [2], uptake of glutathione by isolated vacuoles can only be detected in the presence of MgATP. Uptake was linear for at least 30 min (see also [2]) and was much faster for GSSG than for GSH (Table I, [2]). In the absence of DTT, apparent GSH uptake was slightly enhanced presumably due to the oxidation of GSH to GSSG. The ATP-dependent transport is not inhibited by bafilomycin (Table II), a very specific inhibitor of the vacuolar H^+-ATPase [18] which, along with the vacuolar PPase, drives the secondary active transport across the tonoplast. In contrast, addition of vanadate reduced the uptake rates of GSSG almost to control levels. Vanadate is known to inhibit the plasmamembrane ATPase, which forms a phosphorylated intermediate during ATP hydrolysis, as a transition state analogue [19]. The non-hydrolysable ATP analogue adenylylimidodiphosphate (AMPPNP) is not able to stimulate the uptake of oxidized glutathione. On the other hand, AMPPNP inhibited the ATP-stimulated GSSG uptake. This analogue must be therefore able to bind to the ATP binding site of

Table I. ATP-dependent uptake of GSH and GSSG into barley vacuoles. Vacuoles were incubated in the presence of [^{35}S]-GSH and [^{35}S]-GSSG (0.4 mM) in the absence and in the presence of 3 mM MgATP. GSH uptake was determined in the presence of 3 mM DTT. Uptake rates were calculated by subtracting the radioactivity taken up after 2 min from the values measured after 20 min. The values are means of two experiments, each with five replicates. Standard deviation within one experiment was less than ± 8%.

Species	−ATP	+ATP
	nmol × $(10^{-7}$ vacuoles$)^{-1}$ × min^{-1}	
GSH	0.12	0.54
GSSG	0.16	4.80

Table II. Effect of inhibitors on the uptake of GSSG into barley vacuoles. Isolated barley vacuoles were incubated in the presence of 0.4 mM [^{35}S]-GSSG and the compounds listed in the table. Uptake rates were calculated by subtracting the radioactivity taken up after 2 min from the values measured after 20 min. Means of two to three experiments (*n*), each with five replicates. 100% values ranged from 3.1 to 5.6 nmol × 10^{-7} vacuoles × min^{-1}.

Treatment	Transport (% of control)	(*n*)
no ATP added	3 ± 2	
ATP (3 mM)	100	
ATP (3 mM) + bafilomycin (0.1 μM)	101 ± 5	(3)
ATP (3 mM) + vanadate (1 mM)	14 ± 4	(3)
ATP (3 mM) + NH_4Cl (5 mM)	103	(2)
AMPPNP (3 mM)	4	(2)
ATP (1 mM)	101	(2)
ATP (1 mM) + AMPPNP (2 mM)	36	(2)

the carrier. Dissipation of the ΔpH by NH_4Cl (Table II) had no effect on ATP-dependent GSSG transport.This demonstrated independence of the transtonoplast proton gradient provides good evidence that the transport is indeed directly energized by ATP and not mediated by an ATP-stimulated antiport system which exchanges GSSG for protons. At an external concentration of 100 μM GSSG, a more than four-fold accumulation of the compound within the vacuoles was observed after 40 min, both in the presence and absence of bafilomycin (data not shown). The ATP-dependent GSSG transport is a saturable process (Fig. 1), with an apparent K_m of 0.4 to 0.6 mM. GSSG uptake was competitively inhibited by the GS-conjugate of metolachlor, an acetamide herbicide, with a K_i of approximately 70 μM (Fig. 2a). A similar K_i (75 μM) was determined for the inhibition of the uptake of NEM-GS by metolachlor-GS (Fig. 2b).

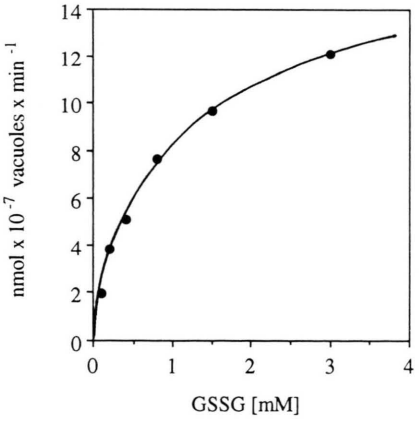

Fig. 1. [^{35}S]-GSSG uptake into isolated barley mesophyll vacuoles as a function of its concentration. Vacuoles were incubated in the presence of 3 mM MgATP and 18.5 kBq × ml^{-1} [^{35}S]-GSSG at the indicated GSSG concentrations. Uptake rates were calculated by subtracting the radioactivity taken up after 2 min from the values measured after 20 min. Each point represents the mean of five replicates. Standard deviation was not more than ± 8%.

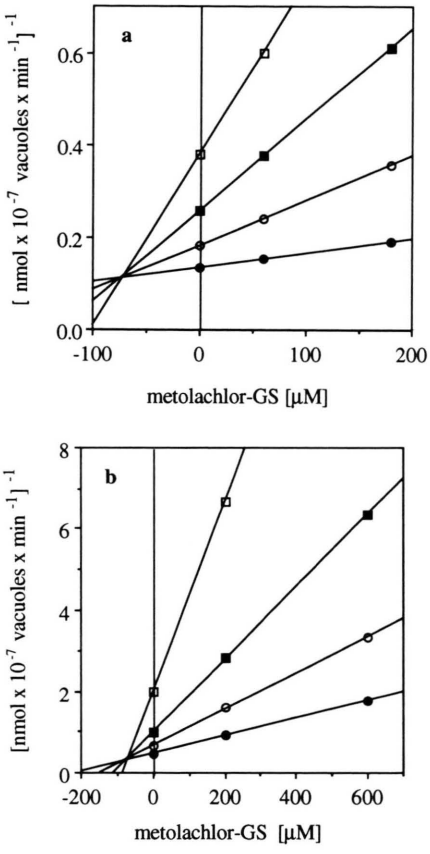

Fig. 2. Competitive inhibition of the uptake of GSSG (a) and of NEM-GS (b) by metolachlor-GS. Isolated vacuoles were incubated in the presence of [^{35}S]-GSSG (0.2 mM (●), 0.4 mM (○), 0.6 mM (■), 1.0 mM (□)) or [^{14}C]-NEM-GS (0.1 mM (●), 0.25 mM (○), 0.5 mM (■), 1.0 (□)) in the absence or presence of metolachlor (60 and 180 μM for GSSG and 200 and 600 μM for NEM-GS respectively). The K_i determined from the respective Dixon plot is 70 μM for the inhibition of GSSG and 78 μM for the inhibition of NEM-GS. Each point represents the mean of five replicates. Uptake rates were calculated by substracting the radioactivity taken up after 2 min from the values measured after 20 min.

Discussion

ATP-dependent uptake of solutes into plant vacuoles has generally been thought to be mediated by secondary active transport processes [6]. ATP-dependent, but not energy requiring transport of amino acids has also been reported [20, 21]. Very recently, direct energization of the transport of glutathione S-conjugates [7] and of bile acids [22] into plant vacuoles has been observed. These ATPases exhibit many similarities to the glutathione S-conjugate- and the bile acid-ATPases described for the canalicular membrane of liver [8–12]. Whereas in liver, GSSG and GS-conjugates are thought to be excreted by the same ATPase, for erythrocyte membranes two distinct ATPases have been postulated. One with a high affinity is thought to transport both, GS-conjugates as well as GSSG, while a low affinity system does not accept GSSG as substrate [12, 13]. As shown above, in plant vacuoles the energetics of GSSG transport are similar to those described for glutathione S-conjugates and taurocholate, suggesting that GSSG, too, is taken up into vacuoles by a direct energization. Uptake is not inhibited by bafilomycin, a very specific inhibitor of the tonoplast ATPase [18], and accumulation of GSSG within the vacuoles could be observed within the vacuoles even in the presence of this inhibitor. The results obtained with the non-hydrolysable ATP analogue AMPPNP suggest that hydrolysis of ATP is required to drive GSSG transport. This hypothesis is also supported by the inhibition observed in the presence of vanadate. Since NH_4Cl, which dissipates the ΔpH between the incubation medium and the vacuole, has no effect on GSSG transport, an ATP activated antiport mechanism can also be ruled out. The apparent K_m observed for GSSG uptake is 0.4 to 0.6 mM. This K_m corresponds approximately to the apparent K_m observed for the glutathione S-conjugate NEM-glutathione [7]. However, the maximal rate of uptake of GSSG is three to four-fold higher than that observed for NEM-GS. Metolachlor-GS has an approximately ten-fold higher affinity (K_m 60 μM), and a similar V_{max} as observed for NEM-GS. Since metolachlor-GS inhibits the transport of NEM-GS and of GSSG with the same K_i, we must assume that the uptake of GSSG and glutathione-conjugates into vacuoles is mediated by the same ATPase, as appears to be the case in the canalicular membrane of liver.

It has been shown that in the cytosol glutathione is present mainly in the reduced form. In the vacuole, the total glutathione concentration is about 50 times lower than in the cytosol, and the GSH : GSSG ratio reported is close to units [2]. In the endoplasmic reticulum of animal cells, the ratio of GSH to GSSG is shifted towards the oxidized form as compared to the cytosol, and a less reducing environment may be important for protein folding [4]. For plant vacuoles this argument is invalid, but the possibility must be considered that the vacuole is involved in the maintenance of a reduced environment in the cytosol. Nothing is known about the metabolism of glutathione in the vacuole. Different possibilities can be envisaged: i) GSSG taken up by vacuoles is irreversibly deposited in this compartment as GSSG. This possibility is less likely since no accumulation of GSSG was observed in the vacuoles under normal growth conditions [2]; ii) GSSG is degraded by peptidases within the vacuoles; iii) GSSG may be reduced in the vacuole and released to the cytosol as GSH. However, such a reducing activity has not yet been demonstrated in vacuoles. It will therefore be an interesting task to investigate the fate of GSSG in vacuoles.

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