γ-Glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae* and its role in the vacuolar transport and metabolism of glutathione

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In the yeast *Saccharomyces cerevisiae*, the enzyme γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2) is a glycoprotein that is bound to the vacuolar membrane. The kinetic parameters of GSH transport into isolated vacuoles were measured using intact vacuoles isolated from the wild-type yeast strain Σ1278b, under conditions of γ-GT synthesis (nitrogen starvation) and repression (growth in the presence of ammonium ions). Vacuoles devoid of γ-GT displayed a K_m (app) of 18 ± 2 mM and a V_{max} (app) of 48.5 ± 5 nmol of GSH/min per mg of protein. Vacuoles containing γ-GT displayed practically the same K_m, but a higher V_{max} (app) (150 ± 12 nmol of GSH/min per mg of protein). Vacuoles prepared from a disruptant lacking γ-GT showed no increase in V_{max} (app) with nitrogen starvation. From a comparison of the transport data obtained for vacuoles isolated from various reference and mutant strains, it appears that the yeast cadmium factor 1 (YCF1) transport system accounts for approx. 70% of the GSH transport capacity of the vacuoles, the remaining 30% being due to a vacuolar (H^+) ATPase-coupled system. The V_{max} (app)-increasing effect of γ-GT concerns only the YCF1 system. γ-GT in the vacuolar membrane activates the Ycf1p transporter, either directly or indirectly. Moreover, GSH accumulating in the vacuolar space may exert a feedback effect on its own entry. Excretion of glutamate from radiolabelled GSH in isolated vacuoles containing γ-GT was also measured. It is proposed that γ-GT and a L-Cys-Gly dipeptidase catalyse the complete hydrolysis of GSH stored in the central vacuole of the yeast cell, prior to release of its constitutive amino acids L-glutamate, L-cysteine and glycine into the cytoplasm. Yeast appears to be a useful model for studying γ-GT physiology and GSH metabolism.

Key words: enzymes, fungi, metabolism, peptide thiol, tonoplast.

INTRODUCTION

In micro-organisms, plants and animals GSH is associated with different cellular functions, including sulphur metabolism, regulation of enzyme activity and responses to various stresses. It is notably involved in the detoxification of xenobiotics and toxic metabolic by-products. [1–4]. γ-Glutamyl transpeptidase (γ-GT; EC 2.3.2.2) catalyses the transfer of the γ-glutamyl moiety of GSH and other γ-glutamyl compounds to amino acids and peptides. It also catalyses the hydrolytic release of L-glutamate from GSH, various γ-glutamyl compounds and S-substituted derivatives [5]. In animal and plant cells, γ-GT plays a central role in the γ-glutamyl cycle of GSH synthesis and degradation [4,5]. Whether a complete γ-glutamyl cycle exists in micro-organisms is questionable [2,6]. In *Saccharomyces cerevisiae*, for instance, only a truncated version has been found, consisting of the biosynthetic enzymes γ-GT and L-Cys-Gly dipeptidase (CGase; EC 3.4.13.6) [6]. Furthermore, γ-GT synthesis in *S. cerevisiae* is regulated by the nitrogen source: ammonium ions cause repression and nitrogen starvation leads to de-repression [7].

In *S. cerevisiae*, γ-GT and CGase are vacuolar-membrane-bound enzymes [8]. It has been suggested that γ-GT might play a role in amino-acid transport in yeast [8], but this has been ruled out as far as bulk transport of amino acids in *S. cerevisiae* is concerned [9–11]. Nevertheless, a role in the vacuolar transport of GSH and related derivatives is not excluded [8].

GSH is stored in the central vacuole of yeast through the action of the yeast cadmium factor 1 (YCF1) transporter system [12]. Its degradation may supply amino acids for cell growth and maintenance during nitrogen or sulphur starvation [13,14]. It has been proposed that γ-GT and CGase could be the enzymes responsible for vacuolar GSH metabolism [14]. In the present study we show that in the yeast *S. cerevisiae*, γ-GT does play a role in the vacuolar transport and metabolism of GSH.

EXPERIMENTAL

Materials

Unless otherwise stated all of the reagents were purchased from Fluka or Sigma–Aldrich. Products for yeast growth media were obtained from Difco or Gibco.

Yeast strains and growth

The *S. cerevisiae* strains used in the present study are listed in Table 1. Strain KMY097 is a haploid segregant with a disrupted *CIS2* gene (synonym of *ECM38*), reported to code for a yeast homologue of human γ-GT [15]. Confirmation that *CIS2* is the gene encoding yeast γ-GT is provided in the Results section (activity measurements in Table 2). Strain KMY097 was created by replacing the open reading frame (ORF) of the *CIS2* gene in diploid Σ1278b with a disruption cassette containing *kanMX2* as a selectable marker. PCR synthesis of the disruption cassette with long flanking regions homologous to *CIS2* was performed according to the long-flanking-homology-PCR procedure developed by Wach et al. [16]. The procedure is fully described elsewhere [17]. *S. cerevisiae* was routinely maintained on YPD

Abbreviations used: ALP, alkaline phosphatase; Blast, basic local alignment search tool; CGase, L-Cys-Gly dipeptidase; γ-GT, γ-glutamyl transpeptidase; ORF, open reading frame; SBM, standard basal medium; V-ATPase, vacuolar (H^+) ATPase; YCF1, yeast cadmium factor 1.

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Table 1 Strains used in the present study

Strain of *S. cerevisiae*	Genotype	Source
Σ1278b	Wild-type (Mat α)	Our laboratory
KMY097	γ-GT disruptant (Mat α Δcis2) derived from Σ1278b	Our laboratory
DT165	Mat α, leu2-3, his3-Δ200,ura3-52,trp1-Δ901,lys2-Δ801,suc2-Δ	Dr Szczypka*
DT167	Mat α, leu2-3, his3-Δ200,ura3-52,trp1-Δ901,lys2-Δ801,suc2-Δ,ycf1::hisG	Dr Szczypka*
W303-1B	Mat α, leu2, his3, ade2, trp1, ura3	Dr Gadd†
ScVatB	Mat α, leu2, his3, ade2, trp1, ura3, Δvma2	Dr Gadd†

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Table 2 Kinetic parameters for GSH transport into native vacuoles prepared from reference and mutant strains of *S. cerevisiae*

See Table 1 for strain characteristics. Vacuoles were prepared from the cells grown as described in the Experimental section. nd, below limit of detection.

Strain	Growth conditions	Specific activity of γ-GT in the vacuoles (units/mg of protein)	V_{max}(app) (nmol of GSH/min per mg of protein)	K_m (app) (mM)
Σ1278b	SBM + nitrogen	nd	48.5 ± 5.1	18 ± 2.0
	Nitrogen starvation	39.2 ± 2.8	150 ± 12.3	18.2 ± 2.2
KMY097	SBM + nitrogen	nd	47.7 ± 6.2	17.9 ± 3.4
	Nitrogen starvation	nd	48.7 ± 3.4	18.4 ± 2.6
DT165	SBM + nitrogen	nd	49.2 ± 7.0	17.8 ± 2.4
	Nitrogen starvation	38.0 ± 3.7	162.3 ± 19.4	18.7 ± 1.9
DT167	SBM + nitrogen	nd	14.7 ± 2.4	18.3 ± 2.7
	Nitrogen starvation	40.1 ± 2.5	13.9 ± 1.5	18.8 ± 3.6
W303-1B	SBM + nitrogen	nd	48.7 ± 4.5	17.4 ± 2.2
	Nitrogen starvation	39.5 ± 3.8	156 ± 17.0	17.9 ± 1.7
ScVatB	SBM + nitrogen	nd	33.9 ± 3.5	18.4 ± 2.6
	Nitrogen starvation	38.2 ± 2.8	132.9 ± 9.3	17.6 ± 2.0

medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose], and grown in standard basal medium (SBM) supplemented with appropriate amounts of amino acids and nucleic bases and with 10 mM $(NH_4)_2SO_4$ as the nitrogen source. SBM is a mineral medium containing 3 % (w/v) glucose as the sole carbon source, supplemented with vitamins and trace minerals [14]. In experiments using nitrogen-starved cells, yeast cells pregrown on SBM and $(NH_4)_2SO_4$ were centrifuged at 28 °C, washed once with SBM, resuspended in fresh SBM and incubated at 29 °C for 4 h. The medium was solidified with 2 % (w/v) agar.

Unless otherwise stated, *S. cerevisiae* was grown at 29 °C with orbital shaking at 150 rev./min.

DNA sequence comparisons

Blast (basic local alignment search tool) sequence-alignment software (version 2.06) was used to determine identity between gene sequences [18]. A hydropathy plot procedure was used to find potential transmembrane domains [19].

Enzyme, GSH and protein assays

γ-GT was assayed as described previously [7]. α-Mannosidase, alkaline phosphatase (ALP), glucose-6-phosphate dehydrogenase, protease B, citrate synthase and CGase were assayed as described by Jaspers et al. [6], and Jaspers and Penninckx [8]. Enzyme units are expressed in nmol of product/min. Specific activities are expressed in units/mg of protein. Protein was measured according to the method of Lowry, using BSA as a standard. GSH was estimated according to Elskens et al. [13].

Isolation and characterization of vacuoles

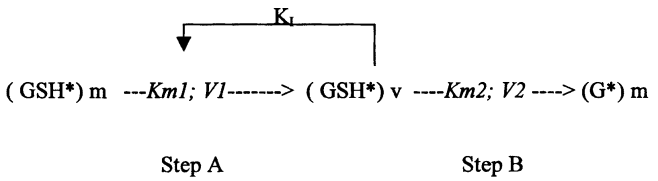
S. cerevisiae strains were grown to an attenuance (D_{660}) of 0.6–0.9. Native vacuoles were isolated by combining the procedures described by Jaspers and Penninckx [8], and Yoshihida and Anraku [20]. Unless otherwise stated, all operations were performed at 4 °C. Yeast cells (1 g wet weight of cells) were washed twice with distilled water and resuspended in 3.5 vol. of 0.1 M Tris/HCl buffer (pH 8.0) containing 0.5 mM mercaptoethanol and 0.1 M sodium EDTA. After incubation for 30 min with slow shaking, the cells were harvested by centrifugation at 4500 ***g*** for 15 min. The pellet was washed twice with distilled water and once with 20 mM KH_2PO_4 buffer (pH 6.5) supplemented with 1.1 M sorbitol and 0.5 mM $CaCl_2$ (buffer S). The cells were centrifuged at 4500 ***g*** after each wash. The recovered cells were resuspended in 4 vol. of buffer S containing 0.5 mM PMSF, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A and 6 mg of Zymolyase type 5000 (Seikagaku Kogyo Ltd, Tokyo, Japan) per g (wet weight) of cells. After incubation for 20 min at 30 °C with gentle shaking, 90 % conversion of yeast cells into spheroplasts was achieved. The spheroplasts were recovered by centrifugation for 10 min at 2500 ***g***, washed once with buffer S and resuspended in 3 vol. (v/w) of a solution containing 20 mM KH_2PO_4 (pH 6.5), 1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 18 % (w/v) Ficoll 400 (buffer L). The spheroplasts were disrupted in a Potter–Elvehjem Teflon/glass homogenizer (model 1561154; Belgolabo, Leuven, Belgium) with ten strokes of the pestle. Heavy debris and unbroken cells were removed by low-speed centrifugation at 750 ***g*** for 10 min. The supernatant was subsequently separated into a particulate fraction and a soluble fraction by centrifugation at 40000 ***g*** for

60 min. The particulate fraction was gently resuspended in 5 vol. (v/w) of buffer L containing 11% (w/v) Ficoll and layered on top of 15 vol. of buffer L containing 14% (w/v) Ficoll. After centrifugation at 3000 *g* for 60 min, a vacuole-enriched fraction was recovered, using a Pasteur pipette with a curved tip, at the interface between the two layers. This fraction was diluted 10-fold with buffer L containing 13% (w/v) Ficoll and centrifuged for 45 min at 6000 *g*. The pellet obtained apparently only consisted of vacuoles. By phase contrast microscopy with the green light of an interference filter, these appeared as darkish, rose–violet round objects. This preparation was found to be considerably enriched in vacuolar markers: the ratio of the activity measured in the vacuole preparation to that measured in total spheroplast lysates was 4.6–5.1 for α-mannosidase (depending on the strain), 5.9–6.2 for ALP, 5.3–5.7 for γ-GT, 6.0–6.4 for CGase and 12.7–13.1 for protease B. For the mitochondrial marker, citrate synthase, and the cytoplasmic marker, glucose-6-phosphate dehydrogenase, the ratio was only 0.18–0.21 and 0.02 respectively.

GSH transport and metabolism by isolated vacuoles

Transport was measured as the uptake of radiolabelled substrate into intact vacuoles collected by rapid filtration on a 0.45 μm Millipore filter under vacuum, essentially as previously described [12]. The standard assay mixture (200 μl) consisted of 25 mM Mes/Tris (pH 7.5), 10 mM $MgCl_2$, 25 mM KCl and an ATP-regenerating system (10 mM phosphocreatine, 100 μg/ml creatine kinase and 5 mM MgATP). Vacuoles were added to this mixture (the amount added corresponded to 20–60 μg of vacuolar-membrane protein). After preincubation at 30 °C, the reaction was started by addition of labelled substrate ([glycine-2-^{3}H]GSH; 50 μCi; NEN Life Science Products). In order to prevent GSH oxidation, the GSH was dissolved in 10 mM dithiothreitol. The reaction was stopped by diluting the mixture with 5 ml of ice-cold stopping buffer [10 mM Mes/Tris (pH 7.5), 5 mM $MgCl_2$ and 25 mM KCl]. The intact vacuoles were recovered on a membrane filter (Millipore; 0.45 μm) and were washed with 5 ml of ice-cold stopping buffer. The radioactivity taken up by the intact vacuoles was determined with a toluene-based scintillation cocktail [6]. Three types of controls were included: (1) controls without vacuoles; (2) controls without MgATP; and (3) controls where ATP was replaced with the non-hydrolysable analogue adenosine 5′-[β,γ-imido]triphosphate. In controls (2) and (3), no GSH transport was detected. On the basis of the controls, all results were corrected for non-specific binding of the radioactive label.

A similar procedure was used to study GSH metabolism, except that the volume of the incubation medium was 1 ml and the labelled substrate was 250 μCi of L-[*glutamyl*-U-^{14}C]GSH instead of the tritiated tripeptide. Aliquots of incubation medium were taken at different times, filtered on a 0.45 μm Millipore filter, and washed with 5 ml of ice-cold stopping buffer (described above). Both the filtrate (pooled with the washing filtrate) and the filtered vacuoles were saved. The radioactivity taken up by the intact vacuoles was determined by scintillation counting as described above. Alternatively, the filtrate was concentrated with a Speed Vac concentrator (Savant, Fullerton, CA, U.S.A.) and aliquots were subjected to an HPLC procedure after amino acid pre-derivatization [13]. In this filtrate, radioactivity was found associated with excess GSH, of course, but apart from that mainly with glutamate. The labelling data for GSH and glutamate were compared with the predictions of a two-compartment model assuming coupling of glutamate production and export from the vacuole



where m and v refer to the incubation medium and the vacuolar space respectively, and V corresponds to V_{max}. Step A was assumed to be mediated by the Ycf1p GSH transporter, anstep B by γ-GT. GSH* corresponds to L-[*glutamyl*-U-^{14}C]GSH and G* corresponds to [U-^{14}C]glutamate. In this model, the complete system is described by three ordinary differential equations:

$$\frac{\mathrm{d}\,GSH\mathrm{m}}{\mathrm{d}t} = -v_T \times f_I \quad (1a)$$

$$\frac{\mathrm{d}\,GSH\mathrm{v}}{\mathrm{d}t} = v_T \times f_I - v_D \quad (1b)$$

$$\frac{\mathrm{d}\,G\mathrm{m}}{\mathrm{d}t} = v_D \quad (1c)$$

where GSHm and GSHv are the concentrations of GSH in the incubation medium and the vacuole respectively, and Gm is the concentration of glutamate produced in the incubation medium.

$$\mathrm{v}_T = V_1 \frac{GSH\mathrm{m}}{K_{m1} + GSH\mathrm{m}} \quad (2)$$

is the rate of transport of GSH from the medium to the intravacuolar space.

$$f_I = \frac{K_i}{K_i + GSH\mathrm{v}} \quad (3)$$

is an inhibition factor describing the effect of GSH accumulated inside the vacuole on its transport by YCF1. The function was assumed to be purely non-competitive, i.e. acting only on the V_{max} and not on the K_m of the transporter.

$$\mathrm{v}_D = V_2 \frac{GSH\mathrm{v}}{K_{m2} + GSH\mathrm{v}} \quad (4)$$

is the rate of production of L-glutamate in the incubation medium. It reflects coupled γ-GT-mediated GSH degradation and glutamate transport towards the medium. Degradation of GSH by γ-GT [eqn (4)] is assumed to be the limiting step.

The software GLUTA.EXE (available on request) was designed in order to perform kinetic simulations and to compare theoretical curves and experimental data. GLUTA.EXE was complemented by DATAFILO, a program allowing the creation of a database in the required format.

RESULTS

γ-GT has an activating effect on Ycf1p-mediated transport of GSH into native *S. cerevisiae* vacuoles

Rebbeor et al. have shown that the transport of GSH into vacuolar membrane vesicles is largely mediated by the YCF1 system [12]. In the present study we compared GSH transport into native vacuoles from nitrogen-starved and ammonia-grown wild-type cells, the former containing active γ-GT and the latter being devoid of it. Native vacuole preparations of both types displayed a high rate of ATP-dependent [glycine-2-^{3}H]GSH uptake, following Michaelis–Menten saturation kinetics. The K_m (app) was 18 ± 2 mM and the V_{max} (app) was 48.5 ± 5 nmol of

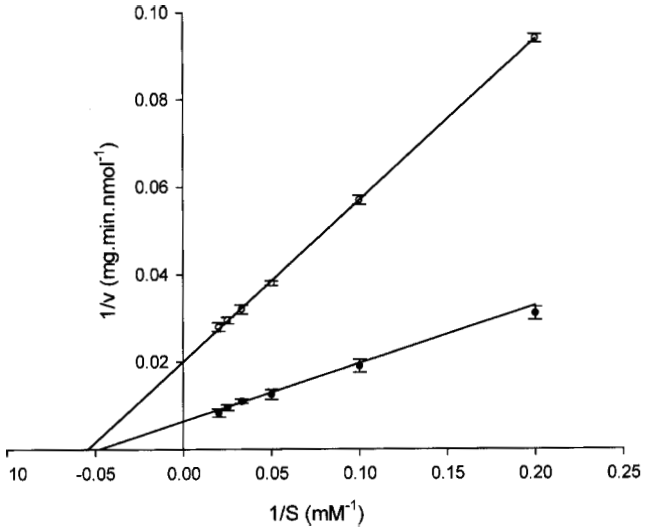


Figure 1 Initial rate of GSH uptake into native vacuoles of *S. cerevisiae* Σ1278b as a function of GSH concentration

Initial GSH uptake rates were determined using native vacuoles incubated for 1 min in GSH transport medium with various concentrations of tritiated GSH. The data, corrected for non-specific retention in the absence of MgATP, as described in the Experimental section, are presented as double-reciprocal plots. ○, Vacuoles devoid of γ-GT prepared from cells grown on SBM + $(NH_4)_2SO_4$. ●, Vacuoles with γ-GT activity prepared from nitrogen-starved cells. The values are presented as means ± S.E.M. ($n = 3$).

GSH/min per mg of protein for Σ1278b vacuoles devoid of γ-GT (Figure 1 and Table 2). Σ1278b vacuoles containing γ-GT showed practically the same K_m (app) value, but a higher V_{max} (app) value (150 ± 12 nmol of GSH/min per mg of protein). No increase in V_{max} (app) was observed with nitrogen-starved vacuoles prepared from the γ-GT-deficient disruptant strain KMY097 (Table 2). The presence of γ-GT in the vacuolar membrane thus increases the V_{max} (app) of vacuolar GSH transport, either directly or indirectly.

The same experiment was repeated with vacuoles isolated from strain DTY167, lacking functional Ycf1p. The measured V_{max} (app) and K_m (app) values were 14.5 ± 1 nmol of GSH/min per mg of protein and 18.5 ± 2.1 mM respectively (Table 2). The kinetic parameters for the isogenic reference strain DTY165 were similar to the values determined for Σ1278b. An increase in V_{max} (app) upon nitrogen starvation, resulting from γ-GT insertion into the vacuolar membrane, was observed in the parental strain DTY165 but not in the Ycf1p-deficient strain DTY167.

ATP-dependent GSH uptake was observed with native vacuoles purified from strain ScVatB lacking the B subunit of the catalytic sector of the vacuolar (H^+) ATPase (V-ATPase). The V_{max} (app) value determined for this mutant was approx. 70 % of the value obtained with the parental strain W303-1B. The K_m (app) value was apparently unaffected by the V-ATPase mutation. Both the mutant and the parental strain displayed the V_{max} (app)-increasing effect of γ-GT. The effect of the V-ATPase mutation was reproduced by addition of Bafilomycin A, a specific non-competitive inhibitor of the V-ATPase-dependent transport pathway [12]. Addition of a saturating amount of this inhibitor to vacuoles of the Ycf1p-deficient strain DTY167 almost totally suppressed the remaining 30 % of ATP-dependent GSH transport (results not shown).

From these experiments we conclude that the stimulating effect of γ-GT is most probably restricted to the YCF1 system.

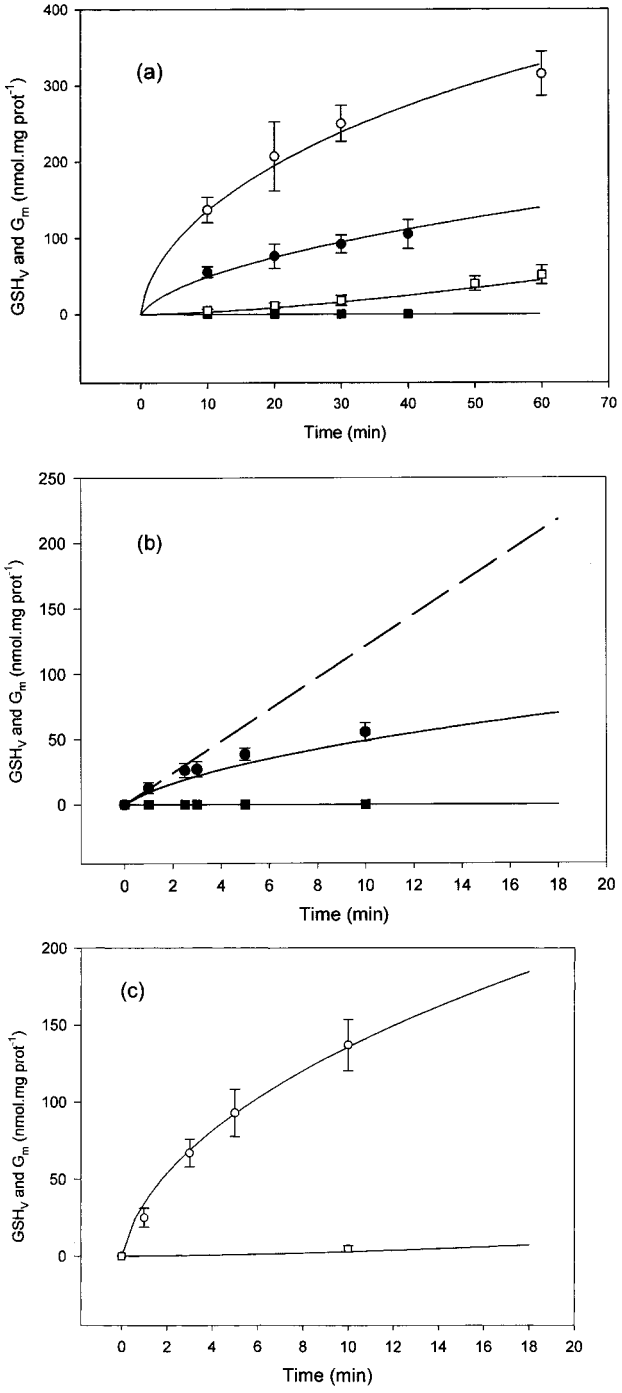


Figure 2 Comparison of the GSH transport and metabolism data with the predictions of the GLUTA.EXE two-compartment model

The assays were performed on native vacuoles treated with 1 μM Bafilomycin in the presence of 10 mM GSH as described in the Experimental section. (**a**) Fitting of the experimental values over a 1 h period with a feedback effect on GSH entry. (**b**) Fitting of the experimental data over the early 10 min period with vacuoles devoid of γ-GT with a feedback effect on GSH entry (continuous line), and prediction of the model without the feedback effect (broken line). (**c**) Same as in (**b**) but with vacuoles containing γ-GT. ○, Concentration of GSH in vacuoles (*GSH*v) containing γ-GT prepared from nitrogen-starved Σ1278b cells; ●, *GSH*v in vacuoles devoid of γ-GT prepared from nitrogen-starved cells of the KMY097 disruptant; and □ and ■ are the respective values for glutamate (*G*m) measured in the filtrate. Values are presented as means ± S.E.M. ($n = 3$). Typical values for the parameters are $K_{m1} = 18$ mM; $K_{m2} = 1.5$ mM; $K_l = 16.5$ nM; $V_1 = 34$ nmol/min per mg of protein in the case of vacuoles devoid of γ-GT and $V_l = 133$ nmol/min mg of protein in the case of vacuoles containing the enzyme; and $V_2 = 5$ μmol/min per mg of protein.

Whether γ-GT acts directly or indirectly remains to be determined. γ-GT might act, for example, by mediating a conformational change in Ycf1p.

GSH metabolism in isolated native vacuoles

The metabolism of vacuolar GSH was studied *in vitro* with intact vacuoles incubated in the presence of L-[*glutamyl*-U-^{14}C]GSH and ATP. The data obtained were compared with the predictions of the two-compartment model described in the Experimental section.

Vacuoles deficient in γ-GT, obtained from yeast cells growing on the repressive medium SBM + $(NH_4)_2SO_4$ or from the γ-GT-deficient strain KMY097, accumulated GSH but did not excrete glutamate (Figures 2a and 2b). In the accumulation profile, a significant deviation from linearity was observed after 1 min of incubation. This has also been reported by other investigators [12]. The deviation could be reproduced in the mathematical model by including an inhibitory factor [see eqn (3) in the Experimental section] acting on the V_{max} (app) of the Ycf1p transporter with a K_i of 16.5 nM (Figure 2b). This supports the hypothesis that GSH exerts an inhibitory feedback effect on its own entry.

Vacuoles containing γ-GT were found to excrete [U-^{14}C]-labelled glutamate when incubated in the presence of GSH and ATP (Figures 2a and 2c). In the model described by eqns (1–4), it was assumed that GSH accumulating in the intravacuolar space is further degraded by the action of membrane-bound γ-GT, and that the L-glutamate produced is directly excreted into the incubation medium, without transient accumulation in the vacuolar space. Vacuole samples having accumulated [U-^{14}C]-labelled GSH were ruptured and an HPLC method for low-molecular-mass thiols [13] was used to identify labelled compounds within the vacuoles. Significant radioactivity (over 96% of the total amount taken up) was found associated exclusively with the GSH elution peak. In the absence of ATP, GSH was not taken up and no glutamate was found in the incubation medium (results not shown). These experiments validate the model and indicate that the active site of γ-GT is orientated towards the vacuolar lumen. In conclusion, the model fitted quite accurately with the experimental data. This strongly supports the hypothesis that γ-GT is involved in the hydrolytic release (into the cytoplasm) of L-glutamate from intravacuolar GSH [14].

DISCUSSION

S. cerevisiae γ-GT has been identified as a vacuolar-membrane-bound enzyme with an apparent molecular mass of approx. 90000 Da [8,22]. The molecular mass predicted for the non-glycosylated form of the enzyme on the basis of its amino-acid sequence is 73162 Da (Figure 3). The small chain of yeast γ-GT located at the C-terminal moiety contains the TAHFSIVDSH-GNAVSLTTTINLLFG sequence (residues 470–494) that matches the γ-GT signature T(STA)HX(ST)X_4G(SN)XV(STA)-

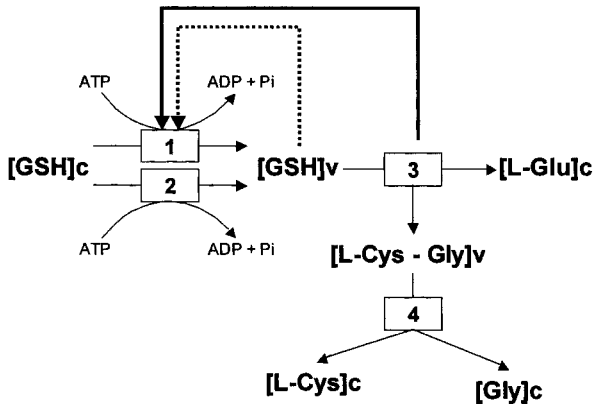


Figure 4 Tentative model for GSH transport and metabolism in the yeast central vacuole

1, YCF1; 2, V-ATPase-linked GSH transport; 3, γ-GT; 4, CGase; c, cytosolic compartment; v, vacuolar compartment; →, activation of Ycf1p by γ-GT; and ⟶, feedback effect of *GSH*v on its transport by Ycf1p.

XTXT(LIVM)(NE)$X_{1,2}$(FY)G (PS00462). A Blast search revealed that *S. cerevisiae* γ-GT shares sequence homology with the γ-GTs of *Escherichia coli* (p18956; 27% identity over 486 residues), rat (p073114; 30% identity over 557 residues) and *Homo sapiens* (p19440; 32% identity over 575 residues). *S. cerevisiae* γ-GT and its human and rat homologues each contain at their N-terminus a hydrophobic sequence that may serve as a transmembrane domain. In the mammalian liver and kidney, γ-GT is a membrane-bound glycoprotein with the N-terminus of the large subunit anchored to the extracellular surface of the plasma membrane [5]. In *E. coli*, the mature enzyme is confined to the periplasmic space [23], but the highly conserved primary structure of γ-GT (Figure 3) suggests that the physiological functions of the enzyme evolved quite early towards GSH mobilization and amino-acid trafficking. In contrast with the *E. coli* enzyme [24], yeast γ-GT lacks an N-terminal signal peptide. It is more similar to mammalian γ-GTs. As in the rat and human enzymes, one potential transmembrane helix α domain, preceded by a short hydrophilic tail in the N-terminal region, has been located in *S. cerevisiae* γ-GT (Figure 3). The organization of γ-GT is highly similar to that of ALP, a typical integral yeast vacuolar membrane protein. ALP also lacks a standard signal sequence at its N-terminus and possesses a single hydrophobic domain of approx. 20 amino acids near the N-terminus [25]. By analogy, the yeast transpeptidase can be expected to have the topology of a type-II integral vacuolar membrane protein, with its short hydrophilic tail extending into the cytoplasm and with its C-terminus bearing the active site [5] inside the vacuolar lumen (Figure 4). This view is supported by experiments reported in the Results section (Figure 2) and also by an additional observation that incubation of isolated yeast vacuoles with

Figure 3 Schematic representation of several γ-GTs

Large and small subunits are coloured in dark grey and white respectively. Black boxes represents putative transmembrane domains, the light grey box represents a peptide signal sequence, and the dashed box represents the γ-GT signature sequence.

acivicin, a specific inhibitor acting at the catalytic site of γ-GT [26], does not detectably inhibit GSH degradation (results not shown).

Excretion of L-glutamate from vacuoles towards the cytoplasm might require either a special orientation of γ-GT, creating a transmembrane exit canal for L-glutamate, or co-operation of an amino-acid efflux transporter dedicated to this amino acid (Figure 4). To date no mechanism of efflux of acidic amino acids from the vacuole towards the cytoplasm has been identified in yeast [27,28]. In contrast, L-arginine efflux from the vacuole is well characterized [14,28]. A more detailed study of the role of γ-GT in the efflux of L-glutamate from the vacuole could be of great basic interest as a case where metabolism and transport are mechanistically inseparable [29].

The γ-GT-deficient *S. cerevisiae* mutant could be a useful model for the study of γ-GT physiology and GSH metabolism. It has been reported that a genetic γ-GT deficiency in a mouse mutant resulted in severe glutathionuria [30]. The glutathionuric phenotype was explained by failure to recover GSH from the renal glomerular filtrate, preventing operation of the γ-glutamyl cycle in the epithelial membrane of the renal tubules.

The *YCF1* gene was isolated for its ability to confer cadmium resistance to yeast. Its product, the Ycf1p transporter, has been found to transport GSH S-conjugates [31]. This pump was first anticipated [14] and later shown [12] to transport GSH itself into reconstituted vacuolar membrane vesicles. In the present study we have confirmed these results with native vacuoles. We have further shown that a second, parallel pathway driven by V-ATPase may contribute significantly to GSH import into native vacuoles. This pathway is most probably related, if not identical, to the YCF1-independent anion uniport system involved in the transport of GSH S-conjugates in *S. cerevisiae* [31]. The physiological reason for the presence of two distinct vacuolar GSH transport systems is unclear at present, but it seems highly significant that membrane-bound γ-GT exerts its activating effect on the YCF1 system only. To observe this activating effect, one must ensure that γ-GT is delivered correctly to the vacuolar membrane via its post-Golgi translocation pathway. When vacuoles were incubated with purified γ-GT, the enzyme did not insert into the vacuolar membrane and no activation of the YCF1 system was observed (results not shown).

As indicated in the Results section, activation of the YCF1 system might result from the direct or indirect action of γ-GT. Although no experimental data are currently available to make a conclusion, it is noteworthy that in a two-hybrid system the product of *ECM38* (identified in the present study as γ-GT) interacts with another yeast membrane protein of unknown function [32].

The YCF1 system was also possibly subjected to feedback inhibition by intravacuolar GSH (Figure 2). Feedback inhibition has been demonstrated in *S. cerevisiae* for lysine transport across the plasma membrane [33], and more recently for the yeast uracil permease [34].

Although it may not fully represent the true *in vivo* situation of GSH metabolism in yeast, the mathematical model exploited in the present study seems to accurately describe the general features of *in vitro* vacuolar GSH transport and degradation. Under growth conditions where γ-GT was absent, GSH appeared to be virtually inert in whole cells (half-life > 900 min) [6]. This was also true in the present study on isolated vacuoles devoid of γ-GT (Figure 2). A lower half-life ($\leqslant 200$ min) has been calculated for GSH in whole cells under conditions of γ-GT de-repression [6,13,14].

In the tentative model depicted in Figure 4, CGase is assumed to catalyse the last step in GSH hydrolysis. This activity is reported to be present in *S. cerevisiae* and associated with the vacuolar membrane [6,8]. A variety of L-Cys-Gly-hydrolysing peptidases are produced by micro-organisms and mammals, such as the yeast vacuolar aminopeptidase I (EC 3.4.11.22) [35] and the mammalian enzymes aminopeptidase M (EC 3.4.11.2) [36] and microsomal dipeptidase precursor 1 (EC 3.4.13.19), both components of the renal brush border [37]. Yet a Blast search for microsomal dipeptidase precursor 1 and aminopeptidase M counterparts in *S. cerevisiae* revealed no similarities to known hydrolases in yeast. The ORF coding for yeast CGase thus remains to be identified.

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