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PATHWAYS OF GLUTATHIONE DEGRADATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Key Word Index—*Saccharomyces cerevisiae*; yeast; glutathione; γ-glutamyl peptides; γ-glutamyltranspeptidase; γ-glutamyl cycle; glutathione turnover; amino acid uptake; transpeptidation.

Abstract—The degradation of glutathione (GSH) in the yeast *Saccharomyces cerevisiae* appears to be mediated only by γ-glutamyltranspeptidase and cysteinylglycine dipeptidase. Other enzymes of the γ-glutamyl cycle, γ-glutamyl cyclotransferase and 5-oxo-L-prolinase, are not present in the yeast. *In vivo* transpeptidation was shown in the presence of a high intracellular level of γ-glutamyltranspeptidase, but only when the de-repressing nitrogen source was a suitable acceptor of the transferase reaction. In contrast, when the de-repressing source was not an acceptor of the transferase reaction (e.g. urea), only glutamate was detected. Intracellular GSH is virtually inert when the level of γ-glutamyltranspeptidase is low. Possible roles for *in vivo* transpeptidation are discussed.

INTRODUCTION

Numerous studies have been devoted to glutathione in animal cells [1] but, in contrast, relatively few papers treating the subject in higher [2] and lower plants [3] have appeared. The only demonstrated role for glutathione in the yeast *Saccharomyces cerevisiae* appears to be linked to the detoxication of endogeneous aldehydes via the glyoxalase pathway [4]. A role for glutathione in amino acid absorption by the yeast cell is currently being debated. Previous claims [5, 6] that glutathione is involved in the bulk transport of amino acid have been contradicted [7–9]. In this paper we demonstrate that glutathione degradation in *S. cerevisiae* is not mediated by a complete γ-glutamyl cycle as previously reported by others [10]. On the contrary, only a circumstantial degradative pathway involving γ-glutamyltranspeptidase and leading to the formation of glutamate and γ-glutamyl amino acids was shown *in vivo*.

RESULTS

Table 1 shows that neither carbon, nitrogen sources, nor anaerobiosis influenced the intracellular levels of glutathione synthetase (EC 6.3.2.3) and glutathione (GSH). In contrast, and as also previously shown by us [11], γ-glutamyltranspeptidase (EC 2.3.2.2) is strongly repressed by ammonium ion, whereas high cellular specific activities are promoted by the use of certain amino acids or proline and urea as nitrogen sources. We reasoned that if γ-glutamyltranspeptidase is involved in the biosynthesis of γ-glutamyl amino acids, one might detect such compounds in the yeast when a high cellular level of

Table 1. Specific activities of enzymes and GSH intracellular concentration in *Saccharomyces cerevisiae*

Carbon source	Nitrogen source	Specific activities of enzymes*						GSH intracellular concentration (mM)
		GS	γ-GT	γ-GCT	5-OP	DP	CP	
Glucose	NH_4^+	0.013	0.011	N.D.†	N.D.	0.022	N.D.	4.0
Glucose	Glutamate	0.012	0.055	N.D.	N.D.	0.025	N.D.	3.1
Glucose	Urea	0.016	0.061	N.D.	N.D.	0.020	N.D.	4.1
Glucose	Proline	0.015	0.059	N.D.	N.D.	0.022	N.D.	4.2
Glucose	Casein amino acid	0.015	0.022	N.D.	N.D.	0.023	N.D.	3.8
Glucose‡	NH_4^+	0.012	0.013	N.D.	N.D.	0.026	N.D.	3.9
Glycerol	Casein amino acid	0.016	0.020	N.D.	N.D.	0.025	N.D.	4.3
Pyruvate	Casein amino acid	0.016	0.019	N.D.	N.D.	0.019	N.D.	4.1

*Data are the means of three independent assays. Units of enzyme activity are expressed in nkat/mg protein. GS, GSH synthetase; γ-GT, γ-glutamyltranspeptidase; γ-GCT, γ-glutamylcyclotransferase; 5-OP, L-5-oxoprolinase; DP, cysteinylglycine dipeptidase; CP, GSH carboxypeptidase.

†N.D. = Not detected.

‡Anaerobiosis in the growth conditions of Pennickx *et al.* [4].

Table 2. Effect of the nitrogen source on the intracellular content of γ-glutamyl compounds and on the half-life of GSH in *Saccharomyces cerevisiae*

Nitrogen source	Specific activity of γ-GT (nkat/mg protein)	Intracellular concentration of γ-glutamyl derivatives (mM)*				Half-life of intracellular GSH (min)†
		GSH	GSSG	γ-Glu-Glu	γ-Glu-Gln	
NH_4^+	0.011	4.6	0.02	N.D.‡	N.D.	990
NH_4^+ + urea	0.017	3.9	0.02	N.D.	N.D.	670
NH_4^+ + glutamate	0.019	4.0	0.03	N.D.	N.D.	680
Urea	0.061	4.1	0.02	N.D.	N.D.	230
Glutamate	0.055	3.8	0.04	0.07	0.08	220

*Intracellular concentrations of γ-glutamyl derivatives were determined by HPLC after calibration with known amounts of the compounds (see Experimental). Data are the means of three independent assays.

†The half-life of GSH was determined in a period of 1 hr from the data reported in Figs. 1A and 1B.

‡N.D. = Not detected.

transpeptidase prevails. TLC and HPLC experiments were performed on intracellular pools of *S. cerevisiae* growing on different nitrogen sources. γ-Glutamylglutamate and γ-glutamylglutamine were detected when L-glutamate served as nitrogen source (Table 2). No γ-glutamyl compounds, except GSH and a small amount of oxidized glutathione (GSSG), were detected when ammonium, urea or ammonium plus urea were used as nitrogen sources. The peptides were also identified by HVPE. Acid hydrolysis (5.7 M HCl, 12 hr, 110°) of peptides recovered by TLC and HVPE gave only glutamate, as shown by amino acid analysis.

γ-Glutamyl cyclotransferase (EC 2.3.2.4) and 5-oxo-L-prolinase, both enzymes of the γ-glutamyl cycle [1], were not detected in the yeast (Table 1). This contrasts with a previous report [10]. In order to clarify this problem, we performed direct labelling experiments. Yeast cells growing respectively on ammonium, glutamate or urea were loaded with L-[U-^{14}C-glutamyl]glutathione. When L-glutamate or urea served as the nitrogen sources, the radioactive label appeared first in GSH then in glutamate but not in 5-oxo-L-proline (Fig. 1A). GSH was the only compound labelled when ammonium served as the nitrogen source (Fig. 1B). Turnover values of GSH were deduced from labelling data (Table 2). The peptide was virtually inert when ammonium served as the sole nitrogen source. Its half-life was significantly decreased in the presence of L-glutamate or urea. In another experiment we showed that yeast cells are unable to degrade 5-oxo-L-proline into glutamate and that the cyclic compound is metabolically inert ($t_{1/2} > 1000$ min, not shown; see Experimental for the procedure).

Cysteinylglycine dipeptidase (EC 3.4.13.6) was the only other enzyme of the γ-glutamyl cycle that was detected in *S. cerevisiae* (Table 1) and it was not affected by the different experimental conditions applied. Another pathway of GSH degradation involving a carboxypeptidase-releasing glycine from the tripeptide was suspected in higher plants [12] but this enzyme was not detected in the yeast (Table 1).

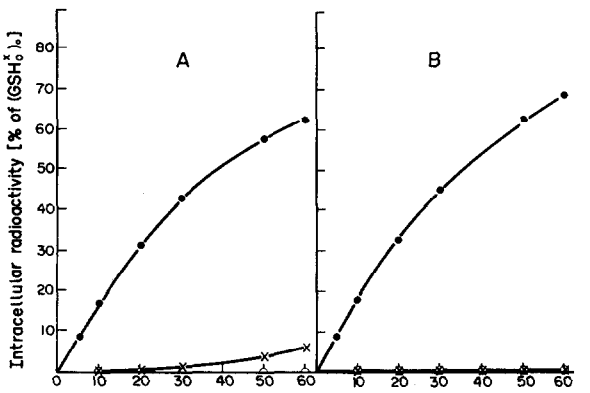

Fig. 1. Intracellular labelling pattern of *Saccharomyces cerevisiae* cells in the presence of L-[U-^{14}C-glutamyl]GSH. The results given are the means ± s.e.m. of three experiments (the procedure is reported in the Experimental). Data with L-Glu as the nitrogen source are not shown. (●) GSH; (×) glutamate; (○) 5-oxoproline. (A) Urea as nitrogen source; (B) NH_4^+ as nitrogen source.

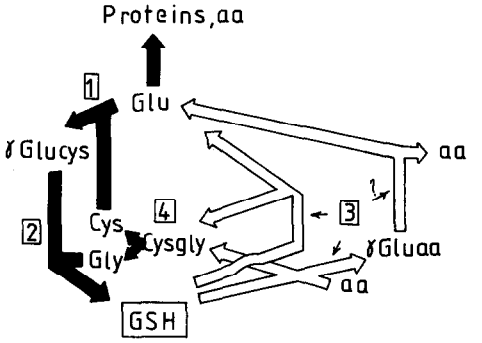

Fig. 2. Metabolism of GSH in the yeast *Saccharomyces cerevisiae*. (➡) Constitutive metabolism; (⇨) metabolism submitted to regulation. (1) γ-Glu-Cys synthetase; (2) GSH synthetase; (3) γ-glutamyltranspeptidase. aa = Amino acid.

DISCUSSION

Figure 2 depicts the conclusion that can be drawn from

this investigation on the metabolism of GSH in the yeast *S. cerevisiae*. This scheme is radically at variance with a previous communication of Mooz and Wigglesworth which reported the presence of a complete γ-glutamyl cycle in the yeast [10]. These authors based their conclusions solely on enzyme level measurements. At the present time, we have no clear explanation for this discrepancy, but variations between strains appear highly unlikely. As shown here by labelling experiments, GSH degradation is largely circumstantial. The tripeptide is virtually inert when ammonium serves as the sole nitrogen source or in combination with amino acids or urea. Degradation of GSH appears to be only significant when high intracellular levels of γ-glutamyltranspeptidase prevail in the yeast cell. Nevertheless, two different situations were encountered with respect to the nature of the nitrogen source promoting the de-repression of γ-glutamyltranspeptidase. When urea, which is not an acceptor of γ-glutamyltranspeptidase [13], was supplied to the cell, only glutamate was detected as a degradation product. In contrast, transpeptidation products were detected in the presence of glutamate, a good acceptor of the transferase [14] and an immediate precursor of glutamine. This picture is consistent with a dual role of γ-glutamyltranspeptidase functioning *in vivo* either as a hydrolase or a transferase of GSH, both situations that are encountered *in vitro* [15].

A role for GSH-synthesizing enzymes in the *in vivo* formation of γ-glutamyl amino acids may probably be ruled out in the yeast. Indeed, the intracellular levels of GSH synthetase and GSH are not affected by the nitrogen source. However, the exact role of *in vivo* transpeptidation is still obscure. In fact, from the measured quantity of GSH present, the turnover of the peptide and the velocity of influx of L-glutamate [16], it can be calculated that 15–200 molecules of L-glutamate are absorbed for every molecule of GSH degraded. This observation makes an uptake of glutamate via γ-GT unlikely. Respective values of 30–100 and 50–600 for the influx of L-methionine in *Candida utilis* and *S. cerevisiae* were reported [7, 17].

In a different approach, Payne and Payne [9] have shown that chemical inactivation of γ-glutamyltranspeptidase in whole cells of *S. cerevisiae* does not affect the rate of uptake of amino acids. The same authors postulated that a membrane form of transpeptidase could supply γ-glutamyl derivatives for use as specific messengers in transmembrane regulation [9]. In this paper we have shown that γ-glutamyltranspeptidase might indeed supply such γ-glutamyl derivatives, but we can offer an alternative hypothesis for its role. We have recently reported that vacuoles of *S. cerevisiae* contain γ-glutamyltranspeptidase [18]. This enzyme could be implicated as a carrier in the facilitated diffusion of amino acids between the cytoplasm and the central vacuole. Indeed, at least *in vitro*, the vacuolar transport of amino acids does not appear to be energy-linked [19]. The small concentrations of γ-glutamyl amino acids that were found in the yeast cell could be steady-state values reflecting the interplay between biosynthesis at the vacuolar membrane and subsequent hydrolytic release of amino acids in the vacuolar sap.

EXPERIMENTAL

Materials. L-[U-^{14}C-Glutamyl]glutathione (oxidized) and L-[glycine-2-^{3}H]glutathione were purchased from New England Nuclear, Boston. 5-Oxo-L-[U-^{14}C]proline was prepared from L-[U-^{14}C]glutamate (Radiochemical Centre, Amersham) by heating for 6 hr at 140° at pH 3 followed by purification on Dowex 50 (H^+) [20]. L-γ-Glutamyl-L-α-aminobutyric acid was synthesized as described previously [8]. All other reagents were of A.R. grade.

Culture of yeast. Saccharomyces cerevisiae Σ 1278b [21] was grown at pH 4.4 as described previously [4]. All experiments were conducted during exponential growth.

Analytical procedures for enzymes. Glutathione synthetase was determined according to ref. [22] with L-γ-glutamyl-L-α-aminobutyrate and [U-^{14}C]glycine as substrates. γ-Glutamyltranspeptidase activity was estimated by the method of ref. [11] using L-γ-glutamyl-*p*-nitroanilide as a donor and L-methionine as an acceptor. γ-Glutamylcyclotransferase activity was assayed with L-γ-glutamyl-L-α-aminobutyrate as substrate [23]. 5-Oxo-L-prolinase was assayed according to ref. [24] with 5-oxo-L-[U-^{14}C]proline as substrate. Cysteinyl glycine dipeptidase was estimated by the method of ref. [25]. A search for carboxypeptidase activity releasing glycine from GSH was made with L-[glycine-2-^{3}H]glutathione according to ref. [26]. Several experimental conditions were tried for the detection of γ-glutamylcyclotransferase, 5-oxo-L-prolinase and carboxypeptidase but without success: use of permeabilized cells obtained by nystatine treatment [27], desalting of the crude extract on a G25 Sephadex column, variations of the pH between 5 and 9, nature of the buffer (Na citrate, KPi, imidazole, Hepes, Tris–HCl) and variation of the ionic strength ($\Gamma/2 = 0.1$ to $\Gamma/2 = 0.5$ M KCl). GSH intracellular concns were determined by the method of ref. [8]. The procedure of ref. [28] was used for the estimation of protein.

Identification of the intracellular γ-glutamyl compounds synthesized by the yeast. Ca 500 mg dry weight of *S. cerevisiae* cells growing in exponential phase was harvested by filtration through a Millipore membrane (0.45 μm pore size). The cells were immediately added to 10 ml H_2O. After allowing the mixture to stand for 10 min at 100°, the extract was separated by a further Millipore filtration. The soln was applied to a Dowex acetate (AG1 × 2) column (1 × 10 cm). Elution with 25 ml 0.05 M HOAc removes all the amino acids except small amounts of glutamate and aspartate. Nevertheless, this procedure permits the complete retention of γ-glutamyl derivatives. These compounds were eluted with 20 ml 3 M HOAc and the recovered eluate was lyophilized; 0.1 ml H_2O was added to the residue. Analyses of γ-glutamyl compounds were performed with a Waters HPLC using an amino acid pre-column derivatization method [29]. The reverse-phase column (Waters C18 Bondapak) was developed using the conditions of ref. [30] with the following changes: solvent A: THF–MeOH–0.05 M NaOAc (1:19:80) (pH 5.9); solvent B: MeOH–0.05 M NaOAc (8:2) (pH 5.9); gradient programme: 0% solvent B (100% solvent A) from 1 min from the initiation of the programme. Linear step to 50% B in 14 min. Isocratic step at 50% B of 5 min. Linear step to 100% B in 5 min. Flow rate: 1.4 ml/min. Analyses of the Dowex eluate containing the γ-glutamyl derivatives were carried out by ascending TLC on silica gel with *n*-BuOH–HOAc–H_2O (4:5:1).

Prep. TLC of the presumed γ-Glu-Glu and γ-Glu-Gln was carried out with *n*-BuOH–HOAc–H_2O (4:5:1). The peptides were recovered by scraping off the sheets and were further eluted three times with 0.05 M HOAc. Identification of the γ-glutamyl peptides was also performed by HVPE (30 V/cm) in C_5H_5N–HOAc–H_2O (1:10:289). Several criteria were used for the identification of the HPLC peaks with γ-glutamylglutamate and γ-glutamylglutamine. In fact, they had the expected retention times and were eluted from the column with authentic samples of the peptides. Samples of the peptides previously identified as γ-glutamylglutamate and γ-glutamylglutamine by TLC were obtained by a preparative procedure and further reinjected on the

HPLC column. Again, they corresponded to the expected compounds.

Labelling experiments. L-[U-^{14}C-Glutamyl]GSH (20 μCi) obtained by incubation of the oxidized form in the presence of 10 mM dithiothreitol was added to 100 ml of a growing culture of yeast containing 0.1 mg dry wt/ml cells at the start of the experiment. At various times, 10 ml of the culture was withdrawn and filtered through a Millipore membrane (0.45 μm pore size). The filter was washed twice with 1 ml cold H_2O. The supernatant was kept to estimate the remaining L-[U-^{14}C-glutamyl]GSH. The cells on the filter were immediately dipped into 5 ml H_2O containing GSH, L-glutamate and 5-oxo-L-proline (each at 0.2 mg/ml) as carriers. The mixture was allowed to stand for 10 min at 100°. The extract was separated by Millipore filtration and applied to a Dowex 50 (H^+) column (1 × 5 cm). The column was eluted with 10 ml H_2O and the recovered eluate was lyophilized (eluate I). The column was further eluted with 10 ml 3 M NH_3. The eluate was flash-evapd and subjected to performic acid oxidation in the presence of HBr following ref. [31]. The mixture was then evapd and 2 ml H_2O was added to the residue. The soln was applied to a Dowex 50 (H^+) column (1 × 5 cm), which was eluted with 10 ml H_2O. The eluate was lyophilized (eluate II). The column was finally eluted with 10 ml 3 M NH_3 and the eluate was evapd (eluate III). 5-Oxo-L-proline was separated from eluate I by TLC on PEI-impregnated cellulose plates developed with LiCl (0.6 M). GSH sulphonic acid was separated from eluate II by TLC cellulose plates with MeOH–C_5H_5N–1.25 M HCl (37:4:8). Glutamate was separated from eluate III by TLC on silica gel developed with Me_2CO–*n*-BuOH–H_2O (5:3:2). In labelling experiments with 5-oxo-L-[U-^{14}C]proline, 20 μCi of the compound was added to 100 ml of a growing yeast culture, as described above. The radioactivity in each purified fraction was measured by scintillation counting in toluene–Triton X 100–PPO. All measurements were corrected for spill-over and background. It was checked in independent experiments that the recovery of each compound amounted to *ca* 90%. The labelling data were confronted with a 2-compartment system, i.e. GSH and glutamate:

$$(GSH^+)_o \xrightarrow{k_1} (GSH^+)_i \xrightarrow{k_2} (G^+)_i \xrightarrow{k_3} \text{metabolites}$$

GSH^+: L-[U-^{14}C-glutamyl]glutathione

G^+: L-[U-^{14}C]glutamate

The subscripts o and i refer respectively to the outside and inside of the cell; $k_{1,2,3}$ are first-order constants linked to the uptake of glutathione, the degradation of the tripeptide into glutamate, and the metabolic consumption of the amino acid, respectively. Mathematical resolution of the model gives the following relationships:

$$(GSH^+)_o = (GSH_0^+)\exp(-k_1 t) \quad (1)$$

$$(GSH^+)_i = \frac{k_1 (GSH_0^+)_o}{k_1 - k_2}[\exp(-k_2 t) - \exp(-k_1 t)] \quad (2)$$

$$(G^+)_i = (GSH_0^+) = \frac{k_1 k_2}{(k_2 - k_1)(k_2 - k_3)(k_1 - k_3)}[(k_3 - k_2)\exp(-k_1 t) + (k_1 - k_3)\exp(-k_2 t) - (k_1 - k_2)\exp(-k_3 t) \quad (3)$$

$(GSH_0^+)_o$ is the amount of radioactive glutathione in the supernatant at zero time. k_1 was determined experimentally by estimating the percentage of radioactivity remaining in the supernatant at various times and plotting the results according to the following equation:

$$\ln \frac{(GSH^+)_o}{(GSH)_o} = -k_1 t$$

It was shown in independent TLC experiments that glutathione is taken up as the intact molecule by the yeast cells. k_2 and k_3 were estimated by curve-fitting with the aid of eqns. (2) and (3). Half-life of GSH: $t_{1/2} = 0.693/k_2$.

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