An important role for glutathione and γ -glutamyltranspeptidase in the supply of growth requirements during nitrogen starvation of the yeast *Saccharomyces cerevisiae*

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When the yeast *Saccharomyces cerevisiae* Σ 1278b was starved for nitrogen, the total glutathione (GSH) pool increased from 7 to 17 nmol (mg dry wt)⁻¹ during the first 2 h and then declined. More than 90% of the total GSH shifted towards the central vacuole during this time. This transient stimulation was not observed in the presence of buthionine-(S,R)-sulphoximine (BSO), a specific transition-state-analogue inhibitor of γ -glutamylcysteine synthase (γ -GCS), nor in a mutant strain deficient in this enzyme. γ -Glutamyltranspeptidase (γ -GT), a vacuolar enzyme responsible for the initial step of GSH degradation, was derepressed during nitrogen starvation. This mechanism can apparently enable the starved yeast cell to use the constituent amino acids from GSH which accumulate in the vacuole to satisfy its growth requirements for nitrogen.

Keywords: yeast, glutathione, γ -glutamyltranspeptidase, nitrogen starvation, vacuoles

INTRODUCTION

Glutathione (GSH; L- γ -glutamyl-L-cysteinylglycine) is present in high concentration in most living cells from micro-organisms to man, and has been shown to play numerous roles, in particular in the yeast *Saccharomyces cerevisiae*, where it may account for 1% of the cell dry weight (Penninckx & Elskens, 1993). A role of GSH as an endogenous sulphur source in *S. cerevisiae* has previously been shown (Elskens *et al.*, 1991). In this yeast, GSH catabolism appears to be mediated by γ -glutamyltranspeptidase (γ -GT) and cysteinylglycine dipeptidase, and leads to the formation of glutamate, cysteine and glycine (Jaspers *et al.*, 1985). Yeast γ -GT biosynthesis was found to be regulated by at least two apparently distinct pathways involving, respectively, repression by ammonium ion (Penninckx *et al.*, 1980) and GSH (Elskens *et al.*, 1991). Repression of γ -GT by GSH is related to the sulphur storage function of GSH (Elskens *et al.*, 1991). In contrast, no clear physiological function was attributed to the repressive effect of the ammonium ion. A possible role for γ -GT in the bulk transport of amino acids by *S. cerevisiae* (Penninckx *et*

al., 1980) was later contradicted (Robins & Davies, 1981; Jaspers & Penninckx, 1981; Payne & Payne, 1984). Here we show that nitrogen starvation induced a migration of more than 90% of GSH towards the central vacuole (tonoplast) of the yeast cell. This migration was accompanied by a *de novo* synthesis of the tripeptide and a derepression of γ -GT. GSH that had accumulated in the central vacuole was further degraded and seems to play the role of a reservoir to provide the yeast cell with amino acids for subsequent growth requirements.

METHODS

Strains. The wild-type strain Σ 1278b (MATa), was described by Grenson *et al.* (1966) and obtained from the Laboratoire de Microbiologie de l'Université libre de Bruxelles. The GSH-deficient mutant (*gshA-2*) yeast strain was from our laboratory (Elskens *et al.*, 1991).

Growth experiments. Orbital shake batch cultures (0.5 and 1 l) were inoculated from aerobic, stationary-phase, glucose-grown precultures and incubated overnight at 29 °C to obtain *S. cerevisiae* cells in the exponential growth phase. A mineral medium (M 165) with 3% (w/v) glucose added as sole source of carbon, and containing vitamins and trace minerals but no nitrogen source, was used as the standard basal medium (SBM) (Messenguy, 1976). In the experiments on nitrogen starvation, cells of *S. cerevisiae* previously grown in SBM with

Abbreviations: BSO, buthionine-(S,R)-sulphoximine; γ -GCS, γ -glutamylcysteine synthase; γ -GT, γ -glutamyltranspeptidase; SBM, standard basal medium.

10 mM $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source (minimal medium) were harvested at $0.4\text{--}0.5$ mg dry wt ml^{-1} by rapid Millipore filtration (0.45 μm HAWP), washed twice with SBM and transferred to the same medium.

Enzyme and protein assays. Cells were harvested at 4°C by centrifugation at 6000 g for 10 min, washed twice with an appropriate buffer for the enzyme determination and resuspended in the same buffer. The cells were ruptured by sonication for 10 min with a Vibra cell VC 500 ultrasonic processor (Sonics Materials). The homogenate was centrifuged ($27\,000$ g for 15 min) and the supernatant fluid was used as the enzyme source.

GSH synthase (EC 6.3.2.3) and γ -GT (EC 2.3.2.2) were assayed according to Jaspers *et al.* (1985). γ -Glutamylcysteine synthase (γ -GCS; EC 6.3.2.2) was assayed following Kistler *et al.* (1990).

Protein was estimated by the Lowry method using bovine serum albumin as standard. Enzyme units (U) were expressed as nmol product h^{-1} . Specific activities were U (mg protein) $^{-1}$.

Determination of metabolic intermediates. The method of Sakaguchi was adapted for the determination of the cell content of arginine of *S. cerevisiae* (F. Ramos, personal communication). All reagents were kept at 0°C and added, with mixing each time, in the following sequence: 2 ml sample ($25\text{--}400$ nmol arginine), 0.25 ml 40% NaOH, 0.1 ml 0.2% recrystallized α -naphthol (1% in ethanol diluted fivefold with water) with immediate mixing. After 15 min incubation, 0.5 ml hypobromite reagent was added (5 ml Br_2 per 100 ml 10% NaOH). After exactly 10 s, 10 ml 95% ethanol was added at 0°C with immediate mixing, followed by 1 ml 40% (w/v) urea. Absorbance at 520 nm was stable for at least 1 h.

GSH was estimated according to Eyer & Podhradsky (1986), or alternatively by the modified Fahey procedure described by Elskens *et al.* (1991) as indicated in the text.

Total and differential pool extraction. A standard procedure (Ramos *et al.*, 1970) was used for the extraction of total GSH and amino acid pools in *S. cerevisiae*. The cytoplasmic and vacuolar pools were extracted following the method of Ohsumi *et al.* (1988). Briefly, this method was based on the specific loss of the permeability barrier of the plasma membrane of the yeast cell in the presence of cupric ion (Cu^{2+}). The cytoplasmic content of metabolic intermediates was estimated on the leakage fluid. The vacuolar content was then estimated by extraction of the Cu^{2+} -permeabilized yeast with boiling water or trichloroacetic acid. The validity of the differential extraction procedure was checked for the *S. cerevisiae* strains used in the present investigation. Cells of the $\Sigma 1278b$ and *gsb*-A2 strains growing in minimal medium were harvested at $0.5\text{--}0.6$ mg dry wt ml^{-1} and extracted according to the Ohsumi method; 95 ± 1.2 and $49.8 \pm 0.9\%$ (mean $\pm$ SD; $n = 3$) of the respective arginine and GSH pools were found to be associated with the vacuolar compartment. These values are close to the data of Messenguy *et al.* (1980) and Jaspers & Penninckx (1984), obtained by a completely different extraction procedure using cytochrome *c*. Respective values of 59.1 ± 2.3 and 7.1 ± 1 nmol (mg dry wt) $^{-1}$ were found for the total pools of arginine and GSH of $\Sigma 1278b$, by the standard extraction procedure mentioned above. By summing the respective values obtained for the cytoplasmic and vacuolar pools by the differential procedure, total respective values of 58.9 ± 2.6 and 6.9 ± 1.1 nmol (mg dry wt) $^{-1}$ were obtained for arginine and GSH.

Reproducibility of results. Only single values are quoted in the figures illustrating this paper. However, experiments are only

reported that were repeated at least twice and that gave consistent results. Standard deviations were all within 10% of the mean values presented.

RESULTS

Effect of nitrogen starvation on the total GSH pool

In cells of $\Sigma 1278b$ growing exponentially on minimal medium, the total pool of GSH accounted for 7 ± 1 nmol (mg dry wt) $^{-1}$ (Elskens *et al.*, 1991). When cells were transferred from minimal medium to SBM devoid of nitrogen source, the total GSH pool increased gradually during the next 2 h, culminating at 17.2 ± 1.3 nmol (mg dry wt) $^{-1}$ (Fig. 1a). The GSH pool decreased subsequently to attain a stable value of 2.6 ± 0.3 nmol (mg dry wt) $^{-1}$ after 2 h. When cells of *S. cerevisiae* were taken from and returned to minimal medium, the value of the GSH pool remained constant (Fig. 1a). The GSH pools determined by the procedure of Eyer & Podhradsky (1986) were very close to the values obtained by the HPLC procedure of Fahey (see Methods), indicating no interfering thiol in the enzyme method used routinely in this investigation.

As reported by several authors, the nitrogen status of a culture medium of yeast may influence the distribution

Fig. 1. Effect of nitrogen starvation on the total intracellular pool of GSH and enzymes implicated in GSH metabolism. (a) At time zero cells of $\Sigma 1278b$ were transferred into nitrogen-deficient SBM (●) or to minimal medium (○). (b) Effect of nitrogen starvation on the specific activities of γ -GCS (●), GSH synthase (○) and γ -GT (△).

Fig. 2. Effect of nitrogen starvation on the differential pools of GSH and arginine in the yeast strain $\Sigma 1278b$. (a) Relative distribution of GSH between the central vacuole (○) and the cytoplasm (●). □, Evolution of the total pool. (b) Relative distribution of arginine. ○, Central vacuole; ●, cytoplasm; □, evolution of the total pool.

of metabolites between the cytoplasm and central vacuole (Messenguy *et al.*, 1980; Kitamoto *et al.*, 1988). The effect of nitrogen deprivation on the repartition of GSH between the cytoplasm and the vacuole was thus examined.

Differential pool changes

As shown in Fig. 2(a), nitrogen starvation was associated with a shift of more than 90% of the GSH towards the vacuole. The subsequent consumption of GSH during nitrogen starvation was about 90% of the accumulated thiol tripeptide. Both cytoplasmic and vacuolar GSH decreased to stable values of around 1.3 nmol (mg dry wt)⁻¹.

The effect of nitrogen starvation on the arginine pool was also tested for comparative purposes. During nitrogen starvation, the total pool of arginine decreased in the first 2 h from 59.1 to 52.1 nmol (mg dry wt)⁻¹ and to 11.6 nmol (mg dry wt)⁻¹ after 20 h (Fig. 2b). As indicated above, about 95% of arginine was confined to the vacuolar space. In contrast to GSH, there was no repartition of arginine between the cytoplasmic and vacuolar compartments during nitrogen starvation. Arginine stored in the vacuole was apparently utilized for growth during nitrogen deprivation, as has also been shown for other *S. cerevisiae* strains (Kitamoto *et al.*, 1988).

Fig. 3. Effect of inhibition of GSH biosynthesis on the levels of GSH and γ -GT. (a) Effect of BSO. The arrow indicates introduction of BSO into SBM. ○, Pool of GSH; ●, specific activity of γ -GT. (b) The same experiment but with the mutant strain deficient in γ -GCS. ○, Pool of GSH; ●, specific activity of γ -GT.

Enzymes of GSH metabolism in relation to nitrogen starvation

Nitrogen starvation had no apparent effect on the specific activities of γ -GCS and GSH synthase, both enzymes implicated in the biosynthesis of GSH (Fig. 1b). In contrast, the specific activity of γ -GT increased under nitrogen deprivation from a value of 45 to 380 U (mg protein)⁻¹ after 3–4 h starvation. This observation is in accordance with previous results on the repressive effect of ammonium ion (Penninckx *et al.*, 1980) and GSH (Elskens *et al.*, 1991) on γ -GT. γ -GT is the first enzyme of the degradation pathway of GSH (Jaspers *et al.*, 1985; Jaspers & Penninckx, 1985), and is most probably responsible for the decrease in GSH (Figs 1 and 2). The effect of selective inhibition of GSH biosynthesis during nitrogen starvation was studied in order to evaluate the physiological implications of the transient accumulation of the thiol tripeptide.

Effects of the inhibition of GSH biosynthesis

Buthionine-(S,R)-sulphoximine (BSO), a specific transition-state inhibitor of γ -GCS (Griffith & Meister, 1979) lowered the level of GSH in *S. cerevisiae* (Elskens *et al.*, 1991). Cells growing in minimal medium

Fig. 4. Effect of nitrogen starvation on growth. Cells were grown initially in minimal medium in order to attain a dry weight of 0.5–0.6 mg ml⁻¹. At time zero the cells were transferred into SBM. Growth of Σ 1278b wild-type (●) and γ -GCS-deficient strain (○) were compared.

(0.1–0.15 mg dry wt ml⁻¹) were pretreated for 6 h with 1 mM BSO in order to attain a residual intracellular concentration of 1.0 nmol GSH (mg dry wt)⁻¹, and transferred to the nitrogen-deprived SBM supplemented with 1 mM BSO. As shown in Fig. 3(a), no transient stimulation in GSH biosynthesis was observed. At time zero of the transfer procedure, the specific activity of γ -GT attained 75 U (mg protein)⁻¹, coming from a basal value of 45 U, observed characteristically on the minimal medium (Penninckx *et al.*, 1980). This higher value reflected the enzyme derepression caused by the lowering of intracellular GSH (Elskens *et al.*, 1991). γ -GT attained a maximal value of 380 U (mg protein)⁻¹ after about 4 h nitrogen starvation (Fig. 3a). A very similar pattern was obtained with the *gshA-2* mutant strain defective in γ -GCS and having a residual GSH pool of 0.5 nmol (mg dry wt)⁻¹ (Fig. 3b). These experiments support the hypothesis that the pathway of GSH biosynthesis is responsible for the *de novo* tripeptide increase, and additionally that the repressive effects of GSH and ammonium ion are independent.

Effect of nitrogen starvation on cell growth

During nitrogen starvation, cells of *S. cerevisiae* increased threefold in dry weight (Fig. 4), which corroborated previous observations (Johnston *et al.*, 1977). In contrast, when GSH biosynthesis was blocked either by mutation or by BSO (not shown), the increase was only 40% of the initial value at time zero of starvation (Fig. 4). This last observation points to a major role for GSH in the supply of growth requirements during nitrogen starvation.

DISCUSSION

The response of GSH to starvation seems to depend on the nature of the nutrient deprivation. When the yeast was deprived of sulphate, γ -GT was derepressed twofold and 90% of the total GSH pool (cytoplasmic and

vacuolar) was consumed in about two cell generations (Elskens *et al.*, 1991). In contrast to the situation encountered in nitrogen deprivation, no transient stimulation of GSH biosynthesis and special migration to the vacuole was observed during sulphur starvation. The *de novo* biosynthesis of GSH observed after nitrogen starvation could be explained by the increase of the flux of the tripeptide towards the vacuole, causing a relief of the feedback inhibition of γ -GCS by GSH (Meister & Anderson, 1983; Elskens *et al.*, 1991).

The central vacuole in the yeast has been identified as an important storage area, and several transport systems of amino acids and ions using channels or pumps have been identified in this organelle (Garrill, 1994). Less information is, however, currently available concerning GSH transporters. A GSH transport system appearing under sulphur deficiency was detected several years ago in the fungus *Penicillium chrysogenum* (Hunter & Segel, 1971). A recent study has identified the *S. cerevisiae* gene *YCF1* as coding for a vacuolar glutathione S-conjugate transporter. The protein is a member of the ATP-binding cassette (ABC) family, has similarity to human multidrug resistance protein and was found to confer cadmium resistance on the yeast (Szczyepka *et al.*, 1994). Nothing is known about a possible relationship between the YCF1 protein and the putative GSH vacuolar transporter identified here. Moreover, the activity or/and biosynthesis of the putative GSH transporter would be sensitive to the ammonium ion, as for example observed for a plasma-membrane general amino acid permease (Grenson *et al.*, 1970). *S. cerevisiae* γ -GT, which appears mainly as a membrane-bound enzyme associated with the central vacuole (Jaspers & Penninckx, 1984), might function as an 'exit gate' for the GSH located inside the tonoplast in a manner similar to that illustrated in an early model of renal amino acid transport (Meister, 1973).

As has also been observed for mobilization of GSH as an endogenous source of cysteine (Elskens *et al.*, 1991), only about 90% of the GSH pool was consumed during nitrogen starvation. *S. cerevisiae* thus seems to have developed a general mechanism preventing complete depletion of GSH. A dramatic decrease of yeast viability was in fact observed when the GSH pool dropped below a critical level corresponding to 1% of the wild-type value (Kistler *et al.*, 1986).

Among the environmental parameters that commonly modulate the properties of microbial cells in nature, the concentration of macronutrients is of particular importance (Nyström, 1993). Micro-organisms are routinely exposed to macronutrient insufficiency (Morita, 1993). Previous investigations (Johnston *et al.*, 1977) suggested that the degradation of intracellular macromolecules (RNA and proteins) could supply most of the growth requirements of the yeast during nitrogen starvation. The conclusions of this investigation were based mainly on the fact that both RNA and proteins were extensively degraded during nitrogen starvation. Macronutrient limitation, in particular of nitrogen, is a

complex stress involving a global control system (Gottesman, 1984) where multiple unlinked genes are co-ordinately controlled by a common regulatory signal. As shown here, the vacuolar storage and further catabolism of GSH seems also to play an important and previously unsuspected role in the response of the yeast to the nitrogen stress.

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