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Role of glutathione in detoxification of metal(loid)s by *Saccharomyces cerevisiae*

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Abstract

Cellular glutathione (GSH) was implicated in tolerance to potentially toxic metal(loid)s using two strains of *Saccharomyces cerevisiae*, a wild-type (Σ1278b) and a GSH-deficient mutant strain (*gshA-2*). Both yeast strains exhibited no significant difference in tolerance to tellurite, zinc, cobalt, copper, manganese, nickel and chromate. There was no marked influence of glutathione on the accumulation of Te, Co, Cu, and Mn, although the absence of cellular glutathione significantly increased the cellular content of Zn and Ni, but greatly decreased Cr content without significant alteration of tolerance. These results indicated the independence of cellular glutathione activity from tolerance to Te, Zn, Co, Cu, Mn, Ni, and Cr. However, involvement of glutathione in Zn, Ni and Cr uptake is possible. The glutathione-deficient strain displayed a high sensitivity to selenite and cadmium in comparison to the wild-type strain of *S. cerevisiae.* The minimum inhibitory concentrations of Se and Cd for the glutathione-deficient strain were 980 ± 13 and 32 ±4 μM, respectively, whereas the wild strain tolerated up to 4080 ± 198 μM Se and 148 ± 5 μM Cd. A relationship between tolerance and reduced cellular content of both Se and Cd was also shown: the mutant strain accumulated approximately three-fold more Se and two-fold more Cd than that accumulated by the wild-type strain. This suggests an influence of GSH on cellular uptake of Se and Cd, and also directly confirms the protective action of such a cellular thiol compound against Se and Cd toxicity.

Introduction

Microbial detoxification of metal ions is achieved by several mechanisms including regulation of uptake, transformation into less toxic species and intracellular immobilization (White & Gadd 1987; Gadd 1993; Gharieb *et al.* 1995; Rama Rao *et al.* 1997; Gharieb & Gadd 1998). Inside cells, the major molecules implicated in sequestration of metal ions include the tripeptide glutathione (GSH; L-γ-Glu-Cys-Gly), phytochelatins (≡ cadystins(γ-Glu-Cys)$_n$Gly), and low molecular weight cysteine-rich proteins, metallothioneins (MT) (Rennenberg 1982; Mehra & Winge 1991; Kneer & Zenk 1992). In the plant *Rauvolfia serpentina*, the induction of phytochelatin synthesis by Cu^{2+}, Zn^{2+} Pb^{2+}, Cd^{2+} has been reported (Steffens 1990). This work found that Cd^{2+} entering cells of *R. serpentina* was totally complexed by phytochelatins, and a series of metal-sensitive plant enzymes tolerated Cd^{2+} when in the form of a phytochelatin complex. The yeasts *Schizosaccharomyces pombe* and *Candida glabrata* also responded to Cd and Cu in culture media by synthesizing γ-glutamyl cysteine-rich peptides, (Murasugi *et al.* 1981; Mehra & Winge 1991). GSH, a typical low molecular weight cellular thiol, forms complexes with the anions arsenate (AsO_4^{3-}) and selenite (SeO_3^{2-}) that have been shown to be inducers of phytochelatin synthesis (Grill *et al.* 1987). Moreover, it was postulated that glutathione (GSH) is a direct precursor for phytochelatin synthesis through γ-glutamyl-cysteine dipeptidyl transpeptidase that cata-

lyses the transfer of γ-glutamylcysteine from GSH to another molecule of GSH (Grill *et al.* 1987). GSH in *Saccharomyces cerevisiae* may account for 1% of the cell dry weight (Penninckx & Elskens 1993), where it functions as a storage form of endogenous sulphur and nitrogen (Elskens *et al.* 1991; Mehdi & Penninckx 1997) as well as having a role in protection against biocides and certain metal ions (Perrin & Watt 1971; Singhal *et al.* 1987; Rama Rao *et al.* 1997). The postulated defensive action of GSH against metal toxicity was primarily based on observed cellular accumulation in response to metal ions. The present study was undertaken to directly define the role of such a thiol compound in the detoxification of a variety of potentially toxic metal(loids).

Materials and methods

Yeast strains and cultural conditions

Two strains of *Saccharomyces cerevisiae*; Σ1278b (*MAT*a) and the glutathione (GSH)-deficient mutant *gshA-2* (obtained from Michel J. Penninckx, Université de Bruzelles, Brussels, Belgium) were routinely maintained and grown on MYGP solid medium of composition (g l^{-1}); malt extract (Lab M), 3; yeast extract (Difco), 3; bacteriological peptone (Oxoid), 5; D-glucose, 10 and agar (Lab M, No. 2), 15. For liquid culture, the yeast strains were grown in MYGP liquid medium of the same composition without the agar. For preparation of starter cultures, 50 ml of liquid medium was loop inoculated and incubated in an orbital shaker (100 rpm) at 25 °C for 48 h. The growing cells were then used to inoculate the experimental cultures containing 50 ml medium to an initial $OD_{550nm} \sim 0.1$.

Szybalski density gradient plates

Density gradient plates were made as described by Ramsay & Gadd (1997), modified from Bryson and Szybalski (1952) and Gadd *et al.* (1985). 60 cm^3 of molten MYGP agar was poured at ∼50 °C into a 10 × 10 cm Petri dish and allowed to set at an angle of 30°. After setting, 60 ml of metal-containing molten agar was then poured onto the set base to give a linear metal gradient across the plate surface (Gadd *et al.* 1985). The final concentrations of the metal(loid)s used were: Na_2SeO_3, 5.0 and 10.0 mM; $MnCl_2.4H_2O$, 10.0 mM; $CuCl_2.2H_2O$, 5.0 mM; $CdCl_2.H_2O$, 0.05 and 1.0 mM; $NiCl_2.6H_2O$, 2.5 mM; $ZnCl_2$, 5.0 mM; Na_2TeO_3, 2.5 mM; $CoCl_2.6H_2O$, 1.0 mM; K_2CrO_4, 1.0 mM. By using sterile cotton wool swabs, the plates were then inoculated with ∼50 μl from each experimental culture which was streaked along the metal gradient. Plates were then incubated at 25 °C for 5 d after which time the extent of growth along the metal concentration gradient was measured and used for the assessment of metal toxicity.

Measurement of accumulated metal and minimum inhibitory concentration (MIC)

After the incubation period, cells were collected from identical locations on the surface of the Szybalski gradient plates, where the minimum growth of either the wild-type or glutathione-deficient strains occurred, so that metal(loid) accumulation by both strains was measured after growth at an identical metal concentration. The cells were fixed by suspension in 5 ml 2.5% glutaraldehyde in 5 mM PIPES buffer, pH 6.5, and kept for 24 h at 4 °C. Metal(loid) ions loosely associated with the cell surface were removed by washing 3× with 10 ml 5 mM PIPES buffer, pH 6.5; cells were vortexed and separated by centrifugation (1200 *g*, 10 min) at each step. Cell numbers were determined using a modified Fuchs-Rosenthal haemocytometer after appropriate dilution with distilled water. For determination of accumulated metal(loid), 0.5 ml of 6 M HNO_3 was added to the washed pellet and incubated for 1 h at 90 °C after which 4.5 ml ddH_2O was added. The minimum inhibitory concentrations (MICs) of the metals being tested were measured by removing discs from the growing medium by means of a 1 cm diameter cork borer, and melting each disc in 9.0 ml dd H_2O, using a boiling water bath, for 15 min. Metal content of the digests was determined after appropriate dilution using a Pye Unicam SP9 atomic absorption spectrophotometer (AAS) and appropriate standard solutions.

Results

Assessment of metal(loid) toxicity towards wild-type and glutathione-deficient strains of S. cerevisiae

Density gradient plates (Bryson & Szybalski 1952; Gadd *et al.* 1985) were used to determine minimum inhibitory concentrations (MICs) of the studied metal(loid)s. In this assay, the glutathione-deficient mutant strain *gshA-2* showed no significant reduction in the MIC when compared with the parental control

Table 1. Toxicity of different metal(loids) towards *S. cerevisiae*; the wild-type strain (Σ1278b) and GSH-deficient mutant strain (*gshA-2*), assessed using Szybalski gradient plates. Values shown indicate the minimum inhibitory concentrations (MICs) expressed in μM and are means of 5 replicates ± SEM. Other experimental conditions as described in Materials and methods.

Metal(loid)	*S. cerevisiae* strain Σ1278b (*MAT*a)	*gshA-2*
Tellurium	584 ± 39.7	612 ± 25.8
Selenium	4080 ± 198	980 ± 103
Zinc	1928 ± 99.5	2224 ± 42.6
Cobalt	552 ± 53.9	524 ± 18.3
Copper	2952 ± 84	2848 ± 72.3
Cadmium	148 ± 4.9	32 ± 3.9
Manganese	1496 ± 13.3	1553 ± 46.6
Nickel	733.3 ± 17.6	806.7 ± 73.3
Chromium	452 ± 26.6	524 ± 69.6

strain Σ1278b when treated with Te, Zn, Co, Cu, Mn, Ni and Cr (Table 1). These results indicated that these metals did not exhibit increased toxicity towards the glutathione-deficient mutant strain in comparison to the parental strain. However, tolerance to Se and Cd was significantly different between the two yeast strains, and the MICs of Se and Cd for the parental strain of *S. cerevisiae* were respectively 4080 ± 198 and 148 ± 5 μM, while the growth of the glutathione-deficient strain was totally inhibited at Se and Cd concentrations of ≥ 980 ± 103 and ≥ 32 ± 4 μM, respectively.

Metal(loid) accumulation by wild-type and glutathione-deficient strains of S. cerevisiae

Variation in accumulation of toxic metal(loid)s between the two strains is shown in Figure 1. Te, Cu and Mn were accumulated similarly, while the glutathione-deficient strain accumulated Zn, Co, Ni and Cd approximately two-fold higher than the wild-type strain. The amount of Cd accumulated by the parental strain was 0.07 ± 0.01 nmol $(10^6$ cells$)^{-1}$ cells while the glutathione-deficient strain accumulated 1.5 ± 0.05 nmol $(10^6$ cells$)^{-1}$ cells. For Se, the glutathione-deficient strain showed the most marked accumulation (about three-fold) in comparison with its parental strain; the mutant strain accumulated 3.4 ± 0.2 nmol $(10^6$ cells$)^{-1}$ cells whereas the parental wild strain accumulated 9.5 ± 0.6 nmol $(10^6$ cells$)^{-1}$ cells. The mutant strain behaved conversely with Cr as it accumulated much less Cr than the wild-type strain.

Discussion

Glutathione participates in some significant cellular activities including the protection of cells against toxic metals. The results presented here have shown that the absence of glutathione in *S. cerevisiae* has no marked influence on tolerance towards Te, Zn, Co, Cu, Mn, Ni and Cr. This result suggests the independence of tolerance towards these metal(loids) from the presence of cellular glutathione. It is worth mentioning that during growth on medium-containing tellurite, both the wild-type and glutathione-defective strains produced a black colouration apparently to the same degree. Many bacteria and fungi have been shown to transform tellurite to both elemental and volatile methylated forms (Karlson & Frankenberger 1993). A *Fusarium* sp. and *Penicillium citrinum* tolerated high concentrations of tellurite and both fungal strains transformed tellurite to elemental tellurium, some deposited within vacuole(s) (Gharieb *et al.* 1999). Additionally, although extremely small amounts of Te were volatilized by the *Fusarium* sp., volatile tellurium species were not detected during growth of *P. citrinum.* We have previously shown that detoxification and accumulation of tellurium and chromium by *S. cerevisiae* were significantly influenced by the presence of a functionally active vacuole (Gharieb & Gadd 1998). Hexavalent chromium (chromate) reduction to the trivalent form has been suggested as a resistance mechanism in *Enterobacter cloacae* (Ohtake *et al.* 1990). On the other hand, chromate resistance in the yeasts *Candida* sp. and *Rhodosporium* sp. was attributed to reduced uptake of chromate rather than reduction of Cr(VI) to Cr(III) (Pepi & Baldi 1992). In the present study, it was shown that chromium accumulation by the wild-type strain was significantly higher than the glutathione-defective mutant despite both strains displaying no apparent difference in sensitivity. This could be due to the involvement of glutathione in the regulation of chromate uptake rather than detoxification. The same conclusion could apply for Zn and Ni where the wild-type strain accumulated about half the amount of metal accumulated by the mutant strain despite no difference in sensitivity.

Zn, Co, Mn and Ni were previously documented to be detoxified by mechanisms other than glutathione activity in *S. cerevisiae* such as compartmentation in

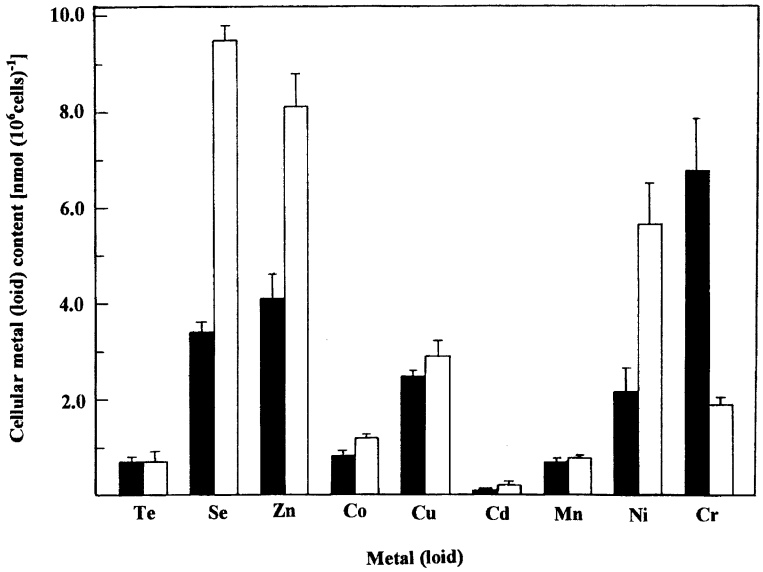

Figure 1. Cellular content of Te, Se, Zn, Co, Cu, Cd, Mn, Ni and Cr accumulated by the parental wild-type (Σ1278b (*MAT*a) (filled bars), and glutathione-deficient mutant (*gshA-2*) (clear bars) strains of *S. cerevisiae.* The yeast strains were inoculated on metal(loid)-containing Szybalski density gradient plates and incubated for 5 d at 25 °C as described in Methods. Cells were harvested from locations on the density gradient plate corresponding to the MIC values for the glutathione-deficient mutant strain. Determination of the metal content was carried out using atomic absorption spectrophotometry (AAS) after acid digestion of the yeast cells. Each bar represents the mean of five replicates ± SEM.

the vacuoles (White & Gadd 1986, 1987; Joho *et al.* 1995; Ramsay & Gadd 1997). Glutathione has been proposed to be involved in Zn resistance of *N. crassa* (Rama Rao *et al.* 1997), although, the present work confirms the lack of a relationship between glutathione and tolerance to Zn, Co, Mn and Ni. Although a Cu-glutathione complex has been previously reported in *S. cerevisiae* (Lin *et al.* 1993), the precise role of glutathione in copper resistance was not established. However, glutathione was implicated in Cu detoxification by *N. crassa* (Germann & Lerch 1987). The present study showed the independence of Cu accumulation and detoxification by *S. cerevisiae* from cellular glutathione availability. In fact, Cu resistance in *S. cerevisiae* is mediated by the induction of cytosolic low molecular weight cysteine-rich metallothionein (Gadd 1993; Kosman 1994).

The present investigation clearly indicates the importance of glutathione in Cd and Se accumulation and detoxification. A relationship between Cd uptake and sensitivity by metal-tolerant yeast mutants has previously been documented (Gadd & Mowll 1983). In another study, glutathione decreased Cd^{2+} toxicity and was also present at low levels in a Cd-tolerant strain of *Datura innoxia* compared with a sensitive strain (Delhaize *et al.* 1989). This was suggested to be due to depletion of glutathione pools for the synthesis of phytochelatins, for which glutathione is a precursor. *S. pombe* mutants that were unable to synthesize phytochelatins from γ-glutamyl-cysteine and glutathione, were found to be Cd-hypersensitive (Mutoh & Hayashi 1988). Singhal *et al.* 1987 reported that intracellular glutathione in mice functions in protection against Cd^{2+} toxicity, and that this tripeptide provides the first line of defence against Cd before induction of metallothionein synthesis occurs. It was suggested that glutathione protects cells, as does metallothionein, by complexation thus decreasing Cd^{2+} availability for toxic interactions, particularly inhibition of thiol-containing enzymes. Among a range of metals, Cd inhibited the enzyme glutathione peroxidase by competition with the donor substrate, glutathione (Splittgerber & Tappel 1979).

Fungi, as well as other microorganisms, tolerate selenium by different mechanisms including transformation of inorganic selenium compounds to methylated derivatives which are volatile (Brady *et al.* 1996; Morley *et al.* 1996), and reduction of selenate and selenite to elemental selenium (Se^0), a process which results in detoxification (Gharieb *et al.* 1995). In dialyzed cell-free extracts of *C. albicans* and *S. cerevisiae*, glucose-6-phosphate, triphosphopyridine nucleotide and oxidized glutathione restored enzymatic reduction of selenite (Nickerson & Falcone

1963). In *S. cerevisiae*, it was also postulated that selenite reacts with glutathione (GSH) through a GS-Se-SG intermediate to form Se^0 non-enzymatically (Dilworth & Bandurski 1977). The present study showed that the glutathione-lacking mutant was significantly more sensitive to selenite than the wild-type strain, and it was noticed that the red colouration characteristic of reduction of selenite to elemental selenium appeared darker in the wild-type strain than in the glutathione-deficient mutant strain. Additionally, the glutathione-deficient strain exhibited accumulation of Se three-fold higher than the wild-type strain which suggests the probable role of this thiol compound in the regulation of selenite uptake, as well as reflecting the relationship between selenium accumulation and toxicity in *S. cerevisiae* (Zhilsova *et al.* 1996; Gharieb & Gadd 1998). In conclusion, the present work presents direct evidence for the involvement of glutathione in Cd and Se detoxification, and its independence for tolerance of Te, Zn, Co, Cu, Mn, Ni and Cr in *S. cerevisiae.*

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