Bull. Environ. Contam. Toxicol. (2006) 77:351–358

DOI: 10.1007/s00128-006-1073-0

Environmental Contamination and Toxicology

Mercury and Organomercurial Resistance in *Rhodotorula rubra:* Activation of Glutathione Reductase

S. K. Ghosh,[1] S. Ghosh,[1] R. Gachhui,[2] A. Mandal[1]

[1] Department of Biochemistry, University College of Science, Calcutta University, 35, Ballygunge Circular Road, Kolkata 700019, India
[2] Department of Life Science and Biotechnology, Jadavpur University, Jadavpur, Kolkata 700032, India

Received: 11 May 2006/Accepted: 25 July 2006

It is well known that microorganisms accumulate environmental mercury. Plasmid-mediated mercury and organomercurial resistance in prokaryotes is well documented. However, very little information regarding mercury resistance in unicellular eukaryotes is known so far. For better understanding of mercury resistance and biological defense mechanism in yeast, studies on cellular and subcellular levels are most essential. Previous report on *Saccharomyces cerevisiae* isolated from Bakers' yeast preparation showed the presence of Hg^{2+} localized in cell-wall fraction and significant amount of mercury was present in cytoplasm (Murray and Kidby 1975). Other workers also observed that Hg^{2+}-resistant mutant of *Saccharomyces cerevisiae* had taken up Hg^{2+} as much as or slightly more than the parental strains did and the cell wall of the mutants retained a large portion of cell-associated Hg^{2+} compared to the parental strain (Ono et al. 1988).

Ionic mercury is highly toxic to all living cells. There are reports that Hg^{2+} ions interact most effectively with thiol groups of proteins, coenzymes, amino acids such as tryptophan and phenyl alanine (Vallee and Ulmer 1972). In heavy metal toxicity cellular redox thiol pool plays an important role in protecting the prokaryotic and eukaryotic cells in metal toxicity (Boyland and Chasseaud 1969; Gachhui et al. 1991; Lash and Zalups 1996; Miszta 1984; Pesti et al. 2002). However, there is no detailed report on the status of the glutathione reductase and the cellular thiol status of the mercury resistant yeast cells. We have been studying the mercury resistance and biological defense against mercury and organomercurials by using *Rhodotorula rubra* isolated from rotten fruits. It has been found that highly Hg^{2+}-resistant *Rhodotorula rubra* took up lesser Hg^{2+} than do weakly Hg^{2+}-resistant *Rhodotorula rubra* (Ghosh et al. 2004). Thus it would be important to explore the mechanism of tolerance in this highly mercury resistant yeast strain. In this paper, we show the presence of significant amount of mercury in the cytosol in case of mercury and organomercurial resistant yeast strains. To investigate the mechanism of cellular protection against this toxic Hg^{2+}, we have determined the effect of cytosolic mercury on the glutathione levels as well as the glutathione reductase activity of highly Hg^{2+}-resistant and weakly Hg^{2+}-resistant yeast strains for the defense of mercury toxicity.

Correspondence to: A. Mandal

MATERIALS AND METHODS

All chemicals and reagents used in the present study were of analytical grade (E. Merck, Germany and British Drug House, UK). All mercury compounds were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

A highly mercury-resistant yeast strain and a weakly mercury-resistant yeast strain, which were identified as *Rhodotorula rubra*, were used for these studies. These microorganisms were isolated from rotten guavas.

To estimate mercury in cytosol in highly Hg^{2+} -resistant yeast strain and weakly Hg^{2+} -resistant yeast strain, the organisms were separately grown overnight in YPD medium on a rotary shaker (200 rpm) at 30°C. After 12 hr the culture was diluted 1 : 10 times with sterile YPD broth to a final volume 200 mL and 3.33 mg of $HgCl_2$, 0.42 mg of phenylmercuric acetate (PMA) or 0.42 mg of thimerosal separately were added. The organism was allowed to grow for 24 hr on a rotary shaker (200 rpm) at 30°C. Then the cells were harvested by centrifugation at 2500 × g for 5 min at 0 - 4°C and washed three times with cold 50 mM phosphate buffer (pH 7.35). The cells were then suitably diluted with phosphate buffer (pH 7.35) to get a cell suspension of OD_{600} = 6.0. The cell suspension was used for the following experiments:

10.0 mL cell suspension (OD_{600} = 6.0) was taken to account for the total mercury bound by the whole cells. Cells were harvested by centrifugation at 2500 × g for 5 min at 4°C, washed twice with cold phosphate buffer (pH 7.35). The cells were then taken in 100 mL volumetric flask and the mercury content of cells was measured by cold vapor atomic absorption spectrometric technique (Ghosh et al. 2004; Hatch and Welland 1968) using Mercury Analyzer [MA 5800D manufactured by Electronic Corporation of India Ltd. (ECIL), Hyderabad] that can measure 20-200 ng of mercury present in the sample.

10.0 mL cell suspension (OD_{600} = 6.0) was taken to estimate mercury present in cell-free extracts of yeast cells. The cells were harvested by centrifugation at 2500 × g for 5 min at 4°C. The pellets were washed twice with cold 50 mM phosphate buffer (pH 7.35) and weighed. Cells were stored at -20°C for 24 hr. The cells were then disrupted mechanically with seasand at 4°C and the disrupted cells were suspended in same cold phosphate buffer and centrifuged at 12.5 × 1000 rpm for 10 min at 0 - 4°C. Seasand along with cell debris was washed three times with cold phosphate buffer (pH 7.35). Cell debris and supernatants after centrifugation were placed in 100 mL volumetric flasks separately and mercury content in cell debris and cell-free extracts were measured by cold vapor atomic absorption spectrometric technique (Ghosh et al. 2004; Hatch and Welland 1968) using Mercury Analyzer (MA 5800D).

In a typical experiment 20 mL of overnight grown yeast culture were added to the 500 mL conical flask containing sterile liquid YPD medium to make the final

volume to 200 mL 3.33 mg of $HgCl_2$ were added to the different flasks so that each flask contained similar concentration (16.8 µg/mL) of $HgCl_2$. The organisms were grown under shaking conditions (130 rpm) at 30°C for 2 hr, 10 hr and 24 hr respectively. Control flasks containing organisms only and no $HgCl_2$ were also shaken similarly. Similar experiments were also done using subinhibitory doses (2.1 µg/mL) of PMA and thimerosal in separate flasks. After incubation for 2, 10 and 24 hr yeast cells were harvested by centrifugation at 2500 × g for 5 min at 0 - 4°C. Cell pellets were washed three times with cold 50 mM sodium phosphate buffer (pH 7.35) containing 0.2 mM EDTA. Cells were disrupted mechanically with sea-sand at 4°C. Disrupted cells were suspended in the same cold buffer and centrifuged at 12.5 × 1000 rpm for 10 min at 4°C. Levels of glutathione reductase (GR) and reduced glutathione (GSH) in cell-free extracts of yeast strains were measured.

The cell-free extract was used to assay GR activity spectrophotometrically at 340 nm as described (Carlberg and Mannervik 1985). The assay mixture contained 1 mM EDTA, 0.1 mM NADPH, 0.1 M potassium phosphate buffer (pH 7.0), 1 mM GSSG (oxidized glutathione) and suitable volumes (10-100 µL) of cell-free extracts to follow the reaction kinetics uniformly for 5 min in a total volume of 1 mL. One unit of GR activity is defined as the amount of enzyme protein in mg that oxidized 1 nmole of NADPH per min.

Cellular reduced glutathione concentration was determined as described (Berendji et al. 1999). 150 µL of cell-free extract was mixed with equal volume of 2 M $HClO_4$ containing 2 mM ethylenediaminetetracetate (EDTA) and kept in ice-bath for 15 min. The suspension was then centrifuged at 5000 × g for 5 min and deproteinized supernatant was neutralized with a solution containing 2 M KOH and 0.3 M MOPS (3 -Morpholinopropane sulphonic acid). After centrifugation at 5000 × g for 5 min, the supernatant was used for the biochemical quantification of GSH by adding equal volumes of DTNB (5,5'-Dithio-bis (2 -nitrobenzoic acid) solution (6 mM DTNB dissolved in 100 mM Na_2HPO_4 per 1 mM EDTA, pH 7.5). GSH concentration was quantitatively measured spectrophotometrically at 414 nm with GSH as standard and was expressed per mg protein.

The protein content of the cell-free extracts were determined following the method of Bradford (Bradford 1976) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Our isolated yeast strains of *Rhodotorula rubra* GVa_5 was highly resistant to $HgCl_2$ (MIC value of 400 nmole per 0.05 mL) but was slightly resistant to PMA and thimerosal (MIC value of 5 nmole per 0.05 mL and 6.25 nmole per 0.05 mL respectively) compared to the sensitive strain. Sensitive strains showed MIC value of 12.5 nmole per 0.05 mL against $HgCl_2$. They were also sensitive towards organomercurial compounds. Among the mercury resistant isolates, *Rhodotorula rubra* App_2 also was slightly resistant to Hg^{2+} (MIC value of 50 nmole per

Table 1. Estimation of mercury in cell-free extracts of Hg^{2+}-resistant yeast strains grown in YPD liquid medium in the presence of mercury and organomercurial compounds.

Yeast strain	Mercury compound	Total mercury bound by cells (μg)	Mercury content	
			Cell-free extract (μg)	Cell-debris (μg)
Rhodotorula rubra App_2	$HgCl_2$	42.1 ± 1.8	4.4 ± 0.3	35.4 ± 2.1
	PMA	15.9 ± 1.2	2.8 ± 0.2	12.3 ± 0.9
	Thimerosal	13.9 ± 1.1	1.5 ± 0.1	11.8 ± 0.8
Rhodotorula rubra Gva_5	$HgCl_2$	24.4 ± 1.4	2.5 ± 0.2	20.8 ± 1.5
	PMA	16.7 ± 1.2	3.1 ± 0.1	13.0 ± 0.9
	Thimerosal	13.5 ± 1.0	1.6 ± 0.1	11.4 ± 0.8

Values represent the mean ± SD for six determinations.

0.05 mL) and they showed resistance pattern towards PMA (MIC value of 3 nmole per 0.05 mL) as well as thimerosal (6.25 nmole per 0.05 mL respectively). As *Rhodotorula rubra* GVa_5 showed high resistance pattern towards this toxic metal ion, we were interested to see the Hg^{2+} content in the cell-free extract of the strain. We also determined the same in case of low resistant strain *Rhodotorula rubra* App_2. Our experimental data (Table 1) showed the presence of significant amount of Hg^{2+} in the cell-free extracts of yeast strains resistant to high and low levels of $HgCl_2$, which indicated the penetration of significant quantity of mercury to the cytoplasm. Total mercury bound by low resistant strain App_2 was also high than the high resistant strain GVa_5. Previous report showed that mercury was associated with both the cell wall and vacuoles in the cytoplasm (Brunker and Bott 1974). Other workers also reported the penetration of mercury to the cytoplasm of yeast strain *Saccharomyces cerevisiae* grown in the presence of $HgCl_2$ (Murray and Kidby 1975). Cell-free extracts of thimerosal-treated cells was found to contain substantial amount of mercury, which was still slightly lower than mercury in cytosol of PMA-treated cells. This may be due to higher lipid solubility of PMA across the cell membrane than thimerosal so that higher concentration of PMA in the cytosol will be more damaging to cellular metabolism of yeast cells than thimerosal. In bacterial system very low concentrations of mercury compounds can readily inhibit microbial growth (Smith 1967) and this inhibition was more pronounced with organomercurial compounds such as PMA, thimerosal, MMC etc because of their high lipid solubility through cytoplasmic membranes (Summers and Silver 1978).

Table 2. Effect of mercury compounds on the specific activity of glutathione reductase and GSH level of highly Hg^{2+}-resistant yeast strain *Rhodotorula rubra* GVa_5 and weakly Hg^{2+}-resistant yeast strain *Rhodotorula rubra* App_2

Strain	Experimental sets	Glutathione reductase (nmole/ min/ mg of protein)	Reduced glutathione (nmole/mg of protein)
Rhodotorula rubra GVa_5	Control	78.8 ± 2.8	72.5 ± 3.5
	$HgCl_2$	108.8 ± 4.1	130.4 ± 6.1
	PMA	80.1 ± 2.4	74.2 ± 4.4
	Thimerosal	82.6 ± 3.9	76.0 ± 3.7
Rhodotorula rubra App_2	Control	88.2 ± 4.9	77.2 ± 3.4
	$HgCl_2$	100.6 ± 4.7	105.1 ± 4.1

Values are mean ± SD of four separate determinations.

Table 2 shows the level of glutathione reductase (GR) and GSH contents in highly Hg^{2+}-resistant yeast strain GVa_5 and weakly Hg^{2+}-resistant yeast strain App_2 grown in the presence of 16.8 µg/mL of $HgCl_2$, 2.1 µg/mL of PMA or 2.1 µg/mL of thimerosal for 24 hr. Levels of glutathione reductase in GVa_5 strain in the presence and absence of PMA or thimerosal were almost the same (78.8 ± 2.8 to 82.6 ± 3.9 nmole/min/mg of protein) and reduced glutathione contents also were

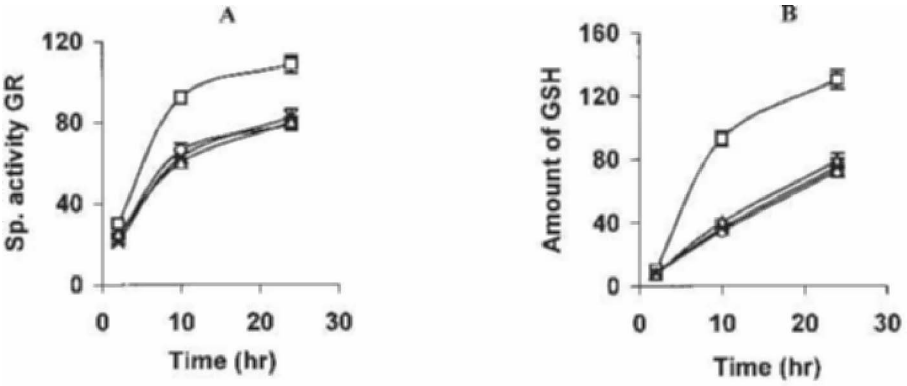

Figure 1. Specific activity of glutathione reductase (A) and GSH level (B) of highly Hg^{2+}-resistant yeast strain *Rhodotorula rubra* GVa_5 after different hours of growth at 30^0C in YPD liquid medium supplemented without (control) (o) or with 16.8 µg/mL of mercuric chloride (□), 2.1 µg/mL of thimerosal (x) or 2.1 µg/mL of PMA (Δ). The results represent mean ± SD of four separate determinations in each case.

almost the same (72.5 ± 3.5 to 76.0 ± 3.7 nmole/mg of protein). But when the mercury-resistant yeast strain GVa_5 was treated with $HgCl_2$, GR activity increased by almost 38.1% over the control level and reduced glutathione (GSH) level also increased by 79.9% with respect to control level. On the other hand it is evident that for weakly Hg^{2+}-resistant yeast strain App_2, GR level in $HgCl_2$-treated cells increased by 14.1% only over control and reduced glutathione contents increased by about 36.1% with respect to control (Table 2). Change of specific activity of glutathione reductase and intracellular GSH contents of GVa_5 strain in the presence of mercury and organomercurials with different times of incubation have been represented in Fig. 1A and Fig. 1B. Specific activity of glutathione reductase of $HgCl_2$ -treated cells increased steeply up to 8-10 hr of incubation over control cells whereas the pattern of increase of specific activities of glutathione reductase was similar in case of thimerosal and PMA -treated cells and untreated cells. After 10 hr of incubation, GR activity of $HgCl_2$-treated cells increased to about 39.9% over the control level (Fig.1A). Reduced glutathione contents of $HgCl_2$ -treated cells of GVa_5 strain also increased steeply up to 8-10 hr of incubation and then the increase was very slow up to 24 hr whereas for thimerosal and PMA -treated cells of GVa_5 strain reduced glutathione level was similar to that of the control cells during the same period (Fig. 1B). These observations indicated that GR and GSH play very important role in detoxification of Hg^{2+} during the log phase of growth of the organism and probably the thimerosal -treated and PMA -treated yeast strains could not synthesize the required level of GSH as well as the glutathione reductase. But in case of weakly Hg^{2+}-resistant yeast strain glutathione reductase activity and GSH level also increased slightly over control levels at stationary phase of growth. No significant change in GR activity and intracellular GSH concentration compared to control levels was observed at 10 hr

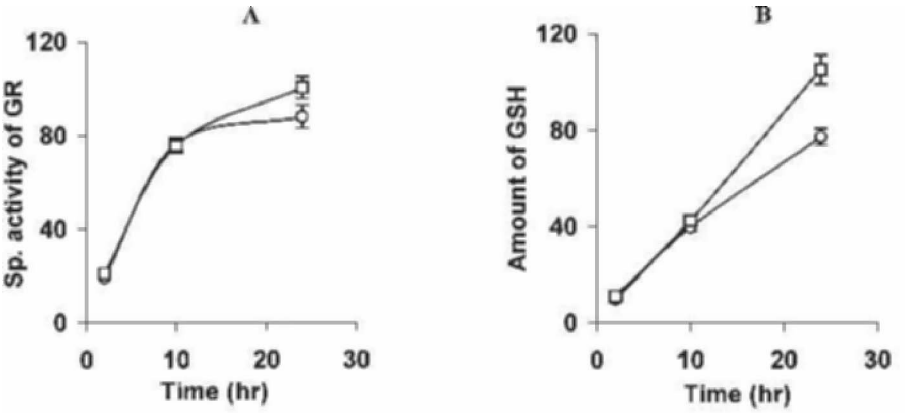

Figure 2. Specific activity of glutathione reductase (A) and GSH level (B) of weakly Hg^{2+}-resistant yeast strain *Rhodotorula rubra* App_2 after different hours of growth at 30^0C in YPD liquid medium supplemented without (control) (o) or with 16.8 µg/mL of mercuric chloride (□). The results represent mean ± SD of four separate determinations in each case.

of incubation (Fig. 2A and B). This data indicate that probably yeast cells are incapable of stimulating GR activity and the level of GSH in the presence of $HgCl_2$ after 10 hr of incubation. Perhaps weakly Hg^{2+}-resistant yeast strains take very long lag period of growth extending to 8-10 hr as a preparative measure for the synthesis of glutathione reductase activity slowly. Thereafter the cells become capable of growing normally and detoxifying the Hg^{2+}-ion toxicity by increasing GSH level and GR activity. GR activity and intracellular GSH concentration of GVa_5 strain was almost same as that of control level when the cells were exposed to PMA and thimerosal. This data could be correlated with the lower resistance pattern of Gva_5 towards organomercurials. Increase in GR activity and GSH level in highly mercury resistant strain Gva_5 are consistent with the observation that *in vivo* exposure to a subtoxic dose of Hg^{2+} is also associated with induction of GSH synthesis and also other key cellular enzymes like glutathione disulfide reductase (GSSG) (Lash and Zalups 1996; Summers and Silver 1978).

Thus from the experimental results it can be concluded that yeast cells may get protection against mercury stress by using cellular thiol pool. As soon as highly mercury-resistant yeast cells are exposed to $HgCl_2$, immediately these cells start synthesizing higher level of reduced glutathione along with the production of GSSG reductase enzyme and the cells are protected from Hg^{2+}-toxicity. As GSH is the most abundant nonprotein thiol compound in all living cell (Boyland and Chasseaud 1969), so it may be directly involved in the detoxification of Hg^{2+} and maintaining the metal homeostasis.

REFERENCES

Berendji D, Kolb-Bachofen V, Meyer KL, Kröncke KD (1999) Influence of nitric oxide on the intracellular reduced glutathione pool: Different cellular capacities and strategies to encounter nitric oxide-mediated stress. Free Rad Biol Med 27: 773-780

Boyland E, Chasseaud LF (1969) The role of glutathione and glutathione S-transferase in mercapturic acid biosynthesis. In: Nord FF (ed) Advances in Enzymology. Interscience Publishers, NY 32: 172-219

Bradford M (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248

Brunker RL, Bott TL (1974) Reduction of mercury to the elemental state by a yeast. J Appl Microbiol 27: 870-873

Carlberg I, Mannervik B (1985) Glutathione reductase. In: Methods in Enzymology, Meister A ed, Acad Press, Inc. Vol. 113 pp. 484-485

Gachhui R, Pahan K, Ray S, Chaudhuri J, Mandal A (1991) Cell free glutathione synthesizing activity of mercury resistant bacteria. Bull Environ Contam Toxicol 46: 336-342

Ghosh SK, Ghosh S, Chaudhury J, Gachhui R, Mandal A (2004) Studies on mercury resistance in yeasts isolated from natural sources. Bull Environ Contam Toxicol 72: 21-28

Hatch WR, Welland L (1968) Determination of submicrogram quantities of mercury by atomic absorption spectrometry. Anal Chem 40: 2085-2089

Lash LH, Zalups RK (1996) Alterations in renal cellular glutathoine metabolism after in vivo administration of a subtoxic dose of mercuric chloride. J Biochem Toxicol 11: 1-9

Miszta H (1984) Mercury and the activity of erythrocyte and bone marrow glutathione reductase (E.C.1.6.4.2.) and glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) in rats. Folia Haematol Int Mag Klin Morphol Blutforsch 111: 638-644

Murray AD, Kidby DK (1975) Sub-cellular location of mercury in yeast grown in the presence of mercuric chloride. J Gen Microbiol 86: 66-74

Ono B, Ohue H, Ishihara F (1988) Role of cell wall in *Saccharomyces cerevisiae* mutants resistant to Hg^{2+}. J Bacteriol 170: 5877-5882

Pesti M, Gazdag Z, Emri T, Farkas N, Koosz Z, Belagyi J, Pocsi I (2002) Chromate sensitivity in fission yeast is caused by increased glutathione reductase activity and peroxide overproduction. J Basic Microbiol 42: 408-419

Smith DH (1967) R-factors mediate resistance to mercury, nickel and cobalt. Science 156: 101-103

Summers AO, Silver S (1978) Microbiol transformation of metals. Ann Rev Microbiol 32: 637-672

Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium and lead. Ann Rev Biochem 41: 94-102