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Cell Free Glutathione Synthesizing Activity of Mercury Resistant Bacteria

Ratan Gachhui, Kalipada Pahan, Satyajit Ray, Jayasri Chaudhuri, and Amalendu Mandal

Department of Biochemistry, University College of Science, 35 Ballygunge Circular Road, Calcutta 700 019, India

Reduced glutathione (GSH) is present in all living cells and is known to have a generalized role in protecting the cells from heavy metal toxicity (Boyland and Chasseaud 1969). Depletion of both GSH and glutathione reductase (GR) level upon treatment with mercuric chloride ($HgCl_2$) is reported in various organs of rat (Addya *et al.* 1984). However, the effect of $HgCl_2$ on glutathione level in bacterial system is not known. Mercury resistant bacteria normally possess enzymes for detoxification of mercury compounds and all of them require a thiol compound for the activity of both mercuric reductase (MR) and organomercurial lyase in *in vitro* systems (Schottel 1978; Summers and Silver 1978). It has already been shown in our laboratory (Pahan *et al.* 1990) that GSH is the most effective thiol compound amongst GSH, sodiumthioglycolate, β-mercaptoethanol, cysteine and dithiothreitol in *in vitro* assay of mercuric reductases isolated from 10 strains, each of a different genus.

In the present communication, we report the results of our investigation on the glutathione status in mercury resistant bacterial cells exposed to $HgCl_2$.

MATERIALS AND METHODS

Chemicals prepared by E. Merck, Germany and Sigma Chemicals Co., USA, were used in our experiments. Mercury resistant bacteria were all isolated from soil and were identified in our laboratory (Gachhui *et al.* 1989). A sensitive strain P2 of the mercury resistant *Flavobacterium* sp strain PR2 was developed by 13 consecutive subculturings in mercury free medium. A laboratory strain of *Escherichia coli* K12 was used as a standard mercury sensitive bacteria.

Minimal inhibitory concentrations (MIC) of $HgCl_2$ on the bacterial strains were determined by agar-disc method following Schottel *et al.* (1974). Bacteria resistant to 12.5 nmols of $HgCl_2$ per disc (4 mm diameter) were considered as mercury resistant (Smith 1967).

Send correspondence/reprint requests to Dr. Amalendu Mandal at the above address.

In the first set of experiment, bacterial cells were grown in mercury free medium while in the second set, cells were induced with $HgCl_2$ for 20 hr so as to produce optimum levels of mercuric reductase. Inocula prepared from cells preexposed to 10 µM $HgCl_2$ were diluted ten fold with sterile medium, and concentration of $HgCl_2$ was maintained at 10 µM. A third addition of 10 µM $HgCl_2$ was made at the early log phase. In both the experimental sets, cells were grown aerobically in nutrient broth at 32°C on a rotary shaker (200 rpm) and harvested at the late log phase by centrifugation at 5,000 x g for 10 min at -4°C. Levels of mercuric reductase and glutathione reductase enzymes, reduced and oxidized glutathione in the cell free extracts of bacterial strains were measured. Net *de novo* synthesis of GSH by the cell free extracts was also determined. Glutathione reductase (GR) assay. Cell pellets were washed three times with cold 50 mM sodium phosphate buffer (pH 7.35) containing 0.2 mM Na_2 EDTA. Cells were disrupted mechanically with sea-sand at 4°C and suspended in the same buffer and centrifuged at 15,000 x g for 30 min at -4°C. The supernatant was used to assay GR activity spectrophotometrically at 340 nm (Pinto and Bartley 1969). The assay mixture contained 2 mM Na_2 EDTA, 0.15 mM NADPH, 0.2 M sodium phosphate buffer (pH 7.4), 2.5 mM GSSG and suitable volumes (10-100 µL) of cell free extracts to follow the reaction kinetics uniformly for 10 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 nmol of NADPH per min.

Mercuric reductase (MR) assay. The cell free extract was prepared as above. 55% of the MR activity in the supernatant was precipitated with 30-50% saturation of $(NH_4)_2SO_4$ at 4°C and the precipitate was dissolved in minimum volume of 50 mM sodium phosphate buffer (pH 7.35) containing 0.2 mM Na_2 EDTA and then dialysed against the same buffer at 4°C for 4 hr. The dialysates were used to assay MR activity spectrophotometrically at 340 nm (Komura *et al.* 1971). The assay mixture contained 5 mM Na_2 EDTA, 2 mM $MgCl_2$, 1 mM GSH, 40 mM sodium phosphate buffer (pH 7.35), 0.15 mM NADPH, 30 µM $HgCl_2$ and suitable volumes (10-100 µL) of dialysates to follow the reaction kinetics uniformly for 5 min in a total volume of 1 mL. One unit of MR activity is defined as the amount of enzyme protein in mg that oxidized 1 µmol of NADPH per min.

Cell free extracts of bacteria, for the estimation of both reduced and oxidized glutathione and *de novo* GSH synthesis, were prepared following the procedure of Samuels (1953). Cell pellets were washed three times with cold 1 mM sodium phosphate buffer (pH 7.0) containing 1 mM cysteine. Cell free extracts were obtained by disrupting the wet cells mechanically with sea-sand at 4°C. The mixture was treated with the same cold buffer and centrifuged at 15,000 x g for 30 min at

-4°C. Aliquots of the supernatant, for GSH and GSSG estimation, was immediately deproteinized by acidification with ice-cold 5% sulfosalicylic acid while the cell free extracts were directly used for the *de novo* GSH synthesis. The incubation mixture contained 1 mL extract, 80 mM sodium phosphate buffer (pH 7.5), 10 mM sodium glutamate, 10 mM glycine, 10 mM cysteine, 0.1 M glucose, 20 µM NAD^+ and 2 mM ATP in a total volume of 3 mL. An aliquot of the incubation mixture was taken out at zero time and the reaction was stopped immediately by acidification with ice-cold 5% sulfosalicylic acid. Another aliquot was taken out after incubation at 37°C for 1 hr and the reaction was stopped in a similar way. GSH was measured in those acidified, protein free, samples by the method of Ball (1966) using Ellman's reagent and glyoxylic acid. The difference between the final (1 hr) and initial (0 hr) values of GSH was the measure of net *de novo* synthesis of GSH by the extract.

Estimation of GSSG was done spectrophotometrically at 340 nm following the method of Pinto and Bartley (1969). The assay mixture contained 2 mM Na_2 EDTA, 0.15 mM NADPH, 0.1 M sodium phosphate buffer (pH 7.4), 10-50 µL of acidified protein free samples and 20 µL of yeast GR (from Sigma, Type III, diluted four fold) in a total volume of 1 mL.

The protein content of the extracts were determined following the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Flavobacterium sp PR2, *Bacillus* sp LR13, *Enterobacter* sp MR4 and *Pseudomonas* sp FR16 were highly resistant to mercury as they had high MIC values of $HgCl_2$, whereas *E. coli* K12 and *Flavobacterium* sp P2 were sensitive to $HgCl_2$. When all the bacteria were grown in the absence of $HgCl_2$, none of them showed any MR activity in the cell free extracts. Levels of GR in all these bacterial strains were almost the same (4.4 to 5.1 U/mg protein). GSH contents were found to be different in different bacterial strains (in the range of 30-62 µg GSH/mg protein) and GSSG contents of these bacterial strains were about 12% of their respective GSH content. For the uninduced bacteria, net *de novo* synthesis of GSH per hr by the cell free extracts at 37°C were almost the same (48-53 µg/mg protein) except for the strain FR16 (34 µg/mg protein).

When the mercury resistant bacterial strains were pre-exposed to $HgCl_2$, MR enzymes, which are usually inducible in nature (Summers and Silver 1978), in these organisms were induced and levels of MR followed MIC values of $HgCl_2$ against these bacterial strains (Table 1 and Table 2). It is to be noted that mercury resistant bacteria volatilized 65% (by the strain FR16) to 98% (by the strain PR2) of total HgCl from the medium

Table 1. Glutathione status of mercury sensitive and mercury resistant bacterial strains grown in mercury free media.

Organism Strain No.	MIC of $HgCl_2$ (nmols/ disc)	Glutathione reductase (GR) (nmoles of NADPH oxidized/mg protein/min)	Oxidized glutathione (GSSG) (μg/mg protein)	Reduced glutathione (GSH) (μg/mg protein)	GSH synthesized (μg/mg protein) by the extracts[a] at 37°C, per hr
Escherichia coli K12	12.5	4.4 ± 0.3	5.14 ± 0.30	62 ± 3	50 ± 3
Flavobacterium sp P2	12.5	5.1 ± 0.3	4.78 ± 0.21	52 ± 3	53 ± 3
Flavobacterium sp PR2	400	4.9 ± 0.3	4.33 ± 0.18	42 ± 2	48 ± 3
Bacillus sp LR13	250	4.7 ± 0.2	4.17 ± 0.25	40 ± 2	50 ± 3
Enterobacter sp MR4	150	4.7 ± 0.2	3.96 ± 0.16	32 ± 1	48 ± 3
Pseudomonas sp FR16	100	4.6 ± 0.2	4.58 ± 0.29	30 ± 2	34 ± 1

a. Values are mean ± SD of 6 experiments.

Table 2. Glutathione status of mercury resistant bacteria in $HgCl_2$-containing media.

Organism Strain No.	Mercuric reductase (MR) (μ moles of NADPH oxidized /mg protein/ min)[a]	Glutathione reductase (GR) (nmols of NADPH oxidized /mg protein/ min)[a]	Oxidized glutathione (GSSG) (μg/mg protein)[a]	Reduced glutathione (GSH) (μg/mg protein)[a]	GSH synthesized (μg/mg protein) by the extracts[a] at 37°C, per hr
Flavobacterium sp PR2	0.25 ± 0.01	10.2 ± 0.6	0.33 ± 0.02	26 ± 1	79 ± 5
Bacillus sp LR13	0.10 ± 0.006	8.9 ± 0.5	0.35 ± 0.03	25 ± 1	73 ± 4
Enterobacter sp MR4	0.06 ± 0.003	9.3 ± 0.5	0.30 ± 0.02	25 ± 1	70 ± 4
Pseudomonas sp FR16	0.05 ± 0.002	9.7 ± 0.5	0.19 ± 0.01	22 ± 1	50 ± 3

a. Values are mean ± SD of 6 experiments.
Mean values are significantly different from control set (Table 1).
$p << 0.001$.

where abiological volatilization of mercury was only 10% (Gachhui *et al.* 1989). However, mercuery sensitive bacterial strains, *E. coli* K12 and *Flavobacterium* sp P2, did not grow in the presence of $HgCl_2$. Comparing the data in Table 1 and Table 2 we found that when the mercury resistant bacterial strains were treated with $HgCl_2$ GR activity increased by almost 100% over the control level whereas GSH level decreased by 20 to 40%. But the rate of GSH synthesis was markedly enhanced (by 45-65%) and GSSG level in these $HgCl_2$ treated cells became very low about 1% of the GSH content.

From our data we can conclude that perhaps higher amount of GSH is necessary for optimization of the activity of MR for which the substrate, as suggested by Summers and Silver (1978) is thiol or dithiol adduct rather than the free ions of mercury. Nevertheless, we found lower levels of GSH in the $HgCl_2$ treated cells as $HgCl_2$ was constantly present in the medium during growth. As GSH is the most abundant non-protein-thiol compound present in all living cells (Boyland and Chasseaud 1969) so it may be directly involved in the detoxification of mercury compounds in organisms showing very high degree of resistance towards $HgCl_2$.

It is also probable that mercury resistant bacteria could survive in the presence of toxic levels of $HgCl_2$ because they could synthesize the required level of GSH along with the production of mercury detoxifying enzymes.

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