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Effect of Thiol Compounds and Flavins on Mercury and Organomercurial Degrading Enzymes in Mercury Resistant Aquatic Bacteria

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Plasmid-determined mercuric and organomercurial resistance in microorganisms has been studied by several workers (Summers and Silver 1972; Schottel *et al.* 1974; Lomovskaya *et al.* 1986; Nakamura *et al.* 1986). Mercury reductase, catalysing the reduction of Hg^{++} to Hg°, depends on sulfhydryl compounds (Summers and Sugarman 1974). Organomercurial lyase that catalyses the splitting of C-Hg linkages also needs thiol compounds for its activity (Schottel 1978). It is reported that thiol specificities of Hg-reductase and organomercurial lyase from a particular bacterial species are different (Izaki *et al.* 1974; Schottel 1978).

Until recently, no study has been reported on thiol specificity of these enzymes from various sources. In the present study, we report on enzymatic volatilisation of $HgCl_2$ by fourteen Hg--resistant bacterial strains (*Klebsiella*, *Streptococcus*, *Micrococcus*, *Alcaligenes*, *Bacillus*, *Proteus*, *Planococcus*, *Shigella*, *Acinetobacter*, *Salmonella*, *Pseudomonas*, *Escherichia*, *Citrobacter* and *Paracoccus*). We also report on that of phenyl mercuric acetate (PMA) and thimersol by two broad-spectrum Hg-resistant bacterial species (*Bacillus* and *Alcaligenes*) (Summers and Silver 1978). There are no published reports on Hg-resistance in *Planococcus* sp. and *Paracoccus* sp. We have also studied thiol specificity of Hg-reductases and organomercurial lyases isolated from the above bacterial species. Hg-reductase is known to have FAD-moiety (Schottel 1978), which stimulates enzyme activity whereas FMN and riboflavin are ineffective in this regard (Izaki *et al.* 1974). The effect of flavins, namely FAD, FMN and riboflavin, on Hg-reductase and organomercurial lyase activity is also reported here.

MATERIALS AND METHODS

All chemicals and reagents used in this study were of analytical grade (E. Merck, U.K.). $HgCl_2$, organomercurials and NADPH (tetra sodium salt) were purchased from Sigma Chemical Co., U.S.A.

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All the Hg-resistant bacterial strains used in this study were isolated from different water sources and identified in our laboratory. Cell-free extracts of these organisms were prepared following the procedure of Summers and Silver (1972). Hg--induced cells were disrupted mechanically with sea-sand at 4°C. Disrupted cells were suspended in cold 50 mM sodium phosphate buffer (pH 7.35) and centrifuged at 15,000 x g for 30 min at -4°C. Most of the Hg-reductase and organomercurial lyase activities were precipitated with 0-50% $(NH_4)_2SO_4$ at 4°C and dissolved in a minimum volume of same cold buffer containing 0.25 mM Na_2-EDTA. Samples were then dialysed overnight against the same buffer at 4°C. The dialysates were used for the assay of Hg-reductase and organomercurial lyase. Hg-reductase activity was determined by measuring Hg^{+2}-dependent NADPH oxidation spectrophotometrically at 340 nm. The reaction mixture contained 5 mM Na_2-EDTA, 2 mM $MgCl_2$, 1 mM thiol compound, 30 µM $HgCl_2$ and 0.50 mM NADPH. Suitable volumes between 10-100 µL cell-free extracts were used to follow the reaction kinetics uniformly and, finally, the volume was made upto 1 mL by 50 mM sodium phosphate buffer (pH 7.35). To determine the activity of organomercurial lyase, a suitable volume of cell-free extract was first incubated for 5 min with 30 µM PMA or thimersol and other ingredients in the assay mixture, except for $HgCl_2$ and NADPH. The oxidation reaction was started by adding 0.5 mM NADPH. To monitor the effects of thiol compounds on Hg-reductases and organomercurial lyases, Na-thioglycollate (Na-TG), β-mercaptoethanol (β-ME), cysteine, glutathione (GSH) and dithiothreitol (DTT) were used separately. To study the effect of flavins, 2 µM of FAD, FMN or riboflavin was used. The effect of N-ethylmaleimide (NEM), a sulfhydryl blocking agent was studied by adding this substance in the assay mixture at different concentrations (0.5 mM - 4 mM). One unit of Hg-reductase activity was defined as the amount of enzyme that oxidised 1 µmole of NADPH per minute in the presence of $HgCl_2$ and one unit of organomercurial lyase activity was defined as the amount of enzyme that oxidised 1 nmole of NADPH per min in presence of organomercurials. Protein was determined by the method of Lowry *et al.* (1951).

Table 1 shows the effects of -SH compounds (Na-TG, β-ME, cysteine, GSH and DTT) on Hg-reductases from different bacterial species. Ten bacterial strains out of fourteen test organisms showed maximum stimulation of Hg-reductase activity with GSH and four of which exhibited maximum enhancement with GSH as well as with one or two other thiol compounds. Hg-reductase from four organisms only were activated most by compounds other than GSH indicating that GSH is the most effective -SH compound to increase the level of Hg-reductase in these bacterial systems.

The effects of different concentrations of thiol compounds on the specific activity of Hg-reductase isolated from four test

RESULTS AND DISCUSSION

Table 1. Effect of various -SH compounds on specific activity of Hg-reductases isolated from different Hg-resistant bacteria

Bacterial strain	Without any -SH compound in the assay mixture	With -SH compounds in the assay mixture				
		Na-TG	β-ME	Cys	GSH	DTT
Klebsiella pneumoniae (KR_2)	0.054	0.063	0.070	0.072	0.083	0.058
Streptococcus faecalis (MR_1)	0.040	0.055	0.055	0.067	0.071	0.055
Micrococcus roseus (MR_3)	0.010	0.016	0.016	0.014	0.016	0.016
Alcaligenes faecalis (DR_1)	0.044	0.065	0.089	0.048	0.044	0.048
Bacillus pasteurii (DR_2)	0.136	0.197	0.180	0.136	0.214	0.146
Proteus mirabilis (GR_3)	0.011	0.011	0.023	0.031	0.031	0.017
Planococcus citreus (GR_5)	0.044	0.048	0.045	0.056	0.067	0.054
Shigella dysenteriae (AR_1)	0.032	0.040	0.050	0.063	0.060	0.058
Acinetobacter sp. (AR_2)	0.027	0.043	0.053	0.076	0.086	0.036
Salmonella sp. (ACR_1)	0.015	0.031	0.015	0.031	0.017	0.021
Pseudomonas aureofaciens (ACR_3)	0.021	0.030	0.029	0.023	0.030	0.030
Escherichia coli (ACR_2)	0.017	0.028	0.019	0.026	0.025	0.019
Citrobacter sp. (ACR_5)	0.031	0.043	0.047	0.055	0.059	0.043
Paracoccus sp. (ACR_6)	0.029	0.046	0.037	0.034	0.046	0.037

Abbreviations used - Na-TG : Na-thioglycollate; β-ME : β-mercaptoethanol; Cys : Cysteine; GSH : Glutathione; DTT : Dithiothreitol.

organisms, namely *Alcaligenes faecalis* (DR_1), *Bacillus pasteurii* (DR_2), *Shigella dysenteriae* (AR_1) and *Escherichia coli* (ACR_2), are shown in Figure 1. For each compound bacterial strain specific to it or the bacterial strains showing the maximum stimulation of its Hg-reductase activity with the particular compound was selected. The highest specific activity of Hg--reductase from *B. pasteurii* (DR_2) was noted with 1.5 mM GSH. The activity gradually decreased with increasing concentration. However, the activity was not suppressed significantly when 60 μM $HgCl_2$ was added to the assay mixture containing 3 mM GSH (data not shown). Activity of Hg-reductase of *A. faecalis* (DR_1) increased with increasing concentration of β-ME until the concentration reached 2 mM, but the activity was neither enhanced nor inhibited with further increase in the concentration of thiols. Both cysteine and Na-TG had maximum stimulatory effect at 1 mM concentration on Hg-reductase of *S. dysenteriae* (AR_1) and *E. coli* (ACR_2) respectively and the stimulatory effect was gradually inhibited with increasing concentrations of these thiols.

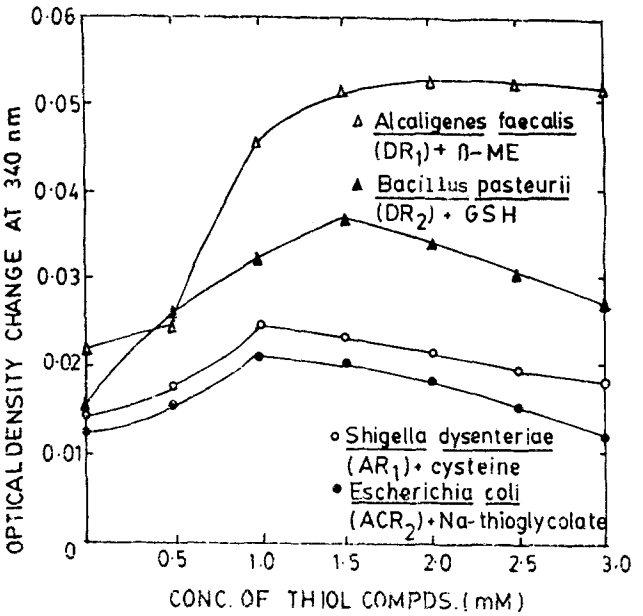

Figure 1 Effect of different concentration of thiol compounds on the specific activity of Hg-reductase isolated from A. faecalis (DR_1), B. pasteurii (DR_2), S. dysenteriae (AR_1) and E. coli (ACR_2)

Table 2 shows the effect of NEM on the specific activity of Hg-reductase of *B. pasteurii* (DR_2). The inhibitory effect was not significant at 0.5 mM concentration, but was remarkably enhanced at successively higher concentration indicating that the -SH compounds may be needed to keep some essential protein -bound -SH group in the reduced state (Brown *et al.* 1983; Fox and Walsh 1983). A requirement of large molar excess of -SH compounds indicates that the true substrate for Hg--reductase is not Hg^{+2} but its thiol or dithiol adducts (Summers and Silver 1978).

Table 2. Effect of N-ethylmaleimide on specific activity of Hg-reductase from *B. pasteurii* (DR_2)

Different concentrations of NEM used	Specific activity	Relation inhibition
0	0.214	0
0.5 mM	0.209	2.7%
2 mM	0.088	58.8%
4 mM	0.022	89.0%

Table 3. Synergistic effect of -SH compounds and FAD on specific activity of Hg-reductases isolated from different Hg--resistant bacteria

Bacterial strain	Without any -SH compound + FAD	With -SH compounds			
		Na-TG + FAD	β-ME + FAD	Cys + FAD	GSH + FAD
Klebsiella pneumoniae (KR_2)	0.058	0.071	0.079	0.079	0.089
Streptococcus faecalis (MR_1)	0.050	0.059	0.063	0.071	0.078
Micrococcus roseus (MR_3)	0.012	0.019	0.018	0.017	0.019
Alcaligenes faecalis (DR_1)	0.048	0.069	0.094	0.055	0.052
Bacillus pasteurii (DR_2)	0.146	0.210	0.197	0.180	0.225
Proteus mirabilis (GR_3)	0.017	0.020	0.033	0.041	0.043
Planococcus citreus (GR_5)	0.048	0.056	0.054	0.067	0.081
Shigella dysenteriae (AR_1)	0.036	0.046	0.057	0.067	0.065
Acinetobacter sp. (AR_2)	0.033	0.058	0.061	0.085	0.103
Salmonella sp. (ACR_1)	0.015	0.031	0.019	0.033	0.017
Pseudomonas aureofaciens (ACR_3)	0.021	0.030	0.032	0.026	0.033
Escherichia coli (ACR_2)	0.019	0.030	0.024	0.028	0.027
Citrobacter sp. (ACR_5)	0.036	0.051	0.053	0.059	0.069
Paracoccus sp. (ACR_6)	0.034	0.049	0.046	0.039	0.052

Abbreviations used - Na-TG : Na-thioglycollate; β-ME : β-mercaptoethanol; Cys : Cysteine; GSH : Glutathione.

The effect of FAD on Hg-reductase activity is shown in Table 3. Addition of FAD caused higher stimulation in almost all cases. FAD, in the absence of any -SH compound in the assay mixture, also enhanced the activity. The effect of FAD appeared to be more pronounced when used together with a -SH compound. FMN and riboflavin showed neither stimulatory nor inhibitory effect of Hg^{+2} -dependent NADPH oxidation (data not shown) when it was studied with 0-50% $(NH_4)_2SO_4$ precipitated cell--free extracts. However, the oxidation was stimulated in all cases when the crude extracts [without any $(NH_4)_2SO_4$ cut] was preincubated with 2 μM of FMN for 5 min prior to the addition of $HgCl_2$. This may be due to the presence of another enzyme, namely FAD-pyrophosphorylase in the crude extract which converts FMN to FAD in the presence of ATP and Mg^{+2} (Methods in Enzymology, Vol. **XVIII**). But if FMN is added to the crude-extract along with $HgCl_2$, no enhancement of Hg^{+2} --reducing activity occurs indicating inhibition of FAD-pyrophosphorylase. This stimulation of Hg-reductase activity with

Table 4. Effect of -SH compounds and FAD on specific activity of organomercurial lyases isolated from B. pasteurii (DR_2) and A. faecalis (DR_1)

Bacterial species	Organo-mercurial used as substrate	Without any -SH compound in the assay		With -SH compounds and with or without FAD in the assay mixture								
				Na-thio-glycollate		β-Mercapto ethanol		Cysteine		GSH		Dithio-threitol
		-FAD	+FAD	-FAD	+FAD	-FAD	+FAD	-FAD	+FAD	-FAD	+FAD	-FAD
B. pasteurii	PMA	11.01	22.30	34.18	37.00	34.18	37.00	39.07	42.10	43.95	49.30	22.30
(DR_2)	Thimersol	9.80	16.90	29.30	32.10	29.30	34.20	24.42	26.00	39.07	42.10	24.42
A. faecalis	PMA	3.00	7.32	8.14	9.76	10.58	12.90	7.32	8.14	5.69	7.32	7.32
(DR_1)	Thimersol	3.43	6.51	10.58	11.39	11.39	13.40	9.04	11.39	6.51	7.32	9.76

FMN does not occur in the cell-free extract obtained after $(NH_4)_2 SO_4$ precipitation, suggesting the elimination of FAD--pyrophosphorylase activity.

Data in Table 4 indicate the effect of -SH compounds and FAD on organomercurial lyases isolated from *B. pasteurii* (DR_2) and A. *faecalis* (DR_1). Lyases isolated from *B. pasteurii* (DR_2) showed the highest specific activity with GSH whereas that isolated from A. *faecalis* (DR_1) had highest specific activity with β-ME. In both instances, addition of FAD to the assay mixture enhanced specific activity of this enzyme. Next to GSH, cysteine was the most effective stimulator of the lyases although it had the minimal stimulatory effect on Hg-reductase from *B. pasteurii* (DR_2) (Table 1).

The present study indicates that both Hg-reductase and organomercurial lyase from various Hg-resistant aquatic bacteria require exogenous thiol compounds for optimum enzyme activity. Thiol specificity of Hg-reductase and organomercurial lyase isolated from different bacterial strains is different for different thiol compounds, indicating synthesis of dissimilar enzymes by different bacteria.

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