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Purification and properties of mercuric reductase from *Azotobacter chroococcum*

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S. GHOSH, P.C. SADHUKHAN, J. CHAUDHURI, D.K. GHOSH AND A. MANDAL. 1999. Mercury resistance determinants in bacteria are often plasmid-borne or transposon-mediated. Mercuric reductase, one of the proteins encoded by the mercury resistance operon, catalyses a unique reaction in which mercuric ions, Hg (II), are reduced to mercury metal Hg(O) using NADPH as a source of reducing power. Mercuric reductase was purified from *Azotobacter chroococcum* SS_2 using Red A dye matrix affinity chromatography. Freshly purified preparations of the enzyme showed a single band on polyacrylamide gel electrophoresis under non-denaturing conditions. After SDS-polyacrylamide gel electrophoresis of the freshly prepared enzyme, two protein bands, a major and a minor one, were observed with molecular weight 69 000 and 54 000, respectively. The molecular weight of the native enzyme as determined by gel filtration in Sephacryl S-300 was 142 000. The Km of Hg^{2+}-reductase for $HgCl_2$ was 11·11 μmol l^{-1}. Titration with 5,5′-dithiobis (2-nitrobenzoate) demonstrated that two enzyme–SH groups become kinetically accessible on reduction with NADPH.

INTRODUCTION

Mercury resistance determinants in bacteria are often plasmid-borne or transposon-mediated (Summers and Silver 1972, 1978; Summers 1986; Silver and Misra 1988; Misra 1992). Genes conferring resistance to mercury compounds are clustered in an operon in most known naturally occurring Hg-resistant systems. Mercuric reductase (Mer A), one of the proteins encoded by the mercury resistance (mer) operon, catalyses a unique reaction in which mercuric ions Hg(II) are reduced to mercury metal Hg(O) using NADPH as a source of reducing power. This enzyme is a soluble cytosolic flavoprotein (Furukawa and Tonomura 1972a, b; Schottel 1978; Fox and Walsh 1982) that contains both FAD and a redox active disulphide that catalyses the reaction.

Mercuric reductase has been purified and cloned from both Gram-negative and Gram-positive bacteria and shown to be a member of the family of flavin-dependent disulphide oxidoreductases, the parent of which is glutathione reductase (Fox and Walsh 1982). The enzyme has been well characterized from *Escherichia coli* J 53 harbouring a broad spectrum Hg-resistant plasmid R831 (Schottel 1988), *E. coli* KP 245 (Rinderle *et al*. 1983), *Pseudomonas aeruginosa (*PAO 9501) (Fox and Walsh 1982), *Bacillus* sp. strain encoded by plasmid RC607 (Moore *et al*. 1989) and *Yersinia enterocolitica* 138 A14 (Blaghen *et al*. 1993). Several recent studies have provided new data to clarify the mechanism of enzymatic reduction of mercuric ions. It was therefore of interest to characterize the mercuric reductase from *Azotobacter chroococcum* and, in particular, to study the similarity and difference, if any, of this enzyme with other mercuric reductases from other sources.

Here, purification and physicochemical properties of mercuric reductase from *Azotobacter chroococcum* SS_2, a broad-spectrum nitrogen-fixing Hg-resistant bacterial strain isolated from soil, are reported. In this connection, it should be noted that there is no published report of studies on purified Hg-detoxifying enzymes from an N_2-fixing bacterial system.

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MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used in this study were of analytical grade (Merck). NADH (disodium salt), NADPH (tetra sodium salt) and mercury compounds were purchased

from Sigma. Red A gel matrix affinity chromatography medium was purchased from Amicon Inc. (Beverly, MA, USA) and Ampholine was purchased from LKB Pharmacia; Sephacryl S-300 was purchased from Pharmacia.

Organisms

Broad spectrum, mercury-resistant *Azotobacter chroococcum* was isolated from agricultural farms and identified in this laboratory according to *Bergey's Manual of Determinative Bacteriology* (Holt *et al.* 1994).

Media and growth conditions

Bacterial cells were grown aerobically overnight (12 h) on a rotary shaker (200 rev min^{-1}) at 32 °C in nutrient broth supplemented with 20 μmol l^{-1} $HgCl_2$ for inducing mercuric reductase. The bacterial cultures were diluted 10-fold with the same sterile medium, and the concentration of $HgCl_2$ was maintained at 20 μmol l^{-1}. A third addition of 20 μmol l^{-1} $HgCl_2$ was made at the early log phase (4 h). Cells were harvested at the late log phase (8 h) by centrifugation at 4 °C and washed three times with cold 50 mmol l^{-1} sodium phosphate buffer containing 0·5 mmol l^{-1} Na_2EDTA and 0·1% 2-mercaptoethanol (pH 7·35) (buffer A). Washed cells were stored at −20 °C until used (Summers and Silver 1972; Fox and Walsh 1982; Blaghen *et al.* 1993).

Enzyme preparation

Cells (7 g wet weight) were thawed, disrupted mechanically with sea-sand at 4 °C, suspended in 65 ml buffer A and centrifuged at 15 000 *g* for 30 min at 4 °C. The supernatant fluid thus obtained was termed crude extract.

Enzyme purification

Most of the mercuric reductase activity present in the crude extract obtained was precipitated with 30–50% saturation of $(NH_4)_2SO_4$ at 4 °C. The precipitate was separated by centrifugation, dissolved in a minimum volume of cold 50 mmol l^{-1} sodium phosphate buffer (pH 7·35) containing 0·5 mmol l^{-1} Na_2EDTA and 0·1% 2-mercaptoethanol, and dialysed against the same buffer for 4 h at 4 °C with two changes. The dialysate was applied to a Red A gel matrix column (1·5 cm × 5 cm) at a flow rate of 0·4 ml min^{-1}. The column was washed at 1 ml min^{-1} with several column volumes of buffer until the absorbance at 280 nm was nearly zero. Mercuric reductase was eluted with 200 μmol l^{-1} NADPH in the above buffer. Active fractions were pooled and concentrated on a centricon PMIO membrane (Millipore Corporation, Bedford, MA, USA) and dialysed against buffer A.

Enzyme assay

Mercuric reductase activity was determined by measuring Hg^{2+}-induced NADPH oxidation spectrophotometrically at 340 nm following the standard method (Izaki *et al.* 1974; Ghosh *et al.* 1996a, b, c). Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard (Lowry *et al.* 1951). One unit of mercuric reductase activity was defined as the amount of enzyme protein in milligrams that oxidized one micromole of NADPH per minute in the presence of $HgCl_2$.

Molecular weight determination

The molecular weight of the native mercuric reductase was determined by gel filtration through a column (1·5 cm × 70 cm) of Sephacryl S-300 equilibrated in 50 mmol l^{-1} sodium phosphate buffer, pH 7·4, and 0·5 mmol l^{-1} Na_2EDTA using the following standards: catalase (232 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (32 kDa).

Polyacrylamide gel electrophoresis

The purity of the enzyme was determined by native gel electrophoresis. Subunit molecular weight was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis of proteins was run under non-denaturing conditions. Bromophenol blue was used as the tracking dye; the gels were run at about 3–4 mA/tube and then stained with Coomassie blue. To assay enzyme activity, unstained gels were sliced into 20 mm pieces and incubated at 4 °C for at least 12 h in a small volume of 50 mmol l^{-1} sodium phosphate buffer (pH 7·35). The eluted protein was then assayed.

SDS-PAGE was carried out according to the method of Laemmli (Laemmli 1970). The samples were dissolved in SDS-sample buffer and denatured by boiling for 3 min prior to loading. Electrophoresis was performed in a 10% polyacrylamide gel containing 0·1% (w/v) SDS. Each lane contained 10–12 μg protein. The gel was run at 20 mA until the tracking dye reached its lower edge. Proteins were visualized by the Coomassie staining method.

Flavin determination

Thin-layer chromatography was used to detect the presence of FAD-containing compounds as part of mercuric reductase according to the method of Blaghen *et al.* (1993) and Faeder and Slegel (1973).

Isoelectric focusing

Isoelectric focusing of enzyme preparation was carried out according to the procedure of Schottel (Schottel 1978). Tube gels (110 mm) containing a final concentration of 5·1% acrylamide, 0·44% glucose, 0·03% riboflavin and 2% Ampholine (pH 4–6) were run at 1 mA/tube for 30 min at 4 °C, before adding the sample, in order to establish the pH gradient. After layering the sample, the gels were run at 300 V for 3 h; during that time, the amperage dropped from 8 mA to about 2·5 mA. Sulphuric acid (0·2%) was used in the anode chamber and 0·4% triethanolamine was used in the cathode chamber.

The gels were stained for protein by immersing in 10% trichloroacetic acid for 1·5 h, in 5% trichloroacetic acid for 1·5 h and 0·1% Coomassie blue dissolved in water : acetic acid : ethanol (9:2:9) for about 2 h. The gels were de-stained in water : acetic acid : ethanol (13:1:6) for about 24 h with two changes in de-staining solution.

Thiol titration

The procedure used was developed by Mathews *et al*. (1974). Mercuric reductase (2·7 nmole) was incubated with $\pm$220 nmol of NADPH. The solution was brought to a final concentration of 5 M of guanidine HCl, 70 mmol l^{-1} Tris-HCl, pH 8·0, and 3 mmol l^{-1} Na_2EDTA. Exposed thiols were quantified by the addition of 400 nmol 5,5′ dithiobis (2 nitrobenzoate). An extinction coefficient of 13·6 mmol l^{-1} cm^{-1} at 412 nm was used for the released thiolate anion (Beutler *et al*. 1963; Fox and Walsh 1982; Blaghen *et al*. 1993).

RESULTS AND DISCUSSION

A total of 201·5 mg of protein corresponding to 25·187 units of mercuric reductase was obtained from crude extract of disrupted cells (7 g). After 30–50% $(NH_4)_2SO_4$ saturation, 37·648 mg of protein containing mercuric reductase activity were recovered. Affinity chromatography of the sample on Red A gel matrix column followed by elution with 200 μmol l^{-1} NADPH resulted in a recovery of 16·49 units of mercuric reductase in 0·9 mg of protein (Table 1). The enzyme was purified to 146·6-fold with an overall yield of 65·47%.

In one set of experiments, crude cell-free extract was directly applied to a Red A gel matrix column for a better yield of protein, but the results were not satisfactory, unlike those described by Schultz *et al*. (1985) on Orange A gel matrix in which the enzyme was purified in the homogeneous state.

Freshly purified preparations of the enzyme showed a single band on polyacrylamide gel electrophoresis under non-denaturing conditions (data not shown). After SDS-polyacrylamide gel electrophoresis of the freshly prepared enzyme, two protein bands, a major and a minor one, were observed with molecular weights of 69 kDa and 54 kDa, respectively (Fig. 1).

The molecular weight of the native enzyme as determined by gel filtration on Sephacryl S-300 was found to be 142 000 $\pm$ 2000. This criterion indicates a dimeric structure, in contrast to the trimeric structure suggested for *E. coli* J 53, (R831) plasmid (Schottel 1978) and *Yersinia enterocolitica* 138 A 14 (Blaghen *et al*. 1993).

The characteristics of mercuric reductase have been studied in greater detail in the plasmid-bearing *E. coli* (Schottel 1978; Rinderle *et al*. 1983), in soil *Pseudomonas* K62 (Kurukawa and Tonomura 1972a, b) and in *Pseudomonas aeruginosa* PA09501 carrying the plasmid pVS1 (Fox and Walsh 1982). Mercuric reductase from different Gram-negative bacterial sources indicates the presence of two distinct types of the enzyme, one of which appears to be trimeric and the other, dimeric (Schottel 1978; Fox and Walsh 1982; Blaghen *et al*. 1993). There was no significant difference in the enzymatic properties of the trimeric enzyme produced by *E. coli* J 53 (R831) (Schottel 1978) and *Yersinia enterocolitica* YE138 A14 (Blaghen *et al*. 1993), and the dimeric enzyme produced by *Ps. aeruginosa* PAO 9501 (Fox and Walsh 1982). Mercuric reductase purified from *Azotobacter chroococcum* SS_2 also did not show any noticeable difference in enzymatic properties compared with the enzymes reported from other sources. However, the enzyme isolated from *A. chroococcum* SS_2 differs from the Hg^{2+}-reductases from other sources in molecular weight, subunit molecular weight, Km value, enzyme inhibitor and isoelectric point. All the mercuric reductases produced by different bacterial strains contained bound FAD, required sulphhydryl compounds for enzymatic activity and utilized NADPH as electron donor.

The larger molecular weight band of mercuric reductase of *A. chroococcum* SS_2 contained 80–85% of the enzyme protein. However, the ratio of the two bands (major and minor) shifted on storage at 4 °C. A similar observation was also reported by Fox and Walsh (1982). In *Pseudomonas aeruginosa* (PAO 9501) the enzyme is also composed of two subunits of 56 kDa and 62 kDa, respectively (Fox and Walsh 1982).

By isoelectric focusing through a linear gradient of Ampholine, mercuric reductase focused with glucose oxidase, which corresponds approximately to pI 4·25 (data not shown). However, the isoelectric point of this enzyme from *E. coli* K 12 strain J53–1 carrying the plasmid R831 was determined as 5·3 by Schottel (1978). According to Blaghen *et al*. (1993), the isoelectric point of this enzyme from *Yersinia enterocolitica* 138 A14 was 4·7.

Optimum pH and optimum temperature of the enzyme were 7·35 and 45 °C, respectively. According to Fox and Walsh (1982), Km and Vmax were 12 μmol l^{-1} and 12·7 μmol min^{-1} mg^{-1} protein, respectively. However, in our system, the Km and Vmax were 11·11 μmol l^{-1} and 25 μmol min^{-1} mg^{-1} enzyme protein, respectively. The Km value of

Table 1 Purification of mercuric reductase from *Azotobacter chroococcum* SS_2

Fraction	Volume (ml)	Protein		Activity		Purification	
		mg ml^{-1}	Total (mg)	Specific activity	Total activity	Fold of purification	Overall yield (%)
Crude	62	3·25	201·5	0·125	25·187	1	100
$(NH_4)SO_4$ cut	5·2	7·24	37·648	0·401	15·11	3·2	60
Affinity chromatography on Red A gel matrix	3	0·3	0·9	18·32	16·49	146·6	65·47

Specific activity is defined as μmole of NADPH oxidized per minute per milligram of enzyme protein.

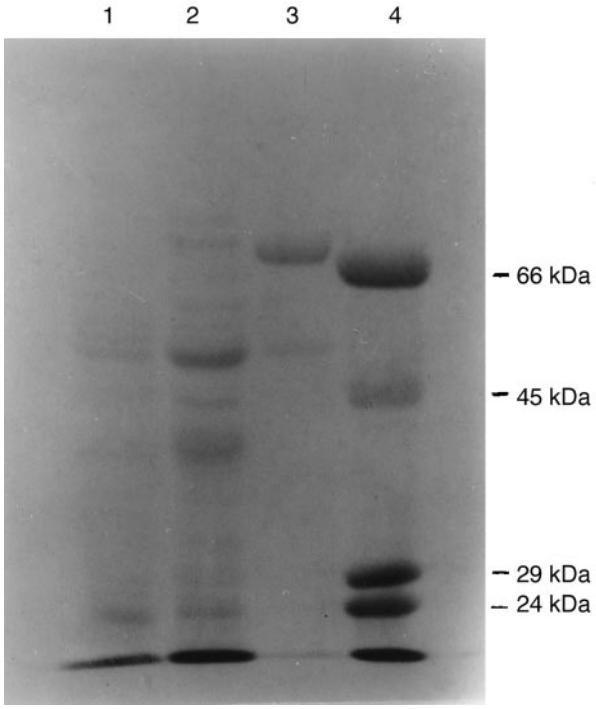

Fig. 1 SDS-PAGE of freshly prepared mercuric reductase from *Azotobacter chroococcum* SS_2. The sample 15 μg of mercuric reductase was applied to a 10% slab gel and electrophoresed as described in Materials and Methods. Lane 1: crude cell-free extract; lane 2: $(NH_4)_2SO_4$ cut preparation; lane 3: purified mercuric reductase; lane 4: molecular weight markers: bovine serum albumin (BSA) (66 kDa); egg albumin (45 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa)

Hg^{2+} reductase was 13 μmol l^{-1} and pH optimum was 7·5, as determined by Schottel (Schottel 1978). In our system also, the enzyme showed the optimum pH as 7·4.

Thin layer chromatography showed that the relative mobilities of the yellow supernatant fluids obtained by centrifugation of purified enzyme after boiling and of FAD were the same. This result indicates that FAD is the coenzyme of the mercuric reductase. Fluorimetric analysis for FAD confirmed that mercuric reductase contained 2·05, 1·96, 2·0 and 2·02 mole of FAD per mole of the enzyme.

When the enzyme (without NADPH) was titrated with 5–5′-dithiobis (2 nitrobenzoate) (DTNB), two thiols per dimer were exposed to titration. Upon reduction of the enzyme with NADPH, another two thiols were found to be exposed to DTNB titration.

The mercuric reductase requires the presence of a sulphhydryl compound such as 2-mercaptoethanol for its activity. At a fixed concentration of 2-mercaptoethanol, mercuric reductase activity increases with the increase of $HgCl_2$ concentration. This activity rapidly reaches a maximum at a certain concentration of $HgC1_2$ and then is inhibited by $HgC1_2$ exceeding that concentration of $HgC1_2$. However, the addition of an excess of 2-mercaptoethanol serves to displace the substrate ($HgC1_2$) inhibitory effect of higher concentrations of $HgC1_2$ on mercuric reductase (data not shown). With increasing concentration of 2-mercaptoethanol, the level of enzymatic activity also increased and a Km value of 11·11 μmol l^{-1} and Vmax of 25 μmol min^{-1} mg^{-1} enzyme protein were obtained (data not shown). Without any thiol agent, only 50% of the enzyme activity was obtained. 2-mercaptoethanol was the best thiol compound at 2 mmol l^{-1} concentration for optimum enzyme activity. FAD greatly stimulated the enzyme activity. The presence of Mg^{2+} was required for activity of the enzyme as only 60% activity was obtained without any Mg^{2+}. Fifteen percent less activity was observed when no Na_2EDTA was used in the assay mixture of the enzyme. The enzyme was NADPH specific as NADH, even at 150 mmol l^{-1}, could not act as electron donor for this system (Table 2). The enzyme kinetics of mercuric reductase from *A. chroococcum* SS_2 in the presence of 2-mercaptoethanol and $HgCl_2$ showed that the activity of the enzyme was inhibited by an excess of 2-mercaptoethanol. It was also observed that, at a fixed concentration of $HgC1_2$ (30 μmol l^{-1}), the rate of reduction was not constant when the concentration of 2-mercaptoethanol varied, and the higher the concentration of 2-mercaptoethanol used during the experi-

Table 2 Cofactor requirement of the mercuric reductase activity in *Azotobacter chroococcum* SS_2

Cofactors used	Relative activity of mercuric reductase
Complete Assay Mixture (CAM)[a]*	100
CAM − thiol compound	50
CAM − 2-mercaptoethanol + Na-thiaglycolate	85
CAM − 2-mercaptoethanol + L-cysteine	90
CAM − 2-mercaptoethanol + GSH	91
CAM + FAD	100
CAM − $MgCl_2$	60
CAM − EDTA	85
CAM − NADPH + NADH	4

[a] Complete assay mixture contained in a total volume of 1 ml: 30 μmol l^{-1} $HgCl_2$, 5 mmol l^{-1} Na_2 EDTA, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} 2-mercaptoethanol (or other thiol compounds), 0·15 mmol NADPH and 20 μg enzyme protein in 50 mmol l^{-1} sodium phosphate buffer (pH 7·35). The reaction was started by adding enzyme and NADPH to the reaction mixture which was pre-incubated for 10 min at 32 °C.

* Original value of specific activity was 18 units mg^{-1} enzyme protein.

ment the lower the rate of reduction of Hg^{2+} to Hg^{o} (data not shown). This could be attributed to the fact, as suggested by Blaghen *et al*. (1993), that the Hg^{2+} chelation at the active sites is likely to become difficult due to the presence of both Hg^{2+} and 2-mercaptoethanol, at that concentration and the probability for ligand exchange via the tridentate intermediate decreases dramatically. Titration with 5,5′ dithiobis-(2 nitro benzoate) demonstrated that two enzyme-SH groups become kinetically accessible upon reduction with NADPH. A similar observation was also reported by Fox and Walsh (1982).

None of the metal ions except Ag^{+} could effectively block the purified mercuric reductase at 10^{-4} M concentration; Cd^{2+} and Co^{2+} showed partial inhibition of the enzyme activity whereas Pb^{2+} (60%), Bi^{3+} (40%), Cu^{2+} (44%), Zn^{2+} (60%) and Ni^{2+} (62%) showed some inhibition of the enzymatic activity. N-ethyl maleimide at 2 mmol l^{-1} showed 60% inhibition and 2% NaN_3 showed 50% inhibition of the enzymatic activity. Therefore, enzyme activity was sensitive to Ag^{+} and was slightly sensitive to Cd^{2+}, Co^{2+}, Pb^{2+}, Bi^{3+}, Ca^{2+} and Zn^{2+}; Ni^{2+} also showed some inhibition.

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