The Isolation of a Mercuric Ion-reducing Flavoprotein from *Thiobacillus ferrooxidans*

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A mercuric ion-reducing flavoprotein was purified from *Thiobacillus ferrooxidans* TFI 29 by using dye-matrix affinity chromatography. The isolated enzyme had a molecular weight of 130,000 and was composed of two subunits (54,000 and 62,000). The visible absorbance spectrum of the oxidized enzyme had a maximum at 455 nm. Upon addition of NADPH a new absorbance at 540 nm appeared, which was attributed to a charge transfer complex. The *K*ₘ for mercuric chloride was determined to be 8.9 μM and the enzyme was shown to have a turnover number of 746 min⁻¹ per FAD. A comparison of these physical properties, as well as metal ion inhibition and pH profiles, indicate that the enzyme from *T. ferrooxidans* is very similar in structure and function to mercuric reductases isolated from other bacterial sources.

**INTRODUCTION**

The chemosynthetic bacterium *Thiobacillus ferrooxidans* has become an important industrial micro-organism which is used primarily in the leaching of low-grade copper- and uranium-containing ores (Brierley, 1978). Although it can grow under very acidic conditions using only reduced sulphur or iron (Fe²⁺) as an energy source, the sensitivity of *T. ferrooxidans* to mercury salts (Norris & Kelly, 1978) may limit the usefulness of this micro-organism under field conditions. It was, therefore, of interest when Olson *et al.* (1981) reported the isolation of a *T. ferrooxidans* strain which was resistant to Hg²⁺ at concentrations up to 5 μM. In another report (Olson *et al.*, 1982), the mercury resistance was shown to be due to the presence of a mercuric reductase enzyme which was capable of reducing Hg²⁺ to Hg⁰. Although no attempt was made to purify the protein, cell-free extracts of this strain were shown to have many properties similar to the mercuric reductase previously isolated from plasmid-bearing strains of *Pseudomonas* (Furukawa & Tonomura, 1972; Tezuka & Tonomura, 1976; Fox & Walsh, 1982) and *Escherichia coli* (Izaki *et al.*, 1974; Schottel, 1978; Rinderle *et al.*, 1983). However, antisera against the purified mercuric reductases from *E. coli* and *Pseudomonas* (which inactivated mercuric reductase from a number of different sources) did not inactivate the enzyme from *T. ferrooxidans*, suggesting significant differences (Olson *et al.*, 1982). In order to clarify the significance of these observations, we have purified the mercuric reductase from *T. ferrooxidans* TFI 29. The results presented demonstrate that this enzyme is similar in structure and function to the mercuric reductase previously described.

**METHODS**

Organism and culture. *Thiobacillus ferrooxidans* TFI 29 (Martin *et al.*, 1981), originally isolated in Quebec, Canada, was a gift from the collection of Dr O. H. Tuovinen, Microbiology Dept, Ohio State University, Columbus, Oh. USA. The strain was grown at room temperature on ferrous sulphate medium containing (g l⁻¹): K₂HPO₄, 0.524; MgSO₄·7H₂O, 0.4; (NH₄)₂SO₄, 0.4; FeSO₄·7H₂O, 33.3. The medium was acidified to pH 1.5 with sulphuric acid. The cells were grown adaptively in this medium with increasing concentrations of HgCl₂ up to 10 μM.

Batches of bacteria (200 l) were grown in 20 litre carboys containing the above medium and 10 μM-HgCl₂. The
cells were harvested after depletion of 70% of the available Fe\(^{2+}\) by using a Pellicon cassette system (Millipore) with 0.45 μm filters. Final yield was 12.6 g (wet wt) of cells, which were stored at \(-20^\circ C\).

**Enzyme preparation.** The cells were thawed, washed several times in 50 mM-sodium phosphate (pH 7.0), containing 0.5 mM-EDTA and 0.2 mM-MgSO\(_4\), resuspended in 50 ml of this buffer, and disrupted by two passages through a French pressure cell at 1400 kg cm\(^{-2}\). Cell debris was then removed by centrifuging at 48000 g for 15 min. Further purification steps were similar to the procedure of Fox & Walsh (1982) with the exception that 0.2 M-KCl was added to the buffer used to wash the Orange A Matrex gel affinity column.

Mercuric reductase from *E. coli* was prepared according to the procedure of Rinderle *et al.* (1983).

**Enzyme assays.** Reaction mixtures contained 50 mM-sodium phosphate, pH 7.0, 100 μM-HgCl\(_2\), 100 μM-NADPH, 0.5 mM-EDTA, and 1.0 mM-mercaptoethanol in a total volume of 2.5 ml at 30°C. The reactions were initiated by the addition of 0.01-0.05 ml of enzyme and the decrease in absorbance was continuously monitored at 340 nm. A unit is defined as that amount of enzyme which catalyses the formation of 1 μmol NADP\(^{+}\) in 1 min.

**Protein concentration.** This was determined by the Lowry method. The concentration of mercuric reductase was determined from the flavin content, using an extinction coefficient of 11.3 mM\(^{-1}\) cm\(^{-1}\) at 456 nm.

**Molecular weight determination.** This was done by the method of Andrew (1964) using a column (0.9 × 30 cm) of Sephacryl S-200 equilibrated in 50 mM-sodium phosphate buffer (pH 7.0). The column was calibrated with alcohol dehydrogenase (yeast, 141,000), glutathione reductase (yeast, 101,000), and serum albumin (bovine, 68,000). The subunit molecular weight was determined by SDS-PAGE by using the procedure of Weber & Osborn (1969) and Bio-Rad (Richmond, Calif., USA) low molecular weight standards.

**RESULTS**

**Enzyme purification**

Using a procedure similar to that described by Fox & Walsh (1982), we were able to purify the enzyme from *T. ferrooxidans* to homogeneity with a good overall yield (92%). The key to the purification was the Orange A Matrex gel affinity column which was washed exhaustively with 0.2 M-KCl and finally eluted with NADPH. The results of a purification (12.6 g wet wt bacteria) are summarized in Table 1. The enzyme prepared in this manner was stable at 4°C after extensive dialysis in order to remove any excess NADP\(^{+}\)/NADPH.

The molecular weight of the purified enzyme was determined to be 130,000 ± 10% by gel filtration. Electrophoresis of the enzyme on SDS polyacrylamide gels showed two protein bands with molecular weights of 54,000 and 62,000 (Fig. 1). The results indicate that mercuric reductase from *T. ferrooxidans* is most closely associated with the dimeric class of mercuric reductase as proposed by Silver & Kinscherf (1982). The presence of two protein bands on SDS polyacrylamide gels corresponding to mercuric reductase appears to be a common characteristic of the enzyme, since two bands were also observed in both the *E. coli* (Schottel, 1978; Jackson & Summers, 1982) and the *Pseudomonas* (Fox & Walsh, 1982) preparations. Although both bands are equally active (Fox & Walsh, 1982), the smaller protein appears to represent the product of post-translational proteolytic cleavage (Jackson & Summers, 1982). The significance of the cleavage is not yet understood.

**Spectroscopic properties**

The spectrum of the oxidized mercuric reductase from *T. ferrooxidans* is characteristic of a flavoprotein with an absorbance maximum at 455 nm (Fig. 2). Upon the anaerobic addition of an excess of NADPH (Fig. 2, curve B), the absorbance at 455 nm decreased by about 30% and the absorption maximum shifted to a lower wavelength. Also, a new absorption maximum appeared at 540 nm. The further addition of an excess of methyl mercury (methyl mercury is not a substrate, but binds at the active site: Marshall *et al.*, 1983) eliminated the absorption at 540 nm (data not shown). Similar spectral changes have been observed with mercuric reductase from *E. coli* and *Pseudomonas* (Fox & Walsh, 1982; Rinderle *et al.*, 1983). The identity of the enzyme-bound flavin was verified as FAD by measuring the change in fluorescence upon the addition of snake venom phosphodiesterase to a solution of boiled (and filtered) enzyme. The fluorescence increased seven- to eightfold as would be expected when FAD is cleaved to FMN (Weber, 1950).

These results indicate that mercuric reductase from *T. ferrooxidans*, like the enzyme from other sources, belongs to a group of flavoproteins all of which contain a redox-active disulphide
Mercuric reductase from *Thiobacillus*

Fig. 1. SDS-polyacrylamide gel electrophoresis of the enzyme from *T. ferrooxidans*. The gel on the left contains mercuric reductase and the Bio-Rad low molecular weight standards. The other gel contains 25 μg of purified mercuric reductase. Both were 10% gels. On the right is a semi-log plot of these results. The open circles indicate the positions of the standard proteins and the arrows correspond to the bands of mercuric reductase.

Table 1. Purification of the mercuric reductase from *T. ferrooxidans*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity [units (mg protein)^{-1}]</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>45.0</td>
<td>17.2</td>
<td>423.0</td>
<td>0.04</td>
<td>–</td>
</tr>
<tr>
<td>Heat step (65 °C)</td>
<td>49.0</td>
<td>19.1</td>
<td>79.6</td>
<td>0.24</td>
<td>111</td>
</tr>
<tr>
<td>Orange A Matrex gel column</td>
<td>10.5</td>
<td>15.8</td>
<td>1.23</td>
<td>12.80</td>
<td>92</td>
</tr>
</tbody>
</table>

The preparation of crude extract and enzyme purification were done as described in Methods.

in their active site (Williams, 1976). During catalysis, these enzymes cycle between the fully oxidized (E) and 2-electron reduced (EH₂) forms. In most cases, the EH₂ form is characterized by a charge transfer complex between a thiolate anion of a cysteine residue and the oxidized FAD which has an absorption at 530–540 nm (Williams, 1976). The charge transfer complex was also present in the reduced mercuric reductase from *T. ferrooxidans* (Fig. 2), thus demonstrating the mechanistic similarities between these enzymes. The fact that methyl mercury eliminated the absorption at 540 nm (due to binding to the thiolate anion in the active site) supports this conclusion.
Fig. 2. Spectra of oxidized and reduced mercuric reductase from *T. ferroxidans*. Curve A, 3·0 μM oxidized enzyme; curve B, after reduction with 4 equivalents of NADPH.

Fig. 3. Optimum pH for mercuric ion reduction by purified enzyme. ○, Mercuric reductase from *T. ferroxidans*; △, mercuric reductase from *E. coli*. The buffer contained 0·05 M-2-(N-morpholino)ethane sulphonic acid (MES), 0·05 M-Tris, and 0·05 M-2-(N-cyclohexylamino)ethanesulphonic acid (CHES), and was adjusted to the appropriate pH with HCl.

Table 2. *Kinetic properties of mercuric reductase from different bacteria*

<table>
<thead>
<tr>
<th>Organism</th>
<th>$K_m$ for HgCl$_2$ (μM)</th>
<th>Turnover no. (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO 9501</td>
<td>12</td>
<td>800</td>
</tr>
<tr>
<td><em>E. coli</em>† KP 245</td>
<td>14</td>
<td>1044</td>
</tr>
<tr>
<td><em>T. ferroxidans</em> TFI 29</td>
<td>8·9</td>
<td>746</td>
</tr>
</tbody>
</table>


Table 3. *Inhibition of mercuric reductase by metal ions*

Each assay was done at 100 μM-HgCl$_2$ (control) and 100 μM of each of the compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$</td>
<td>70</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>50</td>
</tr>
<tr>
<td>AgNO$_3$</td>
<td>30</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>15</td>
</tr>
<tr>
<td>CuCl</td>
<td>12</td>
</tr>
</tbody>
</table>

*Kinetic properties*

Mercuric reductase from *T. ferroxidans* under standard assay conditions, (see Methods) followed normal Michaelis–Menten kinetics, yielding a $K_m$ for HgCl$_2$ of 8·9 ± 0·8 μM and a turnover number ($V_{max}$) of 746 ± 20 min$^{-1}$ per FAD. Table 2 shows a comparison of these kinetic constants with values determined for mercuric reductase from *Pseudomonas* and *E. coli*. If EDTA was omitted from the reaction mixture, the kinetics of NADPH oxidation were distinctly biphasic, which has also been seen with mercuric reductase from other sources (Fox & Walsh, 1982; Rinderle et al., 1983). Although NADPH was the preferred substrate, 25% of the reaction rate could be obtained with an equal concentration of NADH.
The metal ion specificity of the mercuric reductase was also determined. Although none of the metal salts tested were found to be substrates for the enzyme, some metal ions were shown to be effective inhibitors (Table 3). The most notable inhibition was shown by copper and silver salts, while ferric and cobalt chloride also showed some inhibition. Other compounds which were tested but had no inhibitory effect under the conditions used were CdCl₂, MnSO₄, ZnSO₄, NiSO₄, FeSO₄, Pb(acetate)₂ and Na₂Cr₂O₇.

Finally, the pH activity profiles of mercuric reductase from *T. ferrooxidans* and *E. coli* were determined (Fig. 3). The enzyme from both sources had a typical bell-shaped profile covering a broad pH range. The maximum enzymic activity occurred at about pH 7.5 in both cases.

**DISCUSSION**

In almost every mercury resistant strain studied to date, the resistance has been found to be associated with the flavoprotein mercuric reductase, which reduces Hg²⁺ to Hg⁰. In this report, we demonstrate that mercury resistance in *T. ferrooxidans* TFI 29 is also due to a mercuric reductase which is similar to those previously described in *Pseudomonas* and *E. coli*. The most notable difference between mercuric reductase from *T. ferrooxidans* and the enzyme from other bacteria appears to be its metal ion specificity. Cu²⁺, Cu⁺ and Ag⁺ are strong inhibitors of all the enzymes tested. Cd²⁺ does not inhibit the *T. ferrooxidans* mercuric reductase, but is a strong inhibitor of the *E. coli* enzyme. Similarly Co²⁺ and Fe³⁺ were found to be inhibitors of the *T. ferrooxidans* enzyme, but had no effect on the enzyme from *E. coli* (Rinderle et al., 1983). These results suggest that there are subtle, yet significant, differences between mercuric reductases from these two sources. This is verified by the immunological results reported earlier (Olson et al., 1982).

Mercuric reductase in other bacteria has been associated with the presence of plasmids or transposons (Weiss et al., 1978). Although this has not yet been shown to be the case with *T. ferrooxidans* TFI 29, this organism is known to contain three plasmids of different molecular weights (Martin et al., 1981). The identification of mercury resistance (a selectable marker) with one of these plasmids would be a significant advance in developing recombinant DNA technology for this micro-organism.

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**REFERENCES**


