Purification and Biochemical Characterization of Tellurite-Reducing Activities from *Thermus thermophilus* HB8

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Cell-free extracts of *Thermus thermophilus* HB8 catalyze the in vitro, NADH-dependent reduction of potassium tellurite (K₂TeO₃). Three different protein fractions with tellurite-reducing activities were identified. Two exhibited high molecular weight and were composed of at least two different polypeptides. The protein in the third fraction was purified to homogeneity and had a single polypeptide chain of 53 to 54 kilodaltons, with an isoelectric point of 8.1. Each enzyme was thermostable, the temperature optimum was 75°C, and 30 mM NaCl, 1.5 M urea, or 0.004% sodium dodecyl sulfate caused 50% inhibition of the enzymes. However, 2% Triton X-100 did not have an inhibitory effect. The enzymes were also able to catalyze the reduction of sodium selenite and sodium sulfite in vitro. NADH was replaceable by NADPH. Divalent cations, such as Ca²⁺ and Ba²⁺, had no effect on the activity, while similar concentrations of Zn²⁺, Ni²⁺, and Cu²⁺ abolished the activity. This reductase activity could enable these bacteria both to reduce K₂TeO₃ and to increase their tolerance toward this salt.

It has been reported previously that oxyanions of selenium and tellurium—selenite and tellurite—have a toxic effect for most organisms (6, 7, 9, 10, 22-24). Tellurite can act as a strong oxidant, a property which may be related to its toxicity. The relative toxicity of acid anions of tellurium and its selenium analogs is as follows: TeO₃²⁻ > SeO₂⁻ > TeO₂⁻ > SeO₂²⁻ (21). In general terms, tellurite affects gram-negative bacteria to a greater extent than it affects gram-positive bacteria (21). Resistance to tellurite is not a common property of bacteria. Rare examples of naturally occurring resistant strains include *Corynebacterium diphtheriae* (23), *Streptococcus faecalis* (28, 29), and some staphylococci (10).

Many microorganisms produce black intracellular deposits when grown in media supplemented with potassium tellurite (14, 15, 23, 25, 28-31). Even sensitive bacteria are capable of forming jet-black colonies when growing at sub-inhibitory tellurite concentrations (25). X-ray diffraction studies demonstrated unequivocally that this precipitate is pure tellurium metal (28, 29).

Summers and Jacoby (25) reported that tellurite and tellurate resistance may be conferred by R plasmids in gram-negative bacteria. Bradley (2) found that *Escherichia coli* cells carrying some variant IncP plasmids can transfer tellurite resistance at the same frequency as plasmid-determined drug resistance. Transferable tellurite resistance is generated by plasmids RP4, pUZ8, R26, and pMG22 but not by plasmids belonging to subgroup IncPB. Recently, Taylor and Bradley (26) mapped the tellurite resistance determinant of RP4. Also, Jobling and Ritchie (11) cloned the tellurite resistance determinant from *Alcaligenes* spp.

Although tellurite resistance has been studied biochemically, its mechanism is still unclear. Terai et al. (27) showed that cell-free extracts of *Mycobacterium avium* were able to reduce K₂TeO₃ in the presence of NADH or malate and malic dehydrogenase. A similar tellurite-reducing activity was found in cell-free extracts of *S. faecalis* (J. W. Thomas, M. D. Appleman, and F. L. Tucker. Abstr. Annu. Meet. Am. Soc. Microbiol. 1963, P119, p. 124), and such activity in *E. coli* is probably also enzymatic in origin (4).

We recently reported that the extreme thermophiles *Thermus thermophilus* HB8 and *Thermus flavus* AT-62 also produce black intracellular deposits when they are grown in the presence of K₂TeO₃ (3). This reducing activity was correlated with the presence in cell-free extracts of a tellurite reductase; it could also be related to the intrinsic ability of these bacteria to grow in K₂TeO₃-containing media (>10⁻⁴ M). For these thermophiles, SeO₂⁻ was more toxic than TeO₂⁻ (3). In this paper, we report the purification and biochemical characterization of such an enzyme in an effort to elucidate the molecular basis of tellurite resistance.

**MATERIALS AND METHODS**

**Cells, media, and growth conditions.** The extreme thermophiles *T. thermophilus* HB8 (ATCC 27634) (17) and *T. flavus* AT-62 (ATCC 33923) (19) were grown as described previously (32, 33). Stationary-phase cells were collected by centrifugation and were stored frozen at −25°C.

**Chemicals.** NADH and Triton X-100 were from Sigma Chemical Co., St. Louis, Mo. Potassium tellurite, sodium selenite, and sodium sulfite were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany.

**Purification of tellurite-reducing activity.** Cells (10 g [wet weight]) were suspended in 42 ml of buffer A (20 mM sodium phosphate [pH 7.2], 4 mM 2-mercaptoethanol, 5% [vol/vol] glycerol) and disrupted by sonic treatment. Cell debris were discarded by centrifugation at 48,000 × g at 2°C for 30 min. The supernatant was brought to a 2% (wt/vol) final concentration of streptomycin sulfate, and after 30 min in an ice bath, nucleic acids were pelleted at 30,000 × g. The supernatant was dialyzed against buffer A and was considered as the crude extract. All the subsequent steps were carried out at room temperature, except those in which a dialysis took place; these were performed at 4°C.

The crude extract (50 ml) was loaded onto a phosphocelulose (Sigma) column (4.5 by 10 cm) stabilized with buffer.
The reductase activity did not bind to the resin, and it was immediately poured onto a DEAE-cellulose (DE-52; Whatman, Inc., Clifton, N.J.) column (2.7 by 9 cm) also equilibrated with buffer A. After the column was washed, adsorbed proteins were eluted with a linear gradient (500 ml) of 0 to 0.45 M NaCl in buffer A. Active fractions were pooled, diluted with buffer A to 0.1 M NaCl, and applied to a hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) column (2.7 by 1.5 cm). The column was washed with buffer A and developed with a linear gradient (140 ml) of 0.02 to 0.5 M phosphate in buffer A. Reductase activity was resolved into two peaks (fractions 1 and 2), which were separately concentrated by centrifuging them at 2,000 × g in ultrafiltration cones (CF25; Amicon Corp., Lexington, Mass.). Both activities were independently chromatographed in a Bio-Gel A5m (Bio-Rad) column (0.75 by 45 cm) previously equilibrated with buffer A and standardized with blue dextran (Sigma) and the following molecular weight standards: catalase (240,000), γ immunoglobulin (160,000), bovine serum albumin (66,000), myoglobin (17,000), and cytochrome c (12,500). All these proteins were from Sigma.

Enzymatic assay. In addition to the extract, reaction mixtures (250 μl) contained 10 mM Tris hydrochloride (pH 7.5), 1 mM K2TeO3, and 1 mM NADH. Both substrates were saturating under assay conditions. After incubation at 75°C for 15 min, reactions were halted with an equal volume of a 2 M NaCl solution. Reduction of tellurite was determined by measuring the increase in A500 or the decrease in A340. Reactions were started by the addition of the enzyme. Controls were performed with boiled enzyme and were subtracted from activity values. One unit of enzymatic activity was defined as the amount of enzyme which caused an increase of 0.001 units of A500 min⁻¹ ml⁻¹. Specific activity was expressed as units per milligram of protein. The protein concentration was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of samples was carried out by the method of Laemmli (12). Minigels (8 by 10 by 0.03 cm) were run at 200 V (constant current) for 30 min and immediately immersed in a staining solution containing Coomassie brilliant blue R-250. After 1 h at room temperature or 15 min at 60°C, gels were destained and stored in 10% acetic acid.

Isoelectric focusing. Isoelectric focusing of samples was performed by the method described by Pearce et al. (18). Reductase activity was revealed in situ by immersing the cylindrical gels in a mixture of 10 mM Tris hydrochloride (pH 7.5), 1 mM NADH, and 1 mM K2TeO3 for 30 to 60 min at 72°C.

**RESULTS**

Cell-free preparations of *T. thermophilus* HB8 and *T. flavus* AT-62 catalyzed the reduction of potassium tellurite to metallic tellurium at the expense of NADH oxidation. The specific activity of this enzyme was approximately constant when *T. thermophilus* HB8 cells were grown in culture media with different concentrations of K2TeO3.

**Purification of the tellurite-reducing activity.** The chromatographic behavior of the tellurite reductase activity from *T. thermophilus* HB8 is shown in Fig. 1. The activity was resolved into two components (hereafter designated fractions 1 and 2) upon chromatography on hydroxylapatite (Table 1). Filtration of fraction 1 through Bio-Gel A5m gave

**Table 1. Purification of *T. thermophilus* HB8 tellurite-reducing activities**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>627</td>
<td>40,280</td>
<td>64.2</td>
<td>1</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>414</td>
<td>49,000</td>
<td>118.4</td>
<td>1.8</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>99</td>
<td>21,500</td>
<td>217.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>2.94</td>
<td>7,200</td>
<td>2,452</td>
<td>38.2</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.20</td>
<td>5,800</td>
<td>29,000</td>
<td>452</td>
</tr>
<tr>
<td>Gel filtration of 1</td>
<td>1.70</td>
<td>4,660</td>
<td>2,741</td>
<td>42.7</td>
</tr>
<tr>
<td>Gel filtration of 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 2a</td>
<td>0.01</td>
<td>2,648</td>
<td>264,800</td>
<td>4,125</td>
</tr>
<tr>
<td>Fraction 2b</td>
<td>0.06</td>
<td>1,679</td>
<td>27,983</td>
<td>436</td>
</tr>
</tbody>
</table>

The activity was assayed as described in Materials and Methods. The values represent the averages of at least six different purifications.

Fractions 1 and 2 refer to hydroxylapatite chromatography (see text for details).
FIG. 2. SDS-PAGE of the different fractions obtained during the purification of the tellurite-reducing activities from *T. thermophilus* HB8. Lanes: A and J, molecular mass standards in kilodaltons (KDa); B, crude extract; C, phosphocellulose fraction; D, DEAE-cellulose fraction; E, fraction 1; F, filtration of fraction 1 through Bio-Gel A5m; G, fraction 2; H and I, fractions 2a and 2b, respectively. The conditions of electrophoresis as well as those for staining and destaining the gels are described in Materials and Methods.

a single component of high molecular weight, whereas the second hydroxyapatite peak was resolved into two activities which differed in molecular weight. One of them exhibited high molecular weight (fraction 2a), and a second one (fraction 2b) showed a lower molecular weight.

**Molecular weight estimations.** The molecular weight of these three reductase activities was determined by gel filtration and SDS-PAGE. For hydroxyapatite fraction 1, a molecular mass much larger than 240 kilodaltons (kDa) was estimated by gel filtration. However, SDS-PAGE showed two principal bands of 51 and 53.6 kDa (Fig. 2).

Fraction 2a had a molecular mass slightly larger than that of fraction 1 as determined by Bio-Gel A5m fractionation. SDS-PAGE showed that it was composed of two protein bands of 53 and 55 kDa. Fraction 2b had a molecular mass of 54 kDa upon filtration and was composed of a single polypeptide of 53 kDa as determined by SDS-PAGE (Fig. 2).

The 53-kDa polypeptide from fraction 2b had an isoelectric point (pI) of 8.1. Isoelectric focusing of fraction 2a gave two bands which exhibited in situ reductase activity and whose pIs were 5.0 and 8.2 (Fig. 3).

**Properties of the tellurite-reducing activities.** The enzyme activities in fractions 1 and 2 had the same pH optima (7.5) and temperature optima (75°C). Ionic strengths equivalent to a concentration of 0.2 M NaCl inhibited the enzymes almost completely (Fig. 4).

**Thermostability of the reductases.** The heat stability of these activities was determined by preincubating them for different periods of time at 40, 75, and 90°C and measuring the remaining activity. After being cooled on ice, each sample was assayed at 75°C under standard conditions. Preincubations of 60 min at 90°C caused a decrease of only 10 to 20% of the activity (Fig. 5).

**Substrate specificity.** In addition to reducing *K₃TeO₃*, fractions 1 and 2 were able to reduce sodium selenite and sodium sulfite in vitro. The oxidation of NADH was dependent on the concentrations of these anions in the assay mixture (Table 2). Selenite was probably reduced to elemental selenium, since an orange-red color typical of this element appeared upon incubation. At least for *K₃TeO₃*, NADH can be replaced by NADPH and the reduction reaction was dependent on the concentration of this nucleotide (Table 2).

**Effect of divalent cations.** The effect of several divalent cations (at 10 mM concentration) on the reductase activities of *T. thermophilus* HB8 was tested. Ca²⁺ and Ba²⁺ had little or no effect on the activity, while Mg²⁺, Mn²⁺, and Co²⁺ inhibited the enzymes between 20 and 80%. Zn²⁺, Ni²⁺, and Cu²⁺ caused greater than 90% inhibition. The presence of 2 mM EDTA in the assay medium caused the activity of fractions 1 and 2 to increase threefold.

**FIG. 3.** Densitometric tracings of the electrophoresed tellurite reductase activities from *T. thermophilus* HB8. Activity was revealed in situ as described in Materials and Methods. A and B, Fractions 2a and 2b, respectively. OD₅₂₅s, Optical density at 525 nm.

**FIG. 4.** Effect of sodium chloride on the activity of tellurite reductases from *T. thermophilus* HB8. Reactions were carried out at the indicated NaCl concentrations as described in Materials and Methods. Bars represent the standard deviations.

**FIG. 5.** Isoelectric focusing in a pH gradient of 3 to 10. Fractions 1 and 2 were analyzed for each protein band corresponding to the two activities. The activities decreased at temperatures higher than 40°C.
Crude extracts of *T. thermophilus* HB8 cells are able to reduce K$_2$TeO$_3$ to metallic tellurium in vitro (3). This activity seems to be constitutively expressed, since its specific activity is constant when the cells are grown at different K$_2$TeO$_3$ concentrations. Here we demonstrate that this tellurite-reducing activity can be separated into three components.

Two tellurite-reducing fractions (1 and 2a) were macroaggregates composed of at least two different polypeptide chains. The 53- to 54-kDa protein in fraction 2b comigrated with one of the principal polypeptides of fraction 2a in SDS-PAGE (Fig. 2). It is possible that the 55-kDa protein, which formed aggregates with the 53- to 54-kDa polypeptide, stabilizes the oligomer. The oligomer had a 10-fold-higher specific activity than the single-peptide fraction 2b. The 55-kDa protein could have a stabilizing role or it could be an activator.

All the reductase activities were highly thermostable and resistant to protein-denaturing agents, which are characteristics of enzymes from thermophiles (1, 5). Activation of the enzymes by EDTA was probably due to complexing of contaminant-inhibitory divalent cations from the medium. The inhibition of these reductases by heavy metals suggests that sulphydryl groups could participate in the catalysis, as has been shown for other enzymes capable of reducing selenite and tellurite in vitro (6, 16, 27). However, additional experimental work is required.

Some investigators have studied the mechanism of resistance to selenite (6-8, 16, 20, 21). Working with a selenite-resistant strain of *Candida albicans*, Nicksenson and Falcone (16) isolated an enzymatic activity which reduces in vitro selenite to elemental selenium by using NADH or NADPH as a cofactor. Other investigators proposed that the detoxification of selenite in *E. coli* occurs via its reduction through the sulfate reduction pathway (20). It has been observed that the addition of L-methionine to the culture medium increases selenite toxicity (20). Tellurite toxicity seems to be affected in the same way (21). This has been interpreted as a decrease in the sulfate reduction rate, which indirectly decreases those of selenite and tellurite, leaving them available to exert their toxic effects (21). However, this is not the case in *T. thermophilus* HB8. We have grown these cells in the presence of concentrations of potassium tellurite and sodium selenite which varied between 3 x 10$^{-4}$ and 3 x 10$^{-6}$ M. Parallel cultures supplemented with 0.1 mM L-methionine gave the same viable-cell numbers, showing that the toxicities of these salts are not increased (unpublished observations). Hence, at least for these microorganisms, tellurite as well as selenite would be detoxified in a different manner than that described for sulfate reduction.

It is possible that in these *Thermus* strains the resistance to K$_2$TeO$_3$ is related to the ability to reduce it. This has not been determined with certainty, but some observations support this conclusion. Tucker et al. (28) reported that when different strains of streptococci are grown at subinhibitory TeO$_2^{2-}$ concentrations, resistant cells produce blacker colonies than do sensitive ones. When *T. thermophilus* HB8 or *T. flavus* AT-62 cells are grown in the presence of increasing tellurite concentrations, the black color produced is proportional to the salt concentration, i.e., the higher the concentration, the blacker the cells. In the particular case of *T. thermophilus* HB8, there is a good correlation between cell viability and the in vitro reductase activity. Enzyme activity increases as tellurite concentration in the assay.
medium is raised, a maximal level being reached at about 0.1 mM (unpublished results). This value coincides with the maximal amount of potassium tellurite that these cells can tolerate without their viability being affected (3).

Further investigations are being undertaken to determine the specific role of these tellurite reductases and the way in which the resistance to K₂TeO₃ is achieved.

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LITERATURE CITED