RESEARCH ARTICLE

Identification of a TeO_3^{2-} reductase/mycothione reductase from *Rhodococcus erythropolis* PR4

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One sentence summary: A novel enzyme selective for tellurium reduction was isolated from an environmental sample and characterized.

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ABSTRACT

A *Rhodococcus erythropolis* bacterium that tolerates normally lethal concentrations of Fe(II), Cu(II), AsO_3^{2-} , SeO_3^{2-} , TeO_3^{2-} , Cd(II) and Zn(II) was identified from an environmental isolate. In characterizing the molecular basis for metal tolerance, a mycothione reductase (Mtr) with remarkable selectivity for TeO_3^{2-} reduction over SeO_3^{2-} was identified. In equimolar concentrations of TeO_3^{2-} and SeO_3^{2-} , the enzymatic product contains a 7-fold excess of Te. This selectivity is remarkable because the standard reduction potential of SeO_3^{2-} is 0.20 V more favorable for reduction than TeO_3^{2-} . Selectivity of the enzyme for TeO_3^{2-} decreases with increasing assay pH. Homology modeling of the enzyme identifies four aromatic residues near the active site, including two histidine residues, that are not present in a related SeO_3^{2-} preferring reductase. On the basis of more favorable π -interactions for Te than for Se and the pH dependence of the selectivity, the Te-selectivity is attributed in part to these aromatic residues. The resulting Te^0 enzymatic product resembles Te nanowires.

Keywords: biogenic nanoparticles; tellurium reductase; substrate selectivity; environmental isolates; electron microscopy

INTRODUCTION

Cellular resistance to toxic metal ions manifests through several pathways typically involving ion sequestration, the conversion of biologically active soluble species to less active insoluble materials, volatilization or adaptive regulation of ion transportation. (Magos, Tuffery and Clarkson 1964; Choudhury and Srivastava 2001; Puskárová et al. 2002; Sarret et al. 2005; Ollivier et al. 2008; von Rozicky and Nies 2008; Llorens et al. 2012; Lal et al. 2013; Arenas et al. 2014) These pathways typically operate through multiple protein cascades wherein toxins are processed in a stepwise manner. However, in several instances discrete enzymes have demonstrated independent activity towards metals leading to improved cell survivability in challenged environments. Cysteine-rich metallothioneins have been identified for their role in metal sequestration. (Ecker et al. 1986; Huang

et al. 1987; Klaassen, Liu and Choudhuri 1999; Schor-Fumbarov et al. 2005; Reddy et al. 2014; Li et al. 2016) Cystathione γ -lyase is capable of extracting a sulfur group from cysteine which is capable of binding and precipitating Cd^{2+} . (Yang et al. 2015, 2017) Mercuric Reductase (MerR) has been identified in the reduction of Hg(II) to elemental Hg which rapidly volatilizes. (Fox and Walsh 1982; Engst and Miller 1998; Freedman, Zhu and Barkay 2012) Dihydrolipoamide dehydrogenase, nitrate reductase and catalase have demonstrated reducing activity towards selenite, selenate, tellurite and tellurate to form less toxic inorganic precipitates. (Avazéri et al. 1997; Sabaty et al. 2001; Calderón et al. 2006; Castro et al. 2008) Chromate reductase is responsible for the reduction of labile and toxic Cr(VI) to less bio-active Cr(III), although no precipitate is formed in this instance. (Zhou et al. 2017) In current literature there are more documented examples of organisms and specific enzymes capable of performing

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Se-reduction with respect to other metal and metalloid species. (Turner, Weiner and Taylor 1998; Sarret et al. 2005; Avendaño et al. 2016; Elahian et al. 2017)

Tellurium specialized microorganisms have been previously described. In general, zerovalent tellurium is much less toxic than tellurite, so reduction of tellurite is a common strategy for tellurite tolerance. (Chasteen et al. 2009) Several organisms have been described that reduce tellurite, with intracellular Te nanorods observed in *Rhodococcus aetherivorans* and *Rhodobacter capsulatus*. (Presentato et al. 2018; Borghese et al. 2020) Three distinct tellurite reductases have been identified from three different species. (Moore and Kaplan 1992; Maltman, Donald and Yurkov 2017a,b; Maltman and Yurkov 2019) While some enzymes are observed for preferring tellurite substrates over selenite substrates, the molecular mechanism for distinguishing among substrates is obscure.

The identification of additional enzymes and enzymatic pathways for metal oxidation and reduction is of both fundamental and applied interest. Fundamentally, the metabolism of metals is incompletely understood, especially those metals that are non-essential and even toxic. The enzymes and enzymatic pathways revealed in fundamental studies may be subsequently adapted for bio-remediation of areas of metal contamination, and also possibly as reporter genes, producing nanoparticles that impart contrast in imaging modalities such as electron microscopy and x-ray imaging. (Presentato et al. 2018)

Herein, we report the identification and characterization of a mycothione reductase specialized for Tellurite reduction. This enzyme is found in an environmental isolate identified as *Rhodococcus erythropolis* PR4 that was cultured from mine site seepage in the Colorado Mining Belt. Cultured *R. erythropolis* PR4 showed high resistance to several metal ion species, including typically toxic concentrations of Fe(II), Cu(II), AsO_3^{2-} , SeO_3^{2-} , TeO_3^{2-} , Cd(II) and Zn(II). Upon screening of lysate from *R. erythropolis* PR4, a mycothione reductase homolog was isolated possessing selective NADPH-dependent reductase activity towards TeO_3^{2-} to form Te^0 nanoparticulate. The isolated mycothione reductase homolog (Mtr) showed high reduction specificity towards Te-based substrates compared to Se-based substrates. This is a surprising finding on the basis of reduction potentials for the two substrates. The standard reduction of potential for the reaction $\text{TeO}_3^{2-} + 3 \text{H}_2\text{O} + 4\text{e}^- \leftrightarrow \text{Te} + 6 \text{OH}^-$ is -0.57 V vs Hydrogen. The corresponding reduction of SeO_3^{2-} is -0.366 V . Thus, SeO_3^{2-} is the preferred substrate for reduction in the absence of a mechanism for substrate selectivity.

MATERIALS AND METHODS

Materials

PCR and ligation components, restriction enzymes and T7 Expression lysY/I^q Competent *E. coli* cells (Cat # C30131) were purchased from New England Biolabs, Inc. (Ipswich, MA) Primers were purchased from Integrated DNA Technologies (Coralville, IA). The expression vector pD441-CH was originally purchased from ATUM (Dna Twopointo Inc., Newark, CA). Antibiotics were purchased from GoldBio (St. Louis, MO). Na_2SeO_3 , HNaSeO_3 and K_2TeO_3 were purchased from Alfa Aesar (Stoughton, MA). NADPH was purchased from BioVision (Milpitas, CA) and Coomassie Plus Bradford Reagent from Thermo Scientific (Coon Rapids, MN). GTP was purchased from Chem-Impex Int'l Inc. (Wood Dale, IL) GeneJet Plasmid Miniprep Kit (Cat# K0503) and PCR Cleanup Kit (Cat# K0702) were purchased from ThermoFisher Scientific (Waltham, MA).

Metal screening of lysates

Lysate metal screens were then conducted to try and identify specific enzymes responsible for the reduction of the metals tested. A total of 25 mL cultures of the environmental isolates were grown to density, cells were collected, and lysed using intense agitation with glass beads. Lysate was collected and run in a native-PAGE gel for crude separation of the lysate. The PAGE gel was then cut into vertical strips and incubated in 50 mM HEPES buffer with 5 mM of a metal salt and either NADPH or NADH as a cofactor. Reactions were run in an inert atmosphere and the next day, gels were observed for possible metal band development, indicating metal salt reduction. Corresponding bands in the gel were then excised and sent for LC-MS/MS.

LC-MS/MS sample preparation

Gel fragments were subjected to in-gel trypsin digestion and LC-MS/MS as previously described. (Saveliev et al. 2013) Briefly, the gel pieces were washed with 200 μL of LC-MS Grade Water (Optima LC-MS, Fisher Scientific, Pittsburgh, PA) for 30 s and destained with $2 \times 200 \mu\text{L}$ of 50% Acetonitrile (ACN; Optima LC-MS Grade)/50 mM Ammonium bicarbonate at 60°C , with intermittent mixing. The pieces were dehydrated with 100% ACN and allowed to air dry. Proteins were reduced and alkylated, in-gel with 25 mM DTT in 50 mM ammonium bicarbonate (60°C for 20 min) and 55 mM IAA or IAH in 50 mM ammonium bicarbonate at room temperature in the dark for 20 min. Gel pieces were then washed with Optima water and dried. The dried gel pieces were rehydrated in 20 μL 12 ng/ μL MS-grade Trypsin (ThermoPierce, San Jose, CA)/0.01% ProteaseMAX surfactant/50 mM ammonium bicarbonate mixture for 10 min at room temperature, overlaid with 30 μL 0.01% ProteaseMAX surfactant/50 mM ammonium bicarbonate and incubated at 50°C for 1 h. Extracted peptides were transferred and the digestion halted by addition of 10% trifluoro-acetic acid to a final concentration of 0.5%. Peptide extracts were dehydrated then resuspended in 5% ACN/0.1% formic acid. Once resolubilized, absorbance at 205 nm was measured on a NanoDrop (ThermoScientific, Coon Rapids, MN) and total peptide concentration was subsequently calculated using an extinction coefficient of 31 (Scopes 1974).

LC-MS/MS mass spectrometry analysis

A total of 1 μg of peptides were purified and concentrated using an on-line enrichment column (Thermo Scientific 5 μm , 100 μm ID x 2 cm C18 column). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Thermo Scientific EASYnano-LC, 3 μm , 75 μm ID x 100 mm C-18 column) using a 30-min linear gradient from 5 to 30% buffer B (100% ACN, 0.1% formic acid) at a flow rate of 400 nL/min. Peptides were eluted directly into the mass spectrometer (Orbitrap Velos, Thermo Scientific) equipped with a Nanospray Flex ion source (Thermo Scientific) and spectra were collected over a m/z range of 400–2000, positive mode ionization, using a dynamic exclusion limit of 2 MS/MS spectra of a given m/z value for 30 s (exclusion duration of 90 s). The instrument was operated in FT mode for MS detection (resolution of 60 000) and ion trap mode for MS/MS detection with a normalized collision energy set to 35%. Compound lists of the resulting spectra were generated using Xcalibur 3.0 software (Thermo Scientific) with a S/N threshold of 1.5 and 1 scan/group.

LC-MS/MS data analysis

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by ProteoWizard MsConvert (version 3.0). Spectra from all samples were searched using Mascot (Matrix Science, London, UK; version 2.3.01) against the customized Uniprot_Ecoli_rev.08_2416 database (unknown version, 8630 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 PPM. Oxidation of methionine, carbamidomethyl of cysteine and carboxymethyl of cysteine were specified in Mascot as variable modifications. Search results from all samples were imported and combined using the probabilistic protein identification algorithms implemented in the Scaffold software (version Scaffold.4.5.1, Proteome Software Inc., Portland, OR; Keller et al. 2002; Searle, Turner and Nesvizhskii 2008). The peptide threshold (95%) was set such that a peptide FDR of 0.0% was achieved based on hits to the reverse database (Käll et al. 2008). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Plasmid construction

Colony PCR (cPCR) was used to amplify the gene for all the NADPH-dependent proteins that resulted from LC-MS/MS. Briefly, gene sequences were identified using an amino acid sequence BLAST against the genome and three plasmids of *R. erythropolis* PR4. Sequences then directed the design of primers that would be used for gene amplification. PCR reactions were then set up as follows, 100 nM final concentration of each primer, 200 μ M dNTPs, 10 μ L of Q5 buffer, 10 μ L of GC Enhancer, 1U of NEB's Q5 High-Fidelity Polymerase were brought up to 50 μ L with milli-Q water. To each PCR reaction tube, an aliquot of *R. erythropolis* was added to provide a genomic template for the reaction. Denaturing of the template occurred at 98°C for 2 min followed by 37 cycles of a 10 s denaturing at 98°C, 65°C for 20 s and extension at 72°C for 2 min and 45 s. A final 2 min extension at 72°C to finish any incomplete extensions. PCR product was cleaned using the Thermo Gene-jet PCR purification kit and eluted with 50 μ L of milli-Q water for future digestion and ligation. 1 μ g of PCR product was then digested at 50°C for at least 1 h using 10 U of corresponding restriction enzyme, 5 μ L corresponding NEB Buffer and a final volume of 50 μ L using milli-Q water. The restriction digestion was again cleaned using the Thermo purification kit and eluted using 40 μ L of milli-Q water. Insertion of the genes into the expression vector pD441-CH with a C-terminal His-tag purchased originally from ATUM were performed using T4 Ligase and resulting plasmids were transformed into BL21(DE3) cells for protein isolation. Gene insertion was confirmed through sequencing.

Mycothione reductase Isolation

A started culture of BL21(DE3) containing Mtr with a C-terminal His tag was grown overnight at 37°C and 225 RPM. The next day a 1.2 L LB growth flask supplemented with chloramphenicol and kanamycin was inoculated with the starter culture and grown until an OD₆₀₀ ~0.5 at which time the culture was induced using 500 μ M IPTG and grown at ~30°C overnight in aerobic conditions.

The next day cells were collected, resuspended in 20 mM TEA-HCl, pH 7.6 and lysed on ice using sonication. Insoluble material was removed through centrifugation and the resulting lysate, which was red in color due to the presence of concentrated Mtr. Fast Protein Liquid Chromatography (FPLC) was then used for initial isolation of Mtr using a Ni-NTA column. Briefly, a HisTrap FF column was equilibrated with TEA-HCl, pH 7.6, 2.5 mM imidazole. Lysate was then loaded on the column for 30 min mL/min. Once lysate was loaded onto the column a gradient from 2.5 mM imidazole to 150 mM imidazole over 60 min at 5 mL/min. Fractions were collected every minute and the presence of Mtr was indicated by a bright yellow color. These fractions were then combined and run on a HiTrap DEAE FF column to further isolate Mtr using a gradient from 0 to 500 mM NaCl over 60 min. Mtr, an enzyme with a pI of 5.1 was eluted from the DEAE column above 400 mM NaCl. Protein purification was checked using PAGE gel analysis.

Kinetics studies

Reduction reactions were performed using 15 μ g of Mtr in either 20 mM HEPES pH 7.0, 20 mM TEA-HCl pH 7.6, or PBS pH 7.4. Alternatively, pH of each buffered solution was adjusted to pH 6.0, 7.0, 8.0 or 9.0 with HCl or NaOH as appropriate. Varying concentrations of K₂TeO₃, or HNaSeO₃, were added to the reaction and final volumes were brought to 1.0 mL. Reactions were then initiated by the addition of 200 μ M NADPH. The oxidation of NADPH corresponding with the decrease in absorbance at 340 nm was correlated to the activity of the enzyme reaction velocity. At pH 6 and 7, the solubility of TeO₃²⁻ diminished (insoluble precipitates formed), although this does not appear to impact enzyme function. Kinetic data was fit with the Michaelis-Menten model in Origin (Northampton, MA, originlab.com).

Scanning electron microscopy

Samples for SEM were prepared by the following: 15 μ g of Mtr and 1.0, 3.0 or 7.0 mM K₂TeO₃ brought to 1 mL PBS pH 7.4. Reactions initiated with an excess amount of NADPH and run overnight at room temperature. The next day, aliquots (4 μ L) were deposited on C-coated Cu TEM grids and imaged using a JEOL JSM-6500-F Scanning Electron Microscope at an accelerating voltage of 15 kV.

ICP-MS

Mtr (50 μ g), 1 mM K₂TeO₃ and either 1, 5 or 10 mM HNaSeO₃ were combined in PBS pH 7.4. Reactions were initiated with 500 μ M of NADPH and brought to a final volume of 1 mL. Reactions were run overnight at room temperature. The next day, reactions were centrifuged to collect the particulate formed during the reduction. Particulate was washed with 4 $\times$ 1 mL milliQ water before the particulate was dissolved in 70% HNO₃. The solution was then diluted using 2% HNO₃ until concentrations of Te and Se fell within predetermined standardized curves.

Selectivity assay

Mtr (15 μ g), 2 mM K₂TeO₃ and 2 mM HNaSeO₃ were combined in PBS pHs 6.0, 7.0, 8.0, 9.0 or 10.0 in triplicate. Reactions were initiated with the addition of 500 μ M NADPH, brought to a final volume of 1.0 mL and run overnight at room temperature. Samples were centrifuged at 15 000 g for 10 min to collect particulate formed during the reduction, washed 4x with 1 mL milliQ

Figure 1. (A) Native-PAGE gel sections incubated in indicated metal/loid ions and NADPH. The arrow indicates reduced insoluble Te⁰ particulate. Cu(II) was precipitated in gel with and without protein. (B) Proteins associated with the excised Te(0) by LC-MS/MS. Entries in *italics* indicate the presence of an NAD(P)H binding domain.

water, then dried. Samples were then delivered to the Science Exchange, Inc. Biophysics and Spectroscopy Core at the University of Nebraska, Lincoln for ICP-MS and prepared with 450 μ L 2% nitric acid and 50 ppb Ga.

RESULTS AND DISCUSSION

Identification and selectivity of a Te-reductase

The Mtr enzyme was isolated from an environmental *R. erythropolis* isolate. The bacterium was found in multiple metal-contaminated mine runoff in Clear Creek County, Colorado. This is a county in the Colorado Mineral Belt that contains over 1500 abandoned mine sites dating to the 1851 Colorado Gold Rush. More information on this can be found in the Supplemental Information. *Rhodococcus erythropolis* PR4 was of interest because of its tolerance to ordinarily lethal concentrations of Fe(II), Cu(II), AsO₃²⁻, SeO₃²⁻, TeO₃²⁻, Cd(II) and Zn(II). Full data on the metal tolerance of this isolate, including minimum inhibitory concentrations (MIC) can be found in Figure S1 and Table S1 (Supporting Information). Of note, the MIC for TeO₃²⁻ was found as 4.5 mM.

Initial identification of Mtr as a TeO₃²⁻ reducing enzyme was in a previously reported in-situ screen developed for identifying metal-reducing enzymes. (Presentato et al. 2018) Briefly, in a non-denaturing-PAGE, all soluble lysate proteins from *R. erythropolis* PR4 were fractionated. The gel was then incubated in metal salts and enzyme cofactors. Visible bands in the gel are attributed to enzymatic reduction of metal salts to nanoparticulate form. Since *R. erythropolis* PR4 exhibits tolerance to several metal salts, we opted to vertically section native-PAGE gels and expose them to 5 mM concentrations of K₂TeO₃, HNaSeO₃, CuCl₂, ZnCl₂ and FeCl₂. Each in-situ reaction was supplemented with either NADPH or NADH, since many known metal oxidoreductases depend on these biological electron sources.

We observed a single band develop when incubated in TeO₃²⁻ and NADPH (Fig. 1A). This was surprising given that no band developed with SeO₃²⁻, a chemically similar chalcogen oxyanion that has a redox potential 0.2 V more positive. (Rankin 2009) Another band was observed when a gel was incubated with TeO₃²⁻ and NADH although to a far lesser extent indicating the enzyme was preferential to NADPH as the cofactor. Bands were not observed when cofactors were omitted from the assay.

Excision of the bands, followed by proteolytic digestion and LC-MS/MS, led to identification of 13 proteins which are

Figure 2. Elemental composition of precipitates resulting from enzymatic reactions in indicated ratios of TeO₃²⁻ and SeO₃²⁻, as determined by ICP-MS. All reactions were performed with 500 μ M NADPH.

listed in Fig. 1B. Of those 13 identified proteins, five possessed NAD(P)H binding domains. We focused initial characterization on the mycothione reductase identified in this screen, due to its functional homology (metalloid reduction) to a Glutathione Reductase-like Metalloid Reductase (GRLMR) we previously identified from the most Se tolerant organism described to date. (Ni et al. 2015; Nemeth et al. 2018)

Mtr was initially amplified by PCR from the genome of *R. erythropolis* PR4. Primers were based upon the known genomic sequence of *R. erythropolis* PR4, which was initially isolated south of Okinawa Island, Japan in the Pacific Ocean. (Komukai-Nakamura et al. 1996; Sekine et al. 2006) The gene was then inserted into an expression vector from ATUM, p-D441CH plasmid possessing a C-terminal His tag and Kanamycin resistance. Mtr was expressed in T7 Express *E. coli* (C25661) from New England Biolabs (NEB). Expression was confirmed in the soluble lysate after induction for several hours using SDS-PAGE. Mtr was then isolated using a 5 mL HisTrap Fast Flow column and further purified on a 5 mL HiTrap DEAE Fast Flow column. Purity of Mtr was determined on SDS-PAGE, shown in Fig. 2C.

Specificity of Mtr for TeO₃²⁻ reduction over SeO₃²⁻ reduction was established by assaying the elemental ratio of Te: Se in the reaction product of reactions performed in mixtures of TeO₃²⁻ and SeO₃²⁻. Experiments with equimolar amounts of TeO₃²⁻ and SeO₃²⁻ were tested, as well as 5- and 10-fold excesses of SeO₃²⁻. The ratio of Te to Se in each insoluble reaction product was established by ICP-MS. Fig. 2 illustrates that Te content dominates enzymatic products when both TeO₃²⁻ and SeO₃²⁻ are simultaneously available as reactants. At equimolar reactant concentrations, over 6 times more Te than Se is found in the enzymatic product. When Se is present in a 5-fold molar excess, Te is still found in the enzymatic product at 2.5-fold excess to Se. Even when Se is in 10-fold excess to Te, the resulting insoluble fraction still possessed more Te. Combined, these experiments show Mtr exhibits a strong substrate preference for TeO₃²⁻ over SeO₃²⁻.

Kinetics study of Te-reducing Mtr

The enzyme was further characterized for its kinetic rate with substrates TeO₃²⁻ and SeO₃²⁻. Reduction experiments were performed in PBS, 20 mM TEA, and 20 mM HEPES pHs 6–9 to test

Figure 3. A screen for how reaction pH effects Mtr substrate selectivity, using PBS buffer. The y-axis indicates initial velocities for Mtr with substrates TeO_3^{2-} and SeO_3^{2-} . Each substrate was present at 2 mM. The pH screen suggests that selectivity decreases as pH increases.

effects of varying buffers and pH ranges with respect to enzyme kinetics. The enzyme kinetics were established by monitoring the loss of the NADPH substrate spectrophotometrically. This substrate absorbs distinctly at 340 nm, and the corresponding product (NADP^+) does not. Substrates tested were 2 mM starting concentrations of SeO_3^{2-} , TeO_3^{2-} and initial velocities were determined through monitoring the degradation of 200 μM NADPH at 340 nm.

Initial kinetic rates of the enzyme toward each reactant in PBS, HEPES and Triethyl Amine (TEA) buffers, with pH adjusted to values in between 6 and 9. Initial kinetic rates were essentially buffer-independent; The initial rates of reaction in PBS for each substrate at varying pH values are shown in Fig. 3. This pH screen shows that the reduction of TeO_3^{2-} is substantially faster than the reduction of SeO_3^{2-} , independent of pH. Results for HEPES and Triethylamine (TEA) buffers are shown in Figure S3 (Supporting Information). The K_M and k_{cat} of Mtr were also calculated using initial velocities of Mtr with a range of 0.5–10 mM of either TeO_3^{2-} or SeO_3^{2-} in PBS (pH 7.4), 20 mM TEA-HCl (pH 7.6) and 20 mM HEPES (pH 7.0). Plots of the V_0 at varying substrate concentrations for HEPES buffered reactions are shown in Fig. 4 and the calculated values shown in Table 1. In all buffers tested, Mtr possessed a more favorable K_M for TeO_3^{2-} compared to SeO_3^{2-} . The catalytic efficiency (k_{cat}/K_M) is 10-fold higher for TeO_3^{2-} over SeO_3^{2-} . We note that we did not find a substrate concentration of SeO_3^{2-} that achieves a steady-state reaction (Fig. 4), allowing an accurate determination of enzymatic V_{max} for this substrate. This may impact the accuracy of the Michaelis-Menten kinetic fit. However, this difficulty in observing V_{max} for SeO_3^{2-} substrates is a further indication of the adaptation of this enzyme for TeO_3^{2-} . Indeed, the highest concentrations of SeO_3^{2-} tested (40mM) are decidedly non-physiological. To emphasize this point, we found an MIC of 2.5mM SeO_3^{2-} for this microbe.

When examining the activity of the enzyme toward different substrates, we calculate that the activity of the enzyme is higher for TeO_3^{2-} than SeO_3^{2-} for all substrate concentrations tested that were 5 mM or less. For instance, in HEPES buffer at

5 mM substrate concentration, we calculate the activity of the enzyme as 1095.8 ± 34.6 μmol NADPH consumed per minute per mg of enzyme (μmol in g) for TeO_3^{2-} , whereas this value is calculated as 680.7 ± 6.50 μmol NADPH /mn /m of enzyme for SeO_3^{2-} .

Initial kinetics studies (Fig. 3) suggested that selectivity for Te over Se may diminish at higher pH. Because this observation has implications for the mechanism of selectivity, we compared relative amounts of Mtr reduction product when equimolar concentrations of TeO_3^{2-} and SeO_3^{2-} were present as equimolar reactants. Figure 5 depicts the relative molar product ratios of Te and Se as determined by ICP-MS. This experiment confirms that selectivity of the enzyme for TeO_3^{2-} over SeO_3^{2-} diminishes as pH increases. This diminishment of selectivity at higher pH implicates the protonation state of residues near the active site in substrate selectivity.

A structural basis for Te selectivity

A homology model for the structures of Mtr and GRLMR were generated with Phyre2. (Kelley et al. 2015) The most similar empirical structure used in the homology model is the mycothione reductase from *Mycobacterium tuberculosis* which is 65.64% similar (Patel and Blanchard 1999; Kumar et al. 2017; Kumar, Manimekalai and Grüber 2018). 3D structural alignment to GRLMR in Pymol (Kelley et al. 2015) was used to identify the putative active site of Mtr. The active site of GRLMR and related glutathione reductases can be defined by a pair of redox-active putatively catalytic cysteine residues.

An examination of both structures reveals several mutations near the active site. These are T259V, T339A, G203H, Y175F K49T, R305Y, P310H and V434I, Fig. 6 shows the active site of both the Selenite selective GRLMR and the Tellurite selective Mtr. What is apparent in examining these active sites is the abundance of aromatic residues in Mtr that are absent in GRLMR. For Mtr, these residues include Y307, H311, F178 and H206. In contrast, GRLMR contains no aromatic residues in or near its active site. Indeed the aromatic residues and the larger size (to accommodate a larger substrate) in Mtr are the chief differences between the two active sites.

Within the chalcogens, π -interaction potential decreases going towards heavier elements, $\text{S} > \text{Se} > \text{Te}$. (Chivers, Laitinen and Tellurium 2015) The residues H206 and Y307 are predicted as pointing towards the opening and could be involved in substrate interactions that are more favorable for TeO_3^{2-} as compared to SeO_3^{2-} , due to the more favorable π -interactions with Te. The pH dependence of selectivity further implicates H206 and possibly H311 in selectivity for Te over Se, as these residues are protonated at lower pH and deprotonated at higher pH.

Mtr mediated Te-nanostructures

The Mtr reaction product from TeO_3^{2-} substrates is solid-phase Te^0 . Examination of the Te^0 by electron microscopy (EM) reveals nanoparticles and nanostructures of distinct morphology. The size and shape of these Te nanoparticles or nanostructured Te depends sensitively on the concentration of TeO_3^{2-} substrate. Concentrations of 1, 3, and 7 mM of K_2TeO_3 with excess of NADPH were reduced in the presence of 15 μg of Mtr. Reactions proceeded for 16 h and the resulting Te^0 nanostructures were imaged by Scanning Electron Microscopy (SEM). In 1 mM TeO_3^{2-} reactions, the product is a prolate ellipsoid of approximately 50 nm in the axial dimension (Fig. 7, panel A). In 5 mM TeO_3^{2-} , the reaction product resemble bundled Te nanowires (Fig. 7,

Figure 4. Plots of enzymatic rate (min^{-1}) v. substrate concentration (mM) for Mtr. Solutions were buffered in HEPES, pH 7.4. Substrates were either TeO_3^{2-} or SeO_3^{2-} , as indicated on each plot.

Table 1. Kinetics data for Mtr.

	20 mM HEPES, pH 7		20 mM TEA-HCl, pH 7.6		PBS, pH 7.4	
	TeO_3^{2-}	SeO_3^{2-}	TeO_3^{2-}	SeO_3^{2-}	TeO_3^{2-}	SeO_3^{2-}
K_M (mM)	0.517 ± 0.050	41.14 ± 11.69	0.779 ± 0.050	35.23 ± 4.51	0.413 ± 0.141	43.9 ± 14.7
k_{cat} (min^{-1})	59.8 ± 1.07	327.4 ± 56.7	88.6 ± 5.40	497.1 ± 36.9	11.9 ± 0.68	215.8 ± 44.9
k_{cat}/K_M	16 ± 11.4	7.9 ± 2.65	14 ± 10.1	14.1 ± 2.09	28.9 ± 9.99	4.9 ± 1.94

Figure 5. Ratio of Te: Se in enzymatic products, as determined by ICP-MS for reactions performed at pH values between 6 and 9.

panel B). In 7 mM TeO_3 , the resulting nanostructure appears as a fractal-like deposit composed of large aggregates of fused nanowire bundles (Fig. 7, panel C). Te-nanostructures observed *in vitro* also corresponded well with the structures observed in *R. erythropolis* PR4 when incubated with K_2TeO_3 (Figure S2, Supporting Information).

The growth of Te nanowires from amorphous Te, as we observe in this work, is previously described by others in abiotic synthesis. The formation of nanowires from amorphous Te

Figure 6. Homology models of Mtr and GRLMR shown as an overlay of each structure. The active-site cysteine residues are shown in brown (carbon) and yellow (cysteine). Aromatic residues in the active site cavity and within 15 Å of the active site that are present in Mtr but not in GRLMR are shown in Magenta.

results from the high surface energy of amorphous Te, which rearranges spontaneously to trigonal Te nanorods. The conversion from amorphous to trigonal Te is slow at room temperature. The trigonal Te nanorods then grow perpendicular to the [001] plane. (Liu et al. 2003, 2004; Gautam and Rao 2004)

SUMMARY AND CONCLUSION

Microorganisms isolated from metal contaminated mine runoff in the Colorado Mining Belt have provided several opportunities to study enzymes that enable cell survival in a wide range

Figure 7. Scanning electron micrographs of Te-nanostructures produced by Mtr with substrate concentrations of (A) 1 mM, (B) 3 mM and (C) 7 mM K_2TeO_3 . Scale bar in each image is 500 nm.

of toxic metal salts. Screening of cell lysates identified a Te-reducing mycothione reductase identified from *R. erythropolis* PR4. Mtr has notable selectivity for tellurite reduction over selenite reduction, which we attribute to the presence of aromatic residues near the active site.

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SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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