**Structure and mechanism of the chalcogen-detoxifying protein TehB from *Escherichia coli***

Hassanul G. CHOUDHURY, Alexander D. CAMERON, So IWATA and Konstantinos BEIS†

Membrane Protein Laboratory, Imperial College London, Diamond Light Source, Chilton, Oxfordshire OX11 0DE, U.K.

The oxyanion derivatives of the chalcogens tellurium and selenium are toxic to living organisms even at very low levels. Bacteria have developed mechanisms to overcome their toxicity by methylating them. The structure of TehB from *Escherichia coli* has been determined in the presence of the cofactor analogues SAH (S-adenosylhomocysteine) and sinefungin (a non-hydrolysable form of S-adenosyl-L-methionine) at 1.48 Å (1 Å = 0.1 nm) and 1.9 Å respectively. Interestingly, our kinetic data show that TehB does not discriminate between selenium or tellurium oxyanions, making it a very powerful detoxifying protein. Analysis of the active site has identified three conserved residues that are capable of binding and orientating the metals for nucleophilic attack: His₁⁷⁶, Arg₁⁷⁷ and Arg₁⁸⁴. Mutagenesis studies revealed that the H176A and R184A mutants retained most of their activity, whereas the R177A mutant had 65% of its activity abolished. Based on the structure and kinetic data we propose an S₂N₂ nucleophilic attack reaction mechanism. These data provide the first molecular understanding of the detoxification of chalcogens by bacteria.

Key words: chalcogen, detoxification, methyltransferase, nucleophilic attack, selenium oxyanion, tellurite oxyanion.

**INTRODUCTION**

The chalcogens selenium and tellurium can be found as trace elements in the environment. The oxyanions of tellurite (TeO₃²⁻), selenite (SeO₄²⁻) and selenite (SeO₃³⁻), are highly soluble in water and are known to be toxic to biological systems, whereas the elemental tellurium (Te⁰) and selenium (Se⁰) are insoluble in water and are almost non-toxic.

Bacteria have developed mechanisms for the detoxification of the oxyanion metals by reduction to the elemental state or by methylating them so that they become volatile. The bacteria *Paracoccus denitrificans* and *Rhodobacter sphaeroides* f. sp. *denitrificans* can reduce selenite to selenium (Se⁰) [1,2]. The *Escherichia coli* NapA, a periplasmic nitrate reductase, is capable of reducing tellurite with the aid of a membrane-bound nitrate reductase [2,3]. Reduction of tellurite results in precipitation of tellurium within cells, which are seen as black deposits of elemental tellurium [4,5]. Bacteria can also detoxify selenium and tellurium by SAM (S-adenosyl-L-methionine)-dependent methylation mechanisms to produce volatile compounds. Volatile methylated selenium oxyanions have been detected by GC–MS headspace chromatography analysis from *Pseudomonas syringae* cultures that were spiked with sodium selenite, selenate and (methyl)selenocysteine [6,7]. bTPMT (bacterial thiopurine methyltransferase) can methylate inorganic and organic selenium (selenocysteine) to dimethylselenide and dimethylselenide [8]. GC–MS analysis of headspace gases of *E. coli* harbouring bTPMT showed the presence of volatile dimethylselenide and dimethylselenide. bTPMT can also confer resistance to tellurite by methylating to dimethyltelluride [9].

Volatile methylated tellurium compounds have been identified in tellurite-resistant marine microbes of the order Bacillales; GC–MS analysis of headspace gas showed the presence of mixed species of dimethyltellurite, dimethyltelluride and dimethyltellurenyl sulﬁde [5].

The Challenger mechanism has been proposed for the methylation of arsenic compounds [10,11] and for selenium compounds [10]; selenite has to be reduced to selenide and then methylated in the presence of SAM. The methylselenide can be further reduced to methylselenenic acid and methylated to dimethyl selenone. It has been proposed that this mechanism can be adapted for tellurite compounds.

In *E. coli* the protein TehB has been identified as responsible for the methylation of TeO₃²⁻ [12]. In vivo assays did not establish any volatile methylated tellurium, but the kinetic data with purified TehB showed that loss of TeO₃²⁻ was SAM-dependent at 350 nmol of TeO₃²⁻/mg of TehB per min. However, the detailed mechanism of microbial selenium and tellurium oxyanion methylation in *E. coli* by TehB is poorly understood. The present paper is the first report showing that the TehB protein can methylate selenium oxyanions in addition to tellurium oxyanions. We have obtained the structure of TehB from *E. coli* in the presence of the cofactor analogues SAH (S-adenosylhomocysteine) and sinefungin (a non-hydrolysable form of SAM) at 1.48 Å (1 Å = 0.1 nm) and 1.9 Å respectively. Kinetic studies and mutagenesis allow us to probe a mechanism for the methylation of selenium and tellurium oxyanions by TehB.

**MATERIALS AND METHODS**

**Protein expression and purification**

We have previously reported the cloning, expression and purification of TehB from *E. coli* MG1655 [13].

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Abbreviations used: bTPMT, bacterial thiopurine methyltransferase; r.m.s.d., root mean square deviation; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-L-methionine

† To whom correspondence should be addressed (email kbeis@imperial.ac.uk).

Co-ordinates and structure factors have been deposited in the RCSB Protein Data Bank with PDB ID code 2XVM for TehB–SAH and 2XVA for TehB–sinefungin.
 eqns (1–3): two points on the linear portion of the curve was calculated from the equations below. The difference in the absorbance between purification. can be accounted for by the SAH that the protein carries from 7 min. The initial absorbance for the reaction is not zero and this We observed that the reactions had an initial delay of approx. activity was calculated as the expression of percentage activity. shaking at 290 K and measurements were taken every 12 s for 1 h. A colorimetric-based enzyme-coupled assay was used to measure the methylation activity of TehB at 510 nm (SAM Activity ($\mu$mol/min per ml) = $\frac{\Delta$Abs/min}{15 mM$^{-1}$} × $\frac{0.2 ml}{0.01 ml}$ (2) where 15 mM$^{-1}$ is the molar absorption coefficient of 3,5-dichloro-2-hydroxybenzenesulfonic acid in a 0.577 cm well. Percentage activity = untreated − substrate untreated × 100 (3) Crystallography We have reported the crystallization and data collection of the TehB protein previously [13]. Molecular replacement of TehB–SAM against a putative methyltransferase from Salmonella enterica serotype Typhimurium LT2 (PDB code: 2I6G) was performed using Phaser [14]. The two sequences share 91% homology and 84% identity. Molecular replacement located two copies of the putative methyltransferase within the asymmetric unit of TehB–SAM and four copies in the TehB–sinefungin with a Z score of 33.4 and 39.5 respectively. After rigid body refinement in REFMAC5 [15] the $R_{work}$ and $R_{free}$ were 35% and 38% respectively (TehB–SAH). Restrained refinement lowered the $R_{work}$ to 30% and $R_{free}$ to 34%. Manual electron density inspection revealed electron density for the cofactors. The SAH and sinefungin molecules were built in the density in COOT [16] and water molecule addition with ARP/wARP [17] resulted in a complete model with a final $R_{work}$ of 17.0% and $R_{free}$ of 22.9% for the TehB–SAH, and $R_{work}$ of 17.4% and $R_{free}$ of 23.4% for the TehB–sinefungin (Table 1). Figures were prepared using PyMOL (http://www.pymol.org). RESULTS AND DISCUSSION TehB structure We have determined the structure of the E. coli TehB (Figure 1) in the presence of SAH at 1.48 Å and sinefungin at 1.9 Å. SAH is a breakdown product of SAM that was added during crystallization and sinefungin is an analogue of the cofactor.

Table 1 Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data collection</th>
<th>TehB–SAH</th>
<th>TehB–sinefungin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C2</td>
<td>P21</td>
<td></td>
</tr>
<tr>
<td>Beamline</td>
<td>Diamond I02</td>
<td>Diamond I03</td>
<td></td>
</tr>
<tr>
<td>Wavelength ($\AA$)</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Resolution ($\AA$)</td>
<td>28.2–1.48 (1.56–1.48)</td>
<td>42.1–1.9 (2.0–1.9)</td>
<td></td>
</tr>
<tr>
<td>Unit cell parameters ($\AA$, $\degree$)</td>
<td>$a = 130.3, b = 55.6, c = 59.4$</td>
<td>$a = 59.1, b = 55.5, c = 129.7$</td>
<td></td>
</tr>
<tr>
<td>$R_{merge}$ (%)(all/observed)*</td>
<td>6.4 (42.6)</td>
<td>8.3 (41.2)</td>
<td></td>
</tr>
<tr>
<td>Completeness (%)(all/observed)</td>
<td>98.9 (96.2)</td>
<td>94.6 (97.2)</td>
<td></td>
</tr>
<tr>
<td>Unique reflections ($n$)</td>
<td>69781 (9856)</td>
<td>62310 (9309)</td>
<td></td>
</tr>
<tr>
<td>Average I/σ ($I_{1}(hkl)$)</td>
<td>16.1 (2.0)</td>
<td>7.4 (2.1)</td>
<td></td>
</tr>
<tr>
<td>Redundancy</td>
<td>5.3 (2.7)</td>
<td>1.9 (1.9)</td>
<td></td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_{work}/R_{free}$ (%)</td>
<td>17.0/22.9</td>
<td>17.4/23.4</td>
<td></td>
</tr>
<tr>
<td>Bond lengths ($\AA$)</td>
<td>0.021</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Bond angles ($\degree$)</td>
<td>1.912</td>
<td>1.515</td>
<td></td>
</tr>
</tbody>
</table>

* $R_{merge} = \Sigma_{hkl} \Sigma_{i} |I_{i}(hkl)|−<|I_{i}(hkl)>|/\Sigma_{hkl} \Sigma_{i} I_{i}(hkl)$, where $I_{i}(hkl)$ is the intensity of an individual reflection and $<|I_{i}(hkl)>$ is the average intensity.

†$R_{work}$ and $R_{free} = \Sigma_{1}(I_{F_{1}(hkl)})−|F_{1}(hkl)|/\Sigma_{1}(|F_{1}(hkl)|)$. $R_{free}$ was calculated using 5% of the data.

Mutagenesis

TehB mutants were generated using the Stratagene QuikChange® II site-directed mutagenesis kit (Agilent Technologies). The primers used for mutagenesis were 5′-GTGCGCGCAGCTTGCCCAGACCCGACGAC-3′ (H176A forward), 5′-CGTGGTCGGCGGCCAGCTCGCAGT-3′ (H176A reverse), 5′-CCGCGCGACGACGGCGACGACCCGACCAGAC-3′ (R177A forward), 5′-GGTGGCGGTGGCGTGCGACGTCGCGC-3′ (R177A reverse), 5′-CCGCGCGACCCGGTAATGCTATTAAACTGCGTTTC-3′ (R184A forward) and 5′-GAAACCGCTTTATATAAGCATATTACGGTTGCGTGCGG-3′ (R184A reverse). The mutated plasmids were confirmed by DNA sequencing. Expression and purification of the mutant proteins was performed in a similar manner to the native protein [13].

TehB kinetic assay

A colorimetric-based enzyme-coupled assay was used to measure the methylation activity of TehB at 510 nm (SAM methyltransferase kit, Merck). The assay was performed according to the manufacturer’s protocol, except that the substrate was added at the end of the reaction. The amount of TehB present was 177 μg, with a 50-fold excess of tellurium, selenite, or selenium dioxide. The reaction was incubated with shaking at 290 K and measurements were taken every 12 s for 1 h. The reaction was monitored at 510 nm and the methyltransferase activity was calculated as the expression of percentage activity. We observed that the reactions had an initial delay of approx. 7 min. The initial absorbance for the reaction is not zero and this can be accounted for by the SAH that the protein carries from purification. The percentage methyltransferase activity was calculated using the equations below. The difference in the absorbance between two points on the linear portion of the curve was calculated from eqns (1–3):

$$\Delta\text{Abs/min} = \frac{(\text{Abs at } t_2) - (\text{Abs at } t_1)}{t_2 (\text{min}) - t_1 (\text{min})}$$

$\text{Activity (}\mu\text{mol/min per ml)} = \frac{\Delta\text{Abs/min}}{15 \text{mM}^{-1}} \times \frac{0.2 \text{ml}}{0.01 \text{ml}}$ (2)

where 15 mM$^{-1}$ is the molar absorption coefficient of 3,5-dichloro-2-hydroxybenzenesulfonic acid in a 0.577 cm well.

Percentage activity = untreated − substrate untreated × 100 (3)

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The overall structure and fold of TehB resembles that of other SAM-dependent methyltransferases. The TehB–SAH and TehB–sinfungin structures can be superimposed with an r.m.s.d. (root mean square deviation) of 0.32 Å over 190 out of 197 Ca atoms, indicating that there are no significant overall differences between the two structures. Therefore most of the discussion will be based on the structure with sinfungin since this molecule resembles SAM more closely (an amine-carbon group instead of methyl-sulfur group at the sulfur position). The TehB protein has three motifs conserved among methyltransferases that are involved in the binding of the cofactor SAM. TehB is an α/β/α protein that belongs to the SAM methyltransferase superfamily.

It contains seven β-strands and six α-helices. The first six β-strands are flanked by α-helices and adopt a Rossman-like fold. Similarly to other methyltransferases, the first six β-strands are parallel and β7 is antiparallel. In the PDB there are two unpublished structures that are annotated as TehB-like structures with methyltransferase activity: PDB code 3CGG from Corynebacterium glutamicum (A.T.C.C. 13032 Kitasato) and PDB code 3M70 from Haemophilus influenzae. The closest homologue of the E. coli TehB is the putative methyltransferase from Salmonella Typhimurium LT2 (PDB code 2I6G) of unknown function; the two sequences share 91% homology and 84% identity. A sequence alignment between the four sequences is shown in Figure 2. The overall fold is very similar to the E. coli TehB and these structures do not contain any cofactor or substrates in the active site. It has been shown previously

<table>
<thead>
<tr>
<th>E. coli</th>
<th>S. typhimurium</th>
<th>H. influenzae</th>
<th>C. glutamicum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>consensus&gt;50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Conserved residues are shown in grey boxes. The secondary structure is shown above the sequence. The alignment shows a high degree of conservation for the residues responsible for binding the cofactor SAM.
Figure 3  Electron density maps

Electron density maps for (a) SAH \(2F_o - F_c\) contoured at 1.7 \(\sigma\) and (b) sinefungin \(2F_o - F_c\) contoured at 1.2 \(\sigma\) after molecular replacement are shown as a blue mesh. The structures for SAH and sinefungin are shown as sticks for clarity, but are not present in the initial refined model. Carbon, yellow; oxygen, red; nitrogen, blue; sulfur, green.

Figure 4  Stereo representation of the interactions between the sinefungin molecule (dark gray) and the TehB protein active site

Water molecules are shown as spheres and hydrogen bonds are shown as broken lines.

that the TehB from \textit{H. influenzae} is capable of restoring tellurite resistance in an \textit{E. coli} \textit{tehB} mutant [18]. Interestingly, the \textit{H. influenzae} TehB has an extra domain at the N-terminus (residues 1–94) of unknown function. The TehB from \textit{E. coli} can be superimposed with the \textit{H. influenzae} TehB with an r.m.s.d. of 0.74 Å over 190 \(C\alpha\) atoms. Attempts to solve the structures with chalcogen substrates trapped in the active site have so far failed.

SAH- and sinefungin-binding site

Inspection of the electron density maps for both cofactor data revealed density for the SAH and sinefungin molecules (Figure 3). The binding of SAH and sinefungin molecules is mediated through hydrogen bonds and van der Waals interactions with conserved residues of the main and side chains of the TehB (Figure 4). There are no significant side-chain movements in the active site between the SAH- and sinefungin-bound structures. The sinefungin molecule is bound in a cleft formed by \(\alpha 4, \beta 1, \beta 4\) and a loop connecting \(\beta 4\) to \(\alpha 5\). The adenosine ring nitrogen N1 forms hydrogen bonds with the main chain of Leu87 (2.9 Å) and the exocyclic N6 with the side-chain carboxy oxygen of Asp86 (2.9 Å). The ribose moiety oxygens O2’ and O3’ are hydrogen-bonded to the conserved Asp59. Previous mutagenesis studies have shown that a D59A mutation results in reduced tolerance of \textit{E. coli} cells to tellurite harbouring the mutated gene [12]. The rest of the cofactor molecule is hydrogen-bonded to Gly38, Arg43, Asn44 and Thr102. The sinefungin amine group (at the sulfur position in SAM) is hydrogen-bonded with the main chain of Val103 (3.1 Å). In the SAH structure the methyl group is absent and a water molecule
Figure 5  Methyltransferase activity
(a) Activity for the wild-type TehB in the presence of different chalcogens.  ◆, Tellurium; ■, selenium dioxide; ▲, selenate; and ●, selenite. (b) Activity of TehB mutants in the presence of tellurite. ○, Wild-type; □, H176A; △, R177A; and +, R184A. An initial delay of approx. 7 min is observed for all reactions due to the toxicity of the chalcogens.

Table 2  Relative activity of TehB and its mutants
The activity (%) of the mutants and chalcogens was calculated relative to that of the methylation of TeO$_3^{2-}$ by the wild-type protein. n/d, not detected.

<table>
<thead>
<tr>
<th>TehB mutant</th>
<th>TeO$_3^{2-}$</th>
<th>SeO$_3^{2-}$</th>
<th>SeO$_4^{2-}$</th>
<th>SeO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>68.4</td>
<td>68.4</td>
<td>27.3</td>
</tr>
<tr>
<td>H176A</td>
<td>94.7</td>
<td>31.6</td>
<td>44.2</td>
<td>28.7</td>
</tr>
<tr>
<td>R177A</td>
<td>35.2</td>
<td>40.0</td>
<td>19.6</td>
<td>n/d</td>
</tr>
<tr>
<td>R184A</td>
<td>73.7</td>
<td>31.0</td>
<td>37.9</td>
<td>21.7</td>
</tr>
</tbody>
</table>

occupies the position. The amine group is pointing towards the open active site. The sinefungin and SAH molecules also make hydrogen bonds with water molecules in the active site.

Kinetics
It has been shown previously that the detoxification of tellurite by TehB is SAM-dependent in E. coli cells [12]. We wanted to investigate whether the purified TehB protein can detoxify other chalcogens in addition to the tellurite. The activity of the TehB was measured using a colorimetric enzyme-coupled assay (Table 2). This showed that the purified TehB protein is capable of methylating tellurium. Interestingly, the kinetic data in the presence of selenium oxyanions clearly showed that the protein can methylate selenium oxyanions almost as effectively as the tellurite oxyanions (68 % relative activity); the specificity is much higher for tellurite and lower for selenium dioxide and selenite. A rather interesting observation of the assay is that when 50-fold excess tellurite or selenium compounds are added, these inhibit one of the enzymes involved so that the assay only proceeds when these compounds have been ‘detoxified’ by TehB (Figure 5). In controls with similar amounts of tellurite added, the reaction does not proceed. TehB is not capable of methylating selenate as effectively. This would require a reduction step from selenate to selenite; the 27 % activity that we observe can be accounted for by small amounts of selenite (decomposition product), at a 25-fold excess no activity can be detected. Since this assay does not directly measure the methylation of the chalcogens but rather the decomposition of SAM to SAH during the methylation step, it is not surprising that there is residual activity when selenite compounds are used. From Figure 5(a) it is obvious that the selenate does not reach the same final colorimetric product as the other chalcogens, as would be expected if the selenate were reduced. From the structure there are no obvious residues in the active site that could catalyse the reduction. TehB appears only to catalyse the methylation of chalcogens, as assays in the presence of arsenic compounds did not show any activity (results not shown).

Reaction mechanism
The active site is lined by conserved charged residues, which are able to bind and neutralize the negative charge of the oxyanions. The cavity is rather large, enabling it to accommodate metal oxyanions of different sizes. In the sinefungin structure the amine group (methyl group in SAM) at the sulfur is positioned ready for SN2 nucleophilic attack. Both TeO$_3^{2-}$ and SeO$_3^{2-}$ are good nucleophiles with a lone pair for attack. A previous mutagenesis study suggested that the TehB cysteine residues were responsible for mediating the reaction [19]. In our structure the only cysteine residue in close proximity of the active site is Cys$^{143}$, but it is not in a good position to bind or co-ordinate the metals. Sequence alignments and structural analysis of the active site have shown that there are three conserved residues near to the binding site that could be capable of binding and orientating the metals for nucleophilic attack, His$^{176}$, Arg$^{177}$ and Arg$^{184}$. These residues are found in a loop region that cap the active site and form the substrate-binding cleft. Mutating Arg$^{184}$ or His$^{176}$ to alanine did not significantly affect the relative activity of the enzyme in the presence of tellurite (94.7 % and 73.7 % respectively).
Replacing Arg^{177} with alanine on the other hand showed only 35% activity relative to the wild-type protein. In the presence of the selenium oxyanions, the mutations cause more than a 50% reduction in methylation. For the R177A mutant in the presence of selenate we could not detect any activity since the readings were below the detection limits of the assay. None of these mutants completely abolish the activity of TehB. We suggest that the Arg^{177} guanidinium group can bind and neutralize the negative charge of the tellurium oxygens and can also position it near to the methyl group of SAM for nucleophilic attack (Figure 6). In our structure Arg^{177} is positioned away from the sinefungin amine group, but could easily adopt another rotamer position in front of the amine (methyl group in SAM). A possible explanation for the R177A mutant retaining some of the activity is that the His^{176} and Arg^{184} side chains are capable of binding and orienting the chalcogen oxyanions through water-mediated hydrogen bonds to bring them into position for a nucleophilic attack. The E. coli TehB-binding site does not contain any residues that could catalyse the reduction step of selenate to selenite. Selenate detoxification can possibly proceed through a reduction step by a reductase [2,3] and final methylation by TehB.

In conclusion, the E. coli TehB protein is capable of methylating toxic chalcogens with a higher efficiency for tellurite than selenium compounds. The crystal structure in the presence of sinefungin has allowed us to identify the residues responsible for catalysis and propose a mechanism for catalysis with Arg^{177} as the key residue for co-ordination of the oxyanions in the active-site cleft for a nucleophilic attack to the methyl group of SAM.

**AUTHOR CONTRIBUTION**

Hassanul Choudhury performed the experiments and analysed the data. Alexander Cameron and So Iwata helped with the data analysis. Konstantinos Beis designed the experiments, analysed the data and wrote the manuscript.

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**REFERENCES**


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