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To cite this version:

Hassanul Ghani Choudhury, Alexander David Cameron, So Iwata, Konstantinos Beis. Structure and mechanism of the chalcogen detoxifying protein TehB from Escherichia coli. Biochemical Journal, 2011, 435 (1), pp.85-91. 10.1042/BJ20102014 . hal-00576993

HAL Id: hal-00576993

https://hal.science/hal-00576993v1

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Biochemical Journal Immediate Publication. Published on 18 Jan 2011 as manuscript BJ20102014

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

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Running Title: Chalcogen methylation

keywords: tellurite oxyanions, selenium oxyanions, methyltransferase, nucleophilic attack, detoxification

The abbreviations used are: SAM: S-adenosyl-L-methionine, SAH: S-adenosyl-homocysteine, n/d: not determined

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Abstract

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 the TehB from *Escherichia coli* has been determined in the presence of the co-factor analogues S-adenosyl-homocysteine (SAH) and sinefungin (non-hydrolysable form of S-adenosyl-L-methionine) at 1.48 Å and 1.9 Å respectively. Interestingly, our kinetic data show that TehB does not discriminate between selenium or tellurite 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, His^{176}, Arg^{177} and Arg^{184}. Mutagenesis studies revealed that the His^{176}Ala and Arg^{184}Ala mutants retained most of their activity, whereas the Arg^{177}Ala mutant had 65 % of its activity abolished. Based on the structure and kinetic data we propose an S_N2 nucleophilic attack reaction mechanism. These data provide the first molecular understanding on the detoxification of chalcogens by bacteria.

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Introduction

The chalcogens, selenium and tellurium can be found as trace elements in the environment. The oxyanions of tellurite (TeO_3^{-2}), selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}), are highly soluble in water and are known to be toxic to biological systems, whereas the elemental tellurium (Te^0) and selenium (Se^0) are insoluble in water and 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 sp. denitrificans* can reduce selenate to selenite [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 S-adenosyl-L-methionine (SAM) 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]. The bacterial thiopurine methyltransferase (bTPMT) can methylate inorganic and organic selenium (selenocysteine) to dimethylselenide and dimethyldiselenide [8]. GC-MS analysis of headspace gases of *E. coli* harbouring bTPMT showed the presence of volatile dimethylselenide and dimethyldiselenide. 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 of *Bacillales*; GC-MS analysis of headspace gas showed the presence of mixed species of dimethyltellurite, dimethylditellurite and dimethyltellurenyl sulfide [5].

The Challenger mechanism has been proposed for the methylation of arsenic compounds [10, 11] and for selenium compounds [10]; selenate has to be reduced to selenite and then methylated in the presence of SAM. The methyl-selenite can be further reduced to methylseleninic 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 TeO_3^{-2} [12]. *In vivo* assays did not establish any volatile methylated tellurium but the kinetic data with purified TehB showed that loss of TeO_3^{-2} was SAM dependent at 350 nmol of TeO_3^{-2}/mg of TehB/min. However, the detailed mechanism of microbial selenium and tellurium oxyanion methylation in *E. coli* by TehB is poorly understood. This is the first report showing that the TehB protein can methylate selenium oxyanions in addition to tellurium oxyanions. We have obtained the structure of the TehB from *E. coli* in the presence of the co-factor analogues SAH and sinefungin at 1.48 Å 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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Mutagenesis- TehB mutants were generated using the Stratagene QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The primers used for mutagenesis were 5'-GTCGGCGAGCTGGCCCGCACCGACG-3' (His^{176}Ala Forward), 5'-CGTCGGTGCGGGCCAGCTCGCCGAC-3' (His^{176}Ala Reverse), 5'-GCGAGCTGCACGCCACCGACGCCAAC-3' (Arg^{177}Ala Forward), 5'-GTTGGCGTCGGTGGCGTGCAGCTCGCC-3' (Arg^{177}Ala Reverse), 5'-CCGACGCCAACGGTAATGCTATTAAACTGCGTTTC-3' (Arg^{184}Ala Forward) and 5-GAAACGCAGTTTAATAGCATTACCGTTGGCGTCGG-3' (Arg^{184}Ala Reverse). The mutated plasmids were confirmed by DNA sequencing. Expression and purification of the mutant proteins was performed similarly 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 as described in the assay manual, except that the substrate was added at the end of the reaction. 177 μg of TehB was present with 50-fold excess of either tellurium, selenate, selenite or selenium dioxide. The reaction was incubated with shaking at 290 K and measurements were taken every 12 s for 1 hr. The reaction was monitored at 510 nm and the methyltransferase activity was calculated as expression of % activity. We observed that the reactions had an initial delay of around 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 % methyltransferase activity was calculated using the following equations:

The difference in the absorbance between two points on the linear portion of the curve was calculated from:

$$\Delta\text{Abs/min} = \frac{(\text{Abs at t2}) - (\text{Abs at t1})}{\text{t2(min)} - \text{t1(min)}} \quad \text{(Eq. 1)}$$

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

whereas 15 mM^{-1} is the extinction coefficient of 3,5-dichloro-2-hydroxybenzenesulfonic acid in 0.577 cm well.

$$\%\ \text{activity} = \frac{(\text{untreated}) - (\text{substrate})}{\text{untreated}} \text{x100} \quad \text{(Eq. 3)}$$

Crystallography- We have reported the crystallization and data collection of the TehB protein elsewhere [13]. Molecular replacement of TehB-SAM against a putative methyltransferase from *Salmonella typhimurium LT2* (PDB ID: 2I6G) (Joint Centre for Structural Genomics, unpublished) 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 co-factors. The SAH and sinefungin molecules were built in the density in

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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 2). Figures were prepared using PYMOL [18].
Coordinates 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.

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 break down product of SAM that was added during crystallisation and sinefungin an analogue of the co-factor. The overall structure and fold of TehB resembles that of other SAM dependent methyltransferases. The TehB-SAH and TehB-sinefungin structures can be superimposed with an r.m.s.d. of 0.32 Å over 190 out of 197 C_α 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 sinefungin since this molecule resembles SAM more closely (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 co-factor SAM. TehB is an α/β/α protein that belongs to the SAM methlyltrasferase superfamily. It contains 7 β-strands and 6 α-helices. The first 6 β-strands are flanked by α-helices and adopt a Rossmann-like fold. Similarly to other methyltransferases, the first 6 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; entry 3CGG from *Corynebacterium glutamicum* ATCC 13032 Kitasato and entry 3M70 from *Haemophilus influenzae*. The closest homologue of the *E. coli* TehB is the putative methyltransferase from *S. typhimurium LT2* (PDB ID: 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 co-factor or substrates in the active site. It has been shown that the TehB from *H. influenzae* is capable of restoring tellurite resistance in an *E. coli tehB* mutant [19]. Interestingly, the *H. influenzae* TehB has an extra domain at the N-terminus (residues 1-94) of unknown function. The TehB from *E. coli* can be superimposed with the *H. influenzae* TehB with an r.m.s.d. deviation of 0.74 Å over 190 C_α 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 co-factor 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 α4, β1, β4 and a loop connecting β4 to α5. The adenosine ring nitrogen N1 forms hydrogen bonds with the main chain of Leu^{87} (2.9 Å) and the exocyclic N6 with the side chain carboxyl oxygen of Asp^{86} (2.9 Å). The ribose moiety oxygens O2' and O3' are hydrogen bonded to the conserved Asp^{59}. Previous mutagenesis studies have shown that an Asp^{59}Ala mutation results in reduced tolerance of *E. coli* cells to tellurite harbouring the mutated gene [12]. The rest of the

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cofactor molecule is hydrogen bonded to Gly[38], Arg[43], Asn[44] and Thr[102]. The sinefungin amine group (at the sulfur position in SAM) is hydrogen bonded with the main chain of Val[103] (3.1 Å). In the SAH structure the methyl group is absent and a water molecule 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 that the detoxification of tellurite by TehB is SAM-dependant in *E. coli* cells [12]. We wanted to investigate if 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 1). 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 them almost as effectively as the tellurite ones (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 25-fold excess no activity can be detected. Since this assay does not directly measure the methylation of the chalcogens but the decomposition of SAM to SAH during the methylation step, it is not surprising that there is residual activity when selenate compounds are used. From Figure 5a it is obvious that the selenate does not reach the same final colorimetric product as the other chalcogens as it 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 to only catalyse the methylation of chalcogens as assays in the presence of arsenic compounds did not show any activity (data not shown).

Reaction Mechanism- The active site is lined by conserved charged residues, which are able to bind and neutralise 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 S_N2 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 cysteines were responsible for mediating the reaction [20]. In our structure the only cysteine in close proximity of the active site is Cys[143] but it is not in a good position to bind or coordinate 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 Ala 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 Ala 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 Arg[177]Ala mutant in the presence of selenate we could not

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detect any activity since the readings were below the detection limits of the assay. None of these mutants is able to completely abolish the activity of TehB. Though none of the mutations kill the protein. We suggest that the Arg^{177} guanidinium group can bind and neutralise 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 the 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 Arg^{177}Ala mutant retaining some of the activity is that His^{176} and Arg^{184} side chains are capable of binding and orientating 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 mechansim for catalysis with Arg^{177} as the key residue for coordination of the oxyanions in the active site cleft for a nucleophilic attack to the methyl group of SAM.

Acknowledgements
We thank the MPL for access, Diamond Light Source for beamtime allocation and the beamline scientists for technical support.

Funding
This work was partly funded by a Royal Society grant to K.B. [2007/R2].

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Table 1. 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.

	TeO_3^{-2}	SeO_3^{-2}	SeO_2	SeO_4^{-2}
Wild type	100	68.4	68.4	27.3
$His^{176}Ala$	94.7	31.6	44.2	28.7
$Arg^{177}Ala$	35.2	40.0	19.6	n/d
$Arg^{184}Ala$	73.7	31.0	37.9	21.7

Table 2. Data collection and refinement statistics

Data Collection	**TehB-SAH**	**TehB-sinefungin**
Space group	C2	$P2_1$
Beamline	Diamond I02	Diamond I03
Wavelength (Å)	0.97	0.97
Resolution (Å)	28.2 -1.48 (1.56 - 1.48)	42.1 – 1.9 (2.0 – 1.9)
Unit cell parameters (Å, °)	a = 130.3, b =55.6, c = 59.4, α = 90.0, β = 97.7, γ = 90.0	a = 59.1, b =55.5, c = 129.7 α = 90.0, β = 95.9, γ = 90.0
R_{merge} #(%) (all/observed)	6.4 (42.6)	8.3 (41.2)
Completeness (%) (all/observed)	98.9 (96.2)	94.6 (97.2)
R_{merge} #(%) (all/observed)	6.4 (42.6)	8.3 (41.2)
Unique reflections	69781 (9856)	62310 (9309)
Average I/σ (I) (all/observed)	16.1 (2.0)	7.4 (2.1)
Redundancy	5.3 (2.7)	1.9 (1.9)
Refinement		
R_{work}/R_{free} (%) *	17.0/22.9	17.4/23.4
Bond lengths (Å)	0.021	0.015
Bond angles (°)	1.912	1.515

Values in parentheses are for the high-resolution shells

#$R_{merge}=\Sigma_{hkl}\Sigma_I |I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_I I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl)\rangle$ is the average intensity.

*R_{work} and $R_{free} =\Sigma_{hkl} [|F_o(hkl)|-|F_c(hkl)|]/ \Sigma_{hkl} |F_o(hkl)|$

R_{free} was calculated using 5 % of the data

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FIGURE LEGENDS

Figure. 1. Crystal structure of TehB from *E. coli*. Ribbon representation of the TehB protein (light gray). The sinefungin molecule in the active site is shown in stick format (black).

Figure. 2. Sequence alignment of *E. coli*, *C. glutamicum* and *H. influenzae*. Conserved residues are shown in gray box. The secondary structure is shown on top of the sequence. The alignment shows a high degree of conservation for the residues responsible for binding the SAM cofactor.

Figure. 3. Electron density maps for the (a) SAH ($2F_o$-F_c contoured at 1.7 σ) and (b) sinefungin ($2F_o$-F_c contoured at 1.2 σ) after molecular replacement are shown as blue mesh. The structures for SAH and sinefungin are shown as sticks for clarity but are not present in the initial refined model (carbon in yellow, oxygen in red, nitrogen in blue and sulfur in 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 as dashed lines.

Figure. 5. (a) Activity for the wild type TehB in the presence of different chalcogens; tellurium is represented by ◆, selenium dioxide ■, selenate ▲ and selenite ●. (b) Activity of TehB mutants in the presence of tellurite; wild type is represented by ○, $His^{176}Ala$ □, $Arg^{177}Ala$ △ and $Arg^{184}Ala$ +. An initial delay of around 7 min is observed for all reactions due to the toxicity of the chalcogens.

Figure. 6. Proposed reaction mechanism for the TehB protein; Arg^{177} adopts a position to neutralise the oxyanion charge and position the tellurite for nucleophilic attack to the methyl group of SAM. Methylated TeO_3^{-2} and SAH leave the active site, and the enzyme is regenerated.

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Figure 1

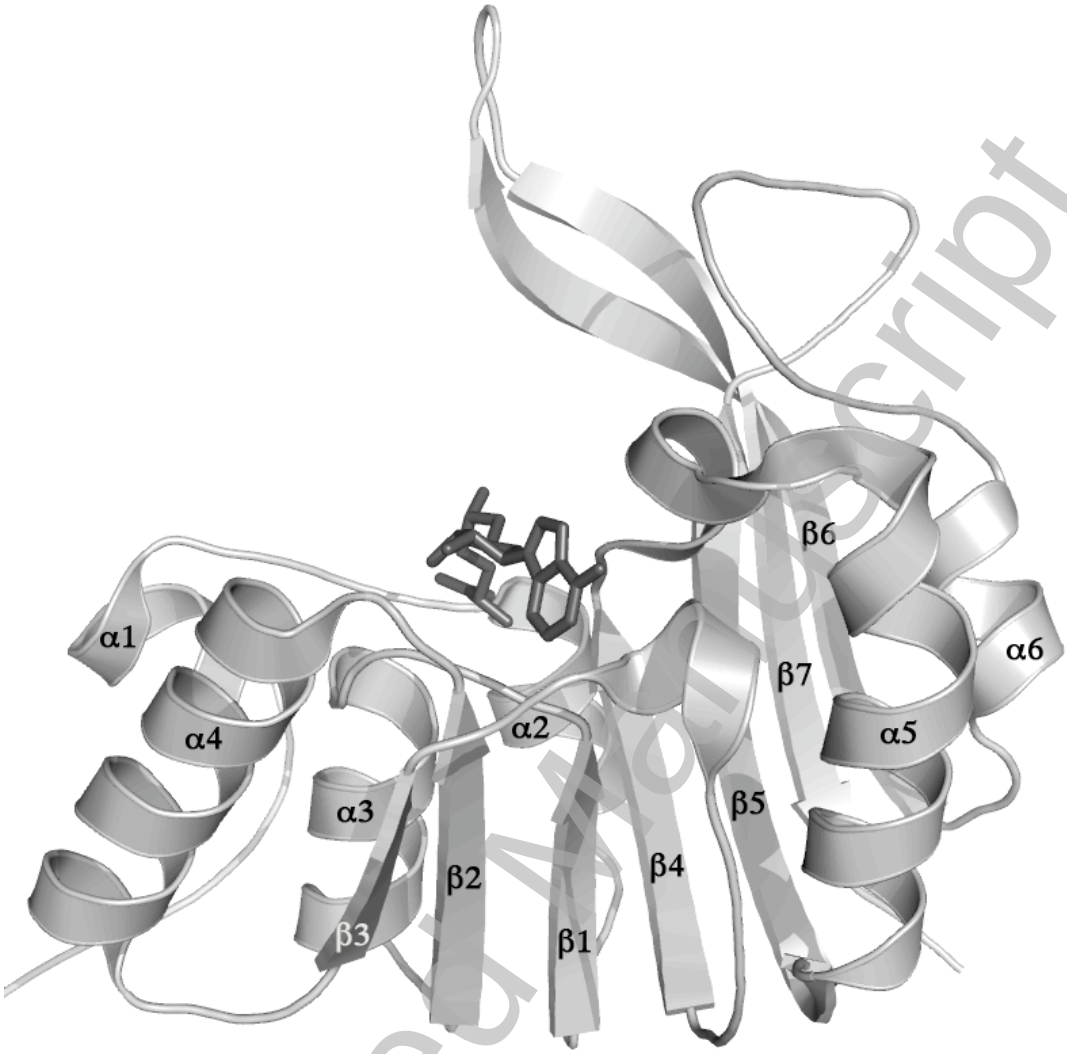

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Figure 2

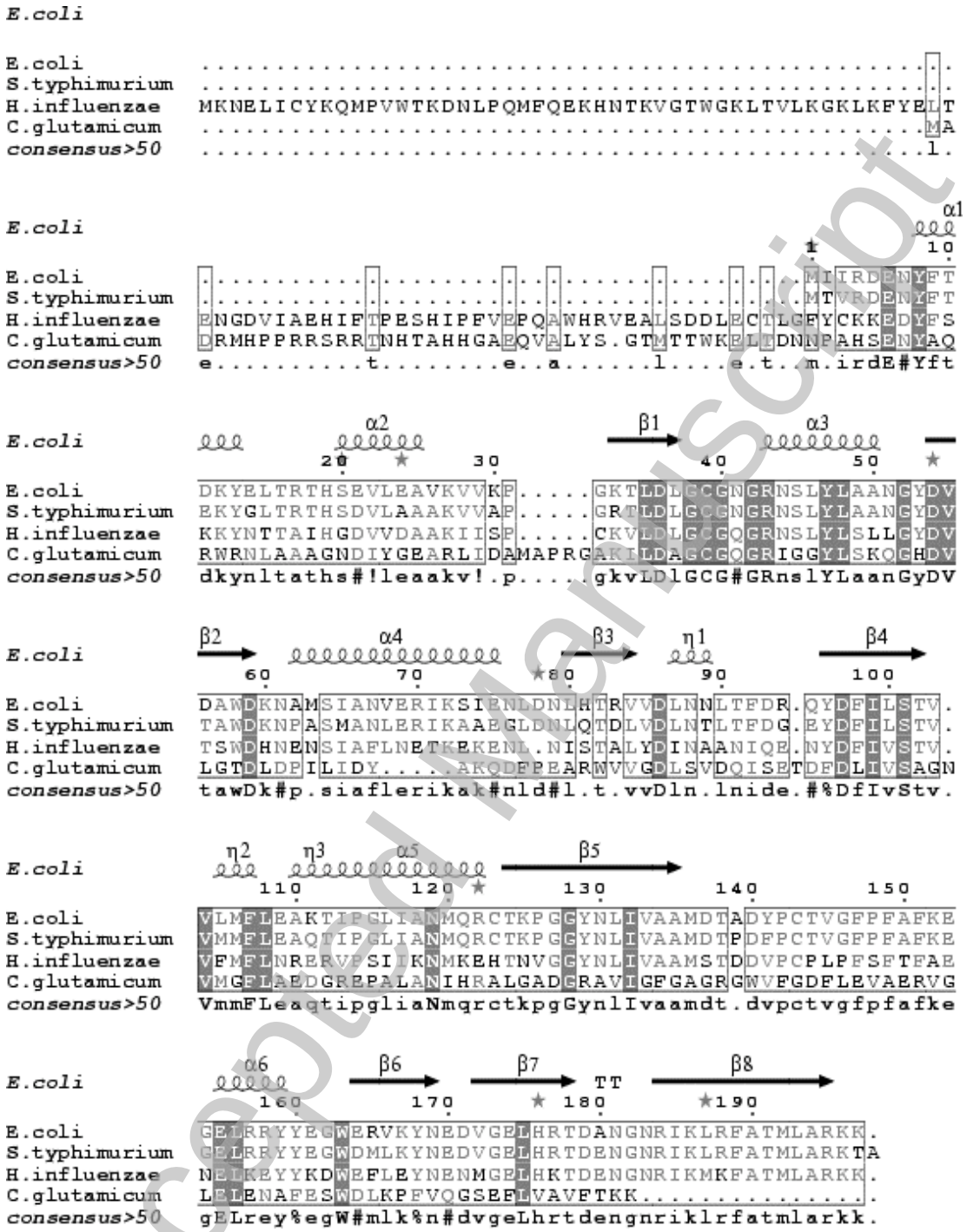

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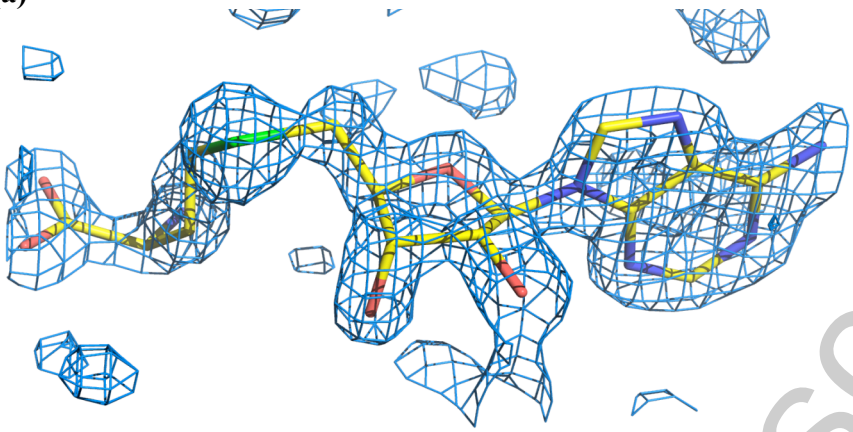
Figure 3

(a)



(b)

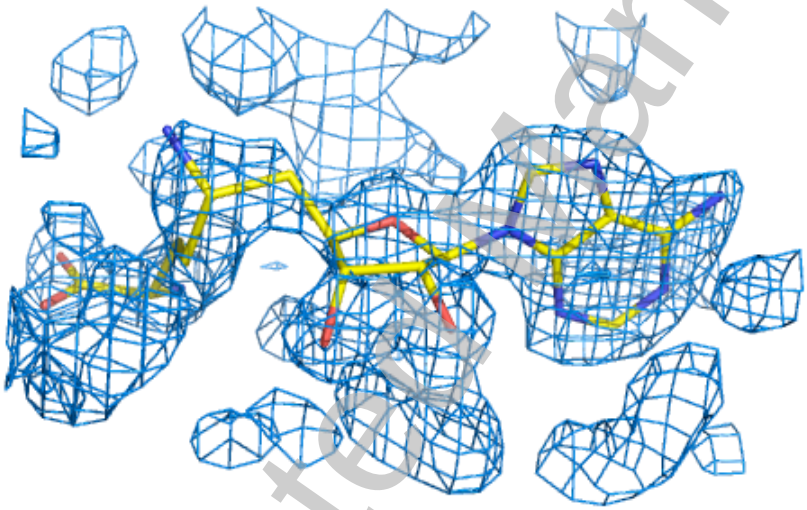


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Figure 4

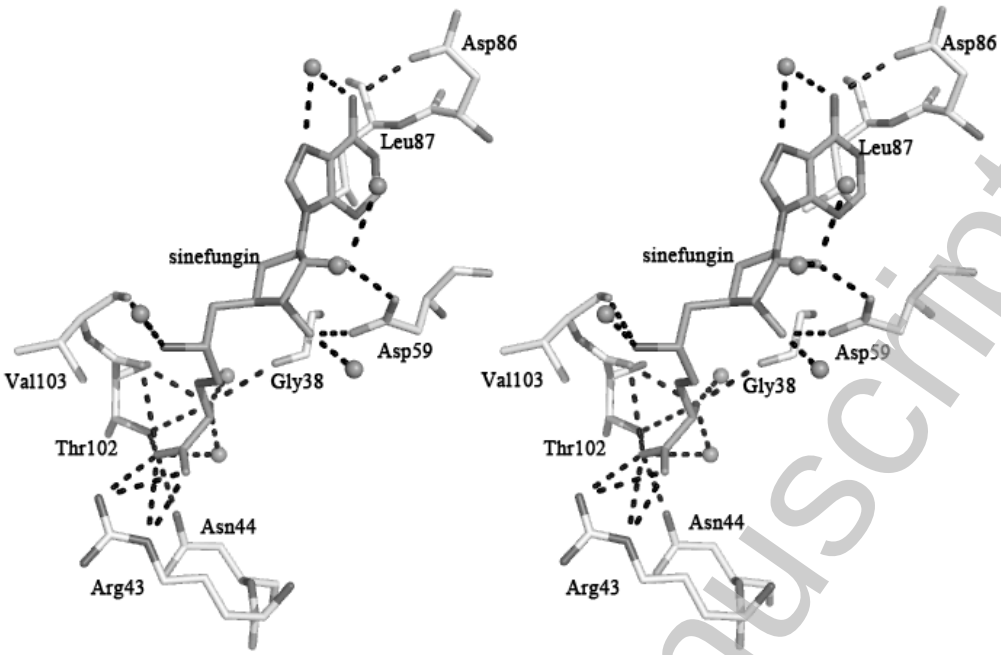

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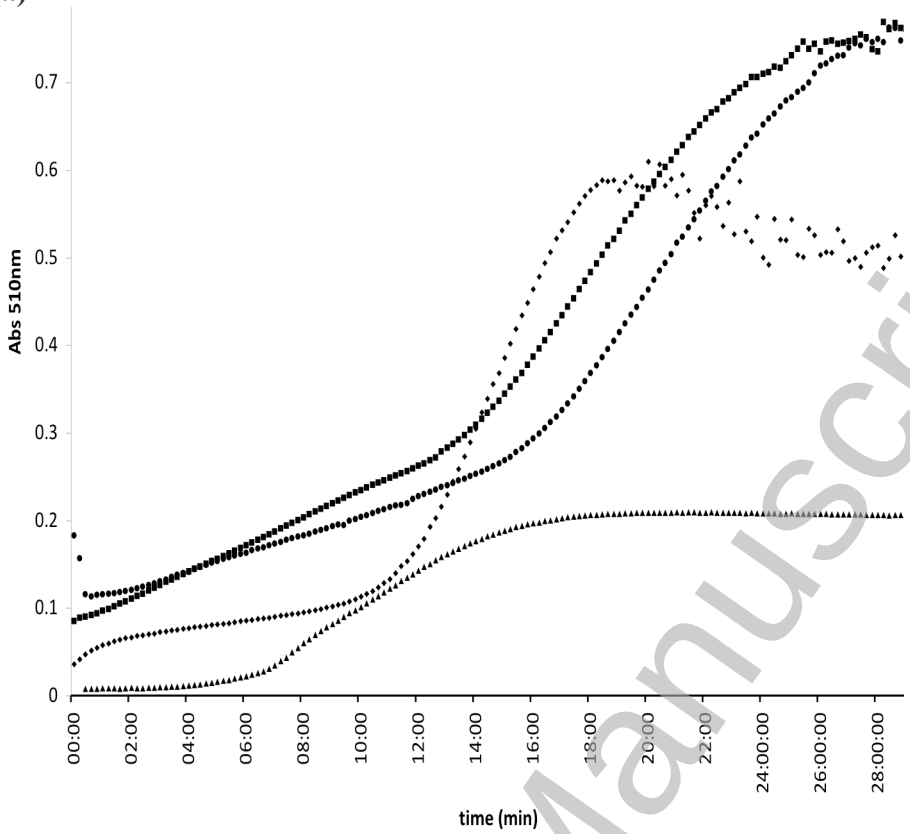
Figure 5

(a)



(b)

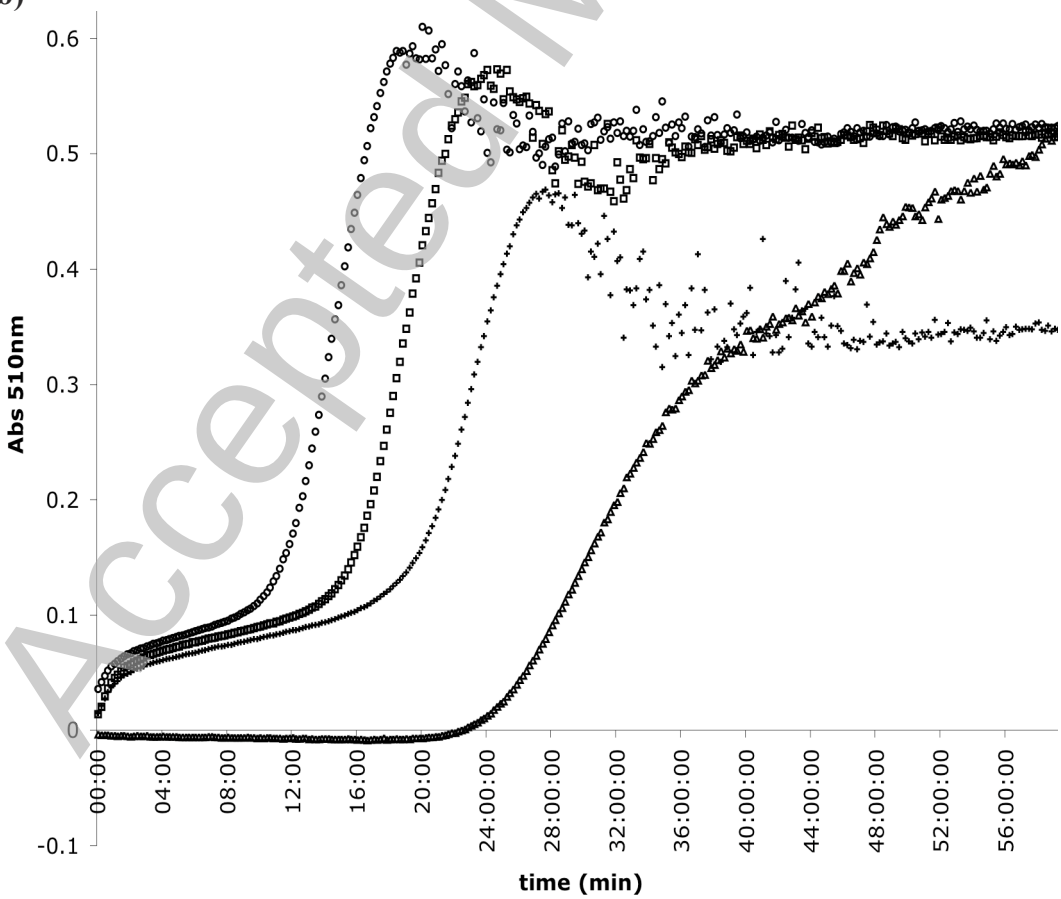


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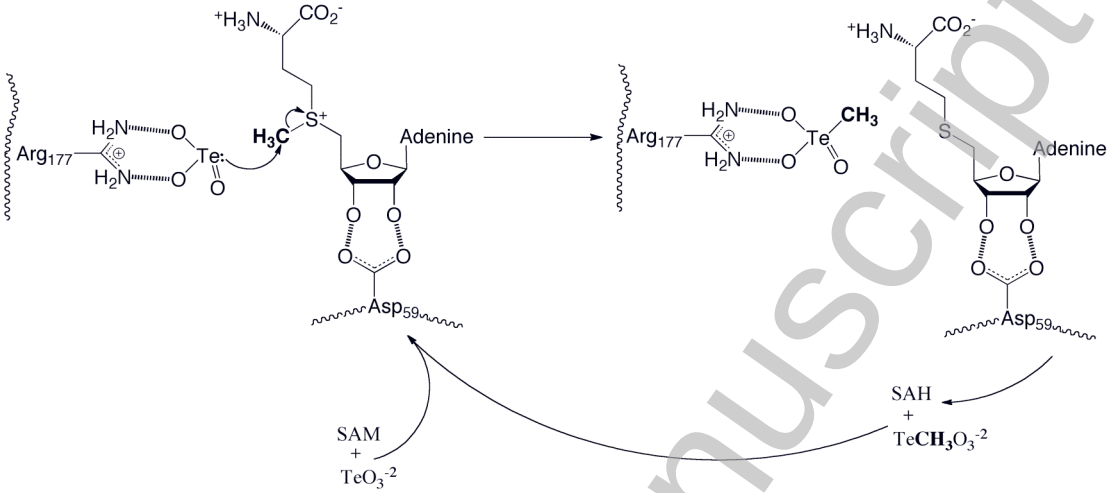
Figure 6



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