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Crystal structure of *Bacteroides thetaiotaomicron* TetX2, a tetracycline degrading monooxygenase at 2.8 Å resolution

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Abstract

We have determined the structure of *Bacteroides thetaiotaomicron* TetX2 at 2.8 Å resolution, and shown that it is a class A flavin dependent oxidoreductase. TetX2 has broad activity against a range of tetracyclines including one of the most recent tetracyclines, tigecycline (Tygacil®). Comparison of TetX2 with that of the weakly homologous *Pseudomonas fluorescens* para-hydroxybenzoate hydroxylase (PHBH) (21% identity) shows substantial differences among residues at the substrate binding site although FAD is positioned in a similar conformation between the two enzymes and is poised to carry out catalysis.

Keywords

TetX2; oxidoreductase; X-ray crystallography; antibiotic resistance

Introduction

Tetracyclines are broad-spectrum antibiotics that are commonly used against both Gram-positive and Gram-negative bacteria as well as in agriculture as growth promoters[1]. Tetracyclines inhibit protein synthesis by non-covalently binding to the ribosome and blocking the binding of aminoacyl-tRNA to the ribosomal acceptor site[2]. Since the discovery of tetracyclines in 1940s, bacterial adaptation has resulted in the diminished effectiveness of these antibiotics. Tetracycline resistance mechanisms have been classified into three groups based on their mechanism of resistance: efflux, ribosomal protection, and more recently discovered, enzymatic modification[1,3]. TetX is a tetracycline degrading enzyme that was originally isolated from *Bacteroides* transposons *Tn*4351 and *Tn*4400[3]. Continued use of tetracycline derivatives in clinical and non-clinical environments suggest that TetX and other novel resistance strategies will continue to arise with important consequences to human health.

Previous *in vitro* studies have shown that TetX hydrolyzes a broad range of tetracyclines including the front line drug, Tygacil® (tigecycline)[4,5]. TetX is a 44 kDa flavin adenine dinucleotide (FAD) containing monooxygenase that requires NADPH, Mg^{2+} and O_2 for enzymatic activity[4]. TetX2, an ortholog of TetX with 99.8% sequence identity to TetX, was originally isolated from transposon CTnDOT in *Bacteroides thetaoiotaomicron*[6]. TetX2

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crystallized in space group $P2_1$ with four molecules in the asymmetric unit. Based upon its modest sequence similarity (21%) to *Pseudomonas fluorescens* FAD-hydroxylase, para-hydroxybenzoate hydroxylase (PHBH) and enzymatic activity, TetX2 was initially classified as a potential class A monooxygenase. Our structure, shows TetX2 to have a tightly bound FAD and as expected for a class A monooxygenase lacks an independent NADPH binding domain[7]. Based on comparative analysis of the TetX2 and PHBH structures, we have identified a putative substrate binding pocket for TetX2. A comparison of PHBH and TetX2 residues within the substrate binding pocket shows little conservation and is consistent with different roles and substrates *in vivo*.

Materials and Methods

Expression and Purification of Recombinant Proteins

An expression vector, pET-28b(+) containing *Bacteroides thetaiotaomicron tet(X2)* (residues 11–388) was a generous gift from Dr. G. D. Wright (McMaster University, Canada). TetX2 was expressed and purified according to protocol modified from Yang et al. (2004)[4]. *Tet(X2)* containing vector was transformed into *E. coli* BL21 (DE3) Star strain (Stratagene). Bacteria were cultured in a shaking incubator at 37°C in 2 × YT media supplemented with 50 μg/ml kanamycin. Seleno-methionine labeled TetX2 (SeMet-TetX2) was expressed according to the methionine inhibition pathway method[8]. The cell culture was grown in M9 Minimal media supplemented with 50 μg/ml of kanamycin. Prior to induction of protein expression 50 mg/L of L-selenomethionine, 100 mg/L of leucine, isoleucine, phenylalanine, and 50 mg/L of threonine, lysine, valine were added to the media. Expression of TetX2 was induced with isopropyl β-D-1-thiogalactopyranoside to 0.5 mM at an optical density of ~0.8 at 600 nm and incubated at 16°C overnight. Cells were harvested by centrifugation at 8000 × g for 10 min, and the pellets were stored at −80°C. Frozen cells were thawed on ice and resuspended in lysis buffer consisting of 20 mM Tris pH 8, 500 mM NaCl, 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM β-mercaptoethanol (BME) and lysed by sonication. Cell lysate was centrifuged at 15,000 × g for 30 min and the soluble fraction was applied on a Ni-His Bind Chromatography column. TetX2 was eluted with 500 mM imidazole containing buffer. An equimolar amount of exogenous FAD was added to the fractions containing TetX2 after each purification step. Pooled fractions were dialysed against 20 mM Tris pH 8, 100 mM NaCl, 0.2 mM PMSF and 5 mM BME overnight. The N-terminal His_6-tag was removed by digestion of TetX2 protein with Thrombin for 24 h at 4°C (Novagen). The TetX2 digest was analyzed on a 15% SDS-PAGE gel and re-applied to a Ni-His Bind Chromatography column. Fractions with cleaved His_6-tag were pooled and dialyzed against 20 mM Tris pH 8, 100 mM NaCl, 0.2 mM PMSF, 5 mM BME, and 1 mM EDTA, overnight. The protein sample was loaded onto a HiTrap Q-XL Sepharose anion exchange chromatography column equilibrated in 20 mM Tris pH 8, 200 mM NaCl, 0.2 mM PMSF, 5 mM BME, and 1 mM EDTA. The protein was eluted with linear gradient of 0–1 M NaCl. Finally, the TetX2 containing fractions were pooled, concentrated and loaded onto HiLoad16/60 Superdex-200 column (GE Healthcare).

Crystallization and Data Collection

Single TetX2 crystals were obtained after 3 days from 2.3 M ammonium sulfate, 0.1 M CHES pH 8.6, and 0.1 M potassium formate at 4°C using hanging-drop vapor diffusion method. SeMet-TetX2 crystals were obtained under similar crystallization conditions using the sitting-drop vapor diffusion method.

Multiple-wavelength anomalous dispersion (MAD) data were collected at Advanced Light Source (ALS) beamline 4.2.2 using a NOIR-1 MBC detector. SeMet-TetX2 diffraction data was processed using HKL2000[9] (Table I). SeMet-TetX2 crystals diffracted to 2.8 Å

resolution. The protein crystallized in space group $P2_1$ with cell dimensions a = 87.65 Å, b = 67.41 Å, c = 152.35 Å and angles α = γ = 90.0° and β = 101.68°.

Structure Determination and Refinement

The initial structure determination by molecular replacement (MR) was performed using BALBES[10]. The best solution was obtained when PhzS from *Psedomonas auerugosa* (PDB ID: 3c96) was used as a search model (21% sequence identity). The solution from MR suggested four molecules in the asymmetric unit. The initial model was submitted to phenix.autobuild and phenix.refine for automatic building and structure refinement[11]. Diffraction data collected at the peak wavelength 0.97889 Å were used for structure determination by Single-wavelength Anomalous Dispersion (SAD) using phenix.autosol[11]. The partial model obtained by MR was used to search for Se sites. Automated model building by phenix.autobuild[11] resulted in successful placement of ~55–60% of the protein structure with the initial R-factor = 45% and R-free = 47.5%. The model was further built manually in COOT[12] and refined by phenix.refine. The initial refinement strategy included rigid-body, positional and group ADP refinement with non-crystallographic symmetry (NCS) restraints. FAD was fit manually into unoccupied density found in all 4 molecules. Additionally, TLS refinement was carried out in phenix.refine[11]. The diffraction was strongly anisotropic and an anisotropy correction using the Diffraction Anisotropy Server was applied to the SAD data set that resulted in a 2% drop in R-factor and R-free after refinement[13]. Structure coordinates were deposited in the Protein Data Bank under accession number 3P9U.

Results and Discussion

The crystal structure of TetX2 shows it is a class A flavin oxidoreductase

The final model consists of 4 molecules (A, B, C, D) with r.m.s.d. = 0.1 Å between copies, 84 water molecules and R-factor and R-free of 24.0 and 28.2 % respectively. Electron density for residues 11, 248–249 and 6 C-terminal residues was weak and could not be modeled. TetX2 shows a very low overall sequence identity with other FAD-dependant hydroxylases (Fig. 1), but shares several conserved motifs associated with formation of an FAD binding pocket and similar overall topology to the class A family of oxidoreductases. TetX2 is a monomer comprised of two domains binding one FAD molecule (Fig. 2A). FAD is stabilized in the active site through a network of hydrogen interactions with residues from both N - and C - terminal domains (Fig. 2B). FAD is in an extended conformation with adenosine positioned close to the Rossmann fold and the isoalloxazine ring extending toward the smaller domain near the putative substrate binding site. The larger (N-terminal) domain contains a Rossmann fold (βαβ-fold) that includes the highly conserved GXGXXG motif required for binding the adenine moiety of flavin[14]. The flavin binding pocket also contains a highly conserved GD motif (residues 310–311) found in most FAD-dependent oxidoreductases[15]. The residues in the GD motif form a specific pocket for the flavin molecule through the formation of hydrogen bond interactions between the Oδ of Asp311 and the O3′ of ribose, and O_{P2} of the pyrophosphate of the FAD to the backbone amide of Asp311. In TetX2, a third DG motif involved in the binding the pyrophosphate moiety of FAD[16], that is found in most FAD – hydroxylases, has an Asn168 in place of Asp resulting in a slightly altered FAD binding pocket. In addition, Arg117 forms highly conserved hydrogen bonds to the ribityl chain of FAD that may orient the flavin toward the substrate in the TetX2 active site[17]. TetX2 lacks an independent NADPH binding domain which is a characteristic of class A monooxygenases and is consistent with the hypothesis that NADPH is immediately released upon FAD reduction [16].

The interface between antiparallel β-sheets (strands $\beta_{5–6}$, $\beta_{16–20}$) of the second domain and the isoalloxazine ring of FAD comprises the binding site for the substrate. We observed additional strong electron density in the (2Fo−Fc) SIGMAA weighted composite maps near the isoallozazine ring of FAD that corresponds to the predicted substrate binding site though the identity of the molecule remains unknown (Fig 2D). The unknown electron density was found for both the $P2_1$ and P_1 (data not shown) crystal forms of TetX2. Although, we were unable to determine the identity of the unknown molecule that gives rise to the strong density seen in the composite electron density map, we speculate based on its position near the isoalloxazine ring of FAD and similar location to the substrate of PHBH that this molecule is a natural *E. coli* metabolite that co-purified with TetX2. The TetX2 structure bound to an unknown substrate is in a 'closed' state and the strong yellow color of the crystals suggests that the flavin is oxidized and therefore unable to carry out further catalysis. This might also explain the extended conformation of FAD which resembles more the FAD conformation of PHBH with substrate bound rather than the FAD in the unbound PhzS structure.

Comparison of the active sites between TetX2 and PHBH—Superposition of TetX2 to the well characterized PHBH (Fig. 2C) (PDB ID: 1cj3) and *Pseudomonas aeruginosa* PhzS (PDB ID: 3c96) results in an r.m.s.d. of 4.1 Å and 2.9 Å, respectively. As shown in Fig. 2C, there are substantive differences in the structure compared to PHBH in the N-terminal domain consistent with the modest sequence identity although the general topology is maintained. The overall comparison of the active sites between crystal structures of TetX2 and substrate bound PHBH shows that the FAD cofactor is oriented in a similar position in both proteins (Fig. 2D) but that the specific residues surrounding the predicted substrate binding site of TetX2 compared to PHBH are poorly conserved, which is not surprising considering that the enzymes bind significantly different substrates. TetX2 maintained a highly conserved proline (Pro318) that is analogous to Pro293 in PHBH that interacts with the phenolic oxygen of the substrate of PHBH. This residue has been suggested to be important for maintaining the proper FAD conformation[18]. The location of the unknown density in the TetX2 structure closely resembles the position of the substrate in PHBH. Arg213 and Met215 in the TetX2 structure are in positions to make hydrogen bonds to the unknown substrate in a manner analogous to residues Ser212 and Tyr201 in PHBH. In addition, the sidechain of His234 and the backbone amide of Gly58 can hydrogen bond with the substrate similarly to Arg214 in PHBH. We speculate that Phe224 is similar to Tyr222 in PHBH, which is also important for substrate binding. A significant shift of a loop (residues 54–59) between the substrate binding site and the isoalloxazine ring of FAD is observed in TetX2 when compared to PHBH loop (residues 42–47), which results in an expanded binding pocket that might be required to accommodate a large substrate, such as tetracycline. While the natural substrate of TetX2 remains unknown, the ability of TetX2 to inactivate tetracyclines in a flavin dependent manner together with the structure presented here confirm the role of TetX2 as a Class A oxidoreductase. The TetX2 structure provides the basis for future structure based drug design and physicochemical characterization towards this mechanism of broad tetracycline resistance.

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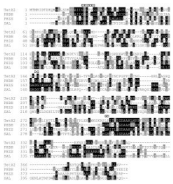

Fig. 1. TetX2 shares common sequence motifs with other FAD-dependant monooxygenases

Multiple sequence alignment of TetX2 from *Bacteroides thetaiotaomicron* (TetX2), p-hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescence*, phzS from *Pseudomonas aeruginosa* (PHZS), and salicylate hydroxylase (SAL) from *Pseudomonas putida* shows that TetX2 shares conserved sequence motifs, GXGXXG, DG, modified GD, with other FAD-monooxygenases. Sequence were aligned with program CLASTALW and represented by BOXSHADE [19].

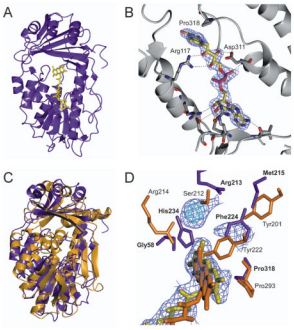

Fig. 2. Crystal structure of wild-type TetX2 from *Bacteroides thetaiotaomicron*, a FAD-dependent monooxygenase determined at 2.8 Å

A) Ribbon representation of crystal structure of TetX2 with bound FAD (yellow). **B)** FAD binding is coordinated through a number of hydrogen interactions with residues surrounding the FAD-binding pocket. **C)** Superposition of structures of TetX2 (blue) and PHBH (orange) results in 4.1 Å r.m.s.d. The overall topology of TetX2 is similar to PHBH and other FAD-dependent hydroxylases, forming two domains with one FAD molecule and of the absence an independent NADHP binding domain. **D)** Overlay of active sites of TetX2 (blue residues) and PHBH (orange residues) with strong unknown density shown in blue and cyan. Residues surrounding the active site are not well conserved with the exception of Pro318 (Pro293 in PHBH). Final (2Fo−Fc) SIGMAA weighted electron density map corresponding to FAD and the unknown density was contoured at 1.5σ (blue) and 3σ (cyan). Figure was made using CCP4mg[20].

Table I

Summary of Data Collection and Refinement Statistics

Data Collection	**SeMet TetX2**
Wavelength (Å)	0.97889
Resolution (Å)[a]	50 – 2.80 (2.85 - 2.80)
Space group	$P2_1$
Unit Cell (Å)	a = 87.70, b = 67.33, c = 153.79 α = γ = 90.0°, β = 100.31°
Total number of reflections[a]	132098
Unique reflections	39866
Average redundancy[a]	3.3 (3.2)
Completeness (%)[a]	92.0 (86.7)
R_{merge} (%)[a,b]	11.8 (28.6)
Output <I/sigI>[a]	16 (3.9)
Refinement	
R_{work} (%)[c]	24.00
R_{free} (%)[d]	28.24
r.m.s.d.[e] from ideality	
Bonds (Å)	0.009
Angles (°)	1.255
Average B-factor (Å2)	25.05
Ramachandran[f]	
favored (%)	92.1
additional allowed (%)	7.9
disallowed (%)	0
PDB accession number	3P9U

[a] Values for the last shell are in parentheses.

[b] R_{merge} $\Sigma|I - \langle I \rangle|/\Sigma I$, where I is measured intensity for reflections with indices of hkl.

[c] R_{work} $\Sigma |F_O - F_C|/\Sigma |F_O|$ for all data with $F_O > 2\ \sigma\ (F_O)$ excluding data to calculate R_{free}.

[d] R_{free} $\Sigma |F_O - F_C|/\Sigma |F_O|$ for all data with $F_O > 2\ \sigma\ (F_O)$ excluded from refinement.

[e] Root mean square deviation.

[f] Calculated by using MolProbity