FEBS Letters 585 (2011) 1061–1066

FEBS Letters

journal homepage: www.FEBSLetters.org

Structural basis for a new tetracycline resistance mechanism relying on the TetX monooxygenase

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ARTICLE INFO

Article history:
Received 28 February 2011
Accepted 4 March 2011
Available online 12 March 2011

Edited by Aleksander Benjak

Keywords:
Antibiotic resistance
Flavin
Tigecycline
Tetracycline degradation
TetX

ABSTRACT

The flavin-dependent monooxygenase TetX confers resistance to all clinically relevant tetracyclines, including the recently approved, broad-spectrum antibiotic tigecycline (Tygacil®) which is a critical last-ditch defense against multidrug-resistant pathogens. TetX represents the first resistance mechanism against tigecycline, which circumvents both the *tet*-gene encoded resistances, relying on active efflux of tetracyclines, and ribosomal protection proteins. The alternative enzyme-based mechanism of TetX depends on regioselective hydroxylation of tetracycline antibiotics to 11a-hydroxy-tetracyclines. Here, we report the X-ray crystallographic structure determinations at 2.1 Å resolution of native TetX from *Bacteroides thetaiotaomicron* and its complexes with tetracyclines. Our crystal structures explain the extremely versatile substrate diversity of the enzyme and provide a first step towards the rational design of novel tetracycline derivatives to counter TetX-based resistance prior to emerging clinical observations.

1. Introduction

One of the most powerful compounds to treat complicated skin and skin-structure infections (cSSSIs) and intra-abdominal infections (cIAIs) is tigecycline (Tygacil®, Wyeth Pharmaceuticals), a recently-approved, 3rd generation tetracycline (Tc). Tigecycline is a glycylcycline with a 9-*tert*-butylglycylamido group [1]. Functional assays demonstrate that tigecycline inhibits ribosomal protein synthesis with 3-fold higher efficacy than the second generation tetracycline minocycline and 20-fold more than the parent compound Tc [2]. Tigecycline was introduced into clinical practice in 2005–2006 and has been of great value as a last line of defense against multi-drug resistant bacteria, including the recently identified Gram negative pathogens bearing the NDM-1 carbapenemase [3]. Importantly, tigecycline is also active in vitro against Tc-resistant bacteria harboring genes responsible for ribosomal protection and drug efflux, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), multi-drug resistant *Acinetobacter* sp., and extended spectrum beta-lactamase (ESBL)-producing Gram-negative organisms [1]. To date, tigecycline resistance in hospital-associated pathogens has been rare. However, an efflux-based resistance mechanism leading to reduced susceptibility for tigecycline was reported recently for *Acinetobacter* [4].

A completely new tigecycline resistance mechanism is conferred by the flavin-dependent monooxygenase TetX. This enzyme catalyzes regioselective hydroxylation of tetracyclines to 11a-hydroxy-tetracyclines (Fig. 1). The hydroxylated tetracyclines degrade non-enzymatically to as yet uncharacterized products [5]. TetX is able to accept tigecycline as substrate in this NADPH and molecular oxygen dependent hydroxylation [6], which makes aerobic strains harboring *tet*X highly resistant against tigecycline. Hydroxylated tetracyclines have significantly reduced antibiotic properties as a result of their fast degradation [6] combined with a weaker coordination of Mg^{2+}-ions, which is crucial for their binding to the ribosome [7,8]. To date, Tc resistance by TetX is not dominant in clinical settings and therefore this is the first time that structural details of resistance are available before the clinical observation of resistance.

Paradoxically, *tetX* was found on transposons Tn*4351* and Tn*4400* [9,10] in the anaerobe *Bacteroides fragilis* during cloning and expression of a clindamycin–erythromycin resistance gene. TetX expression in *B. fragilis* cannot confer Tc resistance because of the requirement of molecular oxygen for enzyme activity [11]. The orthologous genes *tetX1* and *tetX2* were identified on CTnDOT in *Bacteroides thetaiotaomicron*. The amino acid sequence deduced for TetX shares 61.7% and 99.5% sequence identity, respectively, with TetX1 and TetX2. The *tetX* gene was found in aerobic *Sphingobacterium* sp. strain PM2-P1-29 with 99.8% nucleotide and 100%

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doi:10.1016/j.febslet.2011.03.012

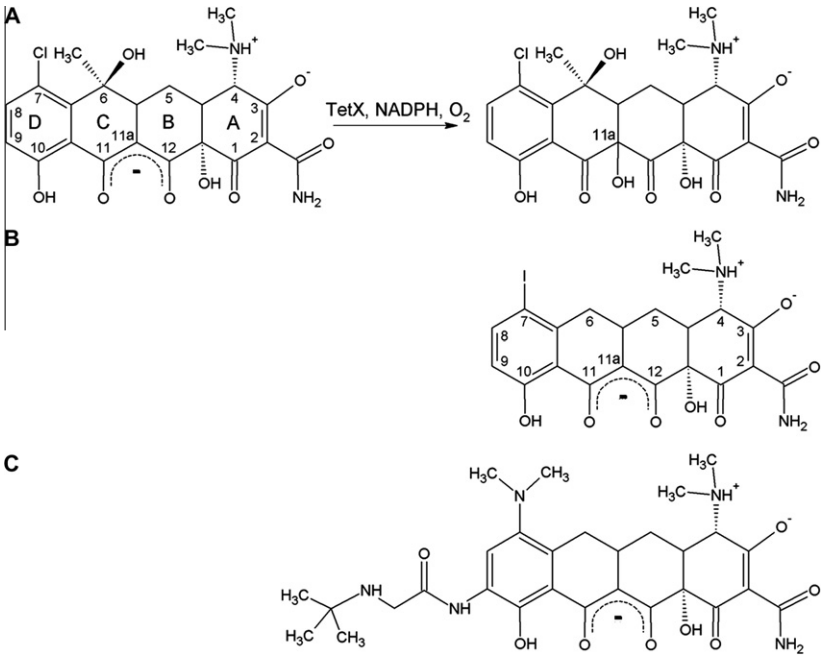

Fig. 1. (A) Structure and nomenclature of 7-chlortetracycline and TetX-mediated reaction to 11a-hydroxy-7-chlortetracycline. (B) Structure of 7-iodtetracycline. (C) Structure of tigecycline which carries a 9-*tert*-butylglycylamido group at the D-ring.

amino acid identity to TetX2 of CTnDOT from *B. thetaiotaomicron*. *Sphingobacterium* harboring *tetX* is resistant to tetracyclines [12]. Because of the low GC content of *tetX* (39%) it is more likely that *Sphingobacterium* sp. (37%) or other, related, soil bacteria are the ancestral source of the *tetX*-genes, not *Bacteroides* (~43%) [12]; this would not be uncommon, given the diversity of the antibiotic resistome and multi-drug resistance in soil bacteria [13]. Therefore it is uncertain whether TetX solely maintains antibiotic resistance or whether it has other unknown functions like biosynthesis or catabolism in ancestral soil bacteria.

2. Methods

The crystal structure of TetX from *B. thetaiotaomicron* was solved by multiple-wavelength anomalous-dispersion using a selenomethionine-labeled protein crystallized in the monoclinic space group $P2_1$ with four molecules in the asymmetric unit [14–17]. The TetX structure was then refined against X-ray diffraction data from a native TetX crystal in space group *P*1 to a resolution of 2.1 Å. These crystals were soaked with different tetracyclines to study substrate recognition. We collected diffraction data of TetX complexes with 7-ITc and 7-ClTc at 2.4 and 3.1 Å resolution, respectively. In all structures the coenzyme flavin adenine dinucleotide (FAD) was clearly recognized in the electron density for each TetX monomer (Fig. S5B). Electron density of tetracycline molecules was clearly identified in the substrate binding pocket close to the FAD and included in refinement of the structural models. Crystallographic details are reported and summarized in supporting online material, Tables S1 and S2 [15].

3. Results

3.1. Overall structure of TetX

The crystal structure of TetX from *B. thetaiotaomicron* was solved by multiple-wavelength anomalous-dispersion using a selenomethionine-labelled protein crystallized in the monoclinic space group $P2_1$ [14,15]. The TetX structure was then refined against X-ray diffraction data from a native TetX crystal in space group *P*1 to a resolution of 2.1 Å. These crystals were soaked with different tetracyclines to study substrate recognition. We collected diffraction data of TetX complexes with 7-iodtetracycline (7-ITc) and 7-chlortetracycline (7-ClTc) at 2.4 and 3.1 Å resolution, respectively. The asymmetric unit of both crystal forms consists of four TetX monomers arranged as pairs of dimers. A search with PISA [18] does not suggest any larger assemblies between monomers nor interfaces that are not crystal contacts. Therefore, it is unlikely that TetX forms quaternary structures in solution; indeed, the enzyme is known to be a monomer in solution [5].

In all structures the coenzyme flavin adenine dinucleotide (FAD) was clearly recognized in the electron density for each TetX monomer (Fig. 3B). Electron density of tetracycline molecules were clearly identified in the substrate binding pocket close to the FAD and included in refinement of the structural models.

The enzyme is composed of two domains where the polypeptide chain (383 amino acid residues) alternates four times between the domains (Fig. 2). Domain 1 exhibits the Rossmann fold [19] responsible for binding of the coenzyme FAD through its adenosine monophosphate component, which is linked to the flavin mononucleotide containing the catalytically active isoalloxazine moiety. The second domain with an extended 7-stranded β-sheet is positioned like a shield on top of the flavin-binding domain covered by five α-helices and is responsible for substrate recognition. A long C-terminal α-helix (10 turns) stabilizes the association of the two domains.

Sequence comparisons and a search with DALI [20] in the Protein Data Bank using the TetX coordinates reveal that TetX belongs to the family of FAD-dependent aromatic hydroxylases which takes its name from *p*-hydroxybenzoate hydroxylase (*p*HBH) of *Pseudomonas aeruginosa* [21] (Fig. S3).

The monooxygenase reaction mechanism relies on the redox properties of FAD. After reduction to $FADH_2$ by NADPH the isoalloxazine binds molecular oxygen at C4a forming a hydroperoxide, which hydroxylates the substrate [22]. The critical point is the FAD-OOH intermediate, which has to be protected against hydrolysis to prevent the alternative oxidase reaction generating H_2O_2.

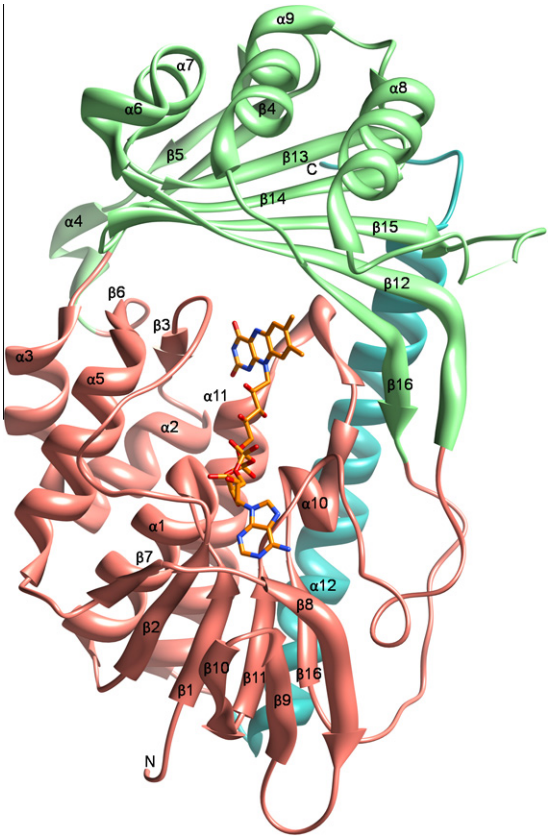

Fig. 2. Ribbon plot of TetX monooxygenase indicating the subdivision into domains by coloring. FAD (stick model) adopts the IN conformation. The *si* side of the flavin is facing the viewer.

According to insights from related monooxygenases with the *p*HBH fold the FAD hydroperoxide is formed after substrate recognition, shielding the hydroperoxide from solvent; subsequently, direct substrate hydroxylation takes place.

3.2. The FAD binding site of TetX

The FAD binding site is well conserved. Characteristically, the turn connecting β1 and α1 is composed of a consensus binding motif GXGXXG responsible for FAD-binding [23]. In TetX, this motif is represented by residues GGGPVG where glycine in position two occurs frequently [24]. FAD is bound non-covalently in an elongated, so-called IN conformation (see below) with the adenine and isoalloxazine moieties distal to each other (Figs. 2 and 3). The dimethylbenzene ring of the flavin is oriented between the methylene chain of P318 on the *re* side and G57/58 on the *si* side. A similar stacking was found for several members of the *p*HBH family, e.g. polyketide oxygenase PgaE from *Streptomycetes* (PDB accession, 2QA1, 16.2% sequence identity) [25]. The 2′ and 3′ hydroxyl groups of the ribose are bound by the carboxylate of the conserved E46 through strong hydrogen bonds (2.65 Å).

In several *p*HBH-members the isoalloxazine moiety of FAD is able to adopt alternatively the so called IN or the OUT conformation to regulate accessibility of the active site [26] and to maintain flavin-reduction by NADPH [27]. The IN conformation of FAD is believed to be necessary for reaction of flavin with molecular oxygen and subsequent hydroxylation of the aromatic substrate. When bound in the OUT conformation, isoalloxazine is bent towards the adenine. This is important to bring C4a of FAD and NADPH in close contact for reduction [28]. In TetX the FAD is found in the elongated IN conformation. The IN conformation is more often observed for oxidized flavin but also for reduced flavin after reduction by NADPH (PDB accession, 3EPT) [29]. In the TetX crystal structure the FAD is bound in its oxidized state leading to yellow protein

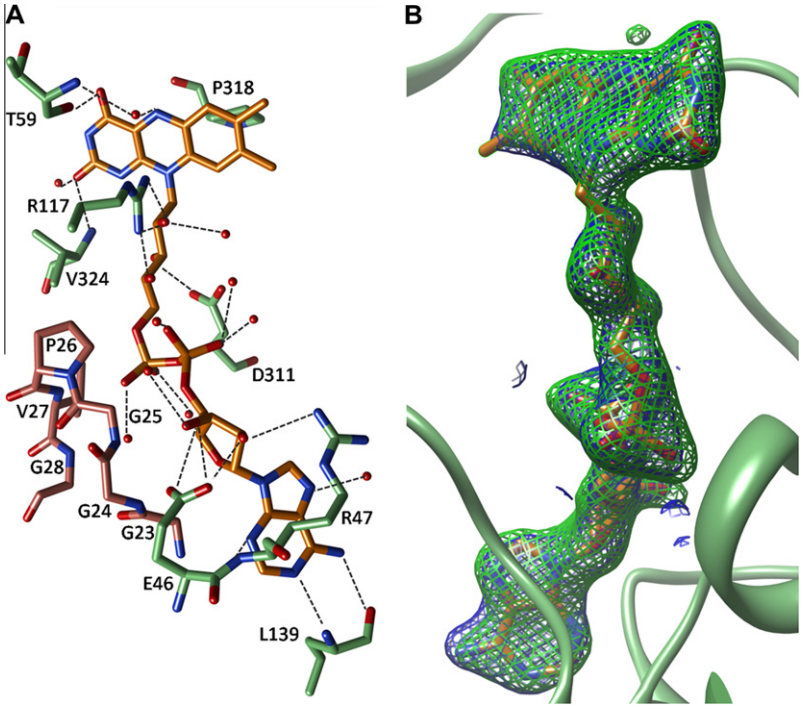

Fig. 3. The FAD binding site of TetX. (A) FAD is shown in orange, protein carbon atoms in green and water as red spheres. The carbon atoms of the conserved GGGPVG motif are highlighted in purple. Hydrogen-bonding interactions are shown as dashed lines. (B) Omit electron density map at 2.09 Å resolution where FAD was omitted from the structure factor calculation (2*Fo*-*Fc*, blue, 1.5 σ; *Fo*-*Fc*, green, 3.0 σ).

crystals, whereas reduction to the 1,5-dihydroisoalloxazine $FADH_2$ results in colorless crystals as reported for RebC [29]. In the case of *p*HBH from *Pseudomonas fluorescens* the IN conformation of the flavin ring is adopted when substrate [30] or NADPH binds ($pHBH_{R220Q}$ from *P. aeruginosa*, PDB accession, 1K0J) [28], leading to a closed conformation of the active site. In contrast to RebC and *p*HBH, the flavin moiety of TetX adopts the IN conformation with well-defined electron density even in the absence of substrate and NADPH. The IN conformation in the absence of substrate was also found for DHPH, MHPCO and PgaE, which has a comparable large substrate binding site. PgaE is involved in the biosynthesis of angucycline, which shares a similar size with tetracyclines [31].

3.3. Tetracycline and oxygen recognition by TetX

Specific tetracycline binding was investigated by diffraction data of tetracycline soaked TetX crystals. Suitable data sets revealed specific contacts of 7-ITc and 7-ClTc to the isoalloxazine of FAD in the large active site cavity. In both crystal structures the tetracycline molecules are unambiguously identified in the electron density maps and refined in all TetX molecules of the asymmetric unit. All Tc molecules adopt a very similar orientation to the FAD cofactor, which adopts the IN conformation similar to the native TetX.

Tetracycline binding in the active site is mediated by hydrogen-bonding interactions of the A-ring with residues Q192, R213, H234 and the backbone carbonyl-oxygen of G236 of domain 2 (Fig. 4A). Furthermore, the hydrophobic part of the Tc framework shows several interactions with hydrophobic side chains (F224, M215, G321 and M375). The hydroxylation site C11a of Tc is at an appropriate short distance (~5.9 Å) to C4a of the isoalloxazine which forms the hydroperoxide by oxygenation (Fig. 4B). The orientation of the substrate to the cofactor is determined by hydrogen bonds of O4 and N5 of FAD to the keto-enol moiety (O12) and the adjacent hydroxyl group O12a of Tc. FAD reduction to $FADH_2$ may strengthen these hydrogen-bond interactions. This binding mode explains the substrate diversity of TetX, tolerating all clinically relevant tetracyclines. All of them have a variable D-ring but a conserved A-ring substitution pattern.

The bound Tc in the active site of TetX closes a strongly hydrophobic pocket near the flavin *re* side in combination with mostly aromatic residues of the 2nd domain in a water-free environment (Fig. 5A and D). This pocket close to C4a of the flavin is composed of residues F319, L222, F224, M375, F376, F380 and F382. The pocket was readily identified upon superposition with the Xenon derivative of 3-hydroxybenzoate hydroxylase *m*HBH from *Comamonas testosteroni* (PDB accession, 2DKI) (Fig. 5B and Fig. S4). Xenon-binding studies on crystals of *m*HBH revealed the putative oxygen-binding site necessary for the oxygenation of $FADH_2$ and stabiliza-

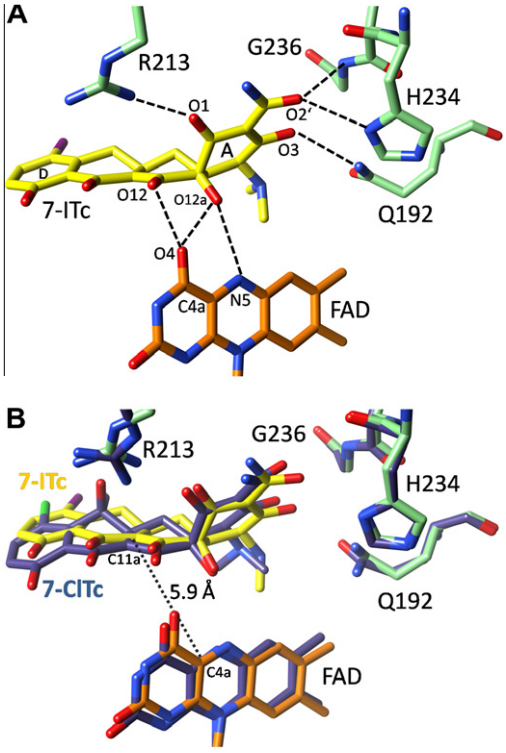

Fig. 4. (A) Active site recognition of 7-ITc by hydrogen-bonding of TetX (protein green, 7-ITc yellow, FAD orange, hydrogen bonds as black dashed lines in the range of 2.7–3.2 Å). (B) Superposition of 7-ITc:TetX with the 7-ClTc:TetX complex (blue) reveals a similar binding mode due to recognition of the conserved A-ring. The hydroxylation target C11a of Tc is at an appropriate short distance (~5.9 Å, dotted line) to C4a of the FAD, which forms the hydroperoxide with molecular oxygen.

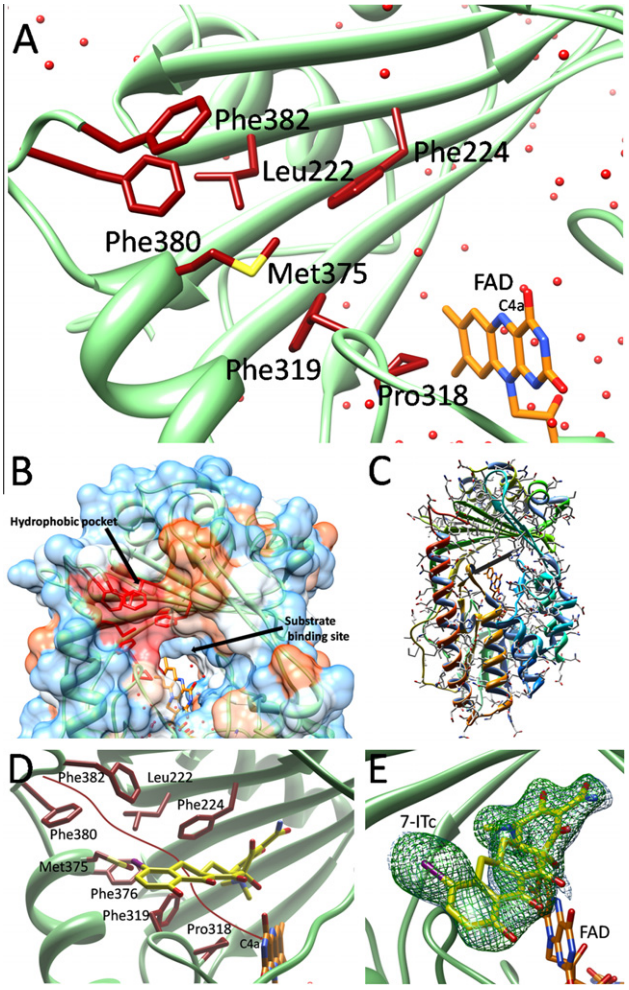

Fig. 5. (A) A putative oxygen binding site can be assigned to a strongly hydrophobic, solvent free pocket near to the active site of TetX with a clear separation to the solvent region (FAD in orange, protein as a ribbon in green, hydrophobic side chains in red, water as red dots). (B) Surface view of the hydrophobic pocket (hydrophobic residues with red surface, hydrophilic blue). (C) The narrowest diameter of the substrate entrance of TetX (shown as ribbons with side chains as sticks) is 8.6 Å (black line). (D) Binding of 7-ITc (yellow) closes the hydrophobic pocket and enables oxygen binding and transport (red line) along the hydrophobic site of 7-ITc towards C4a of the FAD. (E) Omit electron density map where 7-ITc was omitted from the structure factor calculation (2*Fo*-*Fc*, blue, 1.0 σ; *Fo*-*Fc*, green, 3.0 σ).

tion of the FAD-OOH intermediate [32]; the hydrophobic pocket in TetX may play a similar role.

4. Discussion

The antibiotic entrance and the NADPH binding site are rather large, and we predict them to be at opposite ends of the active site tunnel, in contrast to *p*HBH where substrate and NADPH enter through one cavity pointing towards the *re* side, with a second low-affinity substrate-binding site [28]. The TetX structure reveals a large, tunnel-like, substrate binding site connecting bulk solvent and active site, which is shielded at one end by the C-terminal helix α12 and strand β4. The narrowest diameter of the tunnel entrance is 8.6 Å, between NH2 of R213 and CO of A320 (Fig. 5C). We presume the entrance for the antibiotic is here, in contrast to *p*HBH, where active site access is provided through a cavity on the opposite side. Thus, free access to the active site can be easily provided without flavin movement, even for large substrates. The substrate entrance proposed for *p*HBH is blocked in the TetX crystal structure by amino acid residues from strand β13.

Superposition of TetX with the $pHBH_{R220Q}$:NADPH complex (PDB accession, 1K0J; [28]) enables us to assign a putative NADPH binding role to R54, similar to R44 from *p*HBH. R44 stabilizes the 2′-phosphate group via a salt bridge and, together with the isoalloxazine of the FAD, it maintains the NADPH adenine in a sandwich orientation. The guanidinium group of R54 of TetX has to be shifted by about 12 Å to bring NADPH and FAD in close contact. This might be possible, because R54 is located in a long loop connection within the rigid Rossmann fold domain.

Overcoming the TetX resistance mechanism by drug design will be challenging. Tigecycline is the most prominent member of the glycylcyclines, which all have bulky hydrophobic substituents in position 9 of the D-ring. This modification results in increased affinity for the target ribosome and overcomes efflux and ribosomal protection resistance mechanisms, but not TetX-mediated inactivation. Our crystal structures of TetX:Tc complexes show clearly that the D-ring is solvent exposed. Furthermore, the hydrophobic part of the Tc makes few specific interactions with the enzyme. Unfortunately, only these regions of Tc can be varied if the antibiotic function is to be retained. The portion of Tc that makes the specific interactions with TetX has a substitution pattern that is mandatory for antibiotic action (see above) and cannot be easily redesigned. Ring A however may be an area worthy of modification. The carboxamide group on ring A interacts closely with both TetX and TetR, the Tc-dependent repressor of the Tc efflux pump [33]. However, this group interacts with the ribosome only through a single hydrogen bond with the ribose of the nucleotide C1195 [7]. Fourth generation tetracycline antibiotics that incorporate modification of ring D at position 9 as well as modification of the carboxamide group of ring A, which is now chemically possible through new synthetic strategies [34], could overcome all known Tc resistance mechanisms.

5. PDB accession numbers

The X-ray crystallographic coordinates and structure factors of the native TetX, the SeMet-labeled TetX, and complexes of TetX with 7-iodtetracycline and 7-chlortetracycline have been deposited in the Protein Data Bank (PDB) with accession codes 2XDO, 2XYO, 2Y6Q, 2Y6R, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.03.012.

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