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REVIEW ARTICLE

TetR Regulators: A Structural and Functional Perspective

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Abstract | Tetracycline repressor family of transcription regulators (TetR-FTRs) is one of the most predominant families of transcription factors in the prokaryotic system. Classically, they are associated with antimicrobial resistance since they regulate the genes encoding the efflux pumps that export antibiotics out of the cell. Analysis shows that TetR-FTRs adopt a broader role in bacterial function than earlierly envisioned. Apart from efflux of antibiotics these proteins also regulate pathways associated with cell–cell signaling, antibiotic biosynthesis, biofilm formation, etc. Furthermore, an in-depth scrutiny of the available three-dimensional structures of TetR-FTRs and comparison of their various forms (apo, liganded and DNA-bound) helped to obtain valuable insights into the underlying molecular mechanism of action. TetR-FTRs possess a modular architecture with the N-terminal DNA-binding domain comprising canonical DNA-binding helix-turn-helix motif that is mostly conserved, whereas, the C-terminal signal reception domain is evolutionarily more diverse as it is tailored to accept the appropriate ligand. The TetR-FTRs serve as repressors when bound to their target DNA sequence, in the absence of their signaling molecule. On ligand binding, de-repression occurs by the coordinated motions of helices at the interface of the two domains. The DNA-binding domain undergoes a pendulum-like shift along the connecting helix, α4, and this motion transmits the signal. Overall, an understanding of the allosteric mechanism allows these proteins to switch from one state to another, an important transformation of their regulatory function.

Keywords: *Tetracycline repressor, Antimicrobial resistance, Quorum sensing, DNA binding, Allostery, TetR, CprB, Streptomyces*

TetR: Tetracycline Receptors protein is a representative member of the family of proteins engaged in acquiring resistance. In the absence of tetracycline, TetR bind to the DNA and repress the expression of proteins involved in efflux pumps.

1 Introduction

Antimicrobial resistance has now reached a scale where it poses a substantial global threat to public health.[1] There are several mechanisms by which bacteria protect themselves from antibiotics, including (1) restricting the entry of the antibiotic into the cell, (2) degrading/modifying the antibiotics, (3) modification/mutation of the drug target by recruiting enzymes, (4) efflux of the antibiotic out of the cell by recruiting broad spectrum/specific efflux pumps, (5) overproduction of target mimics, and (6) factor-associated protection.[2] One of the most prevalent classes of transcription factors that control the efflux of these antibiotics is the tetracycline repressor family of transcription regulators (TetR-FTRs). Cutherbertson and Nodwell recently reviewed the distribution of these proteins in bacterial genomes and by employing a combination of bioinformatics, structure and genome organization provide insights into the various possible roles of TetR-FTRs in biology.[3] An observation that consistently appears is that a large percentage of TetR-FTRs regulates the antibiotic resistance

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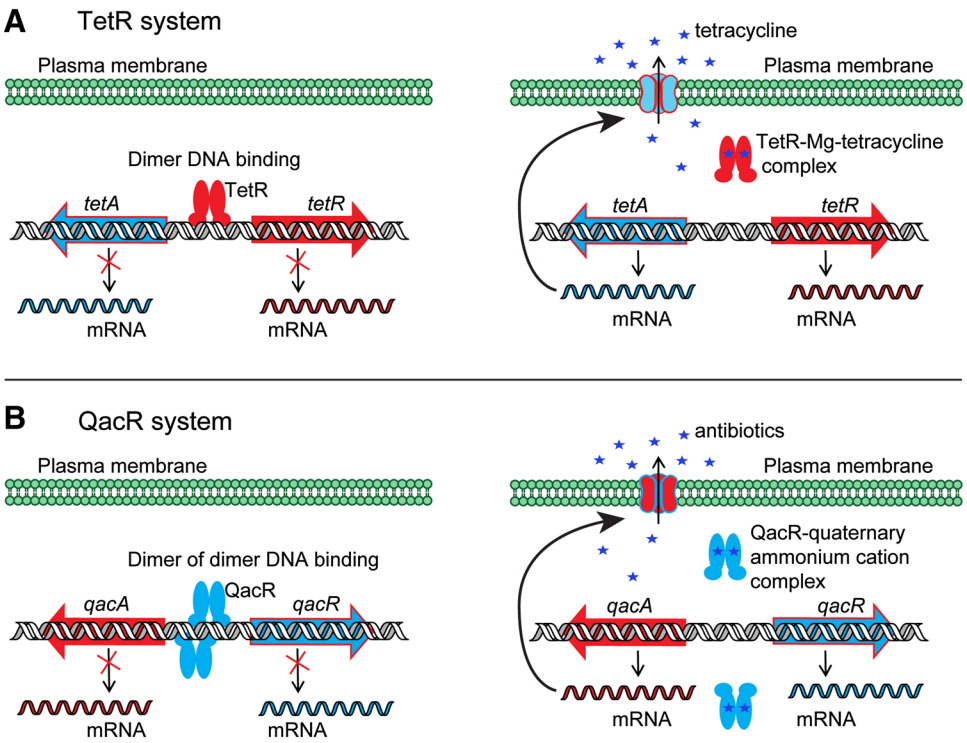

Figure 1: Overall graphical representation of the regulatory mechanism of TetR-FTRs. The mechanisms of repression by **a** TetR and **b** QacR. In both cases, when the effector binds to the ligand-binding domain, it induces conformational change in the repressor leading to the expression of the exporter genes.

efflux pathways in several pathogenic organisms such as *Staphylococcus aureus,*[4] *Mycobacterium tuberculosis.*[5] Moreover, even the founding member of the family encodes for the gene that confers resistance to the broad spectrum antibiotic tetracycline. In the absence of stimuli, TetR binds to the *terR-tetA* intergenic sequence and represses the transcription of the divergent *tetA* and *tetR* genes. Tetracycline–magnesium ion (Tc–Mg) complex on binding with TetR activates the *tetA* gene product TetA, which is a membrane-associated protein that exports the tetracycline out of the bacterial cell,[6–10] as seen in Fig. 1a. Similarly, QacR, another TetR-FTR from *S. aureus,* confers resistance to mono- and bivalent cationic lipophilic antiseptics and disinfectants, such as quaternary ammonium compounds. In the absence of stimuli, QacR represses transcription of the *qacA*, multidrug transporter gene (Fig. 1b). Some of the other family members include *Streptomyces antibioticus* SimR that regulates the expression of the specific simocyclinone efflux pump, SimX, which binds to the *simR-simX* intergenic sequence[11] and EthR that controls the degradation of the tuberculosis drug ethionamide and MtrR that regulates the MtrCDE efflux pump of *Neisseria gonorrhoeae*. It was also shown that CgmR from *Corynebacterium glutamicum* binds to drugs such as ethidium and methylene blue; CgmR also has been proposed as a multidrug resistance regulator.[12] In addition, HrtR regulates the expression of the heme efflux transporter in *Lactococcus lactis.*[13]

Apart from efflux pump regulation, TetR-FTRs are known to govern a wide range of cellular processes. For example, SlmA from *E. coli* partakes in nucleoid occlusion and prevents cytokinetic Z-ring formation during cell division.[14, 15] *Pseudomonas aeruginosa* DesT controls the expression of gene products that maintain the ratio of unsaturated: saturated fatty acid levels in the organism.[16] Ms6564 serves as a master regulator of genes that are responsible for DNA damage/repair mechanism in *Mycobacterium smegmatis.*[17] AmtR is the global nitrogen regulator for the industrial amino acids synthase from the *C. glutamicum*[18] and KstR controls the cholesterol degradation in *Mycobacterium tuberculosis.*[19] Another very important phenomenon cell–cell signalling in *Streptomyces* is also under the control of TetR-FTRs.[20, 21] Here, we focus on the structural analysis of different forms of TetR-FTRs to address the differential modes of DNA recognition and their specificity toward the target

Nucleoid occlusion: It is a defense mechanism that prevent bisection/breakage of chromosome by the cell division septum by forming Z-rings near the nucleoid.

DNA sequence. Furthermore, we also discuss the mechanism of allosteric regulation in TetR-FTRs and the possible existence of cross-talk between them.

2 TetR-FTRs and Quorum Sensing

An organism-wide analysis of TetR-FTRs showed that they are present in large number of bacterial genomes with soil-dwelling bacteria encoding the highest numbers. Many of these are filamentous, Gram-positive bacteria from *Streptomyces* genus. *Streptomyces* are characterized by their ability to produce a wide variety of secondary metabolites, including antibiotics and biologically active substances. Over 70% of commercial antibiotics are produced by these organisms.[22, 23] *Streptomyces* are uniquely placed as they not only produce antibiotics but have also evolved mechanisms to protect themselves from their own antimicrobial agents. It is believed that most of the resistance mechanisms have stemmed from these producer organisms and transferred across species via lateral gene transfer.[24] Therefore, understanding the origins of antibiotic resistance using *Streptomyces* as a model organism is of great importance. *Streptomyces* possess an abundance of TetR-FTRs and use them in several pathways for establishing multilayer regulatory systems. As mentioned earlier a subset of these TetR-FTRs-like molecules in pathogenic strains act as efflux pump regulators. They control the levels of endogenously produced antibiotics. For example, even in *Streptomyces, actR-actA* genes, much like the *tetR-tetA* system, regulate the efflux of the antibiotic actinohrodin, produced by *S. coelicolor.*[25]

However, the status of TetR-FTRs as an ultimate master regulator was established in 1998 by Horonuchi and coworkers. ArpA in S. *gresius* controls secondary metabolism, which is inclusive of both AMR and antibiotic biosynthetic pathways in these species.[26, 27] Unlike the prevalent model of activation via antibiotics this particular TetR-FTR sub-family is triggered by quorum-sensing (QS) molecules γ-butyrolactones (GBL)[28] QS is a specialized cell–cell communicating network, classified as a receptor-based signal transduction system. In this regard, *Streptomyces* cytoplasmically synthesize and release the membrane-diffusing signaling molecules (GBLs/microbial hormones), which ultimately are detected by regulatory proteins.[29] The concentration of these signaling molecules increases as a function of bacterial population density and when it reaches its threshold value, the bacterial co-operative behavior from the collective activities of individual cells is triggered.[30] At this point, the appropriate receptor signal brings about alterations in the complex gene-expression system to adapt to the recurrent and often extreme variations in the host-survival conditions.[31] QS is also implicated in the onset of virulence in several pathogenic bacteria. In Gram-positive bacteria QS occurs via specialized two-component systems, where the membrane-bound extracellular receptors sense the concentration of the secreted autoinducer peptides and activate the cytoplasmic histidine kinase.[32] The kinase auto-phosphorylates and passes the signal via a phosphorylation cascade-activating downstream the QS regulon and virulence genes. On the other hand, Gram-negative bacteria mostly employ the canonical LuxI/LuxR-type QS systems.[33] These systems were first discovered in the bioluminescent marine bacterium *Vibrio fischeri* and here LuxI serves as an *N*-acyl homoserine lactone synthase, where LuxR-type proteins bind the lactones and activate target genes.[34] In contrast, *Streptomyces*, as mentioned above, employs GBLs that freely diffuse into the cell as signaling molecules. These molecules are smaller in size than the signaling peptide-based inducers but are apparently very specific towards their target receptor. The TetR-FTRs sense the GBLs, and thus bring about alterations in the complex gene-expression system by triggering appropriate secondary metabolic pathways such as biofilm formation, antibiotic production and resistance.[35]

Allostery: The process of transferring the effect of binding at one site to the another functional unit in the biological macromolecules (mostly proteins) is termed as allostery.

3 Multilayer Regulation in TetR-FTRs from *Streptomyces*

Apart from ArpA in *S. griseus*, several GBL-responding TetR-FTRs have been discovered over the years in various *Streptomyces* species. These TetR-FTRs have specific molecules that differ from species to species that control their downstream function. Some of these molecules are listed in Fig. 2. *S. lavendulae* FarA, a TetR-FTR, serves as an autoregulator and also controls the biosynthesis of nucleoside antibiotics showdomycin and minimycin by responding to the butanolide, IM-2.[36] In *S. virginiae,* virginiamycin biosynthesis is regulated by BarA and its inducer molecules are virginiae butanolide A–E.[37, 38] One of the most well-studied *Streptomyces* strains, which is used as model system to understand various processes in *Streptomyces* genus is *S. coelicolor* A3(2). Therefore, substantial efforts were undertaken to find the ArpA homolog in *S. coelicolor* A3(2). CprA and CprB (The *coelicolor*

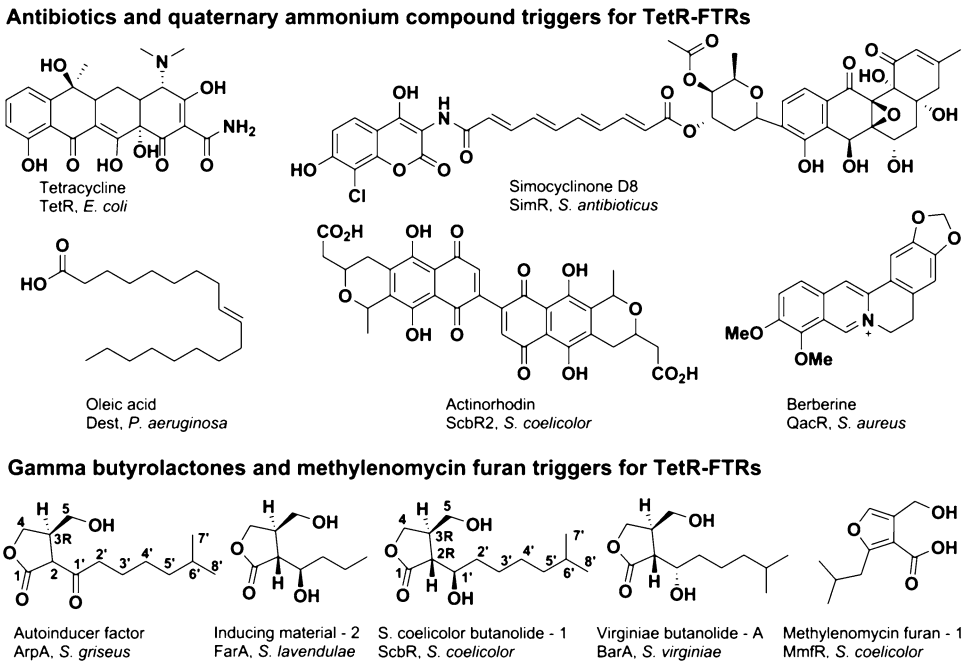

Figure 2: Representative structures of the chemical-signaling molecules for the TetR-FTRs. The producer organism and the name of the signaling molecule along with their receptor are given below their chemical structures.

pigment regulator proteins A and B)[39] happened to be the first two proteins assigned as homologs of ArpA by Onaka et al.

The phenotype of an *S. coelicolor* A3(2) *cprA* and *cprB* deletion-mutant exhibited acute reduction in antibiotic biosynthesis and also altered the time taken for the process of preserving genetic material.[40] However, due to difficulty in working with these proteins and lack of conclusive evidence, the biological role of CprA and CprB was not assigned. More recently, it was established that CprB is an autoregulator and is capable of binding several promoter sequences of various antibiotic precursors synthase gene clusters such as cryptic type-I polypeptide promoters, $kasO_A$ and $kasO_B$. Moreover, through a genomic analysis, it appeared that both CprA and CprB are proximal to gene clusters that may encode a potential efflux pump, and hence they may also play a role in creating efflux pumps in response to QS molecules. In 2001, Takano et al. demonstrated that ScbR (*S. coelicolor* quorum-sensing receptor), another TetR-FTR, also binds to GBLs, SCB1 (ScbR-captured butanolide 1), SCB2 and SCB3.[41] Production of antibiotics such as actinorhodin (Act) and undecylprodigiosin (Red) in *S. coelicolor* A3(2) depends on the concentration of the GBLs. Further, the expression of the GBL synthase gene (*scbA*) is under the tight control of ScbR. ScbR was also shown to regulate the cryptic type-I polyketide synthase gene cluster (*kasO*) by binding at two different positions in the promoter ($kasO_A$ and $kasO_B$),[42, 43] Very recently, another TetR-FTR assigned to this family, ScbR2, was characterized as a pseudo-GBL receptor. ScbR2 binds similar DNA sequences as ScbR, however, instead of binding GBLs, they bind with endogenously produced antibiotics in *S. coelicolor*.[44, 45]

The discovery of around 1000 regulatory proteins, of which 15% are TetR-FTRs, in *S. coelicolor* strengthened the belief of a multilayered regulatory network, where several regulators work in consort to control the secondary metabolic processes. Moreover, the fact that a large number of these TetR-FTRs in *S. coelicolor* control the same set of DNA sequences would seem to indicate that these proteins are likely to engage in cross-talk with one another and are not independent receptors. It is possible that CprA, CprB, ScbR and ScbR2 are connected through some kind of hierarchical control network, whose connections still remain unexplored and unknown. In support of these findings another organism where the hierarchy among TetRs is well established is *Streptomyces fradiae*. Cundliffe and coworkers have

Phenotype: It is the observable result such as physical appearance or biochemical trait of an organism when its genotype interacts with the environment.

Endogenous: Biosynthetic product of the cellular machinery or the processes that are originated from within an organism, tissue, or cell.

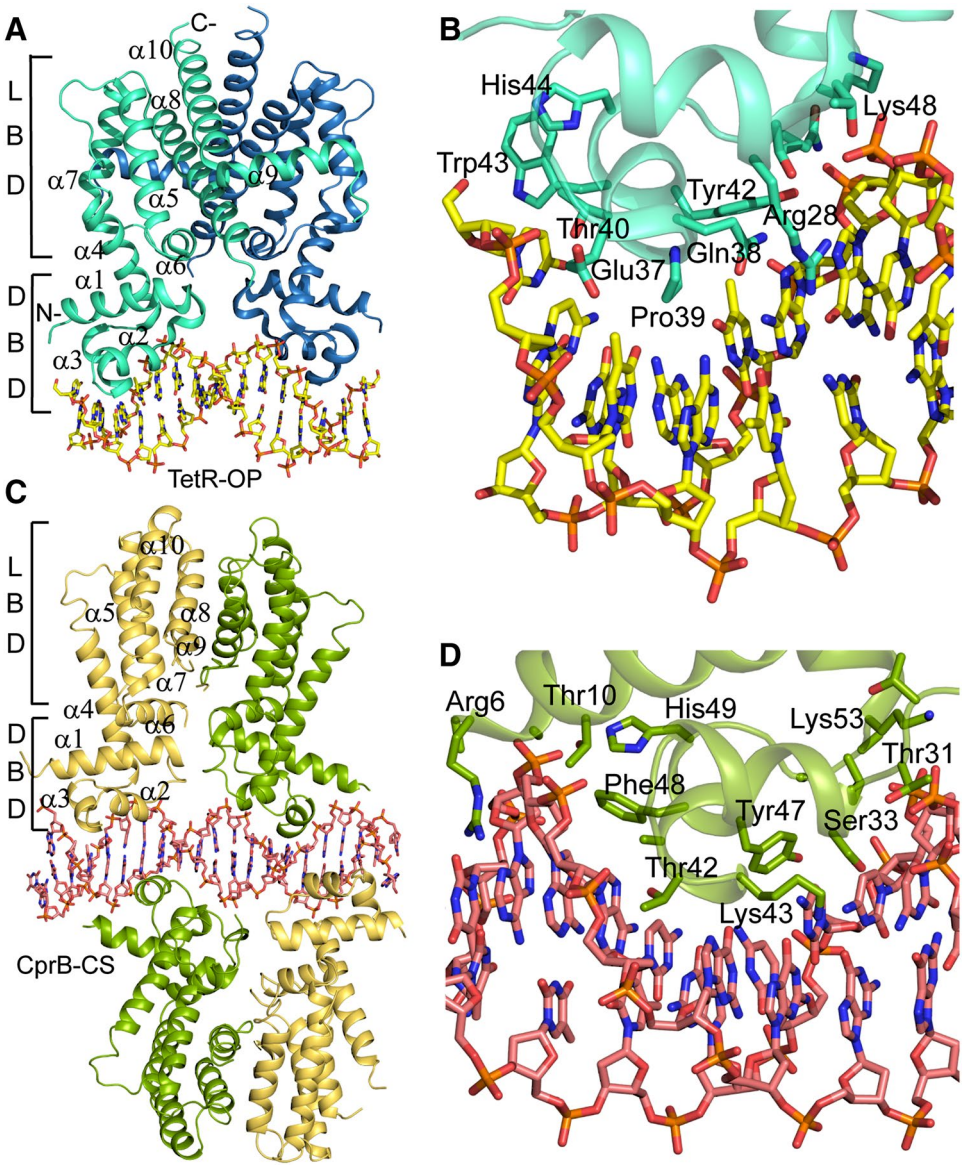

Figure 3: The X-ray crystal structures of TetR-FTRs. **a** The overall architecture of the TetR-operator complex, **b** The zoomed view of the interface between HTH-motif and the DNA major-groove of TetR, **c** Overall structure of CprB-DNA complex, and **d** The zoomed view of the interface between HTH-motif of CprB and the DNA. The ligand- and DNA-binding domains are represented as LBD and DBD, respectively, for each protein and the helices are labeled. Protein is shown in cartoon representation and DNA in sticks model. The interacting residues are highlighted in sticks representation.

established a remarkable network of TetR-FTRs that are proposed to be triggered by GBLs. In *S. fradiae,* five TetR-FTRs that show similarity to ArpA have been shown to control the tylosin biosynthetic gene cluster and also regulate resistance pathways. They form a regulatory circuit with TylP being at the top of the regulatory network, and TylR at the bottom, directly controlling tylosin production.[46] A series of knockouts of individual TetR-FTRs were generated to establish the circuitry of these regulators.[46, 47] Comparison of expression levels through RT-PCR in conjunction with tylosin levels showed that these TetR-FTRS are interdependent and control expression of each other. While proteins such as TylP, which is on top of the signaling cascade, constitutively active others like TylQ, TylS and TylU serve as positive or negative regulators and control expression of TylR, which directly regulates tylosin production in *S. fradiae.*[47, 48] TylP knockouts exhibited very high tylosin levels, whereas, TylR knockouts produced almost none.[46] Therefore, these two genes

with opposing effects seem to regulate tylosin levels. How this cascade is triggered or responds to GBLs is still not well understood and requires further investigation. One thing that, however, is exceedingly clear is that TetR-FTRs interact with each other and it is the fine control that they exert on each other that confers the finesse in their downstream regulation.

4 Structure of TETR-FTRs

4.1 Overall Architecture

Topology: The regular secondary structure of protein, such as the α-helices and β-strands, and their orientation with respect to each other in the protein structure.

TetR-FTRs serve as transcriptional repressors and bind to their cognate DNA with an affinity ranging from low to high nanomolar concentrations. TetR-FTRs harbor a modular architecture with the N-terminal portion involved in DNA binding and the C-terminal domain, tailor-made to host a ligand molecule. Upon binding of the appropriate ligand, TetRs release their DNA, resulting in the activation of the downstream pathways they regulate. To develop a molecular-level understanding of the mechanism of these events, several crystal structures in various stages, apo, ligand-bound and as a protein-DNA complex have been solved by researchers in a number of different laboratories around the globe. Of a total of 350 or so structures of TetR-FTRs deposited in the protein data bank, 250 are of apo and 90 of ligand-bound complexes. Due to difficulty in obtaining good diffracting crystals of protein–DNA complexes, very few, less than a dozen structures, are available for TetR-FTRs in complex with their target DNA fragments. Structural analysis reveals that all TetR-FTRs have an overall "Ω" shape (seen in Fig. 3a) and in general are mostly helical in nature, possessing nine conserved α-helices. The X-ray structures show that in their apo-form they generally exist as homo-dimers with a pseudo-2-fold rotation axis between the monomeric units.[49] The N-terminal DNA-binding domain (DBD) is composed of three helices, where helices α2 and α3 form the conventional helix-turn-helix (HTH)-DNA binding motif. The helix α3 serves as the recognition helix and is involved in making most of the base-specific contacts with the DNA, whereas, helix α2 widens the major-groove to allow for perfect docking of the recognition helix. This 20 amino-acid HTH-motif is the most conserved stretch among all members of the family. On the other hand, the C-terminal ligand-binding domain (LBD) is more diverse and it is here that most of the topological differences occur among various TetR-FTRs. The LBD is composed of helices α4–α9 and is lined with mostly hydrophobic residues. There is huge variation in the pocket volume among the TetR-FTRs. This is because the LBD domain can respond to a spectrum of cues ranging from antibiotics to quorum-sensing molecules to fatty acids, etc. As a result, this domain is variable and is tailor-made according to the needs of the system. The binding pocket complements the cognate ligand. Helix α4 which lies at the periphery of the LBD pocket serves as a connector between DNA binding and the ligand-binding domain and most likely transmits the information between the two domains.[20, 49]

In spite of topological similarity and high degree of amino-acid sequence conservation among various TetR-FTRs in the DBD, small differences relate to the specificity of binding a specific operator DNA sequence. Due to these fine differences in the amino acid sequence, the mode of DNA recognition among the TetR-FTRs varies.[20] Analysis performed on the available DNA-bound crystal structures of the TetR-FTRs shows that they exhibit two modes of DNA recognition. In the first case, the TetR-FTRs bind as a dimer and in the second, they recognize their cognate DNA sequence as a pair of dimers. It was observed that, in general, the TetR-FTRs which bind as dimers display greater specificity towards their operator sequence, whereas, some of the dimer of dimer DNA-binding TetR-FTRs serve as global regulators. This is mostly because the dimer DNA-binding TetR-FTRs distort the structure of the DNA to a higher degree than the dimer of dimer binding by inducing overall bend and also by altering the groove widths. However, the dimer of dimers compensates for the loss of specificity by additional interactions introduced by another pair of monomers.[50]

4.2 Structure of the DNA-Bound Form of TetR-FTRs

To get an in-depth insight into the structure of dimeric TetR-FTRs, the founding member TetR receptor is taken as an example to describe the mode of DNA binding and also the ligand recognition for this sub-class. From the crystal structures of the TetR-DNA complex, it is observed that the HTH-motif is docked on to the major-groove of the promoter sequence with an overall global bend of 15° in the DNA (Fig. 3b). The axis of the recognition helix α3 is almost aligned parallel to the DNA major-groove to make maximum contact. The side chains of the amino acid involved in interacting with the phosphate backbone are Thr26, Thr27, Tyr42 and Lys48 via hydrogen bonding and π–π interactions with the DNA strand running from 3′ to 5′ and for the

complementary strand, the interactions occur via the amide nitrogen atom of Glu37 along with the side chains of residues Thr40 and His44.[49]

The base-specific contacts of TetR are achieved via the involvement of residues Pro39, Tyr42 and Trp43 of recognition helix. The majority of these contacts are base-stacking interactions. In addition, hydrogen-bonding contacts are also made by the side chains of the residues Arg28, Gln38 and Thr40. It was also observed that the HTH-motif tightly docks on to the DNA leaving no space to accommodate any water molecules at the interface.[49]

To illustrate the structural similarities and differences in dimer of dimer DNA-binding subclass with the dimeric one, CprB, is taken as an example. The structures of apo and DNA-bound forms of CprB were used to understand nuances of binding. CprB is the only protein whose structure is available from this QS GBL receptor sub-class of TetR-FTRs (Fig. 3c)[51] The structure revealed that CprB also possesses ten α-helices and similar to other TetR-FTRs, it has an overall omega-shaped architecture.[20, 51] Unlike its apo-form, which is a dimer, the DNA-bound form of CprB comprises a pair of dimers positioned on either side of the DNA. The structure shows that the DNA is sandwiched between the two dimeric units with a slight offset of around 10 Å between the two dimers. To facilitate the interaction of the HTH-motif with the DNA, the spacer helix α2 orients such that it results in a widening of the groove to ~13 Å (ideal B-form has 11.7 Å). This allows the helix α3 to insert and make protein DNA contacts to achieve specificity of binding via an induced fit mechanism which is similar to the TetR. This deformation in the DNA is transmitted along the length of the chain. The side chains of the amino acids involved in interacting with the phosphate backbone include Thr10, Thr31, Ser33, His49, and Lys53 along with the amide nitrogen atom of Leu32. The base-specific contacts of CprB are achieved by the involvement of residues Lys43, Gly44, Tyr47 and Phe48 (which appear to be conserved in the GBL receptor subclass) and are present on the recognition helix α3 (Fig. 3d).[50]

A comparison of the dimer DNA-binding sub-class with the dimer of dimer class shows that distortion in the major-groove width and N-terminal interactions with the minor- groove is common in both the cases. The former mostly distorts the B-form of the DNA by widening the major groove and also by inducing a global bend. As seen in the case of SimR and TetR, the bend due to the HTH-motifs of the dimer is

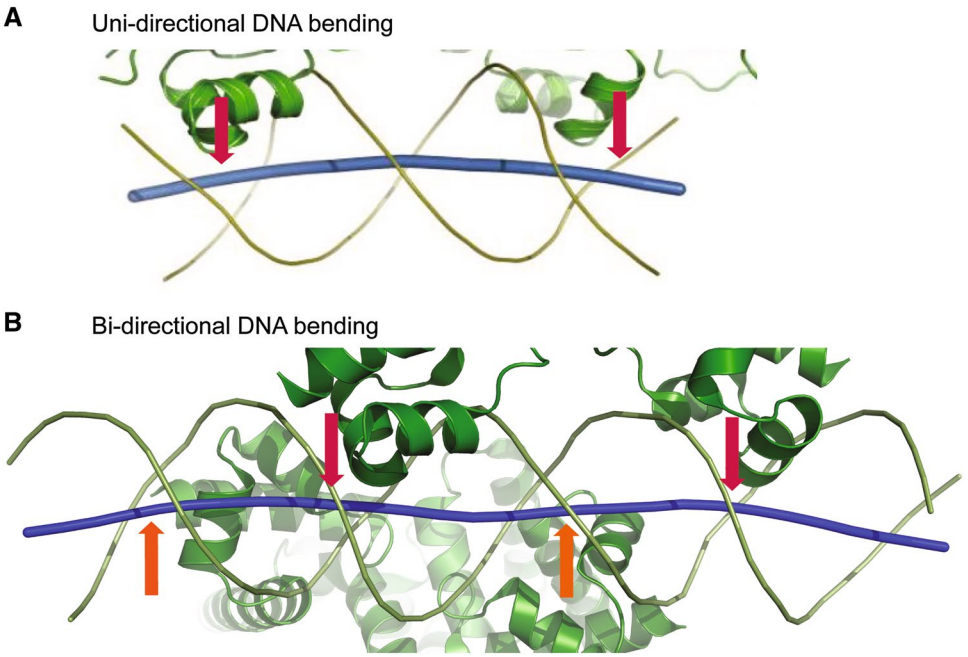

Figure 4: DNA bending by two different sub-classes of TetR-FTRs. **a** Dimer DNA-binding TetR-FTRs show unidirectional bend in the DNA that results in a large degree of distortion in it from the ideal B-form, adapted from Le et al.[11] **b** Dimer of dimer DNA binding exhibit minimal (0–4°) distortion in the DNA, from the ideal B-form. *Red* and *orange arrows* show downward and upward bends, respectively.

Table 1 Analysis of various forms of TetR-FTRs

	DNA-bound		Apo	Effector-bound	
	Distance (α3–α3'), Å	RMSD, Å	Distance (α3–α3'), Å	Distance (α3–α3'), Å	RMSD, Å
TetR	30.4	2.1 (4.5)	35.2	37.9	1.5 (3.1)
DesT	32.7	–	–	37.5	1.9 (8.9)[a]
SimR	32.4	1.9 (6.8)	39.8	40.5	1.5 (6.8)
HrtR	32.1	1.3 (8.6)	31.3	49.5	3.3 (9.4)
AmtR	32.6	7.5 (12.1)	40.5	–	
KstR	33.9	8.8 (15.3)	47.5.4	49.3	1.6 (6.7)
QacR	37.0	1.6 (4.1)	36.0	45.2	3.3 (11.0)
CgmR	35.7	–	–	40.9	1.7 (7.5)[a]
SlmA	37.0	5.2 (10.2)	43.0	–	–
TM1030	36.2	9.3 (17.8)	53.9	59.0	1.6 (4.5)
Ms6564	35.4	4.9 (15.1)	21.1	–	–
CprB	38.2	1.6 (3.8)	40.2	–	–

Distances between the HTH-motifs of a dimer and the root mean-squared deviation (RMSD) in the Cα atoms of ligand and DNA-bound forms are listed. The rows in light green correspond to the dimeric DNA- binding TetR-FTRs and in grey the dimer of dimer DNA binding sub-class

[a] RMSD between the DNA and ligand-bound forms. The values in the parentheses correspond to maximum deviation observed for a particular structure

unidirectionally induced, and the overall bend in the DNA is 15–17°, Fig. 4a.[11, 49] However, in the dimer of dimer, sub-class the bend is no longer unidirectional in nature. This is because, one set of dimers pushes the DNA downwards and the other, binding from the opposite end, pushes it upwards. Hence, the range of net global bend in this scenario is only 0–4°, Fig. 4b.[50, 52] For example, in the case of QacR and CprB, the bend in the DNA is reported to be around 3° in both the protein–DNA complexes. Furthermore, it was observed that the dimerc sub-class prevents another dimer from binding on the opposite side by reducing the distance between the recognition helices within a dimeric unit. Analysis of the available TetR-FTR–DNA complex structures shows that the distance of dimeric DNA-binding proteins is in the range of 30–33 Å, Table 1, whereas, in the case of dimer of dimer DNA-binding proteins, it is around 36–38 Å. The smaller the inter-HTH-motif distance, the larger is the bend resulting in no room for the other dimer to bind on the opposite side of the DNA. The large inter-HTH-motif distance allows the second dimer to interact on the other side of the DNA yielding a dimer of dimer assembly. From this analysis, we can comment on the TetR-FTRs induced distortion in the structure of the B-DNA and also upon the mode of DNA recognition.

Another important feature exclusive to the dimer of dimer DNA-binding sub-class is that the DNA-binding event is cooperative. Based on the results of the electrophoretic mobility shift assays and isothermal titration calorimetric (ITC) experiments, it was observed that both dimer of dimers, QacR and CprB, follow a cooperative

Cooperativity: Here, the event of binding interactions between the partners (protein and DNA) where DNA has more than one binding site and binding of one protein molecule effects the binding of another.

mode of DNA recognition. For CprB, ITC performed with its cognate DNA demonstrates that it binds to DNA in a two-step fashion. The first step is enthalpy driven, therefore, most likely the individual monomers of the dimeric units bind here, whereas, the second step is entropy driven where the other monomer latches on to the DNA and plausibly results in expulsion of water molecules from the protein–DNA interface. Hence, the mode of DNA binding was proposed to be a click-and-clamp mechanism where a monomer clicks on the DNA fragment and primes the second entropy-driven clamping event.[50] The proposed model is based on the cooperativity within the dimer, however, the possibility of the cooperative binding between both the dimers cannot be ruled out. A similar case has been reported for QacR.[52]

4.3 N-terminal Extensions in TetR-FTRs

Analysis of both the dimeric and dimer of dimer class shows that several TetR-FTRs possess ordered N-terminal fingers. It appears that, in general, many of them have positively charged (mostly arginine residues) N-terminal residues in their α1 helix; alternatively, some of them possess an N-terminal extended flexible arm.[11] Structural analysis on select DNA-bound TetR-FTRs reveals that the positively charged extensions are mostly employed by TetR-FTRs to anchor into the minor-groove of the DNA. The positively charged residues interact with the phosphate backbone and impart extra stability and in some cases specificity to these TetR-FTRs. For example, SimR has a 28-residue N-terminal flexible arm that stretches to form interactions with the DNA minor-groove and phosphate backbone.[11] A DNase I footprinting assay for the SimR and the *simR-simX* intergenic sequence shows that SimR masks about 23 base-pairs.[11] N-terminal deletion mutants sharply reduced the binding affinity of the SimR with its operator sequence. In the case of CprB, a similar N-terminal tail stabilization was observed and a deletion of this tail resulted in a reduction in DNA-binding ability. This result shows the importance of the N-terminal flexible arm in acquiring added stability for these complexes. Similarly, the master regulator Ms6564 and the global nitrogen uptake regulator AmtR also show that its arginine-rich N-terminal arm is docked onto the DNA minor-groove forming hydrogen-bonding and hydrophobic interactions with the operator sequence, thereby enhancing the complex stability.[18] There are other TetR-FTRs like DesT,[12] CgmR,[12] KstR,[19] and TM1030 having a short N-terminal tail involved in forming non-covalent interactions with the DNA backbone and the minor-groove.[53] Interestingly, in case of QacR, the positive dipole of the helix α1 partakes in interaction with phosphate backbone.[52] However, an N-terminal interacting extension in case of TetR, SlmA and HrtR is not observed. As a result, TetR-FTRs like AmtR, CprB, SimR, Ms6564, DesT, CgmR, QacR, TM1030 and KstR recognize longer DNA sequences and, therefore, can attain specificity.

Positive dipole: This occur when two atoms in a molecule have substantially different electronegativity and one atom attracts electrons getting more negative charge and the other gets more positive. Here, the nitrogen atom of the peptide bond at the start of the α-helix gets more positively charged and involves in interacting with the phosphate backbone.

4.4 Ligand-Binding Domain of TetR-FTRs

The ligand-binding event in TER-FTRs results in the release of DNA and thereby activates downstream transcription. As mentioned earlier there is very little sequence identity in this region among various TetR-FTRs. The tetracycline receptor is an example of an antibiotic-binding efflux pump regulator that binds tetracycline (Tc)-Mg with nanomolar affinity. The information relating to the binding of tetracycline is transferred to the DBD via the connector helix α4, which then primes the DBD to release the DNA. Structural analysis of the TetR–Tc–Mg complex shows that the pocket size in the LBD is approximately 10 Å in diameter and 25 Å in depth. This large cavity is partly lined with charged residues (His100, Thr103 and Glu147) to chelate the Mg ion, Fig. 5a. The rest of the pocket is lined with hydrophobic residues (Val113, Leu131, Ile134, Leu170 and Leu174), where the tetracycline binds. His64, Asn82 and Phe86 serve as anchoring residues by forming both hydrogen-bonding and hydrophobic contacts with the Tc–Mg complex. The DNA- and ligand-binding sites in the TetR are about 33 Å far apart and the helices α6 and α4 are part of LBD; however, they are at the interface of the DBD. Apart from the ligand-binding pocket, the LBD also has the helices involved in the dimerization. The four-helix bundle (helices α8 and α10 from each monomer) serves as the dimerization unit. There are a large number of non-covalent interactions between the two monomers with hydrophobic interactions being predominant. Helix α9 from each monomer is involved in domain swapping and it extends into the adjacent monomer and partially wraps around the LBD. This extension provides increased dimerization contacts for the coordinative conformational change, which is transmitted from LBD to DBD.[49]

Chelate: Here, the charged/polar amino acids side chains bonded to a central metal atom at two or more points.

Another class of compounds that triggers TetR-FTRs transcription is GBLs. CprB is the only structurally characterized GBL, hence to

understand its ligand-binding domain and to compare and contrast with the well-studied antibiotic class the regulatory domain of CprB was analyzed. It was observed that the regulatory domain is composed of an antiparallel bundle of helices from α4 to α10, with helix α6 situated at the base of the domain. Helix α6 forms non-covalent interactions with the helix α1 of the DBD.[54] The regulatory/effector-binding domain contains a solvent accessible pocket with a depth of approximately 20 Å and a diameter of 5 Å, where the ligand likely binds. When compared with the TetR cavity architecture, CprB has thinner cavity which is evident from the diameter of the pocket; however, the depth of the cavity is more or less similar. The cavity is lined by hydrophobic residues and contains a tryptophan residue at position 127 (Fig. 5b) that is conserved among the other related γ-butyrolactone- binding proteins.[54] Mutation of this conserved tryptophan residue in ArpA is known to abolish its A-factor-binding ability and, therefore, suggests a direct role in binding GBLs.[55] The other residues lining the pocket are Leu86, Leu89, Met93, Leu107, Leu157, Val158, Val161, Val162 and Leu181. TetR has polar residues such as His64, Asn82, His100, Glu147, which are essential to accommodate the positively charged Tc–Mg complex; however, CprB lacks them as it may recognize GBLs, which are hydrophobic. In the absence of the structure of ligand complex of CprB, the structure of apo CprB was docked with a battery of GBLs by varying the aliphatic chain lengths. Docking results show that the five-membered lactone ring stacks against the indole ring of Trp127 enabling the lactone to be stabilized.[56] The aliphatic tail of the GBL moiety likely extends into the rest of the hydrophobic pocket. Furthermore, docking is also suggestive of the likely stereochemistry of the GBL-binding CprB protein. The hydroxyl group at C5 position of GBL hydrogen bonds with the Trp127 backbone carbonyl group and, therefore, favors a stereoisomer with an R configuration at the C3 position. Overall, the ligand-binding site in TetR-FTRs may control the size, shape and stereochemistry of the incoming ligand. Depending on the stringency imposed by LBD, a broad spectrum or a specific ligand is accepted for regulation.[50]

Stereoisomer: The compounds with same chemical formulae and have identical bonding atoms are known as the isomers. The isomers that differ only in the spatial orientation of their component atoms are called stereoisomers.

4.5 Allostery in the TETR-FTRs

TetR-FTRs maintain exclusivity between their two forms and either exist in a ligand- or a DNA-bound form. That is, both monomers are either in the apo form or are ligand bound. In general, the ligand-bound form of TetR-FTRs attains the relaxed state and favors increased conformational stability. In this state, both the HTH-motifs of a dimer are far apart and they are locked in conformational state, where the dimer has lost its shape complementarity to fit into adjacent DNA major-grooves. Conformational restriction in

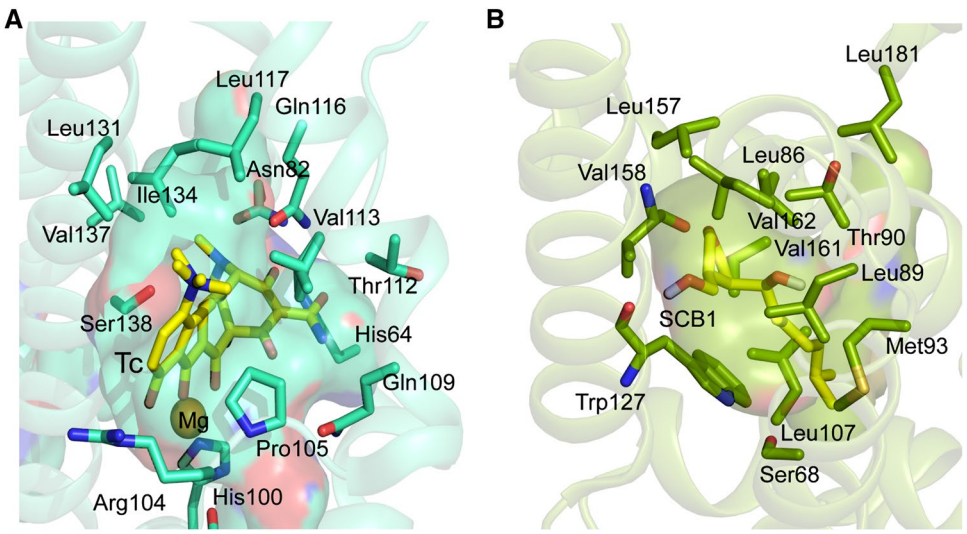

Figure 5: Zoomed view of the active site of TetR-FTRs. **a** The Tetracycline (Tc)-magnesium ion (Mg) complex bound to the TetR in the LBD, **b** The hydrophobic cavity of CprB, docked with a GBL (SCB1). The residues lining the binding pockets, tetracycline and GBL moieties are shown in sticks model, and the magnesium ion in sphere representation. The binding pockets of TetR and CprB are shown as a transparent surface.

TetR-FTRs prevents them from binding both DNA and ligand simultaneously. In general, the DNA-bound state is referred to as the tensed state and the ligand-bound state as relaxed state. Understanding the allosteric mechanism of regulation is of paramount significance as it is the switch between two forms, which drive the function of TetR-FTRs. Toward this end, the available three-dimensional structures can be examined for insights into the mechanism of signal transduction from sensory to functional domain. To date, complex structures of five dimeric TetR-FTRs (TetR, DesT, SimR, HrtR and KstR) have been identified in both the DNA- and ligand-bound forms. Additionally, three dimer of dimer DNA-binding (QacR, CgmR and TM1030) TetR-FTRs have also been solved in both the forms. The ligand-bound forms of AmtR, SlmA, Ms6564 and CprB are not available in the public domain.[3, 49]

In all cases, effector binding resulted in increased separation between the recognition helices of the dimer when compared with their corresponding DNA-bound forms. The connector helix α4 seems to be the trigger between the two domains through which induced conformational change in the structure of TetR-FTRs occurred. This signal transduction may be further accompanied by conformational changes in helix α6, which also directly interacts with functional domain. For example, in the Tc–Mg complex-bound state of TetR, part of the short-helix α6 adopts a type-II β-turn (residues 100–103). This induces shift in the connector helix α4 accompanied by the swing resembling a pendulum-like motion in the DBD. This conformational change is initiated by the inward movement of the pocket of helix α6 resulting in overall DNA release. Residues Val 99, Thr103 of helix α6 are in van der Waals contact with Leu52 and Ala56 of helix α4. These interactions provide the link between the movement in α6 and the corresponding movement of the connector helix α4.[49] To compare the various forms (apo, liganded and DNA-bound) of the TetR-FTRs, the complex forms were superposed using LsqAB of CCP4 program suit. RMSD in the Cα atoms of the TetR–Tc–Mg complex with respect to its apo form is 2.1 Å on average and the maximum deviation was observed to be 4.5 Å in the DBD.

Furthermore, to highlight the pendulum-like shift in the DBD, the LDB of TetR in all three forms was superposed, Fig. 6a. The distance

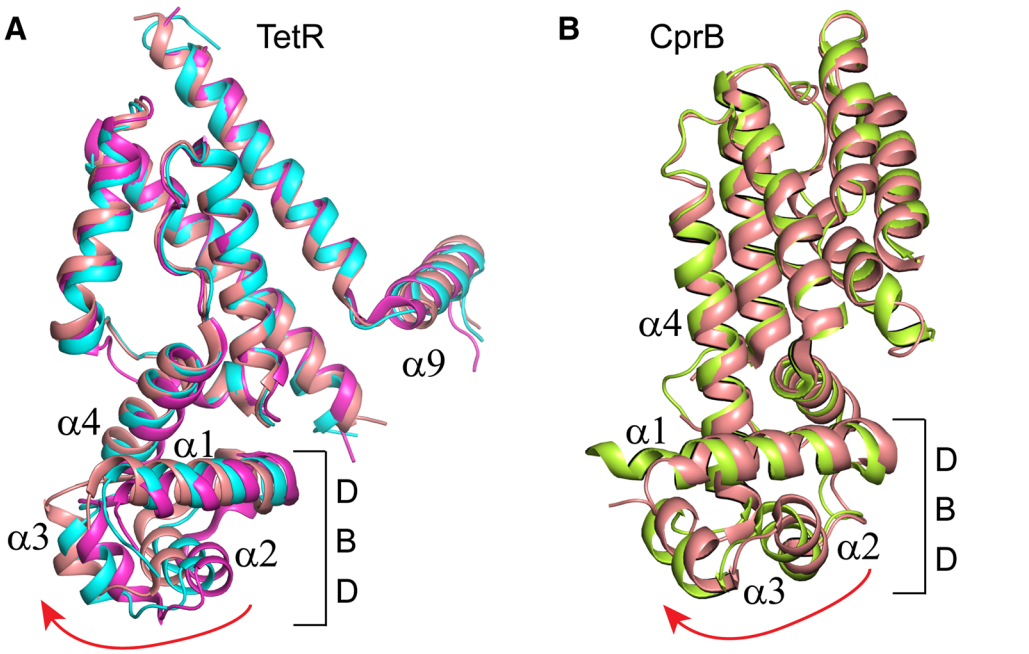

Figure 6: Allostery in the dimer and dimer of dimer DNA-binding TetR-FTRs. **a** The three forms (apo, ligand and DNA-bound) of TetR are superposed to highlight the allosteric changes in the three different conformational states of the TetR. The cartoon representation in *brown*, *cyan* and *magenta* are for apo, TetR–Tc–Mg and TetR-DNA complexes, respectively. **b** The superposition of apo and DNA-bound forms of CprB is shown in cartoon representation, *brown* and *green* respectively. In both **a** and **b** cases, the pendulum-like shift in the DBD is indicated with the *red arrow*.

between the recognition helices (measured from the amine nitrogen atom of the second residue in the recognition helix α3 of each monomer of the dimer) of liganded TetR increased by around 7.5 and 2.7 Å with respect to the DNA-bound and apo forms, respectively. In general, as shown in Table 1, a common variation of these distances among various members could not be proposed based on the available structural information. However, the ligand-bound form has increased the distance in both the dimer DNA binding and dimer of dimer DNA- binding TetR-FTRs.

To explain the allostery in the dimer of dimer DNA-binding sub-class of TetR-FTRs, the DNA-bound form and the apo form of CprB are compared. In case of CprB, the inter-HTH-motif distance was observed to be 38.2 Å in DNA-bound form and 40.2 Å in the apo form. The superposition of the LBD of two forms of CprB helped show the structural changes in the DBD on DNA binding. A pendulum-like motion along the connector helix was observed, Fig. 6b. The RMSD between the two forms of CprB exhibited 1.6 Å in average for the overall structure and the maximum of 3.8 Å deviation from DNA-bound to the apo form. In case of CprB, the shift in the DBD is accompanied by the reorganization of the dimeric interface formed by helices α8 and α9 from each monomer. Upon DNA binding, around 11 hydrogen-bonding interactions were disrupted and 9 new hydrogen-bonding interactions were formed. Interestingly, it was observed that CprB has an inter-monomeric disulphide bond between cysteine 159 residues from each monomer. It was proposed that the disulphide bond serves as a fulcrum for the protein to adopt various conformational states in different forms (i.e., apo, DNA and ligand-bound). Experiments where the cysteine is replaced by serine showed that protein expression is severely affected.[50] However, no change in DNA-binding ability of CprB was observed. Hence, the exact reason for this disulfide bond is still elusive. Additionally, the allostery in the broad spectrum antibiotic efflux pump regulator QacR has also been studied. In case of QacR, the distance between the recognition helix for the DNA-bound and apo form is almost the same, however, the ligand-bound form exhibits an increased distance of 45.2 Å as compared to 38 Å observed for the apo form. This suggests that the apo and the DNA-bound forms of QacR are in an equally tensed state and attain relaxed state when the ligand is bound.

5 Future Perspectives of TETR-FTRs

The TetR-FTRs exhibit a vital role in governing several aspects of physiology of prokaryotes. Although a vast amount of knowledge has been gained from the literature, with plethora of reports of the structural and functional studies performed on TetR-FTRs, so far, a general model to explain the exact allosteric determinants connecting both the sensory and functional domains of the TetR-FTRs remains elusive. As mentioned earlier, there are more than one million TetR-FTRs but there are only a handful of structures with effector and DNA-bound complexes. The pressing need is to establish an evolutionary link governing allostery in TetR-FTRs.

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