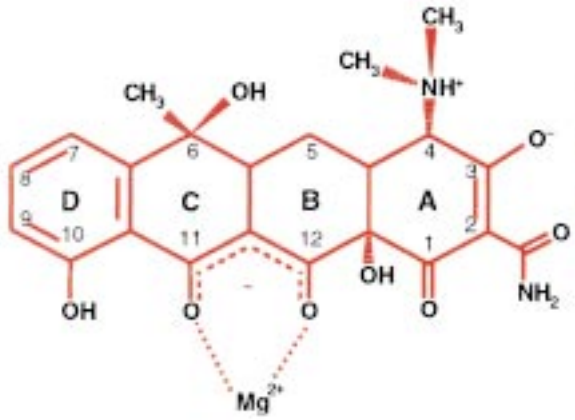
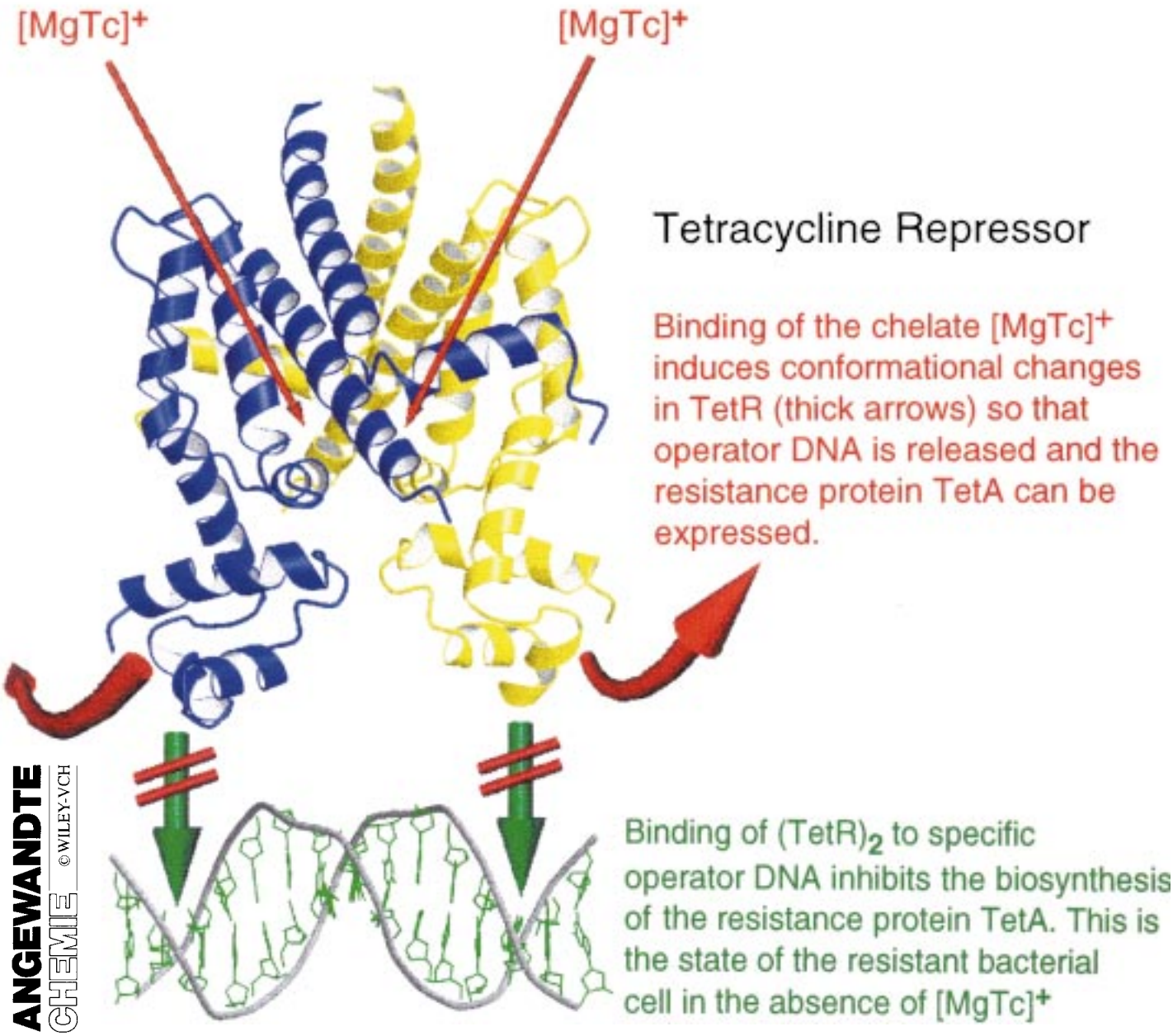
Resistance against the antibiotic tetracycline (Tc) is tightly regulated in gram–negative bacteria by two states of the dimeric tetracycline repressor $(TetR)_2$: bound to operator DNA and, in the "induced" form, bound to $[MgTc]^+$.



If Tc enters the (bacterial) cell, it binds Mg^{2+} to form the biologically active chelate $[MgTc]^+$.



Binding of the chelate $[MgTc]^+$ induces conformational changes in TetR (thick arrows) so that operator DNA is released and the resistance protein TetA can be expressed.

Binding of $(TetR)_2$ to specific operator DNA inhibits the biosynthesis of the resistance protein TetA. This is the state of the resistant bacterial cell in the absence of $[MgTc]^+$

The Tetracycline Repressor—A Paradigm for a Biological Switch

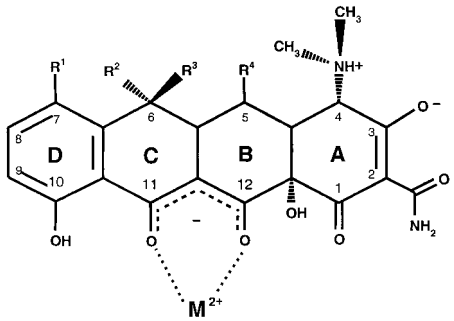
Wolfram Saenger,* Peter Orth, Caroline Kisker, Wolfgang Hillen, and Winfried Hinrichs

The excessive use of antibiotics has enabled bacteria to develop resistance through a variety of mechanisms. The most common bacteriostatic action of the broad-spectrum antibiotic tetracycline (Tc) is by the inactivation of the bacterial ribosome so that the protein biosynthesis is interrupted and the bacteria die. The most common mechanism of resistance in gram-negative bacteria against Tc is associated with the membrane-intrinsic protein TetA, which exports invaded Tc out of the bacterial cell before it can attack its target, the ribosome. The expression of TetA is tightly regulated by the homodimeric Tet repressor $(TetR)_2$, which binds specifically with two helix-turn-helix motifs of operator DNA (*tetO*; $K_{ass} \approx 10^{11} M^{-1}$) located upstream from the *tetA* gene on a plasmid or transposon. When Tc diffuses into the cell it chelates Mg^{2+} and the complex $[MgTc]^+$ binds to $(TetR)_2$ to form the induced complex $(TetR \cdot [MgTc]^+)_2$. This process is associated with conformational changes, which sharply reduce the affinity of $(TetR)_2$ to *tetO*, so that expression of TetA can take place, thus conferring resistance to the bacteria cells against Tc. Crystallographic studies show sequence-specific protein–nucleic acid interactions in the $(TetR)_2 \cdot tetO$ complex and how the binding of two $[MgTc]^+$ to $(TetR)_2$ enforces conformational changes that are stabilized by cooperative binding of two chains of eight water molecules each so that the formed $(TetR \cdot [MgTc]^+)_2$ is no longer able to recognize and bind to *tetO*. Since the switching mechanisms of the TetR/$[MgTc]^+$ system is so tight, it has proven very useful in the regulation of eukaryotic gene expression and may also be applicable in gene therapy.

Keywords: antibiotics · DNA recognition · resistance · tetracycline

1. Introduction: Regulation of Tetracycline Resistance

The ability of bacteria to respond favorably to their environment and to survive under hostile conditions has raised severe problems in contemporary pharmacology and medicine.[1–3] This situation has arisen because almost all pathogenic bacteria have acquired resistances against the presently known and widely used antibiotics. The resistances are associated with proteins encoded by genes located on moveable genetic elements, such as transposons or resistance (R) factors,[4–6] which can be rapidly exchanged and efficiently distributed among bacteria. One of the best known examples of bacterial resistance is directed against the broad-spectrum antibiotic tetracycline (Tc) and its chemically modified analogues (Scheme 1).



Scheme 1. Chemical structure of tetracycline (Tc). In the therapeutically used Tc, substituents may be exchanged as follows: $R^1 = H$, Cl, $N(CH_3)_2$; $R^2 = CH_3$, H; $R^3 = OH$, H; $R^4 = H$, OH. Under physiological conditions a divalent metal ion (M^{2+}; preferentially Mg^{2+}) is chelated by the deprotonated keto-enol group O11/O12$^{(-)}$ to form $[MgTc]^+$.

After Tc has diffused through the cytoplasmatic membrane into bacteria of different species it chelates a divalent cation M^{2+}, preferentially Mg^{2+}. The complex $[MgTc]^+$ (Scheme 1)

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Wolfram Saenger was born 1939 in Frankfurt (Germany). He graduated from the Technische Hochschule Darmstadt in Chemistry and received his Ph.D in 1965 for studies on the kinetics of cyclodextrin inclusion formation with F. Cramer. In his two years postdoctoral work at Harvard University with J. Z. Gougoutas he became acquainted with X-ray crystal structure analysis and joined F. Cramer at the Max-Planck-Institut für experimentelle Medizin in Göttingen. There he set up an independent research group working on structure analyses of oligosaccharides, proteins, and nucleic acids. In 1972, he habilitated at the Universität Göttingen, and since 1981, he has held the chair for crystallography at the Freie Universität Berlin. In 1987, he received the Leibniz Award and in 1989 the Humboldt Award. Besides structural biology, his current interests are in hydrogen bonding and protein aggregation that lead to crystallization.

Peter Orth was born in 1969 in Berlin (Germany). He studied Chemistry at the Lomonossow University in Moscow until 1994. During his Ph.D. he elucidated the machanism of regulation of the tetracycline repressor with crystallographic methods in the group of W. Saenger at the Freie Universität Berlin and obtained his Ph.D. in 1998. He proceeded to work in W. Saengers group on the structural characterization of the membrane protein, photosystem II.

Caroline Kisker was born in 1964 in Berlin (Germany). She studied Biochemistry at the Freie Universtität Berlin and pursued her Ph.D. thesis in the group of W. Saenger with the structural characterization of the tetracycline repressor in the complex with its inducer tetracycline. For this work she obtained the Karl Ramsauer Award. After completion of her Ph.D. thesis in 1994, she joined D. C. Rees' laboratory at the California Institute of Technology in Pasadena. She elucidated the structure of carbonic anhydrase and studied the sulfite oxidase deficiency at the atomic level. For the last two years she has worked as Assistant Professor at the State University of New York at Stony Brook where she pursues crystallographic and biochemical studies of DNA repair enzymes and enzymes containing the molybdenum cofactor.

Wolfgang Hillen was born in 1948 in Osnabrück (Germany). He studied chemistry at the university of Münster and received his Ph.D in 1977 for a thesis on codon–anticodon interaction with H.-G. Gassen. He was a postdoctoral fellow with R. D. Wells from 1978 until 1980 at the University of Wisonsin, Madison, where he studied sequence-dependent tertiary structures of DNA. He returned to the Technische Hochschule Darmstadt where he earned his Habilitation in 1983 with work on the molecular mechanisms of gene regulation. In 1984 he was appointed as Associate Professor of Physical Biology at the University of Erlangen-Nürnberg. He received the Carl Düisberg Award of the GDCh in 1986 and the Max Planck Award in 1997. His current research interests are molecular mechanisms of gene regulation, protein–ligand interactions, signaling between physiological parameters and gene regulators, and allosterical conformational changes of proteins.

Winfried Hinrichs was born 1950 in Hamburg (Germany). After training as a laboratory assistant, he studied chemical engineering (1970–1973), then chemistry (1976–1980), and received his Ph.D. in 1983 at the Universitry of Hamburg with a thesis on organic semiconductors (group of G. Klar). He spent a postdoctoral time at the Rijksuniveriteit te Leyden (The Netherlands) and joined the group of W. Saenger in 1986 at the Institut für Kristallographie in the Freie Universität Berlin (Habilitation 1996). After a one year sabbatical in the group of D. Moras in Strasbourg (Université Louis Pasteur, IGBMC) he became professor for biochemistry at the Ernst-Moritz-Arndt-Universität Greifswald in 1999. His scientific work is dominated by X-ray crystal structure anlayses with the main interests focused on structure–function relationships of biological macromolecules.

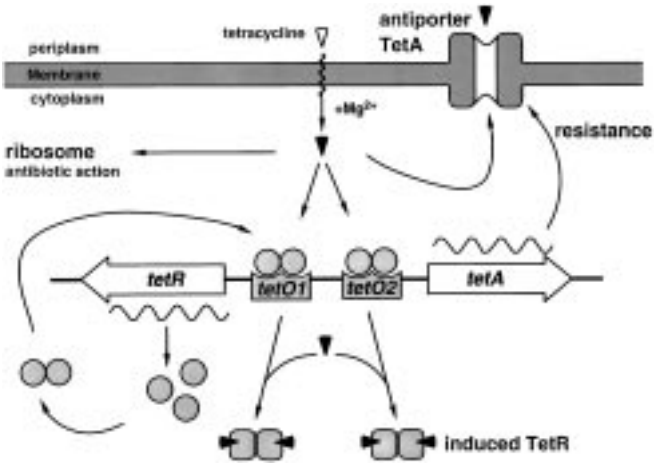
acts by binding to the small (30S) prokaryotic ribosomal subunit ($K_{ass} \approx 10^6\,M^{-1}$), thereby inhibiting protein biosynthesis and stalling the growth of the invaded cell.[7, 8] The presently known resistance mechanisms[6] against the Tc-type antibiotics involve proteins that either protect the ribosome from attack by $[MgTc]^+$,[9, 10] or export invaded Tc out of the bacterial cell.[11–14] Chemical modification of Tc by resistance proteins has also been demonstrated, but this plays no significant role in resistance.[15] The export mechanism is most abundant among gram-negative bacteria. It relies on the transport protein TetA, which is embedded in the cytoplasmic membrane and acts as an antiporter by coupling the export of $[MgTc]^+$ out of the resistant bacterial cell with the uptake of H^+.[16, 17] The expression of TetA is tightly regulated by the tetracycline repressor (Tet Repressor, TetR); TetA would be disadvantageous for the cell in the absence of $[MgTc]^+$, since it interferes with the maintenance of the electrostatic potential across the cell membrane.[18]

1.1. The Tet Repressor (TetR) and Its Induction by $[MgTc]^+$

The tetracycline repressor occurs as the homodimer $(TetR)_2$ in which two identical helix-turn-helix (HTH) motifs bind in the absence of $[MgTc]^+$ to two adjacent major grooves of DNA. $(TetR)_2$ has only a low affinity to nonspecific DNA sequences but binds tightly ($K_{ass} \approx 10^{11}\,M^{-1}$) to two specific DNA operator sequences, *tetO1* and *tetO2*, which have twofold (palindromic) symmetry of the nucleotide sequence,[18, 19] to form the complex $[(TetR)_2 \cdot tetO]$ (here and in the following sections, *tetO* means *tetO1* and/or *tetO2*). This process blocks the transcription of the gene *tetR* (dependent on *tetO1*), which encodes for TetR itself, and of the gene *tetA* (dependent on *tetO1* and *tetO2*), which encodes for the resistance protein TetA (Schemes 2 and 3A). If Tc diffuses into a resistant bacterial cell carrying the genes *tetR* and *tetA* a chelate $[MgTc]^+$ is formed with Mg^{2+}. This chelate binds to $[(TetR)_2 \cdot tetO]$ ($K_{ass} \sim 10^9\,M^{-1}$)[20] and enforces conformational changes in $(TetR)_2$ that weaken its affinity to *tetO* in two steps, depending on conditions: 10^2–10^3-fold for the first and 10^4–10^7-fold for the second bound $[MgTc]^+$ so that the overall affinity of $(TetR)_2$ to *tetO* is reduced by 6–10 orders of magnitude.[19] The "induced" complex $(TetR \cdot [MgTc]^+)_2$ formed between $(TetR)_2$ and two inducers $[MgTc]^+$ releases *tetO*, so that expression of the genes *tetR* and *tetA* can now proceed. The antiporter protein TetA is inserted into the cytoplasmic membrane and exports $[MgTc]^+$, thereby preventing attack of $[MgTc]^+$ at the ribosomal 30S subunit, which has a 1000-fold lower affinity to $[MgTc]^+$ than $(TetR)_2$.

1.2. The Tet Repressor is a Unique Biological Switch

The high affinity of $(TetR)_2$ to the DNA operators *tetO1* and *tetO2* is sharply reduced upon the binding of inducer $[MgTc]^+$ to $(TetR)_2$. This well-defined switching between the two states relevant for regulation of gene expression is outstanding for inducible prokaryotic repressors. This is why



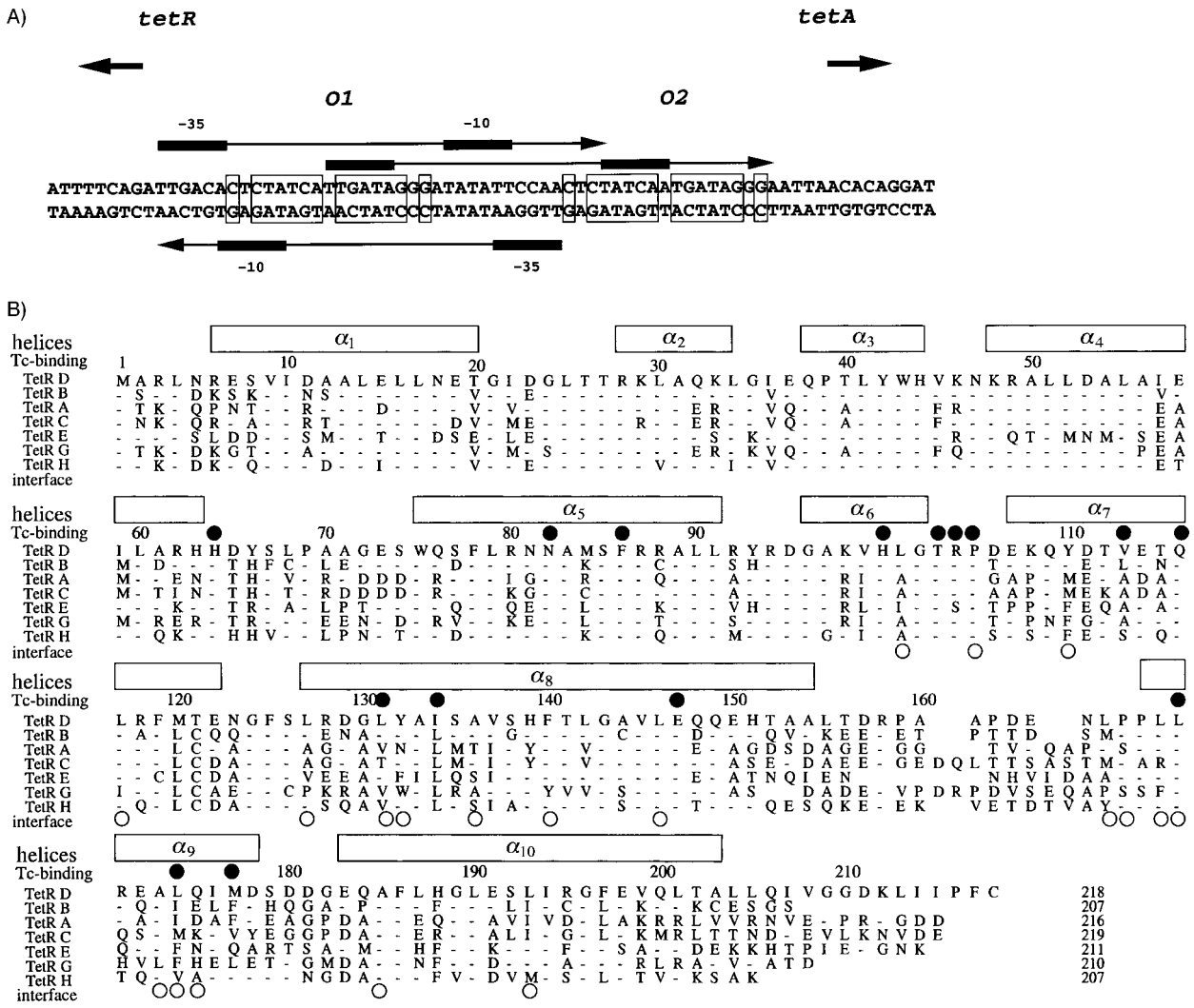
Scheme 2. Schematic description of tetracycline (Tc) resistance. Tet repressor (TetR, depicted as gray spheres) binds as a homodimer to operators *tetO1* and *tetO2*. Tc (open triangle) diffuses into the (bacterial) cell and forms a complex with a divalent cation (Mg^{2+}) to produce $[MgTc]^+$ (black triangles). If $[MgTc]^+$ binds to $[(TetR)_2 \cdot tetO]$, it enforces a conformational change and the "induced" $(TetR \cdot [MgTc]^+)_2$ complexes release the operators so that transcription of genes *tetR* (coding for TetR) and *tetA* (coding for the resistance protein TetA) can now proceed (wavy lines represent mRNA). After translation, TetA is inserted into the cytoplasmatic membrane and exports invaded $[MgTc]^+$ out of the cell so that it cannot attack the ribosome. The export of $[MgTc]^+$ is coupled with the influx of H^+.

$(TetR)_2/[MTc]^+$ is utilized by molecular biologists as an easily controllable switch for the regulation of gene expression in transgenic organisms.[21–23] X-ray crystal structures of $(TetR)_2$ and of its complexes with several chemically modified $[MgTc]^+$ and with oligonucleotide duplexes carrying the operator DNA (*tetO1*) sequence have been determined to elucidate the molecular mechanism of TetR regulation at the atomic level.[24–28] These results form the basis of the following discussion.

2. Biochemical Background: The Tc and TetR Families and the DNA Operators *tetO*

Of the four six-membered rings A–D in the Tc skeleton, only D is aromatic (see Scheme 1). On the "south" side of the Tc molecule there are five hydroxyl/keto groups in a suitable arrangement to coordinate metal ions.[27] The 1,3-keto-enol groups O11/O12 are deprotonated at physiological pH conditions, and have a high affinity for divalent cations. The binding of Mg^{2+} in aqueous solution ($K \approx 10^3\,M^{-1}$) suggests that the complex carrying one net positive charge, namely $[MgTc]^+$, is involved in all relevant biological processes.[29]

At present, seven highly homologous resistance determinants of the TetR-type are known. They are grouped into the classes A to E, G, and H (Scheme 3B) and share 45% to 75% amino acid sequence identity. As expected, the highest identity/homology is found in positions known to be engaged in binding to the inducer $[MgTc]^+$ and to the DNA operator. The high sequence identity of amino acids suggests that the seven TetR share the same overall fold with ten α-helices and no β-strands, as deduced from crystallographic studies of TetR



Scheme 3. A) Nucleotide sequences of operators *tetO1* and *tetO2* for the class $TetR^D$ taken from the resistance factor RA1. The boxed operator sequences are palindromic, with one extra base pair A-T or T-A inserted at the position of the twofold symmetry axis. The promoter of *tetA* is indicated above the sequence, that of *tetR* below; the binding regions for RNA polymerase (TATA- and GC-rich boxes) are indicated by thick lines numbered −10 and −35, respectively. The directions for transcription are marked by thick arrowheads. B) Alignment of the seven amino acid sequences of TetR classes A–E, G, and H. Hyphens (–) indicate amino acids identical to those in the top line ($TetR^D$), every tenth amino acid is numbered. The α-helices $\alpha_1-\alpha_{10}$ are indicated on top of the sequences. Amino acids marked ● in the line "Tc binding" are in contact with the inducer $[MgTc^+]$ in the complex $(TetR \cdot [MgTc]^+)_2$; these amino acids are conserved or type-conserved. Open circles (○) at the bottom line ("interface") show amino acids involved in monomer–monomer contacts in $(TetR^D)_2$. The helix-turn-helix (HTH) motif binding to operator DNA *(tetO)* is formed by α_2 and α_3.

classes B and D, $TetR^B$ and $TetR^D$, respectively.[24–28] There is only one segment, between helices α_8 and α_9, where deletions/insertions are observed and the C-terminus varies; the overall length of the repressor varies from 207 to 218 amino acids. A number of random and site-directed point mutations produced variants of TetR that have contributed to our understanding of the function of these proteins, and these will be discussed in Section 5.

The two DNA operators *tetO1* and *tetO2* are palindromic with a central base-pair inserted on their symmetry axes (see Scheme 3 A). The associated genes *tetR* and *tetA* are arranged in opposite polarity and overlapped by promoters for *tetA* and *tetR* transcription. The distance between the symmetry axes of *tetO1* and *tetO2* depends on the regulon in the different transposons and R factors and varies between 27 and 36 base pairs. As shown by footprint analyses, the recognition helices α_3 and $\alpha_{3'}$ of the homodimeric $(TetR)_2$ insert into two adjacent major grooves on *tetO1* and *tetO2*.[31, 32]

3. Crystallographic Studies Correlate the Structure and Function of TetR

3.1. Architecture of the $(TetR^D)_2$ Homodimer

The X-ray crystal structure analyses of the $(TetR^D)_2$ homodimer in a complex with operator DNA and bound in the induced form as a complex with two molecules of $[MgTc]^+$ have shown that the polypeptide chains do not consist of the 218 amino acids corresponding to the nucleotide sequence of the $tetR^D$ gene, but that the N-terminal Met1 group and the ten amino acids of the C-terminus are missing (confirmed by

N- and C-terminal sequencing and matrix-assisted laser-desorption/ionization (MALDI) mass spectrometry). These two deletions occur either posttranslationally (Met1) or during preparation (ten C-terminal amino acids) and have no effect on the biological activity of $TetR^D$.

The two monomers of the $(TetR^D)_2$ homodimer are identical, each folded into ten α-helices: $\alpha_1-\alpha_{10}$ for one and $\alpha_{1'}-\alpha_{10'}$ for the other monomer (Figure 1). The monomers are related by a twofold rotation axis coinciding with the long dimension of the $(TetR^D)_2$ dimer. The $(TetR^D)_2$ molecule is clearly divided into two N-terminal DNA-binding domains and a regulatory core domain with globular shape.

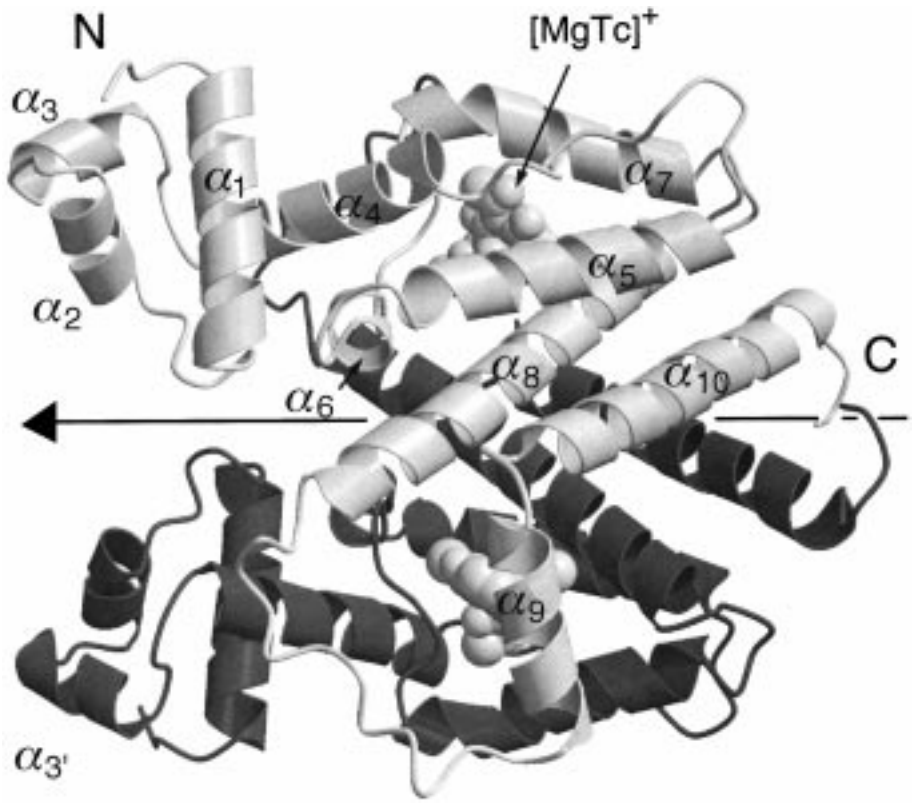


Figure 1. Schematic representation of the $(TetR^D)_2$ homodimer binding two inducers $[MgTc]^+$ to form the induced complex $(TetR\cdot[MgTc]^+)_2$. The two monomers of TetR, one depicted in light gray and the other in dark gray, are related by a twofold rotation axis (horizontal arrow). The α-helices are represented by spirals and labeled α_1 to α_{10}. The termini of the polypeptide chain in light gray are marked N and C. The two $[MgTc]^+$ are shown in a space-filling representation. The DNA binding domains $\alpha_1-\alpha_3$ and $\alpha_{1'}-\alpha_{3'}$ are located on the left. They are of the common helix-turn-helix (HTH) type, with α_3 and $\alpha_{3'}$ the DNA recognition helices; their midpoints are separated by 39.6 Å and only after removal of $[MgTc]^+$ are they able to approach by about 3 Å so that they can insert into adjacent major grooves of the operator DNA, *tetO* (see Figure 2A). The large regulatory core domain of $(TetR)_2$ is located on the right, formed by $\alpha_5-\alpha_{10}$ and $\alpha_{5'}-\alpha_{10'}$, and harbors the two $[MgTc]^+$ binding tunnels. The core domain is connected to the DNA-binding domains through helices α_4 and $\alpha_{4'}$.

The DNA-binding domains consist of the N-terminal three-helix-bundles ($\alpha_1/\alpha_2/\alpha_3$ and $\alpha_{1'}/\alpha_{2'}/\alpha_{3'}$) of which α_2 and $\alpha_{2'}$ are the supporting helices and α_3 and $\alpha_{3'}$ are the recognition helices of classical helix-turn-helix (HTH) motifs.[33] The center-to-center separation of the recognition helices α_3 and $\alpha_{3'}$, which determines the ability of $(TetR^D)_2$ to bind to *tetO*, is controlled by helices α_4 and $\alpha_{4'}$ (amino acid residues 48–63); they link the DNA-binding domains to the $[MgTc]^+$ binding sites in the regulatory core domain (see Sections 3.3.3 and 4).

The central part of the regulatory core domain is a four-helix-bundle consisting of the antiparallel helices α_8 and α_{10}, which cross the dyad related helices $\alpha_{8'}$ and $\alpha_{10'}$ at an angle of approximately 50°.[25] If the three-dimensional structures of free $(TetR^D)_2$,[26] the complex $(TetR^D\cdot[MgTc]^+)_2$, and the complex $[(TetR)_2\cdot tetO]$ are superimposed, then these four α-helices as well as α_5 and $\alpha_{5'}$ are identified as the most rigid and structurally best-conserved part, which constitutes a scaffold that determines the architecture of the core domain of $(TetR^D)_2$. The core domain harbors two tunnellike $[MgTc]^+$ binding cavities formed by $\alpha_5-\alpha_8$, $\alpha_{8'}$, and $\alpha_{9'}$ (and their symmetry-related counterparts $\alpha_{5'}-\alpha_{8'}$, α_8, and α_9 for the other tunnel).[26]

In the regulatory core domain, both monomers are clearly separated in a hand-shake motif. Only α_9 wraps around the "other" monomer at the periphery of $(TetR^D)_2$ and closes the entrance of the $[MgTc]^+$ binding tunnel, like a sliding door, in the final step of the recognition and binding process. The two $[MgTc]^+$ in the induced repressor $(TetR\cdot[MgTc]^+)_2$ are completely buried in the binding tunnels of $(TetR)_2$ far away (at $\approx$33 Å) from the DNA-recognition helices α_3 and $\alpha_{3'}$.

3.2. Structure of the Complex Formed between $(TetR)_2$ and the Operator DNA

$(TetR^D)_2$ was co-crystallized with different oligonucleotide duplexes carrying the specific DNA operator sequence *tetO1*.[34] Crystal structure analyses of complexes containing *tetO1* fragments with blunt ends (13 and 15 base pairs long) or one-nucleotide overhangs (18mer) provide the following picture.[35]

The *tetO* operator in the complex with $(TetR^D)_2$ is in the B-DNA conformation and the overall twofold rotation symmetry is retained for both components (Figure 2A). At the center (base pair 0) of the palindromic *tetO* the minor groove facing $(TetR^D)_2$ is widened and the major groove on the "back side" is correspondingly compressed from 11.7 Å in canonical "straight" B-DNA[36] to 9.5–10 Å. At base pairs +2 and −2 the DNA is bent "away" from $(TetR^D)_2$ because at these positions the recognition helices α_3 and $\alpha_{3'}$ are inserted in the major grooves and widen them to about 14 Å. These distortions of the DNA double helix are compensated for along the trajectory of base pairs 3 to 6 and −3 to −6, respectively, by a bending of the DNA towards the protein so that the overall geometry of DNA appears to be "straight".

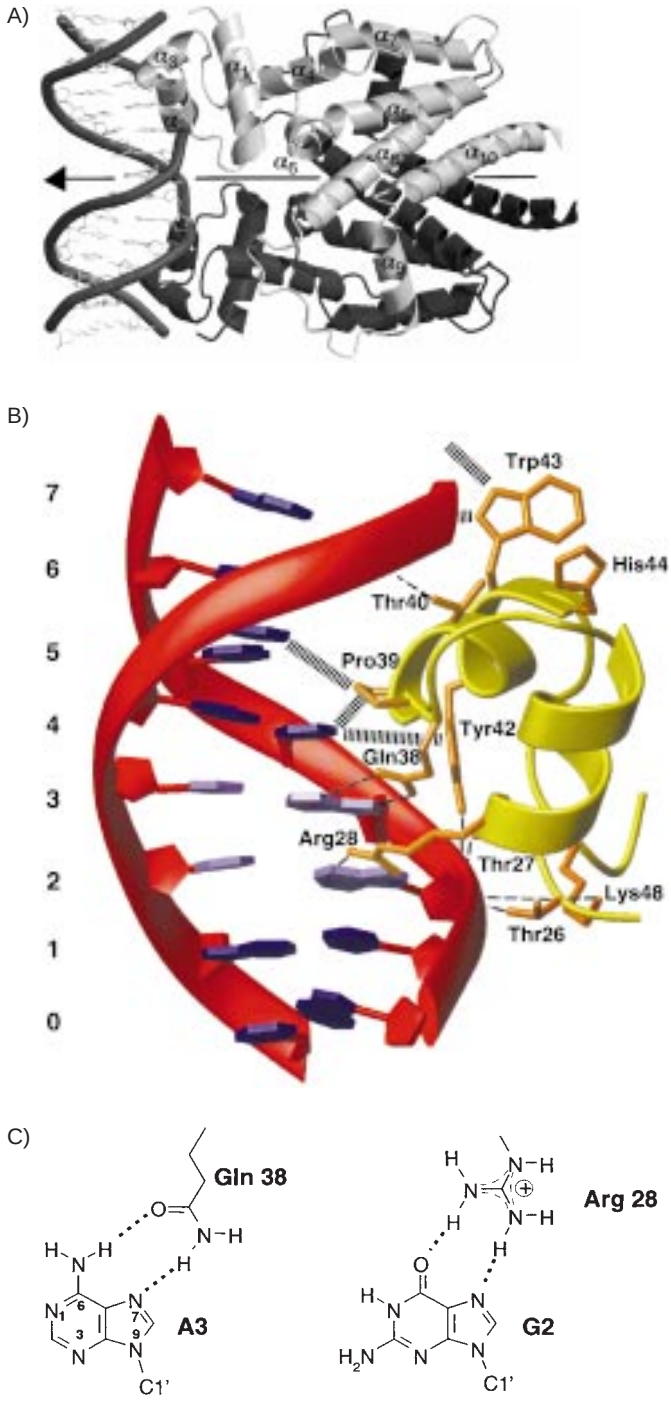


Figure 2. A) Crystal structure of the complex formed between $(TetR^D)_2$ and operator DNA *tetO1*. The α-helices of $(TetR^D)_2$ are indicated schematically by spirals (as in Figure 1) and *tetO1* by two tubes for the sugar–phosphate backbones with attached nucleosides. The complex has twofold rotation symmetry, indicated by the horizontal arrow. Note the distortion of the DNA double helix as shown by kinks in the sugar–phosphate backbone; see text. B) Detailed view of the interactions formed between one half of the operator *tetO1* and the HTH motif (α_2 and α_3). Note the specific hydrogen bonds (–––) formed between conserved Arg28 (N-terminus of α_2), Gln38 (N-terminus of α_3), and bases G2 and A3, respectively, and the van der Waals contacts (||||||) between conserved Pro39, Tyr42, Trp43, and C5-methyl groups of bases T4 and T5. T4 is on one strand of the DNA double helix, T5 on the other. C) Illustration of the hydrogen bonds (••••) in the specific interactions Arg28–G2 and Gln38–A3. Some atoms of the purine ring A3 are numbered.

Sequence-specific contacts between amino acid side chains of the $(TetR)_2$ helix-turn-helix motifs and bases in adjacent major grooves of operator DNA involve G2, A3, and T4 in one strand of the double helix and T5 in the other (Figure 2B). The contacts are contributed by bidentate hydrogen bonding between side chains of Arg28 (on α_2) and Gln38 (on α_3) interacting with bases G2 and A3, respectively (Figures 2B, C), and by van der Waals contacts between side chains of Pro39 and Tyr42 (both on α_3) and the C5-methyl groups of bases T4 and T5. The affinity of *tetO* to $(TetR)_2$ is greatly reduced if these partners are exchanged for others, as shown by a series of genetic experiments.[29] These sequence-specific interactions are augmented by a number of non-specific interactions between $(TetR)_2$ and the sugar–phosphate backbone of *tetO* involving Thr26 and Thr27 on α_2, Thr40 and Trp43 on α_3, and Lys48 at the N-terminus of α_4. Since all these amino acids are conserved among the seven TetR classes (Scheme 3B), the interactions illustrated in Figure 2B are likely to be identical for the entire *tetO*/$(TetR)_2$ system.

3.3. The Induced Complex $(TetR^D \cdot [MgTc]^+)_2$

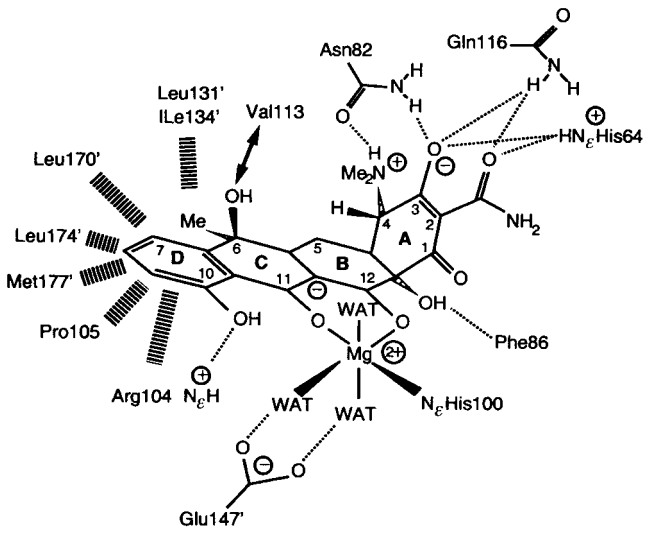
3.3.1. Binding the Inducer $[MgTc]^+$ to $(TetR^D)_2$

If Tc invades a bacterial cell that is resistant to tetracycline, it binds as $[MgTc]^+$ with high affinity ($K_{ass} \approx 10^9\,M^{-1}$) to $(TetR^D)_2$ in the complex formed between $(TetR^D)_2$ and operator DNA.[30] This binding is accompanied by conformational changes in $(TetR^D)_2$ (see Section 3.3.2), which abolish the specific interactions between $(TetR^D)_2$ and *tetO* and reduce the binding affinity to operator DNA by 6–10 orders of magnitude.[19] $(TetR \cdot [MgTc]^+)_2$ dissociates from *tetO* so that gene expression of *tetR* and *tetA* can proceed (Scheme 2).

The inducer $[MgTc]^+$ binds to the two tunnellike cavities in the regulatory core domain of $(TetR^D)_2$.[24–26] The tunnels have a narrower opening formed by α_5 and α_7 ($\alpha_{5'}$ and $\alpha_{7'}$) and a wider opening defined by the loop α_6–α_7 and by $\alpha_{9'}$ ($\alpha_{6'}$–$\alpha_{7'}$ and α_9).[26] Crystallographic studies have shown that $[MgTc]^+$ has to enter the wider opening head-on with ring A of Tc so that hydrogen bonds can form between the functional groups on ring A and the amino acids near the narrower opening of the tunnel (Scheme 4). After $[MgTc]^+$ is fully inserted, $\alpha_{9'}$ (α_9) shifts laterally, like a sliding door, and closes the "entrances" of the tunnels.[26] The complex $(TetR \cdot [MgTc]^+)_2$ is the "induced" form of $(TetR^D)_2$, which is unable to bind to operator DNA.

3.3.2. Binding of $[MgTc]^+$ Triggers a Sequence of Conformational Changes in $(TetR^D)_2$

The amino acids of $(TetR^D)_2$, which are hydrogen bonded to ring A of Tc, are in comparable positions if the crystal structures of free $(TetR^D)_2$,[26] the complex $[(TetR^D)_2 \cdot tetO]$,[35] and the induced form $(TetR^D \cdot [MgTc]^+)_2$[24–26] are superimposed. Larger structural changes are found with amino acids engaged in hydrophobic interactions, especially those located on α_9 (Leu170, Leu174, and Met177, see Scheme 4). The



Scheme 4. Schematic representation of the interactions between $(TetR)_2$ and the inducer $[MgTc]^+$. Amino acids of the two TetR molecules are distinguished by (′), hydrogen bonds are indicated by dotted lines (- - - -), and van der Waals contacts by thick dashed lines (|||||||). Mg^{2+} is octahedrally coordinated by O11 and O12 of Tc, by His 100-N_ε, and by three water molecules (WAT). Note the unusual O–H···π interaction between the OH group of Tc and the aromatic side chain of Phe86; the interaction between Val 113 and OH group at position 6 of Tc is less favorable (indicated by a double arrow).

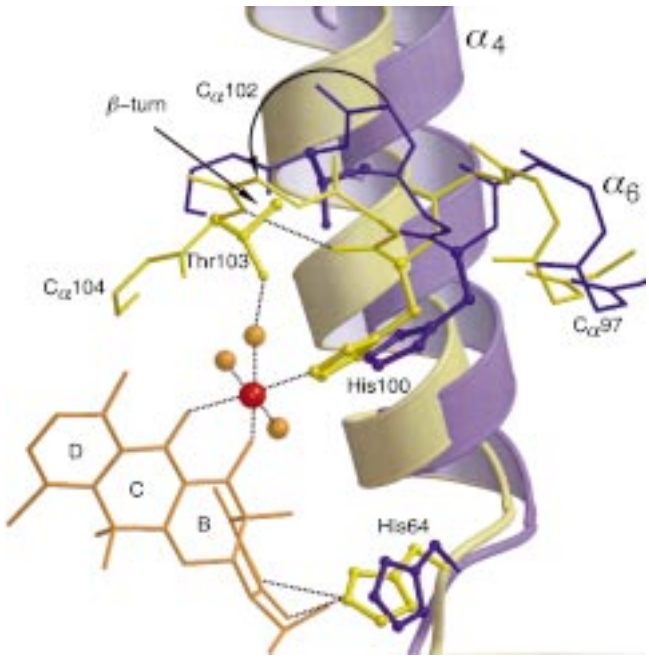


Figure 3. Comparison of the $[MgTc]^+$ binding site without the inducer (blue), showing the arrangement of some of the relevant amino acids, the two-turn-long α_6 (wire representation), and α_4 (continuous spiral) connecting the core domain of $(TetR^D)_2$ with the DNA binding domain. If $[MgTc]^+$ enters the binding site to form the induced complex, the amino acids of TetR move into positions drawn yellow. Note the modifications at the C-terminus of α_6 where an α-helical turn is peeled off to form the type II β-turn (indicated by the straight arrow) and Gly102 flips over (shown by the curved arrow); in addition, α_6 is shifted by about 1.5 Å to the left (in the direction of the C-terminus).

major conformational changes leading to induction of $(TetR^D)_2$, however, concern the coordination of Mg^{2+}.

In the absence of $[MgTc]^+$ the binding tunnel of $(TetR^D)_2$ is filled with (disordered) water molecules that cannot be located in electron density maps. Helix α_6 is only two turns long, being formed by ^{96}Gly-Ala-Lys-Val-His-Leu-Gly^{102}. In the seven classes of TetR these amino acids are conserved or type-conserved (Lys 98, Val 99, and Leu 101; see Scheme 3 B). Upon insertion of $[MgTc]^+$, α_6 is displaced by about 1.5 Å in the direction of its C-terminus, and the C-terminal turn of α_6 is peeled off to form the type II β-turn ^{100}His-Leu-Gly-Thr^{103} (Figure 3). This conformational change is triggered by direct coordination of His 100-N_ε to Mg^{2+} and simultaneous formation of a hydrogen-bonding pattern between Thr 103-O_γ, an aqua ligand of Mg^{2+}, and the peptide oxygen atom of His 100. As a structural consequence, the loop connecting α_6 and α_7 is pushed by about 2.5 Å so that the methylene side chains of Arg 104 and Pro 105 form part of the hydrophobic cavity contacting rings C and D of Tc.[26, 35] In addition, the N-terminus of α_7 is overwound so that the common 3.6_{13} α-helix is changed to a 3_{10} helix. The associated rearrangements of main-chain and side-chain atoms of the loop N-terminal to α_7 are required for the cooperative binding of the chain of water molecules described below.

3.3.3. A Cooperative Zipper of Eight Water Molecules Tightens α_4 in the Induced Position

The conformational changes that occur after induction of $TetR^D$ by $[MgTc]^+$ and dissociate the complex $[(TetR^D)_2 \cdot tetO]$ have also been verified by immunological and genetic studies.[37, 38] Crystallographic analyses show that they are stabilized by two zigzag chains of eight water molecules, one in each of the two monomers in the $(TetR \cdot [MgTc]^+)_2$ complex (Figure 4).[28] The water molecules are linked by cooperative hydrogen bonds and tether α_4, $\alpha_{4'}$, and the DNA binding domains in a position that abolishes or prevents the specific interactions with operator DNA described in Figure 2 A. The zigzag chain of water molecules is hydrogen bonded on one side to the peptide groups of Arg104 in the loop connecting α_6 and α_7, as well as to the side chain of Gln109 on α_7 and the amide substituent at position 2 of Tc and, on the other side to the peptide oxygen atoms of groups 53, 56, 57, 60, and 64 of α_4. These functional groups are thus stitched like a zipper. Compared with the $[(TetR^D)_2 \cdot tetO]$ complex, where in the absence of $[MgTc]^+$ the functional groups of α_4 and the loop connecting α_6 and α_7 are in different positions, α_4 is now rotated approximately 5° about His 64[35] in a pendulumlike movement (see Figure 3). In this rotation, His64 acts as a C-terminal pivot, which is anchored tightly by $[MgTc]^+$ to the side chains of conserved amino acids Asn 82 and Ser 85, both located on the scaffold α_5.

4. Regulation of $(TetR^D)_2$ is Associated with an Induction-Triggered Rotation of DNA Binding Domains

Superposition of the three-dimensional structures of $(TetR^D)_2$ in the complexes with operator DNA *tetO* and with the inducer $[MgTc]^+$ reveals the essential features of $(TetR^D)_2$

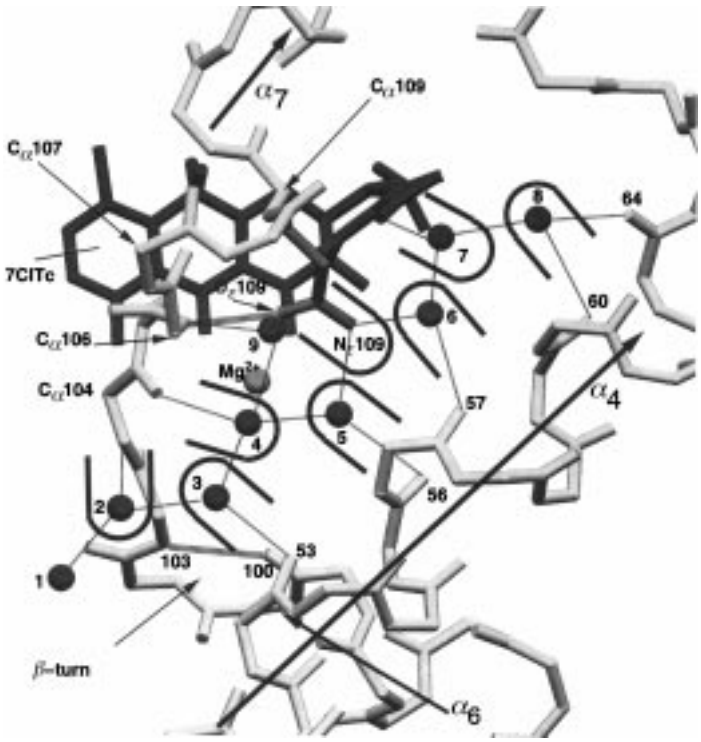
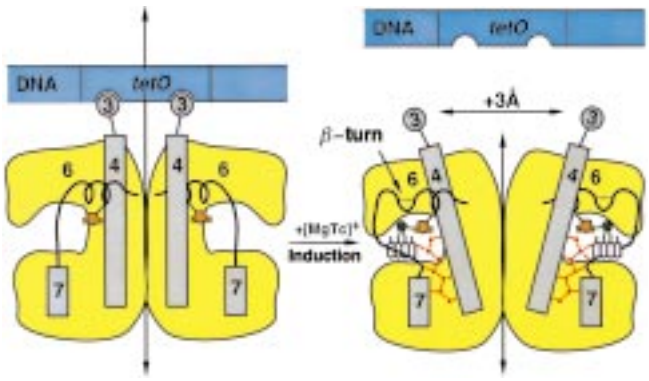


Figure 4. The orientation of α_4 in the induced complex $(TetR^D \cdot [MgTc]^+)_2$ is stabilized by a chain of eight cooperatively hydrogen-bonded water molecules (zipper) labeled 1–8 and marked by U shapes. The chain of water molecules is hydrogen bonded on one side to main-chain atoms of the loop segment connecting α_6 and α_7 (Arg104NH $\cdots$ Wat2 and Arg104O $\cdots$ Wat4, Gln109N$_{\varepsilon}$ $\cdots$ Wat5/Wat6, and at position 2 of $[MgTc]^+$-amide-NH_2 $\cdots$ Wat7) and on the other side by hydrogen bonds to the peptide oxygen atoms of groups of 53, 56, 57, 60, and 64 of α_4.



Scheme 5. The structure–function relationship of $(TetR^D)_2$ (yellow) in the complex with operator DNA *tetO* (left) and with inducer $[MgTc]^+$ (right). $(TetR^D)_2$ is drawn schematically with helices α_3, α_4, and α_7 as cylinders and α_6 as a spiral, His100 is outlined in orange, Tc is indicated by four fused hexagons (right), Mg^{2+} by a green sphere, and the zipper formed by hydrogen-bonded water molecules in red. In $[(TetR^D)_2 \cdot tetO]$ (left), α_3 and $\alpha_{3'}$ of $(TetR^D)_2$ are bound to adjacent major grooves of *tetO*; α_6 is two turns long, α_4 and $\alpha_{4'}$ lie vertical. If two $[MgTc]^+$ enter the binding tunnels in $(TetR^D)_2$ to form $(TetR^D \cdot [MgTc]^+)_2$ (right), His100-N$_{\varepsilon}$ coordinates to Mg^{2+}, the C-terminal turns of α_6 and $\alpha_{6'}$ peel off to form β-turns. These changes trigger pendulumlike movements of α_4 and $\alpha_{4'}$, which are stabilized in their final positions by the water zippers. These movements drive α-helices α_3 and $\alpha_{3'}$ approximately 3 Å apart so that *tetO* is released and gene expression may occur. Both complexes $[(TetR^D)_2 \cdot tetO]$ and $(TetR^D \cdot [MgTc]^+)_2$ show twofold rotational symmetry indicated by the vertical arrows located between $TetR^D$ monomers.

regulation as shown in Scheme 5. The regulatory core domains of these structures are nearly identical except for helices α_6, $\alpha_{6'}$, α_9, and $\alpha_{9'}$. The latter move and close the binding tunnels like sliding doors after insertion of $[MgTc]^+$, whereas the C-terminal helical turns of α_6 and $\alpha_{6'}$ are peeled off during induction to permit coordination to the Mg^{2+} ion of the two bound inducers $[MgTc]^+$. This process triggers rotation of helices α_4 and $\alpha_{4'}$ in the pendulumlike movement that shifts the attached DNA binding domains in opposite directions, following the dictate of the inherent twofold rotation symmetry of the $(TetR^D)_2$ homodimer.

Helices α_4 and $\alpha_{4'}$ are tightly fixed in their positions in the induced complex $(TetR^D \cdot [MgTc]^+)_2$ by two cooperative chains of eight water molecules each. We assume that the latter provides the force to rotate α_4 and $\alpha_{4'}$ as well as the attached DNA-binding domains so that the distance between the midpoints of the DNA recognition helices α_3 and $\alpha_{3'}$ increases from 36.6 Å in the complex $[(TetR^D)_2 \cdot tetO]$ to 39.6 Å in the induced complex $(TetR^D \cdot [MgTc])_2$. Since this is too far to permit binding of α_3 and $\alpha_{3'}$ into adjacent major grooves of B-DNA, *tetO* is released and expression of genes *tetR* and *tetA* can proceed.

5. Point Mutations Support and Extend Structural Studies on $TetR^B$

A set of about 100 different point mutations of $tetR^B$ was used to investigate the in vivo binding to operator *tetO1* and induction by $[MgTc]^+$.[39, 40] The screening system consisted of *tetO1* inserted in the promoter region of the *lacZ* gene, which codes for β-galactosidase, so that binding/release of the $(TetR^B)_2$ variants could be easily monitored by the activity of the expressed β-galactosidase. The amino acid substitutions detected were almost evenly distributed over the entire polypeptide chain of $TetR^B$ except for the DNA-binding domain formed by α_1, α_2, and α_3. This observation suggests that association of $(TetR^B)_2$ with *tetO1* critically responds to any substitution of the amino acids in the DNA binding domain. Several other modifications of the polypeptide chain were accompanied by severe interference in the recognition and binding of $[MgTc]^+$. The variants of $TetR^B$ showed that noninducible phenotypes may be associated with a) reduced affinity of the inducer $[MgTc]^+$ as a result of the amino acid substitutions in $TetR^B$ and b) interference with the conformational changes required for induction.

The binding of $[MgTc]^+$ to $(TetR^B)_2$ is reduced by several orders of magnitude if amino acids His64, Asn82, Phe86, His100, and Gln116, which are engaged in hydrogen bonding to $[MgTc]^+$ (see Scheme 4), are substituted by other amino acids, and consequently these variants are no longer inducible but they still bind to *tetO1*. By contrast, substitution of amino acids engaged in van der Waals contacts to $[MgTc]^+$, such as Arg104, Pro105, Leu113, and Leu117, only marginally reduces the affinity to $[MgTc]^+$, but may largely or even completely abolish induction, which suggests that the conformational changes associated with successful induction are inhibited or even blocked.

Another set of mutations was directed against a few amino acids, such as Asp23, Asp95, Lys98, and Glu150, which connect the regulatory core domain and the DNA-binding

domains through formation of hydrogen bonds. Substitution of these amino acids reduces, but does not abolish, inducibility and has no effect on the affinity of $(TetR^B)_2$ for $[MgTc]^+$. In a comparable approach to probe the monomer–monomer interface in $(TetR^B)_2$, substitution of the amino acids located on the four-helix bundle $\alpha_8/\alpha_{10}/\alpha_{8'}/\alpha_{10'}$ and directed towards the "other" monomer were investigated further. These variants show similar affinities to $[MgTc]^+$ as wild-type $(TetR^B)_2$ but still bind to *tetO1*, that is, the inducibility is largely abolished; the extent is dependent on the steric bulk of the side chains of the substituted amino acids.

These and other[38] mutational studies on $TetR^B$ not only give credit to the biological significance of the crystallographic work, but they extend considerably our understanding of the processes associated with induction by $[MgTc]^+$.

6. The Use of TetR as a Regulatable Switch in Eukaryotic Gene Expression

Transcription of DNA into complementary mRNA is controlled by promoters to which RNA polymerase binds in the presence of a defined number of transcription factors. Since "strong" promoters slow down bacterial growth, a switch was introduced, as in the common *tac* promoter, to initiate transcription at a point suitable to produce proteins in large quantities. This promoter consists of an optimized DNA sequence for enhanced binding of RNA polymerase and an operator DNA that is specific for the Lac repressor.[41] Coexpression of the Lac repressor in a bacterium carrying the *tac* promoter switches off the expression of the gene located downstream of this promoter as it binds the Lac repressor, thereby preventing transcription. If the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) is added, the Lac promoter is released from its operator and gene expression is switched on.

This system works well in prokaryotes, but eukaryotes require concentrations of IPTG several hundred times higher,[42] which interferes with the growth of animal cells. These adverse effects can be avoided with TetR since the affinity for its inducer $[MgTc]^+$ is 1000-fold higher than that of IPTG for the Lac repressor,[21, 22] and the concentration of $[MgTc]^+$ required for induction is so low that it is not toxic to the cell. Another advantage of Tc is that it can pass through the eukaryotic cell membrane without requiring transport molecules.

It proved possible to employ the TetR system for switching on *and* for switching off gene expression when $[MgTc]^+$ was added.[22] In *switching on*, *tetO* was inserted in a promoter close to the TATA box (see Scheme 3A) of a target gene so that binding of $(TetR)_2$ blocks transcription until it is switched on by the addition of $[MgTc]^+$. In *switching off*, a fusion protein formed between TetR and an eukaryotic activator domain is bound in the absence of $[MgTc]^+$ to *tetO* located upstream of the TATA box of a target gene so that transcription is activated. If $[MgTc]^+$ is added, this fusion protein dissociates from *tetO* and transcription is switched off because the activator is now removed.

Another interesting use of TetR in gene regulation has been opened by the "reverse TetR", which *requires* Mg^{2+} complexes of the Tc analogues anhydrotetracycline or doxycycline (in which the OH group at C6 is missing) for optimal, tight binding to *tetO*.[43] In the "reverse TetR", four amino acids are substituted, of which two, in positions 95 and 102, are conserved in the TetR family (see Scheme 3B): Glu 71 Lys, Asp 95 Asn, Leu 101 Ser, and Gly 102 Asp. Since no crystal structures of "reverse TetR" have been determined so far, the molecular mechanisms underlying its phenotype are unknown. From the available structural information on TetR and its complexes with *tetO* and $[MgTc]^+$, however, it is clear that the Gly 102 Asp substitution is of main importance. As shown in Section 3.3.2, the induction of TetR is associated with the formation of the type II β-turn ^{100}His-Leu-Gly-Thr^{103}. For steric reasons this β-turn can only form with Gly 102, and not with Asp 102, so that the "reverse TetR" is not inducible. In addition, the Asp 95 Asn substitution supports this effect since a hydrogen bond between Asn 95 and Glu 23 may stabilize the relative orientations between α_1 (Glu 23) and the regulatory domain (Asn 95) so that induction is less favorable. The reason why the "reverse TetR" binds even tighter to *tetO* in the presence of anhydrotetracycline and doxycycline than in the absence of these Tc analogues remains elusive.

A fusion protein constructed of the "reverse TetR" and a transcriptional activator was constitutively expressed in HeLa cells. In the presence of anhydrotetracycline and doxycycline it bound tightly to *tetO* inserted upstream of a target gene, which was expressed over 1000-fold more than in the absence of this construct.[43] This experiment has shown that "reverse TetR" and TetR will be suited for the regulation of gene expression even in transgenic animals and possibly in gene therapy.

A combination of genes for TetR and "reverse TetR" in the same cell line leads to the possibility of regulating two genes simultaneously;[44] additional specialized constructs are being designed to keep the basal repression level of target genes at a minimum.[45] A very valuable feature of the TetR system is that it shows practically no leaking, that is, the switching function is very precise. For this reason, TetR is frequently employed in the regulation of gene expression in transgenic organisms. At present, it is one of the best understood repressor–operator systems and serves as a solid basis for further studies probing into structure–function relationships.

7. Summary and Outlook

A series of crystallographic studies on the system $(TetR^D)_2$/*tetO*/$[MgTc]^+$ have disclosed how the binding of two inducer molecules $[MgTc]^+$ to the $(TetR)_2$/*tetO* complex leads to the release of *tetO* so that transcription of the genes *tetR* and *tetA* can proceed. The key to the events, which reduce the high affinity of the $(TetR^D)_2$/*tetO* complex ($K_{ass} \approx 10^{11}\,M^{-1}$) by about ten orders of magnitude, is the insertion of $[MgTc]^+$ into the two specific binding tunnels in $(TetR^D)_2$. This process is followed by direct coordination of Mg^{2+} to the His 100-Nε atom, which triggers a number of conformational changes in $(TetR^D)_2$ and culminates in a pendulumlike rotation of helices

α_4 and $\alpha_{4'}$ to which the DNA binding domains are attached; they move about 3 Å apart, thereby releasing *tetO*. The force that stabilizes this particular conformation is provided in part by the formation of two cooperative chains of eight water molecules each (zipper), which form 2×17 hydrogen bonds. This would certainly provide sufficient energy to drive the conformational changes of $(TetR \cdot [MgTc]^+)_2$ that causes the release of *tetO*, but the number of hydrogen bonds has to be taken with caveat as these are not the only hydrogen bonds that are formed or modified during the induction of $(TetR^D)_2$.

The $(TetR)_2$/*tetO*/$[MgTc]^+$ system is unique because the tight, leakage-free repression of the *tetA* and *tetR* genes is abandoned even at low, subtoxic concentrations of $[MgTc]^+$. This favorable property has been utilized in transgenic organisms to regulate the expression of certain target genes. If fusion proteins between TetR and transcriptional activators are employed, $(TetR)_2$ can also be used to switch gene expression on and to switch it off if $[MgTc]^+$ is added. Another variation on this theme is the "reverse TetR", which binds to *tetO* only in the presence of $[MgTc]^+$. With TetR, a versatile tool has been found that has already been applied successfully for gene regulation in a number of transgenic plants and laboratory animals.[46] It has even opened future avenues in gene therapy since Tc is nontoxic to the mammalian cell and extensive documentation exists concerning its safety in its pharmacological application to humans.

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