Structure-Based Design of Tet Repressor To Optimize a New Inducer Specificity†

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ABSTRACT: We constructed a mutant of the tetracycline-inducible repressor protein TetR with specificity for the tc analogue 4-de(dimethylamino)anhydrotetracycline (4-ddma-atc), which is neither an antibiotic nor an inducer for the wild-type protein. The previously published relaxed specificity mutant TetR H64K S135L displays reduced induction by tc but full induction by doxycycline (dox), anhydrotetracycline (atc), and 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline (cmt3). To create induction specificity for tc derivatives lacking the 4-dimethylamino grouping such as cmt3 and 4-ddma-atc, the residues at positions 82 and 138, which are located close to that moiety in the crystal structure of the TetR−[tc-Mg]$^{+}_{2}$ complex, were randomized. We anticipated that a residue with increased size may lead to sterical hindrance, and screening for 4-ddma-atc-specific induction indeed revealed the mutant TetR H64K S135L S138I. Out of 24 exchanges only the addition of S138I to TetR H64K S135L yielded a mutant with a pronounced reduction of affinity for atc and dox, while the one for 4-ddma-atc is not affected. The ratio of binding constants revealed a 200-fold specificity increase for 4-ddma-atc over atc. The contributions of each single mutant to specificity indicate that the tc variants bind slightly different positions in the TetR tc binding pocket.

The transcriptional regulator protein Tet repressor (TetR)[1] combines high affinity for its cognate DNA sequence (*tetO*) with sensitive induction by tetracycline (tc) and especially the more potent analogues doxycycline (dox) and anhydrotetracycline (atc) (*1*). Since, in addition, inducers can penetrate most cells by passive diffusion, there is widespread use of TetR-based gene regulation in pro- and eukaryotes (*2*).

The crystal structures of the [tc-Mg]$^{+}$-bound, the operator-bound and free TetR have been solved (*3*, *4*). TetR is an all α-helical protein with 10 α-helices composing a monomeric unit, and two subunits forming the homodimer. It contains two N-terminal DNA binding domains built of helices α1 to α3 of each subunit. Helix α4 connects the N-terminal domain with the C-terminal domain consisting of helices α5 to α10 of both subunits. The inducer binding region is located inside the C-terminal domain where each chemical function of tc is contacted by the protein (see Figure 1), leading to the nanomolar affinity of the drug for TetR. Despite this intimate interaction, TetR shows an unexpected plasticity for induction by tc variants (*5*). In vitro *tetO* binding data even revealed induction in the absence of Mg^{2+}, although the ion seems to play a crucial role in the structure-based induction mechanism (*6*). Furthermore, the inducer specificity of TetR has been changed from tc to cmt3, a sancycline derivative lacking the 4-dma grouping (see Figure 2 for chemical structures) in the TetR H64K S135L mutant. However, TetR H64K S135L responds also to the high-affinity inducers atc and dox (*7*), classifying it as a relaxed specificity mutant. To create a truly new specificity, we made use of 4-de-(dimethylamino)anhydrotetracycline (4-ddma-atc; see Figure 2 for the structure) with the goal to reduce atc and dox recognition by additional mutations in TetR H64K S135L. We describe the randomization of residues at positions 82 and 138 of that mutant since they are close to the 4-dimethylamino grouping in the TetR−[tc-Mg]$^{+}_{2}$ crystal structure (*3*) (see Figure 3). We anticipated that residues with larger side chains may lead to sterical hindrance with the 4-dma grouping, thereby reducing the affinity for dox and atc while that of 4-ddma atc should not be affected.

EXPERIMENTAL PROCEDURES

Materials and General Methods. Tc was from Merck (Darmstadt, Germany), atc from Acros (Geel, Belgium), and dox from Sigma (Munich, Germany), and 4-ddma-atc was synthesized by Prof. Gmeiner (Pharmazeutische Chemie, Friedrich-Alexander Universität Erlangen-Nürnberg). All other chemicals were from Merck, Roth (Karlsruhe, Germany), or Sigma. Enzymes for DNA restriction and modification were from New England Biolabs (Frankfurt/Main,

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[1] Abbreviations: TetR, Tet repressor; tc, tetracycline; atc, anhydrotetracycline; dox, doxycycline; 4-ddma-atc, 4-de(dimethylamino) anhydrotetracycline; β-gal, β-galactosidase; cmt3, 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline.

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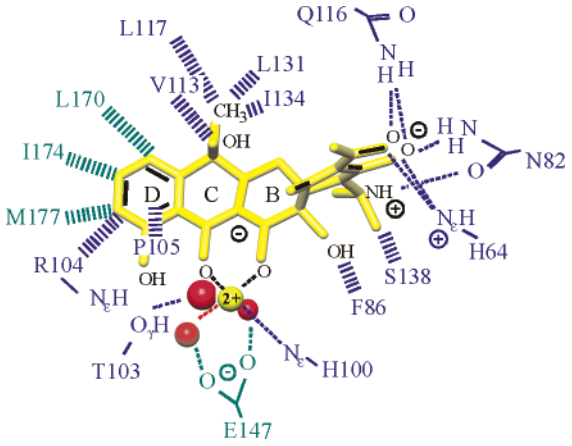

FIGURE 1: Overview of all tc contacting residues in TetR. Tc is shown as a yellow stick model, Mg^{2+} is shown as a yellow ball, and the three coordinating water molecules are shown as red balls. The TetR residues from one monomer are colored blue, and those from the other monomer are green. Hydrophobic interactions are symbolized by dashed lines, H-bonds by broken lines.

Germany), Roche (Mannheim, Germany), or Pharmacia (Freiburg, Germany). Isolation and manipulation was performed as described (*8*).

Chemical Synthesis of 4-De(dimethylamino)anhydrotetracycline. Starting from tetracycline, 4-de(dimethylamino)-anhydrotetracycline (4-ddma-atc) was synthesized by *N*-methylation, reductive removal of trimethylamine, and dehydration (see refs *9* and *10*) when concentrated aqueous hydrochloric acid was used instead of hydrobromic acid. Analytical data: $C_{20}H_{17}NO_7$ (383.4). EIMS: $m/z = 383$ (M^+). Mp: 257 °C. Lit. (*11*) mp: 256−258 °C. IR (KBr): $\nu = 3460, 3385, 3345, 3210, 1635, 1570, 1560, 1375, 1230, 670\ cm^{-1}$. ^1H NMR (360 MHz, pyridine-d_5): $\delta = 2.35$ (s, 3 H, CH_3), 2.82 (dd, $J = 18.2, 6.2$ Hz, 1 H, H-4α), 3.06 (dd, $J = 15.9, 7.8$ Hz, 1 H, H-5α), 3.08−3.15 (m, 1 H, H-4a), 3.38 (br d, $J = 18.2$ Hz, 1 H, H-4β), 3.50 (dd, $J = 15.9, 3.0$ Hz, 1 H, H-5β), 7.08 (d, $J = 7.8$ Hz, 1 H, H-9), 7.39 (d, $J = 8.2$ Hz, 1 H, H-7), 7.59 (dd, $J = 8.2, 7.8$ Hz, 1 H, H-8), 9.82 (s, 1 H, NH), 10.15 (s, 1 H, OH-10). ^{13}C NMR (90 MHz, pyridine-d_5): $\delta = 14.32$ (CH_3), 29.4 (C-5), 36.3 (C-4), 37.1 (C-4a), 78.5 (C-12a), 100.5 (C-2), 109.5 (C-11a), 111.5 (C-9), 113.1 (C-7), 115.4 (C-10a), 122.5 (C-6), 131.9 (C-5a), 133.1 (C-8), 139.8 (C-6a), 159.3 (C-10), 165.0 (C-11), 174.7 ($CONH_2$), 193.4 (C-1), 196.3 (C-3), 201.9 (C-12).

Construction of the Plasmid Pools. *E. coli* DH5α was used for cloning. Mutations for the codons N82 and S138 were introduced as follows. Randomization was done by PCR mutagenesis with the primers N82mut (5′-tca gcg gtc (agct) (agct) (gc) gca atg agt ttc-3′) and S138mut (5′-tta gcg gtc (agct) (agct) (gc) cat ttt act tta ggt gcc-3′) according to the three-primer method (*12*) with *tetR* H64K S135L as template. The *tetR* fragments containing the randomized codons and the H64K S135L mutations were introduced in pWH1411-(BD) (*7*) and pWH1925(BD),[2] respectively, for constitutive expression. Isolation, manipulation, and sequencing were performed as described (*8*).

E. coli Screening System. The mutant pools were tested for inducibility with 0.4 μM 4-ddma-atc and repression with 0.4 μM atc or without inducer in a genetic screen. The *E. coli* strain WH207 containing pWH414 (*13*) was transformed with the pWH1411 H64K S135L S138mut mutant pool. pWH414 contains a *tetA*−*lacZ* fusion expressing β-galactosidase under *tetO* control. pWH1411 constitutively expresses the *tetR* mutants. The cells were plated on M9 minimal medium (*8*) containing 0.2% (w/v) glucose as carbon source and 0.004% (w/v) X-gal. We screened for blue colonies on plates with 0.4 μM 4-ddma-atc and for white on that without inducer and with 0.4 μM atc.

E. coli WH207/λ*tet50* (*13*, *14*) was transformed with the pWH1925 H64K S135L N82mut mutant pool. The *E. coli* strain contains a chromosomal *tetA*−*lacZ* fusion under *tetR* control. The cells were plated on MacConkey Agar Base (Becton Dickinson) containing 14 g/L lactose, 0.0042% (w/v) neutral red, and 0.0014% (w/v) crystal violet. The colonies were screened for their ability to repress β-galactosidase in the absence and the presence of 0.4 μM atc and to be induced on plates containing 0.4 μM 4-ddma-atc.

β-Galactosidase Assays. Repression and induction with different tc analogues was determined in *E. coli* WH207/λ*tet50*. Cells were grown in LB supplemented with 0.4 μM tc, atc, dox, or 4-ddma-atc at 37 °C. β-Galactosidase activities were determined as described (*15*). Three independent cultures were assayed for each mutant, and measurements were repeated at least twice.

Protein Purification. *E. coli* RB791 transformed with pWH610 derivatives (*16*) was used for expression of the TetR mutants. Purification of the TetR mutants to homogeneity was done as described (*16*). Protein concentrations were determined by UV spectroscopy and by saturating titration with atc or 4-ddma-atc, observing the change of fluorescence.

Determination of Mg^{2+}-tc Analogue Equilibrium Binding Constants. The binding constants from Mg^{2+} to the tc derivatives were determined from UV titration as described

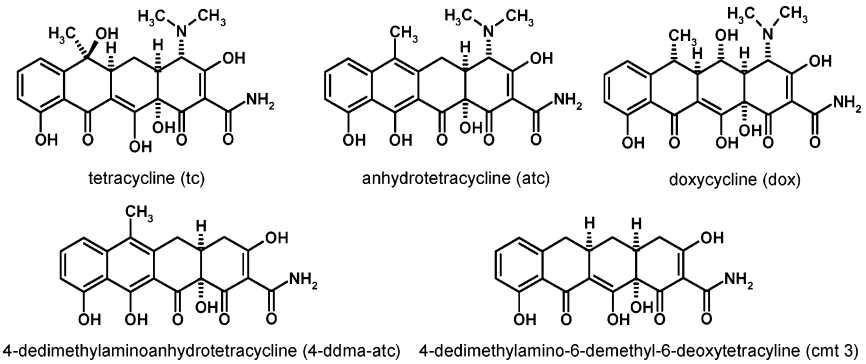

FIGURE 2: Chemical structures of tetracycline derivatives used in this study. The designations and abbreviations (in parentheses) used in this work are indicated.

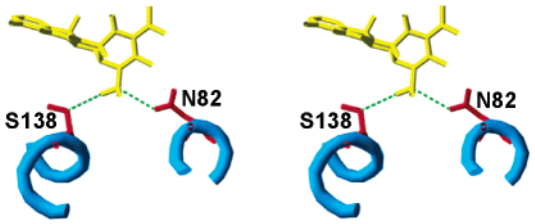

FIGURE 3: Stereoview of the N82 and S138 residues in proximity to the 4-dma moiety. Tc is shown as a yellow stick model, and the side chains of N82 and S138 are shown in red attached to the blue-colored backbones of helices α5 and α8, respectively. The distances between the nearest atoms are indicated by green broken lines. The C^{β} atom of Ser138 is 3.46 Å from the CH_3 of tc, and $O^{\delta 1}$ of Asn82 is 3.26 Å from the nitrogen of the 4-dma function.

(*6*). The following Mg^{2+} binding constants were obtained: atc, 3.4×10^3 M^{-1}; dox, 7.2×10^3 M^{-1}; 4-ddma-atc, 17.5×10^3 M^{-1}.

Fluorescence Measurements. All fluorescence measurements were performed in a Spex Fluorolog with two double monochromators. Atc fluorescence was excited at 455 nm and monitored at 545 nm. For dox, the excitation wavelength was set to 370 nm and detected at 515 nm. To observe 4-ddma-atc fluorescence, we used 420 nm excitation and 540 nm for detection.

Mg^{2+} independent binding constants were determined using a buffer containing 100 mM Tris−HCl, 100 mM NaCl, and 1 mM EDTA at pH 8. Titration and calculation of the equilibrium binding constants were done as described (*6*). TetR−$[tc\text{-}Mg]^{+}_2$ binding constants were obtained from fluorescence titration at limiting Mg^{2+} concentrations as described (*17*). We adjusted free Mg^{2+} concentrations ranging from 10^{-10} to 10^{-3} M using a buffer containing 0.1 mM EDTA as metal chelator. In all cases a 10% molar excess of TetR over the tc derivative and total concentrations of 1.1, 0.11, and 0.011 μM protein were used. Calculation was done as described previously (*6*, *17*) and repeated at least twice.

RESULTS

Effects of Mutations at Asn82 and Ser138 on 4-ddma-atc Specificity. To obtain affinity distinction between tc analogues with and without the 4-dma grouping, we looked for amino acids near that moiety in the TetR−$[tc\text{-}Mg]^{+}_2$ crystal structure (*3*, *4*) since we assumed that a size increase may lead to sterical hindrance in the interaction with tc, atc, and dox. The two most proximal residues Asn82 and Ser138 (see Figure 3) were chosen for randomization. The distance between the C^{β} atom of Ser138 and CH_3 of tc and between $O^{\delta 1}$ of Asn82 and the nitrogen of the 4-dma grouping is less than 3.5 Å.

Randomization of codons 82 and 138 was accomplished by PCR mutagenesis of *tetR* H64K S135L using oligonucleotides with the respective degenerated codons. The resulting PCR fragments were inserted into pWH1925 or pWH1411, respectively, from which they are constitutively expressed. *E. coli* WH207/*λtet50* was then transformed with the mutant pool at position 82, and *E. coli* WH207/pWH414 with that for position 138. *E. coli* WH207/*λtet50* and pWH414 provide *tet*-controllable *lacZ* expression. The transformants were screened for induction on MacConkey agar plates or on X-Gal-containing M9 minimal medium plates, respectively, each containing 0.4 μM 4-ddma-atc. Candidates that are inducible by 4-ddma-atc were rescreened for repression in the presence of 0.4 μM atc and without inducer. This approach yielded S138 substitutions by Ile and Leu as well as N82 substitutions by Val and Trp. In addition, random clones were sequenced, and 9 different residues were found at position 82 and 11 at position 138 (see Figure 4A,B). The induction specificities of these mutants were analyzed in broth cultures by determining repression of β-galactosidase encoded by a chromosomally located *tetO−lacZ* fusion in *E. coli* WH207/*λtet50* in the presence and absence of 0.4 μM 4-ddma-atc, tc, or atc. The results obtained with TetR H64K S135L with mutations at position 82 are shown in Figure 4A. All mutants exhibit a reduction of inducibility. The N82G, N82E, N82S, and N82T mutations in TetR H64K S135L show reduced induction by atc and 4-ddma-atc and no induction by tc. The amino acid exchanges N82L with a hydrophobic side chain; N82F bearing an aromatic amino acid and N82Q yield proteins with no response to all three inducers. The substitutions N82V, N82I, N82M, and N82W result in proteins with strongly reduced inducibility but improved specificity for 4-ddma-atc, being a 4−6-fold better inducer than atc for these repressors. However, maximal induction is less than 15% of that obtained with TetR H64K S135L.

The phenotypes of the S138 exchanges in TetR H64K S135L are shown in Figure 4B. Most variants show only weak effects on inducibility. In particular, S138G and S138A with small residues, S138T and S138Q with polar residues, and S138H are not or only marginally affected. Slightly reduced induction with atc and almost no change of 4-ddma-atc inducibility is seen with C, D, and V at this position. P instead of S at position 138 leads to a mostly inactive protein with almost complete loss of repression. Reduced induction is seen with the S138R variant with all tc analogues. Hydrophobic residues at position 138 cause the strongest effects on induction. The S138V variant exhibits slightly lower induction by atc. S138L and S138W lead to the expected reduced induction by atc and dox, but also by 4-ddma-atc. The only mutant with reduced induction by atc and dox (3% and 7%, respectively) and full induction with 4-ddma-atc contains I at position 138. Thus, TetR H64K S135L S138I exhibits the desired phenotype.

Contribution of the S138I Exchange to Inducer Specificity. To uncover the contributions of the S138I mutation to the phenotype of TetR H64K S135L S138I, all possible single- and double-exchange mutants were constructed and cloned into pWH1925. The steady-state protein levels of all mutants determined by Western blot analysis (data not shown) were indistinguishable. Thus, the repression and induction properties as monitored by β-galactosidase expression reflect the activities of the various mutant proteins and not their stability. The results are shown in Figure 4C. Since pWH1925 leads to slightly different repression compared to the previously used pWH1411 (*7*), the induction properties of the TetR mutants H64K, S135L, and H64K S135L for tc and dox were redetermined in addition to the ones with atc and 4-ddma-atc. TetR S138I is not induced by 4-ddma-atc, while atc and dox induction are decreased to 54% and 20%, respectively. Thus, as expected from the crystal structure, S138I reduces

[2] Scholz, O. *Mol. Microbiol.*, in press.

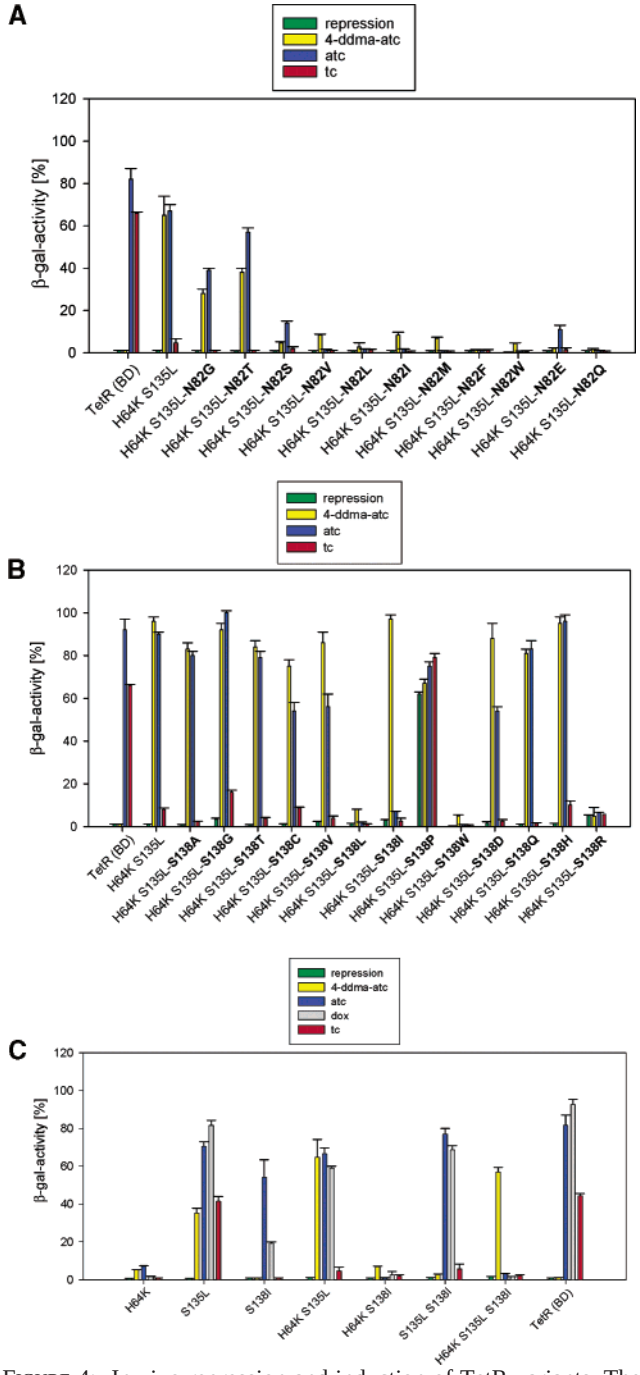

FIGURE 4: In vivo repression and induction of TetR variants. The host strain was *E. coli* WH207/λ*tet*50. β-Galactosidase activities were determined at 37 °C in units according to Miller (*15*). They are given in percent of the value determined in a strain without *tetR* (not shown). (A, top) Mutations at position N82. All *tetR* variants are on plasmids originating from pWH1925(BD). An activity of 100% corresponds to 5400 ± 100 units for repression, 5300 ± 90 units in the presence of 0.4 μM 4-ddma-atc, 5400 ± 100 units in the presence of 0.4 μM tc, and 5100 ± 200 units in the presence of 0.4 μM atc. (B, middle) Mutations at position S138. All *tetR* variants are on plasmids originating from pWH1411(BD). An activity of 100% corresponds to 6200 ± 100 units for repression, 6600 ± 200 units in the presence of 0.4 μM 4-ddma-atc, 4500 ± 500 units in the presence of 0.4 μM tc, and 6500 ± 100 units in the presence of 0.4 μM atc. (C, bottom) Single and double mutants. All *tetR* variants are on plasmids originating from pWH1925(BD). An activity of 100% corresponds to 5400 ± 100 units for repression, 5300 ± 90 units in the presence of 0.4 μM 4-ddma-atc, 5400 ± 100 units in the presence of 0.4 μM tc, 3400 ± 100 units in the presence of 0.4 μM dox, and 5100 ± 200 units in the presence of 0.4 μM atc.

Table 1: Mg^{2+}-Independent Equilibrium Binding Constants of TetR Variants[a]

TetR variant	Mg^{2+}-independent equilibrium binding constant,[b] K_T ($\times 10^7$ M^{-1})		
	4-ddma-atc	atc	dox
TetR (BD)	<0.01[c]	6.5	5.3
S135L	<0.01[c]	338	51.6
S138I		1.7[d]	0.8
H64K S135L	0.6	0.1	0.03
S135L S138I		16.1	11.6
H64K S135L S138I	0.4	<0.01[c]	<0.01[c]

[a] All constants have been determined by fluorescence titrations as described (*6*). [b] The standard deviations typically range from 10% to 40%. [c] The affinity is less than 1×10^5 M^{-1}, which is too low for quantification. [d] The standard deviation is 50%.

tc, atc, and dox induction. TetR H64K exhibits a 5-fold increase in 4-ddma-atc induction and a strong reduction in tc, atc, and dox response (below 10%) as compared to wild-type TetR. The double mutant TetR H64K S138I shows largely reduced atc and dox induction, while the 4-ddma-atc response is the same as that of TetR H64K. The TetR S135L mutation does not interfere with atc, dox, or tc induction, but 4-ddma-atc response is increased more than 30-fold as compared to that of the wild type. TetR S135L S138I is fully induced by atc and dox, while the response to 4-ddma-atc and tc is reduced. The double-exchange mutant TetR H64K S135L is induced by 4-ddma-atc but not by tc, whereas it still responds to atc and dox. Thus, only the triple mutant TetR H64K S135L S138I shows 4-ddma-atc specificity with respect to atc and dox.

Inducer Binding Constants of the TetR Mutants. We overexpressed the TetR mutants, purified them to homogenity as described (*16*), and determined their binding constants to the $[tc\text{-}Mg]^+$ complexes. The binding constants of the tc analogues to Mg^{2+} and their Mg^{2+}-independent binding constants to the proteins are required for fitting the titration curves for the Mg^{2+}-dependent equilibrium binding constants. Therefore, the association constants of the tc analogues with Mg^{2+} (K_M) were determined as described (*6*). Atc binds Mg^{2+} with $K_M = 3.4 \times 10^3$ M^{-1} and dox with a roughly 2-fold and 4-ddma-atc with an about 5-fold increased constant.

Like atc (*6*), dox and 4-ddma-atc bind TetR also in the absence of Mg^{2+}. These Mg^{2+}-free binding constants (K_T) were determined by fluorescence titration of wild-type and mutant TetR with the corresponding tc derivative in a Mg^{2+}-free buffer (Table 1). The K_T values range between 10^5 and 10^{10} M^{-1}. The highest affinity without Mg^{2+} is seen for TetR S135L with atc and dox. The Mg^{2+}-independent binding of atc is 50-fold and that of dox is 10-fold enhanced as compared to that of the wild-type protein.

The binding constants (K_A) of $[tc\text{-}Mg]^+$ with the TetR variants were determined using K_M and K_T for fitting the titration curves (*6*). Examples are shown in Figure 5, and the resulting K_A values are summarized in Table 2. In previous work (*7*), the binding constants for $[tc\text{-}Mg]^+$ and $[cmt3\text{-}Mg]^+$ to TetR H64K, S135L, and H64K S135L were presented, while we are discussing binding of atc, dox, and 4-ddma-atc. The affinity of TetR for $[4\text{-}ddma\text{-}atc\text{-}Mg]^+$ is about 6 orders of magnitude lower compared to the one for $[atc\text{-}Mg]^+$ and 5 orders of magnitude lower than that for $[dox\text{-}Mg]^+$. The binding constant of TetR H64K S135L

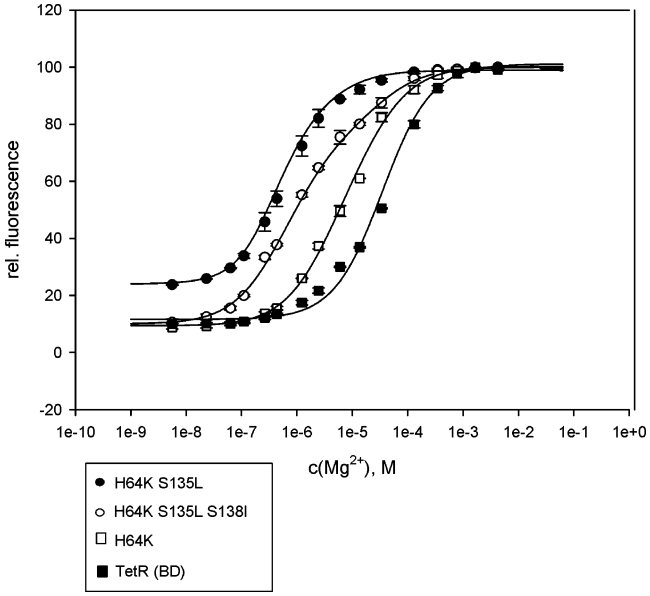

FIGURE 5: Fluorescence titration of TetR variants with $MgCl_2$. Titrations were done at c(H64K S135L) = 0.11 μM monomer and c(4-ddma-atc) = 0.1 μM, c(H64K S135L S138I) = 0.11 μM monomer and c(4-ddma-atc) = 0 1 μM, c(H64K) = 1.1 μM monomer and c(4-ddma-atc) = 1 μM, and c(TetR (BD)) = 1.1 μM monomer and c(4-ddma-atc) = 1 μM. The circles and squares show the relative fluorescence, and the line indicates the fit according to the binding function. The binding constants were calculated as described (*17*) and are $224 \times 10^7 \pm 50$ M^{-1} for TetR H64K S135L, $132 \times 10^7 \pm 48$ M^{-1} for TetR H64K S135L S138I, $4.9 \times 10^7 \pm 1$ M^{-1} for TetR H64K, and $0.3 \times 10^7 \pm 0.06$ M^{-1} for TetR (BD).

Table 2: Tc Derivative Binding Constants of TetR Mutants[a]

TetR variant	Mg^{2+}-dependent equilibrium binding constant,[b] K_A ($\times 10^7$ M^{-1})		
	4-ddma-atc	atc	dox
TetR (BD)	0.3	119 600	16 700
H64K	4.9	42.5	19
S135L	75	240200	14600
S138I	0.5	252	578
H64K S135L	224	260	64
H64K S138I	6.2	0.7	0.7
S135L S138I	1.6	980	14020
H64K S135L S138I	132	1.7	0.9

[a] All constants have been determined by fluorescence titrations under limiting Mg^{2+} concentrations as described (*17*). [b] The standard deviations typically range from 10 to 40%.

S138I to [4-ddma-atc-Mg]$^+$ is increased 440-fold compared to that of the wild-type protein. The [atc-Mg]$^+$ affinity, on the other hand, is 7×10^4-fold and the [dox-Mg]$^+$ affinity 2×10^4-fold lower for the mutant compared to the wild type. The increase in specificity conferred by the additional S138I exchange compared to that of TetR H64K S135L is resembled by almost unchanged affinity for [4-ddma-atc-Mg]$^+$, whereas the drop in affinity is 150-fold for [atc-Mg]$^+$ and 70-fold for [dox-Mg]$^+$. This effect is also seen in the TetR S138I mutant showing 470-fold-reduced [atc-Mg]$^+$ binding compared to the wild type. [dox-Mg]$^+$ binding is 30-fold reduced. The H64K mutation leads to decreased binding of [atc-Mg]$^+$ and [dox-Mg]$^+$, while [4-ddma-atc-Mg]$^+$ affinity is enhanced 17-fold. TetR S135L exhibits 250-fold higher affinity for [4-ddma-atc-Mg]$^+$ than the wild type, while [atc-Mg]$^+$ binding is only slightly affected and [dox-Mg]$^+$ binding is unaffected. Combination of the mutations in TetR H64K S135L leads to adding up the properties of the single mutants (*7*) for [tc-Mg]$^+$ binding, but not for [4-ddma-atc-Mg]$^+$, [atc-Mg]$^+$, or [dox-Mg]$^+$ binding. The binding constant of [4-ddma-atc-Mg]$^+$ is 770-fold increased, and there is detectable [atc-Mg]$^+$ and [dox-Mg]$^+$ binding. The binding constants of the double mutant TetR H64K S138I resemble its in vivo properties. [4-ddma-atc-Mg]$^+$ binding is slightly enhanced compared to that of the single-exchange mutants, while [atc-Mg]$^+$ and [dox-Mg]$^+$ binding is reduced. The TetR S135L S138I protein shows increased [4-ddma-atc-Mg]$^+$ affinity as compared to TetR S138I but decreased binding with respect to TetR S135L. [dox-Mg]$^+$ binding is only slightly reduced compared to that of S135L but strongly increased compared to that of TetR S138I. Binding of [atc-Mg]$^+$ is increased 3-fold compared to that of S138I but 330-fold reduced regarding that of the S135L mutant, while the Mg^{2+}-independent binding constant of atc is increased compared to that of the wild-type protein.

Comparison of the single mutants TetR H64K, TetR S135L, and TetR S138I with the triple mutant shows that S135L mediates recognition of 4-ddma-atc, H64K reduces binding of all tc analogues except 4-ddma-atc, and S138I reduces atc and dox binding but does not interfere with 4-ddma-atc recognition. Only the combination of all three mutations leads to a 4-ddma-atc-specific phenotype. The ratio of binding constants for the inducer pairs 4-ddma-atc and atc or 4-ddma-atc and dox serves as a measure for the specificity of the correponding mutants. Comparing TetR and the H64K S135L S138I triple mutants, this ratio increases 3 $\times 10^7$-fold for atc and 8 $\times 10^6$-fold for dox. This clearly exceeds the 20000-fold specificity gain found in the previous TetR inducer specificity study (*7*).

DISCUSSION

We had recently constructed the inducer specificity mutant TetR H64K S135L which responds to cmt3 but not to tc and attributed this to the lack of the 4-dma grouping in cmt3 (*7*). However, the more potent tc analogues atc and dox are still good inducers for this TetR mutant, which, therefore, has rather a relaxed than a truly discriminating phenotype. This is consistent with the fact that neither of these two mutations is located near the 4-dma function in the crystal structure (*3*, *4*). We assume that the increased basicity resulting from the H64K exchange compensates for the decreased acidity of the 2-OH grouping in 4-dedimethyltetracycline derivatives to strengthen the H-bond found in the crystal structure between the residues at these positions. This assumption would explain the relaxed specificity of the TetR H64K S135L mutant. Consequently, the object of this work was to construct a TetR variant with a truely new specificity by changing residues located in proximity of the 4-dma moiety in the TetR−[tc-Mg]$^+_2$ complex. We reached our goal by adding the S138I exchange to TetR H64K S135L, but it is quite surprising that this is the only exchange at position 82 or 138 leading to a pronounced 4-ddma-atc-specific phenotype. On the basis of the crystal structure, sterical interference with the 4-dma grouping should be accomplished for both positions (Figure 3).

All N82 mutations lead to decreased affinities for tc and atc. An explanation for this result can be the contribution of

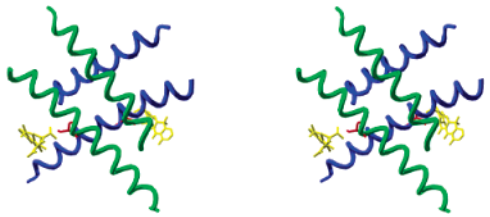

FIGURE 6: Stereoview of the location of S138 in the TetR four-helix bundle. The α-helices forming the four-helix bundle are indicated as blue and green ribbons from each monomer, respectively. S138 is located in helix α8, and the side chain (red) is oriented toward the 4-dma moiety of tetracycline (yellow stick model). Mutation of S138 may influence the dimerization of TetR.

N82 for tc binding. It undergoes two H-bonds to tc, one with the nitrogen of the 4-dma moiety and the other one with O-3 in the A-ring. Removal of these interactions in the TetR N82A mutant leads to almost complete loss of tc affinity, while the affinity for atc drops nearly 4 orders of magnitude (*6*). Since H-bonds are sterically sensitive, they cannot be formed by other residues in most cases. Therefore, any change of N82 weakens inducer binding and inducibility. This is true for all position 82 mutations found in previous studies (*18*, *19*) and in this work, including those with a 4-ddma-atc specificity increase. The latter mutants carry hydrophobic residues (V, I, M, W) at position 82. Thus, hydrophobicity at this position supports 4-ddma-atc more than atc binding, but these mutants are only poorly inducible.

In contrast, no contacts with the inducer are obvious for the S138 residue in the crystal structure, and yet, this position is quite sensitive to changes. It is located in helix α8, which forms part of the dimerization interface (Figure 6). The S138P exchange may alter the conformation of helix α8. Since Pro residues distort α-helices, the repression-deficient phenotype brought about by the S138P exchange may be due to lack of dimerization. Arg instead of Ser at position 138 causes reduced induction. This effect can be explained by the increased size and positive charge of Arg, which may be oriented toward the tc binding pocket clashing with the 10-hydroxide or 11-oxygen function of tc. While the S138I exchange causes a complete shift to 4-ddma-atc specificity, the very similar S138L exchange leads to almost complete loss of induction. Both amino acids are of similar size and should clash with the 4-dma grouping. This assumption is corroborated by the drastic affinity drop of all mutants containing Ile at position 138 for all tc derivatives with 4-dma as opposed to the rather small changes in affinity for tc derivatives lacking 4-dma. However, the different behavior of Ile and Leu highlights the importance of the exact geometry of the side chain for 4-ddma-atc recognition. It may be explained by effects on the conformation of surrounding residues, which are involved in shaping the tc binding pocket. F86, I134, and L142 are in close proximity to the 138 residue (Figure 7). The methyl group at position 6 in ring C of tc forms hydrophobic contacts with Ile 134 (*3*). F86 accepts a hydrogen bond donated by the 12a-hydroxide of tc, and TetR F86L exhibits reduced inducibility (*18*). Albeit L142 is not directly involved in tc contacts, severely reduced induction was seen for TetR L142F and TetR L142R in previous work, indicating the importance of this residue for induction. (*18*). Thus, a reorientation of either one or more of these residues as a result of a mutation in the neighboring 138 position may influence induction.

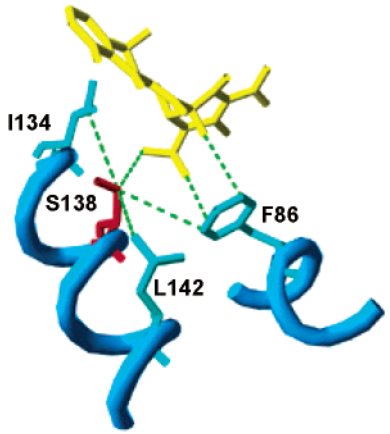

FIGURE 7: Location of the residues F86, I134, and L142 in proximity to S138. Ser138 is shown as a red stick model, and the side chains of F86, I134, and L142 are shown in light blue. The broken green lines indicate the nearest atoms of S138 and F86 relative to tc and of S138 relative to F86, I134, and L142. The distance between these residues is less than 5.3 Å. The 6-methyl group of tc is in hydrophobic contact with Ile 134, and F86 accepts a hydrogen bond from the 12a-hydroxide of tc (*4*). All three residues may be reoriented as a result of a mutation of the proximal residue at position 138, thereby influencing induction.

The amino acid alterations H64K, S135L, and S138I contribute to different degrees to inducer affinity in the TetR mutants, depending on their combinations and the respective inducer. The effects of H64K and S138I on 4-ddma-atc binding determined in the single mutants add up in the corresponding double mutant, while all other combinations do not yield the sum of the effects seen for the respective single mutations. This results in a smaller affinity increase than predicted from the single-exchange mutants. In contrast, an analogous consideration for atc shows that only the affinity changes resulting from the S135L and S138I mutations act independently. The dox affinities of all combined mutations resemble approximately the values predicted from the addition of the single-mutant effects. From these findings we conclude that the tc variants assume different positions in the tc binding pocket of TetR. This is the case for atc compared to tc, and even more pronounced for the tc variant lacking the 4-dma grouping.

Taken together, TetR H64K S135L S138I exhibits 4-ddma-atc specificity in distinction to atc and dox in in vivo and in vitro experiments. The interplay among all three mutations is crucial for the newly created 4-ddma-atc specificity. The isolation of a TetR mutant specifically induced by 4-ddma-atc, which contains no antibiotic activity, may be useful for gene regulation in prokaryotes and eukaryotes. In combination with specific operator binding and different dimerization specificities (*20*−*22*), it will allow fully independent expression control of more than one gene by the Tet system in the same cell.

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