The Role of the Variable Region in Tet Repressor for Inducibility by Tetracycline*

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A set of deletions and substitutions to alanine was introduced into the loop separating helices a8 and a9 of Tn10 Tet repressor (TetR). This region appears as an unstructured loop in the crystal structure of the TetR(D)·([Mg-tc]²)ₙ complex and is the only internal segment of variable length in an alignment of Tet repressors from seven different resistance determinants. In vivo analysis of 10 mutants shows that this loop is important for inducibility by tetracycline (tc), whereas DNA binding is not or only marginally affected. All deletions have an induction-deficient TetR⁸ phenotype, but the corresponding substitutions do not or only slightly affect inducibility. The purified mutant TetR proteins have a reduced affinity for tc in vitro that correlates with their lack of inducibility. The association rate of [Mg-tc]¹ to the TetR mutants is enhanced. Since none of the mutated residues contacts tc directly in the crystal structure, we propose that the length of the loop is important for the structural transition between a closed, tc binding and an open, operator binding conformation of TetR. We propose that the deletions in the loop shift the equilibrium between both forms toward the open, operator binding conformation.

Binding of small effector molecules often triggers conformational changes in proteins. In the absence of bound ligand an open conformation is favored, whereas the presence of a ligand stabilizes the closed form (1). This is also the case for proteins involved in transcriptional regulation like PurR (2, 3), LacI (4), TrpR (5, 6), and the cAMP receptor protein (CRP)¹ (7, 8). The active DNA binding conformation requires the bound ligand for PurR, TrpR, and CRP, whereas the ligand of LacI is an inducer leading to a conformation inactive in DNA binding. When bound, the ligand is mostly buried in a deep pocket in the protein interior and cannot reach or leave its binding site without inducing conformational changes. Thus, the liganded form corresponds to the closed conformation, and the unliganded form represents the open conformation.

Tet repressors (TetR) are well characterized isofunctional proteins with at least two different conformations. They regulate the expression of seven (classes A–E, G, and H) tetracycline (tc) resistance determinants present in Gram-negative bacteria. In the absence of tc, TetR is bound via an α-helix-turn-α-helix motif (HTH) to tetO. In the presence of tc, TetR dissociates rapidly from tetO allowing expression of tc resistance (for a review, see Ref. 9). Two crystal structures of the induced TetR(D)·([Mg-tc]²)ₙ complex have recently been solved (10, 11). The recognition helices of the TetR HTH are separated by 39 Å and enclose an angle of about 110°. These helices must approach each other by 5 Å and at least 50° to be able to bind to B-form DNA (9). The inducer, [Mg-tc]⁺, is bound in a deep pocket formed by residues from both monomers in the TetR dimer (10, 11). Thus, the tc-bound form would correspond to the closed conformation, and the DNA-binding form may represent the open conformation.

Loop movements are often involved in conformational changes in proteins where they contribute to the formation of ligand-binding sites and enzymatically active structures. Owing to their flexibility, these loops are not always defined in crystal structures (12). In TetR(D), the loop from residues Ala-154 and Pro-167 separating helices a8 and a9 is flexible and leads only to weak electron density in the TetR(D)·([Mg-tc]²)ₙ complex (11). Comparison of the residues in this loop in an alignment of TetR sequences (13) shows that it contains a region variable in both primary structure and length (see Fig. 1 for a schematic representation of TetR). Its length ranges from 11 residues in TetR(E) to 18 residues in TetR(C) (11) and is the only internal segment of TetR that is variable in length. Substitutions of single amino acids and deletions of 3 and 16 amino acids in this variable loop have shown that it is important for inducibility by tc but not for DNA binding (13, 14). Mutational analyses suggest that this region might be involved in the conformational change associated with induction (13, 15). Residues in this variable loop do not directly contact tc but might indirectly be involved in tc binding by positioning of a9 (11).

We investigated the role of this variable loop in induction by introducing deletions and substitutions in TetR(B). Deletions led to induction deficiency of TetR(B), and the TetR variants bind tc with a reduced affinity. We suggest that these mutations act by interfering with the initial closure of the tc-binding pocket.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Chemicals were from Merck (Darmstadt), Serva (Heidelberg), Sigma (München), or Roth (Karlsruhe) and of the highest purity available. Tc was from Fluka (Buchs). Enzymes for DNA restriction and modification were from Boehringer Mannheim, Life Technologies, Inc. (Egggenstein), New England BioLabs (Schwalbach), or Pharmacia (Freiburg). Isolation and manipulation of DNA was as described (16). Sequencing was carried out according to the protocol provided by Pharmacia for use with T7 DNA polymerase, with [α-³²P]dATP from Amersham (Braunschweig). Soluble protein extracts were prepared and analyzed in Western blots as described (13).

Bacterial Strains, Plasmids, and Phage—All bacterial strains are

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derived from Escherichia coli K12. Strain DH5α (17). hsdR17(k+ m+) recA1, endA1, gyrA96, thi-1, relA1, supE44, doldaZ Lac25M5, ΔlacZYA-argF(U169) was used for general cloning procedures. Strains J1M01 (18; Δlac-proAB), thi-1, supE44, F′ traD36, proAB, lacF2ΔM51 and RZ1032 (19, 20, 21) were grown in 1 liter of LB medium at 37°C in a 2-liter shaking flask, except for the strain containing TetR(E) that was grown at 28°C. The respective TetR variant was overexpressed by adding isopropyl-1-thio-
β-galactosidase to a final concentration of 1 mM at Ano = 0.9 and incubating the culture for an additional 4 h. Isolation and purification of overexpressed TetR was performed (24). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, München) and by saturating fluorescence titration with tc.

Analysis of TetR-tc Interactions—The concentration of tc was determined by UV spectroscopy using an extinction coefficient of εnm = 13320 m m −1 cm −1 in 0.1 N HCl (32). All fluorescence measurements were carried out in a Spex Fluorolog equipped with double Spex 1680 monochromators. The slit width was 2.2 mm for excitation (370 nm) and emission (515 nm), and an internal standard (8 mg/ml rhodamine B (Kodak, Stuttgart) in 1,2-propanediol (Fluka)) was used to correct for intensity fluctuations of the mercury lamp. Association constants K were determined under equilibrium conditions at 37°C as described (33) by fluorescence titration with limiting Mg2+ concentrations. TetR monomer and equimolar concentrations of dNTP were used in all experiments. Free Mg2+ concentrations ranging from 10−11 to 10−2 M were adjusted using diluted stock solutions of MgCl2 and K buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA, and 5 mM dithiothreitol) containing EDTA as a metal chelator (34). Free Mg2+ concentrations ranging from 10−1 to 10−2 M were adjusted using diluted stock solutions of MgCl2, and O buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM dithiothreitol) without EDTA. A K[s] of 2400 s−1 for the association of Mg2+ to tc was employed to analyze the titration curves (35). The fitting was performed by minimizing S0 = ΣFmax − F0, where Fmax and F0 are the experimental and theoretical fluorescence intensities, respectively. Fits with minimal S0 for a given pair of K and were transformed to K for α = 1 (no cooperativity in tc binding) by the equation K = 1 + α = 1 + α Ktcrt − tc Association constants Kcat were determined at 20°C, 30°C, and 40°C (as described (32) with equimolar concentrations of TetR monomer and tc at 25, 50, and 100 mM in B buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, and 5 mM dithiothreitol). All measurements were repeated at least twice.

RESULTS

Mutants in the TetR Variable Region—We introduced the set of mutations shown in Fig. 1 into the variable region of TetR(B) to evaluate the importance of its length and sequence for DNA binding and inducibility by tc. Deletion of the residues “DSM” in TetRA164–166 caused reduced in vivo inducibility (13). To address the importance of these side chains in a “loop” contact with tetO (36), we exchanged the charges Thr154–Asn165 that is disordered in the TetR(D)([Mg-tc])6 crystal structure (10). These residues were also substituted by alanine (TA162, s161AA, s161AAA).

In Vivo tetO Binding and Inducibility by tc—To determine the in vivo operator binding activity of the tetR mutants, they were transformed as pWH520 derivatives, which constitutively express TetR at a high level (37), into E. coli WH207(Δtet50). Repression of the tetA-lacZ transcriptional fusion and inducibility by tc were determined at 37°C and are shown in the second and third columns of Table I. All tetR variants show wt operator-binding activity in that strain. Class E TetR on pWH620(E) shows a marginally reduced inducibility with 86% β-galactosidase activity compared with TetR(B). The same result was found for TetR(s161AAA). All other mutants with sub-

2 M. Kintrup, personal communication.
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FIG. 1. Mutations in the variable region constructed in this survey. TetR(B) is depicted schematically with its N and C termini indicated. The 10 α-helices are displayed as open boxes and numbered as determined in Kisker et al. (11). The location of the DNA-binding HTH motif is shown above the representation. Positions at which amino acid residues contact tc in the crystal structure (11) are depicted above the representation by filled rectangles. The primary structure of the tc contact site from the second monomer is given below the representation. The mutations introduced are shown below the sequence and, in the case of the deletions, include the residues enclosed by the brackets. Their respective designations are indicated at the left side of the figure.

TABLE I

In vivo DNA binding and inducibility by tc of the mutants in the TetR variable region

The host strain used was WH207(tet50), β-galactosidase activities were determined in units according to Miller (31). They are given as percentages of the values obtained in the strains lacking tetR. These are defined as 100% β-galactosidase activity and correspond to 5844 ± 146 units (pWH520 derivatives), 6296 ± 264 units (pWH510 derivatives), or 4481 ± 237 units (pWH520 derivatives) and 5169 ± 238 units (pWH510 derivatives) after the addition of 0.2 µg/ml tetracycline. 0.0% correspond to less than 23 units. All measurements were performed at 37 °C. The class designation of the respective tetR variant is given in parentheses (42).

| tetR variant | pWH520 derivative | pWH510 derivative |
|--------------|------------------|------------------|
|              | DNA binding      | Induction by tc  | DNA binding      | Induction by tc  |
| None         | 100 ± 2.5        | 100 ± 5.3        | 100 ± 4.2        | 100 ± 4.6        |
| (B)          | 1 ± 0.0          | 92 ± 2.4         | 7 ± 0.1          | 101 ± 3.5        |
| (E)          | 1 ± 0.0          | 86 ± 4.9         | 88 ± 4.4         | 98 ± 8.8         |
| (B)Δ164–166  | 1 ± 0.0          | 1 ± 0.1          | 11 ± 1.3         | 22 ± 1.0         |
| (B)Δ161AAA   | 1 ± 0.0          | 88 ± 2.6         | 11 ± 0.5         | 101 ± 3.5        |
| (B)Δ164PTT   | 1 ± 0.0          | 45 ± 2.2         | 13 ± 3.4         | 96 ± 5.3         |
| (B)Δ161–163  | 1 ± 0.1          | 1 ± 0.1          | 7 ± 0.2          | 47 ± 3.3         |
| (B)Δ161AAA   | 1 ± 0.0          | 86 ± 3.0         | 8 ± 0.1          | 95 ± 5.5         |
| (B)Δ161–162  | 1 ± 0.0          | 2 ± 0.1          | 7 ± 0.3          | 74 ± 4.8         |
| (B)Δ161AAA   | 1 ± 0.0          | 93 ± 1.7         | 8 ± 0.4          | 98 ± 8.3         |
| (B)Δ162      | 1 ± 0.0          | 53 ± 4.1         | 7 ± 0.6          | 98 ± 9.8         |
| (B)TA162     | 1 ± 0.0          | 95 ± 6.4         | 9 ± 0.4          | 102 ± 7.0        |

Amino acid substitutions to alanine are fully inducible. The substitution of residues 164–166 to “PTT” leads to partial inducibility. In contrast, all deletions are less efficiently inducible. Variants with deletions of three residues (Δ161–163, Δ164–166) are not inducible; the variant with a deletion of two residues (Δ161–162) is only 2-fold and that with a one residue deletion (Δ162) is 50-fold inducible.

The DNA-binding activity of all TetR variants and the inducibility of the deletions of two and three residues at the basal limit of lacZ expression with 1% of the β-galactosidase activity in unrepressed strains. The pWH520-based expression system provides no resolution for large defects in inducibility (15) and slight differences in repression (29). To determine differences in the DNA-binding activity and inducibility, we used a second expression system with a better resolution for mutants with wt-like binding activity (29). The mutants were recloned into pWH510 that expresses TetR at an at least 30-fold reduced level (23, 37). The repression mediated by wt TetR in WH207(tet50) at 37 °C is only 14-fold in this construct, as compared with 100-fold in the pWH520-background (Table I, column 2). Repression with the pWH510 derivatives is presented in column four of Table I. TetRΔ161–163, TetRΔ161–162, and TetRA162 bind tetO with the same efficiency as wt, the other mutations lead to a less than 2-fold reduction in repression. Inducibility by tc was also determined and is shown in column 5 of Table I. The increased sensitivity of the pWH510 derivatives reveals that the TetR variants are inducible to different degrees. Only the deletions of three residues have a TetR<sup>5</sup> phenotype. They are 2- (Δ164–166) and 7-fold (Δ161–163) inducible. All other TetR variants tested are fully inducible at a lower expression level.

The variable loop is clearly not important for DNA binding of TetR, since none of the mutations leads to significantly reduced repression of the tetA-lacZ transcriptional fusion. On the other hand, it is important for the inducibility by tc. Deletions of two and three amino acids lead to impaired inducibility, while none of the substitution mutants has a TetR<sup>5</sup> phenotype. We thus conclude that the deletions are mainly responsible for the TetR<sup>5</sup> phenotype. The slightly reduced inducibility of TetRa164PTT demonstrate that the amino acids at the positions deleted contribute to inducibility. Four effects can lead to a TetR<sup>5</sup> phenotype: (i) an increase in the protein steady-state level, (ii) a super-repressor, (iii) reduced binding of tc, and (iv) failure to release tet operator upon tc binding. Therefore, these questions were addressed.

Steady-state Levels of TetR Mutants—The steady-state levels of the TetR variants encoded by pWH520 derivatives in E. coli WH207(tet50) were determined. Extracts of soluble protein were prepared from log-phase cultures grown under the same conditions as for the β-galactosidase activity measurements. Aliquots of these extracts, which allow the detection of 2-fold changes in TetR content (data not shown), were analyzed in Western blots for their TetR content. A typical result is shown in Fig. 2. The protein levels of the TetR mutants are either identical to the one of wt, or only slightly reduced. This rules out (i) as a cause of the TetR<sup>5</sup> phenotype and, in combination
with the wt- or lower DNA-binding activity of the mutants (Table I), also eliminates the super-repressor explanation.

**Binding of [Mg-tc]" to TetR Variants**—The TetR variants of classes B and E, as well as the mutants s164PTT, Δ161–163, and Δ164–166 were overproduced and purified to homogeneity. Their binding constants for [Mg-tc]" were determined under equilibrium binding conditions by fluorescence titration with limiting Mg2⁺ concentrations. The method measures the increase in the intrinsic fluorescence of [Mg-tc]" upon binding of TetR and can be used to determine binding constants between 1 × 10⁻¹⁰ and 1 × 10⁻⁷ M⁻¹ of tc derivatives to TetR (35) and of tc to TetR mutants (15). The titration curves obtained are shown in Fig. 3A. The lowest free Mg2⁺ concentration needed for fluorescence enhancement of tc is observed for TetR(E). Slightly higher Mg2⁺ concentrations are needed for TetR(B) and TetRs164PTT, in that order, while both TetRΔ164–166 and TetRΔ161–163 require about 100-fold higher Mg2⁺ concentration to show fluorescence enhancement. The best fits with the experimental data were obtained with cooperativity values for α ranging from 0.5 (TetRs164PTT) to 18 (TetR(E)). Varying α between these values changed the least square errors S² (see “Experimental Procedures”) less than 5-fold (data not shown).

Fig. 3B shows the theoretical fluorescence calculated for TetR(B) at three different cooperativity values of α. The three values for α chosen were 0.5 (lowest value obtained for all mutants; dashed line), 18 (highest value obtained for all mutants; full line) and 6, the value that lead to the lowest S² for TetR(B) (dotted line). The experimental data fit all three curves reasonably well. This indicates that the binding assay is not sensitive for potential cooperativity. Since TetR(B) binds two molecules of tc without detectable cooperativity as described (32), we determined the equilibrium association constants K for α = 1. They are displayed in the first column of Table II. The equilibrium association constant determined for TetR(B) is with 1.6 × 10⁶ very close to the previously published values of 1.9–3.1 × 10⁵ (15, 32, 33) and that of TetR(E) is about 2-fold higher. All mutants with a TetRβ phenotype have a reduced affinity for tc. While it is just 3-fold lower for the substitution s164PTT, it is diminished about 70-fold for the deletions of three amino acids (Δ161–163, Δ164–166). For all TetR(B) mutants purified, the reduction in tc binding affinity correlates to the severity of their *in vivo* TetRβ phenotypes.

A decrease in the equilibrium binding constant of tc to TetR can be caused by a decrease in the association rate constant, an increase in the dissociation rate constant, or both. The temperature dependence of tc to TetR can be fitted using second-order kinetics for a bimolecular reaction (32). The association rates of tc to the TetR variants were determined by measuring the increase in tc fluorescence upon addition of Tet repressor at temperatures of 20, 30, and 40 °C. They are shown in Fig. 4 and for kₐ at 30 °C in column three of Table II. TetR(B) has the lowest association rate constant of all variants at all temperatures assayed. At 30 °C, kₐ of tc to TetR(B) is 1.4-fold slower than to TetRΔ161–163, 3.4-fold than to TetRΔs164PTT, 5.2-fold than to TetRΔΔ164–166, and 11-fold than to TetR(E). The temperature dependence is smallest for TetR(E), almost identical for TetR(B) and the mutants Δ161–163 and s164PTT,
and largest for the mutant Δ164–166. The activation energies $E_a$ for this reaction were calculated from the association rate constants in Arrhenius plots and are displayed in column four of Table II. They are the same for TetR(B) for TetRΔ164PTT and TetRA161–163, about twice as high for TetRΔ164–166, but only half as high for TetR(E). Thus, the reduction in tc binding affinity is not due to an impaired access of the drug to its binding site, but tc must be retained less efficiently in its binding pocket. We can imagine that the reduction is caused either by the loss of direct contacts to tc or by interfering with the conformational change that leads to the closure of the tc binding pocket.

**Combinations of TetR<sup>S</sup> Mutations with HTH Mutations—**If the deletion mutants interfere with the conformational change leading to the induced structure of TetR, the conformational equilibrium between the DNA-bound and induced structures of TetR will be shifted toward the DNA-bound form. This assumption would imply that more DNA-binding active repressor is present in the mutants than in the wild-type and should lead to enhanced repression in the *in vivo* test system. Screening for second-site suppressors of the TetRS phenotype of TetR Δ164–166 yielded one candidate with a HR44 mutation. The *in vivo* repression conveyed by the double mutant HR44Δ164–166 was 2-fold higher than that of the single mutant HR44 (Table III), even though the mutant with residues 164–166 deleted does not show increased DNA-binding activity in *in vivo* (Table I). This would be consistent with the idea of a shift in the equilibrium toward the DNA-bound form. To determine whether the enhanced DNA-binding activity observed in this case is specific for these two mutations or general for combinations of a mutation in the DNA-binding domain with the deletion Δ164–166, we combined the deletion mutant Δ164–166 with 10 other mutations in the DNA-reading head. They are located in both $\alpha$-helices of the HTH (co: TQ27, LR34, LW34; c3: QG38, TG40, LT41, WG43), the connecting turn (VS36, VW36), and the following $\alpha$-helix 4 (KT48) at positions located either in the protein interior or at the surface (11). These HTH mutations span a wide range of DNA-binding activities, with HR44 being at an intermediate level (20, 29, 30).

To additionally ask if mutations with a TetR<sup>S</sup> phenotype generally enhance the DNA-binding activity of mutations in the HTH, we combined the HR44 mutation as a control with three previously described TetR<sup>S</sup> mutants that have phenotypes similar to Δ164–166 (15) and either contact tc directly (NS82, PT105) or do not form contacts with tc (DG178).

In *Vivo* tetO Binding of the Mutants—The pWH520 derivatives carrying the single and double mutants were transformed into WH207(tet50) and their $\beta$-galactosidase activities determined at 37 °C. The results are shown in Table III. While the $\beta$-galactosidase activities determined for the mutants with a TetR<sup>S</sup> phenotype are identical to that of wt TetR, those of the mutations in the HTH are between 3- and 60-fold higher. In combination with the Δ164–166 mutation, the $\beta$-galactosidase activities obtained for 9 out of the 11 HTH mutants are 1.5–3-fold lower when compared with the respective single mutation. Only the two mutations at position 34 with their unchanged $\beta$-galactosidase activities do not show an increase in repression. Repression is reduced in two of the double mutants involving HR44 (NS82, PT105), while it is increased in the other two mutants (Δ164–166, DG178).

**Steady-state Levels of the TetR Single and Double Mutants—**Soluble protein extracts were prepared from log phase cultures of pWH520 derivatives encoding the TetR variants in *E. coli* WH207(tet50) and analyzed in Western blots for their TetR content. A typical result is shown in Fig. 5. The intracellular protein levels of the two mutants with combinations of a substitution at position 34 and Δ164–166 are strongly reduced. For the other single and double mutants, the intracellular protein levels determined are either identical to the wild-type or only slightly reduced, as judged by their band intensities.

When only the double mutants with roughly unchanged protein levels are considered, the increase in the DNA-binding activity of the HTH mutations after introduction of the Δ164–166 deletion is general and not specific for a certain mutation in the HTH. This effect is not general for mutants with a TetR<sup>S</sup> phenotype, as the data for the double mutants with NS82 and PT105 show.

**DISCUSSION**

Mutations in the variable region of TetR affect DNA-binding activity only marginally (Table I) verifying that they do not introduce major changes into the DNA-binding conformation of TetR. Deletions of two or three residues have an induction-deficient TetR<sup>S</sup> phenotype, and the single residue deletion is slightly impaired in inducibility. All deletion mutants show a clear correlation between the reduction in loop length and the severity of the TetR<sup>S</sup> phenotype. In contrast, the alanine substitution mutants corresponding to the deletions do not affect inducibility and one further substitution mutant (s164PTT)

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The host strain used was WH207(tet50). All tetR variants belong to class B and are derivatives of either pH520 (wt- and Δ164–166 background) or pH1919 (NS82-, PT105-, and DG178 background). β-Galactosidase activities were determined at 37°C in units according to Miller (31). They are given in percentage of the value determined in strain WH207(tet50)/pWH201 (not shown in the table). This strain does not encode TetR. Its β-galactosidase activity was defined as 100% and corresponds to 4610 ± 166 units. 0.0% corresponds to less than 18 units.

| Mutation in the HTH | wt | Δ164–166 | NS82 | PT105 | DG178 |
|---------------------|----|----------|------|-------|-------|
| wt                  | 1 ± 0.1 | 1 ± 0.0 | 1 ± 0.2 | 1 ± 0.1 | 1 ± 0.1 |
| TQ27                | 6 ± 0.7  | 2 ± 0.1  |       |       |       |
| LR34                | 66 ± 2.2 | 71 ± 3.8 |       |       |       |
| LW34                | 15 ± 2.2 | 16 ± 0.8 |       |       |       |
| VS36                | 4 ± 0.5  | 2 ± 0.1  |       |       |       |
| VW36                | 35 ± 1.3 | 15 ± 0.7 |       |       |       |
| QG38                | 23 ± 1.1 | 10 ± 0.9 |       |       |       |
| TG40                | 3 ± 0.3  | 2 ± 0.1  |       |       |       |
| LT41                | 28 ± 1.4 | 10 ± 0.5 |       |       |       |
| WG43                | 40 ± 1.4 | 17 ± 2.1 |       |       |       |
| HR44                | 21 ± 0.3 | 11 ± 0.6 | 30 ± 1.6 | 32 ± 2.6 | 5 ± 0.3 |
| KT48                | 29 ± 1.5 | 20 ± 1.1 |       |       |       |

What leads to the TetR5 phenotype of the deletion mutants? The roughly unchanged steady-state levels of the proteins indicate that the TetR5 phenotype is an intrinsic property of the TetR variants. All TetR5 mutants purified have reduced affinities for tc (Table II) that correlate to the severity of their in vivo induction deficiency (Table I). Therefore, the deletions must change the tc binding pocket. Since none of the mutations affects residues in direct contact to tc, the deletions should indirectly exert their effects on tc binding. The deletion length dependence suggests that positioning of residues within or adjacent to the variable loop may be important for induction. The loop might be indirectly involved in tc binding by positioning a9, so that residues in a9 can form contacts with the D ring of tc (11). The end points of helices a8 and a9 are separated by 24.3 Å in the crystal structure of the TetR(D)([Mg-tc])2 complex (10). A fully extended peptide chain in a β-sheet spans 3.6 Å per residue (38). If we assume that each residue in the loop only spans 2.8 Å, 9 of the 14 residues present would suffice to bridge 25 Å. All deletion mutants would suffice for that. The end points of the segment disordered in the crystal structure are separated by 19.5 Å. This distance could be bridged by only seven residues, whereas nine are present in the two-residue deletion mutant. Thus, if the residues were fully extended they would not alter positioning of a9.

Substitutions at positions 7, 8, or 9 of tc that contact amino acids in a9 affect TetR binding and induction less than 10-fold (35, 39). Twelve induction-deficient TetR mutants were isolated in a9. Müller et al. (15) proposed that they affect inducibility by preventing the structural change associated with induction since, for all mutants, the TetRS phenotypes observed in vivo are more severe than indicated by the merely 1.3–4.5-fold reduced tc binding (see Tables I and IV in Ref. 15). Taken together, the loop deletions may affect tc binding by interfering with the formation of contacts between a9 and tc or by interfering with the conformational changes necessary for induction, or both.

It has been suggested that the lack of induction of several single TetR5 mutants is not due to their reduced inducer binding but to preventing conformational changes (15). Müller et al. (15) proposed a tc-induced reorientation of the four-helix bundle formed by a8 and a10 from both monomers. In this model, a9 serves as a “bolt” locking the TetR(D)([Mg-tc])2 complex in the induced, closed structure. Shortening the variable region by more than one amino acid might thus interfere with the closure of the tc-binding pocket inhibiting the transition between the DNA-binding and the induced conformations. TetR

Fig. 5. Steady-state levels of the TetR variants with combinations of a mutation in the HTH and a mutation with a TetR5 phenotype. Lane 1 (TetR) contains in all four blots 20 μg of purified wt TetR; all other lanes 20 μg each of a soluble protein extract from E. coli WH207(tet50). The strain was either transformed with pWH1201 (2nd lane), pH520 (3rd lane, wt), or the TetR single or double mutants (4th to 10th lanes, the respective designations of the mutants are given above each lane). A, combinations of mutations in the HTH with the deletion Δ164–166. B, combination of the HR44 mutation with the TetR5 mutations.

has only a small effect on induction (Table I). This demonstrates that the location of the deletion and the sequences of the deleted amino acids contribute less to the TetR5 phenotype than the reduced length of the variable region.
exists most likely in an equilibrium between the DNA-binding and the induced structures. Mutations stabilizing the DNA-binding conformation would be expected to show a TetR$^\text{S}$ phenotype, increased in vivo repression in the absence of inducer, and an increased association rate of tc. These are exactly the properties we observed for the deletion mutants. They show a clear TetR$^\text{S}$ phenotype. Owing to the law of mass action, the association and dissociation rate constants for the respective mutants (Table II). Thus, the reduced affinity for tc is not caused by increased protein levels. The facilitated access of tc to the binding pocket is indicated by the increased association rates for [Mg-tc]$^+$.

It is slowest with TetR(B) and faster in all mutants (Table II). Thus, the reduced affinity for tc is not caused by preventing the drug from reaching its binding site in the protein interior but must be due to its increased dissociation from the binding pocket. Mutations of groups I and II periplasmic sugar binding proteins show similar effects. A decrease in both association and dissociation rate constants for the respective ligand was observed and interpreted as an increased rigidity of the protein between closed and open forms of the protein toward the closed form, thereby shifting the equilibrium between closed and open forms of the protein toward the closed form. This assumption was confirmed by structural analysis (40, 41). It therefore seems plausible to assume for the TetR mutants that shortening the variable loop interferes with the closure of the tc-binding pocket, thus shifting the equilibrium between DNA- and tc-binding conformations toward the open DNA-binding conformation.

No correlation was seen between the activation energies of tc association and the in vivo phenotype of the TetR variants. Further experiments are needed to determine why the activation energies for tc association vary among different inducible and non-inducible TetR variants.

Taken together, we suggest that the deletions in the variable region of TetR affect the closure of the tc-binding pocket upon association of tc. This occurs most likely by interfering with the conformational change that locks the TetR(D)\(\text{[Mg-tc]}^+\)^2 complex into the induced structure.

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