Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor

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ABSTRACT

We report for the first time the in vitro characterization of a reverse tetracycline repressor (revTetR). The dimeric wild-type repressor (TetR) binds to tet operator tetO in the absence of the inducer anhydrotetracycline (atc) to confer tight repression. We have isolated the revTetR G96E L205S mutant, which, contrary to TetR, binds tetO only in the presence of atc. This reverse acting mutant was overproduced and purified. Effector and DNA binding properties were analyzed by EMSA and quantified by fluorescence titration and surface plasmon resonance. The association constant KA of revTetR for binding of [atcMg]⁺ is -10⁸ M⁻¹, four orders of magnitude lower than that of TetR. The affinity of TetR for tetO is 5.6 ± 2 x 10⁵ M⁻¹ and that for revTetR in the presence of atc is 1 ± 0.2 x 10⁸ M⁻¹. Both induced forms, the atc-bound TetR and the free revTetR, have the same low affinity of 4 ± 1 x 10⁵ M⁻¹ for DNA. Therefore, atc does not act as a dimerization agent for revTetR. We discuss the structural differences between TetR and revTetR potentially underlying this reversal of activity.

INTRODUCTION

Expression of tetracycline (tc) resistance determinants is strongly regulated in bacteria, mediated by the repressor protein TetR. It binds to the operator tetO, repressing its own expression and that of the efflux determinant tetA. The tightness and efficiency of tet regulation have been exploited for gene regulation setups in pro- and eukaryotes (1). Target genes in prokaryotes can at present only be negatively regulated by TetR. It binds to the operator tetO only in the absence of atc. The TetR portion of the eukaryotic regulator rtTA, the first reported revTetR version, shows a less pronounced phenotype in Escherichia coli [P.Schubert, unpublished (2,3)].

The 46 kDa TetR is a dimer in which each monomer consists of 10 α-helices. The DNA-binding domain is linked via helix α4 to the protein core, where dimerization and inducer binding occur. The inducer, mostly in complex with bivalent metal ions, enters both binding pockets initiating conformational changes leading to an increased distance between two DNA recognition helices and subsequent dissociation from tetO (4–6). Among many tc analogs, anhydrotetracycline (atc) binds TetR with a 500-fold higher affinity and represents the most efficient inducer (7).

We have isolated a revTetR mutant in E.coli, bearing the mutations G96E and L205S. This mutant does not bind tetO unless atc, which now acts as a corepressor, is present. Thus, it exhibits a reverse phenotype compared with TetR and is called revTetR.

Mutations yielding a reverse transcriptional regulator have been described for LacI but were not further characterized (8,9). In this study, we quantify for the first time protein–effector and protein–effector–DNA interactions of a reverse transcriptional regulator.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli DH5α was the host strain for cloning experiments (10). The E.coli strains BL21(DE3) (Stratagene) and RB791 were used for protein overproduction; E.coli WH207 λtet50 bearing a Tn10 tetA-lacZ transcriptional fusion was used for β-galactosidase activity determination which was performed as described (11–14). For tetR(BD) expressions, we used pWH1411 and for expression of the mutant we used pWH1411-(revtetR-G96E-L205S) carrying the gene revtetR-G96E-L205S (13). pWH1411-(revtetR-G96E-L205S) was isolated as described elsewhere (13). For over-expression of the mutant, we used the plasmid pET3c (Novagen, Darmstadt, Germany) carrying a 688 bp HincII fragment containing revtetR-G96E-L205S in NdeI filled in (this work). Standard DNA techniques were used as described (15). All wild-type and mutant tetR genes employed in this study are based on tetR(BD), a chimera consisting of the first 50 codons of tetR(B) fused to the last 158 codons of tetR(D). The detailed properties of this construct and its crystal structure were described (5,6,16,17). For the sake of convenience, TetR(BD) is called wt TetR and the reverse TetR(BD) mutant G96E L205S is called revTetR.

Over-expression and purification of proteins

Escherichia coli BL21(DE3) was transformed with pET3c-G96E-L205S. Cells were grown at the indicated temperatures in LB supplemented with the appropriate antibiotic. Gene expression was induced at an OD₆₀₀ of 0.7 by adjusting the

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broth to 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubation was continued up to 12 h. Cells were harvested by centrifugation, washed, and ruptured by sonification in 20 mM sodium phosphate buffer (pH 5.7) (buffer A). During sonification, the temperature of the sample was kept below 10°C. Crude cell extracts were analyzed by an 10% denaturing polyacrylamide gel. The soluble proteins obtained after centrifugation at 4°C for 45 min at 40 000 r.p.m. were loaded on a POROS HS/M 20 cation exchange column. Proteins were eluted with a linear gradient (50–500 mM NaCl) in buffer A. Fractions were collected and analyzed by SDS–PAGE, and revTetR containing fractions were pooled. This step was repeated and the eluted protein was purified via gel filtration as described (18). The protein concentration was determined via UV spectroscopy.

Electrophoretic mobility shift assays (EMSA)

For EMSA, the synthetic 40 bp tetO-containing fragments designated tetO1. 5′-CTTAATTTTTGTGACACTCTAT-CATTGATAGAGTTATTTACCACCT-3′, and tetO2 containing the complementary sequence (tetO nucleotides in bold) were generated by hybridization. Equal molar amounts of each oligonucleotide were mixed in water, heated at 96°C for 5 min and allowed to cool to room temperature within 2 h. The DNA was incubated with the indicated amounts of atc and protein. An oligonucleotide of the same size containing no palindromic sequence was used as a control (5′-CTTAATTTTTGTGACACTCTAT-CATTGATAGAGTTATTTACCACCT-3′). All samples were incubated in complex buffer containing 0.02 M Tris–HCl (pH 8.0) and 5 mM MgCl2. Atc was added to the sample to a final concentration of 0.1 mM. After incubation for 10 min at ambient temperature, the DNA was electrophoresed on an 8% polyacrylamide gel at 50 V in 20 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl, 0.003 M EDTA and 0.005% surfactant P20.

Kinetic studies were performed at a flow rate of 70 μl/min to avoid mass transfer effects. For corepression and induction studies, atc was used in a 4-fold excess over TetR dimer. MgCl2 (2 mM) was incubated with atc before adding [atcMg]+2 to the protein solution. After incubation at room temperature for at least 5 min, 100 μl samples were injected at the flow rates mentioned above for kinetic measurements. Kinetic analyses were interpreted using the separate fit modus of the BIAevaluation 3.0 program.

Equilibrium measurements were performed at 5 μl/min. For equilibrium analyses, the injected volume was adjusted to the amount needed for a constant response difference indicating equilibrium of interaction. R(eq) is the concentration of the complex and was measured directly as the steady-state response. The concentration of free analyte is equal to the concentration of bulk analyte since it is replenished constantly during sample injection. The difference between the total surface binding capacity (R(max)) and the steady-state response signal [R(eq)] can be treated as equal to the concentration of free ligand [D]. For determination of the equilibrium association constant, Scatchard plot analysis was performed using the equations below.

\[ K_A = \frac{[A-B]([A] \times [B])}{[D] - [D]) \]
\[ D - [D] + [D] \]
\[ [D] = [R_{eq} - [R_{eq}]] \]
\[ [D] = [D] - [R_{eq} - [R_{eq}]] \]
\[ [D] = [R_{eq} - [R_{eq}]] \]
\[ D_{eq} = [R_{eq} - [R_{eq}]] \]
\[ [R_{eq}] = [R_{eq}] \]
\[ [R_{eq}] = [R_{eq}] \]

where R = TetR; I = [atc-Mg]+2; D = [tetO]; D0 = D(total); R_{eq} = [RI-D]; RI = free repressor; and R(max) - R_{eq} = D.

Surface plasmon resonance (SPR) measurements

SPR measurements were performed with a BIACORE X™ instrument (BIAcore, Uppsala, Sweden). All measurements were carried out at 25°C. All TetR concentrations refer to the dimer. As ligand in flow cell 2 (Fc2), we used the 48 bp tetO-bearing fragment described above. Flow cell 1 (Fc1) contained a DNA fragment of the same size without tetO as described above. Both upper strand oligonucleotides were biotinylated at the 5′ end and hybridized as described above with the complementary non-biotinylated lower strand oligonucleotide. Biotinylated DNA was coupled to the streptavidin-coated sensor chip SA as recommended by the manufacturer and yielded about 130 resonance units in each flow cell. Fresh streptavidin-coated SA-sensor chips were prepared for each measurement. The response signal difference Fc2 – Fc1 corresponds to specific interaction between tetO and the analyte. DNA fragments, proteins, MgCl2 and atc were diluted in HBS running buffer containing 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 0.003 M EDTA and 0.005% surfactant P20.

Circular dichroism (CD) measurements were carried out on a Jasco J-175 spectropolarimeter in 0.5 cm cells at a concentration of 5 μM of TetR dimer. Fluorescence measurements were carried out in a Spex Fluorolog 3 (Jobin Yvon) with two double monochromators. For determination of repressor–atc stoichiometry, 2 μM repressor was titrated with up to 17 μM atc in Mg2+-containing buffer. To observe atc fluorescence, the excitation wavelength was set to 455 nm and the emission was detected at 545 nm with a slit width of 4 mm each. TetR dilutions of 1.1 μM mixed with 1 μM atc were adjusted to the MgCl2 concentrations as described (7). All measurements were done at 25°C in titration buffer containing 100 mM Tris–HCl (pH 8.0), 100 mM NaCl and 0.1 mM EDTA. Concentrations of free Mg2+ in titration buffer were calculated as described previously (7,13). All revTetR–[atcMg]+2 binding constants K_A were determined by Mg2+ titrations. Calculations of binding constants K_T for revTetR–atc binding, K_M for atc–Mg2+ binding, K_{T-M} for Mg2+ binding to revTetR and K_A for binding of [atcMg]+2 to revTetR were performed as described (7,13).
**RESULTS**

**In vivo quantification of induction efficiencies**

RevTetR was isolated from a mutant pool generated by DNA shuffling as described (13). Screening of the pool with and without atc yielded several revTetR mutants. RevTetR G96E L205S showed a pronounced phenotype and the best over-production properties. G96 is localized in helix α6, which participates in the conformational change during induction, while L205 is close to the C-terminus behind helix α10 (4). The induction efficiency of revTetR was quantified by β-galactosidase (β-gal) assays using E.coli WH2071ΔtetO transformed with a plasmid bearing either no tetR (pWH1401), tetR or revtetR are shown in the presence (white columns) and absence (black columns) of 0.4 μM atc. β-Gal activity in the absence of tetR was set to 100% and represents ~7000 Miller units.

**Purification and CD spectroscopy of revTetR and TetR**

Over-expression of soluble revTetR in E.coli BL21(DE3) required a growth temperature of 22°C, and yielded 2 mg l⁻¹ of pure protein which is 8-fold less compared with TetR. CD spectra for both proteins show nearly the same shape and reveal two minima at 209 and 222 nm, which is typical for proteins with a high content of α-helices such as TetR (Fig. 2).

Titration of 2 μM revTetR with atc or tc in a buffer containing a fixed amount of Mg²⁺ indicated a higher affinity for atc (data not shown). Therefore, all measurements were carried out with atc. Titration with atc yielded a point of equivalence at an atc concentration of nearly 2 μM, indicating that one molecule of atc binds per monomer (data not shown).

**EMSAs of revTetR–tetO interaction**

EMSA was performed to demonstrate in vitro DNA binding of TetR and revTetR. The results depicted in Figure 3 demonstrate that revTetR binds tetO only in the presence of atc (lanes 1–7 and 11), while the control DNA fragment without tetO (see Materials and Methods) is not bound (lane 8). TetR binds tetO only in the absence of inducer (lanes 9, 10 and 12–17), while the control DNA is not bound (lane 17). A complete shift of tetO is observed with a 3.4-fold excess of TetR over tetO (lane 16), while, even in the presence of a 9-fold molar excess of revTetR[atcMg]⁺² over tetO, the DNA is not entirely retarded. This result suggests a lower affinity of the revTetR–atc complex for tetO compared with TetR.

**Affinity of revTetR for atc**

The atc affinities of TetR and revTetR were determined by fluorescence titration. Protein- and atc-containing samples were titrated with Mg²⁺, and the binding constants were derived as described previously (7,13). No increase of fluorescence was detected in the absence of Mg²⁺. An Mg²⁺-driven bridging effect could be excluded due to extensive study (7,19). K₄ values are listed in Table 1. A remarkably large decrease in the affinity to atc of about four orders of magnitude from 1 × 10⁻¹² M⁻¹ for TetR to 1 × 10⁻⁸ M⁻¹ for revTetR was observed. This is astonishing since neither of the two mutations are near the inducer-binding site in the crystal structure of the TetR-tc complex (5). However, the exchange of hydrogen with the propionic acid group could influence the position of the H100 imidazole moiety contacting the magnesium ion.

**Protein–DNA interaction analysis by SPR**

We performed SPR using a BIACORE X™ to analyse revTetR interaction with tetO. Affinity constants are expressed as K₄ and K₋₄, K₋₂ is the binding constant with both effector-binding pockets occupied, while K₋ represents the one with free effector-binding pockets. The tetO binding constant for revTetR was derived from kinetic measurements since saturation was not accomplished in a feasible concentration range. The data in Figure 4A and B demonstrate the recognition of tetO by revTetR in the presence and absence of atc. The dissociation rate constants showed no dependence on the protein concentration. The resulting DNA binding constant is
The affinity of revTetR for DNA in the absence of atc is low, very high concentrations of revTetR were injected. The high affinity of TetR for tetO allowed determination of \( K_\alpha \) from saturation experiments. Sensorgrams obtained from the titration of tetO with TetR are shown in Figure 5A. Scatchard analysis was used to determine equilibrium constants from the SPR response (equations 1–7) (Fig. 5B) and resulted in \( K_\alpha = 5.6 \pm 2 \times 10^9 \text{M}^{-1} \). All binding constants are shown in Table 1.

**DISCUSSION**

In contrast to the wild-type, revTetR, differing by the two amino acid exchanges G96E and L205S from TetR, requires atc to bind to tetO and to confer transcriptional repression. Although repression is somewhat less efficient compared with TetR, the two amino acid exchanges are sufficient to completely revert the response of this allosteric protein to the inducer atc, turning the latter into a corepressor. Thus, revTetR can apparently not assume the tetO binding conformation in the absence of atc. There are several possibilities to rationalize such a behavior. One consideration assumes that revTetR may need to be stabilized by the dimerization agent atc which interacts with both subunits and may thus support formation of the active dimer. While the difficulties experienced during over-production would corroborate this hypothesis, the lack of improved yield of revTetR in the presence of atc argues strongly against this idea. The similar \( \alpha \)-helical contents of both proteins are also in agreement with a native revTetR structure, since dissociation of the dimer leads to denaturation of TetR (20).

The binding constants of revTetR and TetR for atc differ by four orders of magnitude from \( 1 \times 10^{12} \text{M}^{-1} \) of TetR to \( 1 \times 10^8 \text{M}^{-1} \) of revTetR. This result is quite remarkable because neither G96 nor L205 participate directly in atc binding (4).
However, a neighboring effect of E96 could influence the positioning of H100 which forms part of the inducer-binding pocket; otherwise, it does not have an influence on stoichiometry of atc binding. A possible contribution of L205 to atc binding is not obvious on the basis of the TetR structure. So, revTetR must clearly assume a structure which is less favorable for atc binding than that of TetR.

The revTetR conformation is also unfavorable for tetO binding, as is indicated by its low affinity for the tetO-containing fragment, which resembles that of induced TetR. Binding of [atcMg]$_2^+$ to revTetR increases its affinity for tetO to about $1 \times 10^8$ [M$^{-1}$], about one order of magnitude lower than the apparent TetR–tetO affinity determined under identical conditions. This somewhat lower affinity also agrees with the less efficient gel mobility shift. Although the two mutations are not close to the DNA-binding site of revTetR, they exert a strong influence on DNA binding. Since the revTetR conformation in the absence of atc shows only low tetO affinity, atc binding to revTetR may trigger conformational changes that allow high affinity tetO binding. Another possibility is that the tetO- and atc-binding conformations are the same for revTetR, whereas they are mutually exclusive for wild-type TetR.

We conclude that the DNA-binding domain in revTetR must have a different position relative to the atc-binding core of the proteins so that tetO binding is not possible. This idea is supported by the fact that E96 is located between those two domains. The in vitro characteristics of this revTetR mutant demonstrate that the allosterical change of TetR must be redesigned so that both activities of of the wild-type protein are weakened in the free form of revTetR, but restored when both ligands are present. Thus, the atc-free structure of revTetR must exhibit a conformation which is incompatible with high affinity tetO binding, whereas it forms a ternary complex with tetO in the presence of atc. How this is brought about by the two altered amino acid residues is not obvious at this point and awaits clarification by X-ray analysis.

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REFERENCES


