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Structural Requirements of Tetracycline-Tet Repressor Interaction: Determination of Equilibrium Binding Constants for Tetracycline Analogs with the Tet Repressor

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We used the Tn*10*-encoded Tet repressor, which has a highly specific binding capacity for tetracycline, to probe contacts between the drug and protein by chemical interference studies of the antibiotic. For that purpose, the equilibrium association constants of modified tetracyclines with the Tet repressor and Mg^{2+} cations were determined quantitatively. The results confirm the previous notion that Mg^{2+} probably binds with the oxygens at positions 11 and 12 and is absolutely required for protein-drug recognition. Modifications were introduced at positions seven, six, five, and four of the drug, and anhydrotetracycline was also studied. Substitutions or eliminations of functions at these positions influenced binding to the Tet repressor up to 35-fold. The introduction of an azido function at position seven in 7-azidotetracycline and epimerization of the substituents at position four in 4-epitetracycline lead to a 2- or 25-fold reduction, respectively, of Tet repressor affinity in those compounds. Anhydrotetracycline bound about 35-fold more strongly than tetracycline did, indicating that the oxygen at position 11 may be involved in Tet repressor recognition. This increased binding is in contrast to the lower antibiotic activity of anhydrotetracycline and indicates that the Tet repressor and ribosomes recognize the drug differently.

Resistance to tetracycline (Tc) in enterobacteria is mostly mediated by active efflux of the drug from the resistant cell. This property can be encoded by five related genetic determinants, named classes A through E, which share extensive sequence homology (9). The most frequently found determinant is located on transposon Tn*10*. Resistance to Tc mediated by Tn*10* is inducible on the level of transcription (2). Repression is mediated by a repressor protein (1, 13) which binds as a dimer (5) to two operator sequences in the *tet* regulatory region (7). The inducer is the antibiotic Tc itself, which interacts with the repressor-operator complex, leading to at least a 1,000-fold reduction in operator binding affinity (5, 8). Since Tc inhibits protein synthesis in the bacterial cell (4), the release of repression must be very efficient to allow the synthesis of the resistance protein.

Tc-Tet repressor binding has been studied by nitrocellulose filter retention (6) and fluorescence measurements (16). The equilibrium binding constant of Tc to the Tet repressor could be deduced only from kinetic data and was found to be about 10^9 M^{-1} (16). It was also established that a ternary complex of Tc, Mg^{2+}, and the Tet repressor is formed, while the drug does not bind to the protein in the absence of divalent cations (16). To our knowledge, the Tet repressor is the only protein that shows highly specific binding to Tc and that is available in large amounts and high purity (10). It is thus important to determine the molecular interactions of the drug with this protein. A preliminary X-ray analysis of the Tet repressor-Tc complex has not yet revealed structural information (11).

Our approach to gain information about the functions of Tc that are important for repressor binding made use of many chemical analogs of the drug, which have been synthesized and studied for their effects on antibiotics (14). The chemical structures of the derivatives used in this study are shown in Fig. 1. Since it is a problem to determine high binding constants in the range of 10^9 M^{-1}, in particular, since no radioactive Tc derivatives are available, we developed a new technique which may be generally applicable to ternary complexes with high binding constants.

MATERIALS AND METHODS

General methods. All chemicals used were of the highest purity available and were obtained from Merck, Darmstadt, Federal Republic of Germany; Sigma, Munich, Federal Republic of Germany; or Roth, Heidelberg, Federal Republic of Germany. Tc and the analogs were provided by Lederle Laboratories, Pearl River, N.Y. The Tet repressor was purified from an overproducing *Escherichia coli* strain as described previously (10). Fluorescence measurements were carried out on a Spex instrument equipped with two double monochromators at a slit width of 4 mm for exitation and emmission, and an internal standard was used to correct for intensity fluctuations of the mercury lamp. The concentrations of the Tc analogs were determined by UV spectroscopy by using extinction coefficients, when they were known (16). The concentrations of the other Tc analogs were determined from saturation titration with known amounts of the Tet repressor. Association rates constants were determined, as described previously (16), from the time-dependent increase in fluorescence after mixing known amounts of Tet repressor and Tc.

Analysis of Tc binding curves. All equilibrium binding data

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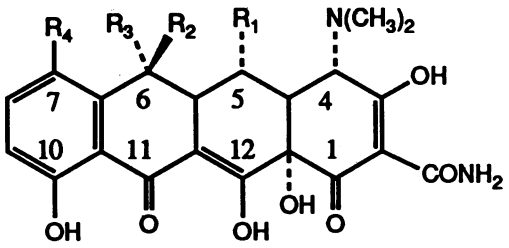
A



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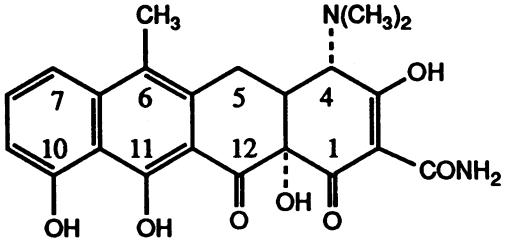

C

	R_1	R_2	R_3	R_4
tetracycline	H	OH	CH_3	H
7-chloro-tc	H	OH	CH_3	Cl
4-epi-7-chloro-tc	H	OH	CH_3	Cl
oxytetracycline	OH	OH	CH_3	H
doxycycline	OH	H	CH_3	H
6-deoxy-6-demethyl-tc	H	H	H	H
7-azido-6-deoxy-6-demethyl-tc	H	H	H	N_3
minocycline	H	H	H	$N(CH_3)_2$

FIG. 1. Structure of Tc and the derivatives used in this study. (A) The chemical structure of Tc. (B) The chemical structure of anhydro-Tc. (C) The substituents and nomenclatures of the derivatives.

were obtained from fluorescence measurements. The intrinsic fluorescence of Tc is enhanced upon binding of Mg^{2+} and is enhanced further upon binding of the Tet repressor (16). The results were analyzed by comparing experimental and theoretical fluorescences. The qualities of these fits were optimized by minimizing $S^2 = \Sigma(F_{exp} - F_{theor})^2$, where S^2 is analysis of variance, F_{exp} is experimental fluorescence, and F_{theor} is theoretical fluorescence. A detailed description of the computer programs will be presented elsewhere. The binding scheme is as follows:

$$T + M \overset{K_M}{\rightleftharpoons} TM$$

$$TM + R_0 \overset{K_1}{\rightleftharpoons} R_1$$

$$TM + R_1 \overset{K_2}{\rightleftharpoons} R_2$$

where T, M, TM, R_0, R_1, and R_2 are free Tc, Mg^{2+}, Tc · Mg^{2+} complex, repressor dimer, repressor dimer complexed with one TM, and repressor dimer complexed with two TMs, respectively. K_M, K_1, and K_2 are the equilibrium association constants of each reaction.

Analysis of Tc · Mg^{2+} binding. Each Tc derivative was titrated with $MgCl_2$ stock solutions, and the change in fluorescence was observed as a function of the Mg^{2+} concentrations. These experiments were carried out by using various concentrations of Tc and its analogs, and the resulting constants were independent of these parameters. The association constants, K_M, were determined from these saturation curves by fitting theoretical curves to them. For that purpose, the concentration of Tc that complexed with Mg^{2+} was calculated for each titration point by using the mass law. Then, the theoretical fluorescence was calculated from the following equation:

$$F = F_0 + \frac{T}{T_t} \times F_1 + \frac{TM}{T_t} \times F_2$$

where F_0 is the fluorescence of the buffer, F_1 is the fluorescence of free Tc, F_2 is the fluorescence of the Tc · Mg^{2+} complex, and T_t is the total concentration of Tc. The fit was optimized by systematically changing K_M and F_2. The best fits were obtained with F_2 values close to the ones determined experimentally from the saturation plateaus of the titration curves (compare Fig. 2). This indicates that the photoreaction of Tc was not significant in these experiments. F_0 and F_1 were determined experimentally. The analysis of variance (S^2; see above) yielded clear minima for the K_M values, indicating that they are determined with a high degree of accuracy.

Analysis of Tc · Mg^{2+} binding to the Tet repressor. Formation of the ternary complex was determined from the change in fluorescence of a mixture containing 1 μM Tc and 0.5 μM Tet repressor dimer upon an increase in the Mg^{2+} concentration. In order to define the free Mg^{2+} concentrations, mixtures of Mg^{2+} and EDTA were used (12). While the Tc fluorescence increased only slightly upon the addition of the Tet repressor in the absence of Mg^{2+}, the addition of the divalent cation led to large enhancements of fluorescence, indicating complex formation. The fluorescence intensities of the ternary complexes were increased over the intensities of the respective Tc · Mg^{2+} complexes. Upon an increase in

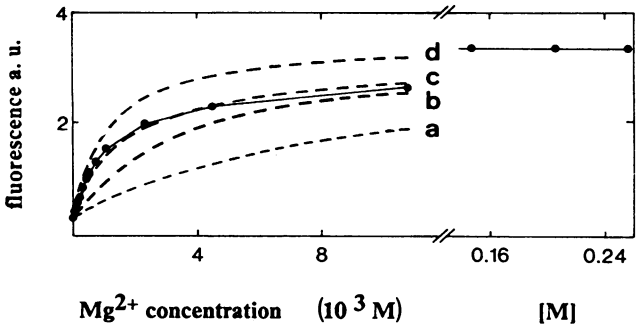

FIG. 2. Fluorescence titration of anhydro-Tc with Mg^{2+}. The fluorescence is given in arbitrary units (a.u.). The excitation and emission wavelengths were 460 and 550 nm, respectively. The Mg^{2+} concentration was increased by adding respective amounts of 1 M $MgCl_2$. The measurements were done in 0.15 M NaCl–0.1 M Tris hydrochloride (pH 8.3)–20 μM EDTA–1 mM dithiothreitol. The solid line represents the experiment result. The dotted lines represent theoretical binding curves. The different association constants (K_M) assumed for their calculation are as follows: 10^2 M^{-1} (a), 3×10^2 M^{-1} (b), $5.6 - 10^2$ M^{-1} (c), 10^3 M^{-1} (d).

the free Mg^{2+} concentration, the fluorescence reached a plateau, and further addition of Mg^{2+} did not lead to changes in fluorescence.

The titration curves measured for the Tc analogs were fitted by an adopted least-squares procedure by using the coupled reaction scheme given above. The concentration of free Mg^{2+}, represented by M in the scheme, was maintained constant by use of an EDTA-Mg^{2+} buffer system (12). The concentration of bound Tc · Mg^{2+} at a given Mg^{2+} concentration was calculated by a numerical solution of the equation system given above with the help of a half-internal method. The theoretical fluorescence was then calculated according to the following equation:

$$F = F_0 + \frac{T}{T_t} \times F_1 + \frac{TM}{T_t} \times F_2 + \frac{R_1 + 2R_2}{T_t} \times F_3$$

where F_3 is the fluorescence of Tc · Mg^{2+} bound to the Tet repressor, and F_0 is the fluorescence of the Tc and Tet repressor mixture in the absence of Mg^{2+}. The other abbreviations are as described above. Since the Tet repressor dimer consists of two identical subunits, the association constants K_1 and K_2 may be described by the cooperativity α and the intrinsic association constant K_A. This was considered by $K_1 = 2K_A$ and $K_2 = \alpha \times K_A/2$. The fit of the theoretical fluorescence to the experimental result was optimized by varying K, α, and F_3. The computer programs used for this procedure will be presented elsewhere.

RESULTS AND DISCUSSION

A typical set of calculated titration curves of Tc with Mg^{2+}, by assuming different K_M values, is displayed in Fig. 2. The four different fits displayed in Fig. 2 indicate that the deviation of the theoretical curves from the experiment is already significant when K_M is reduced by only 50%. This indicates that K_M values were determined with a degree of high accuracy. The resulting K_M values for all Tc analogs used here are given in Table 1. Spectroscopic measurements have shown that divalent metal ions interact with Tc in aqueous solution, probably via the oxygens at positions 11 and 12 (Fig. 1) (15). Since these functions were not altered in the Tc analogs studied here, it is not surprising that the binding constants for Mg^{2+} did not vary by more than sixfold between the strongest and weakest binders. These variations can be rationalized by considering the electronic effects of the modifications on the oxygens at positions 11 and 12. The 7-chloro substitution may have an electronegative effect on the BCD chromophore, weakening the interaction with Mg^{2+}. This is even more pronounced in the 7-azido derivative. The elimination of the 6-hydroxyl residue to yield an additional double bond in the BCD chromophore adds an aromatic six-membered ring in anhydro-Tc when the O-11 is tautomerized to OH. Since this leads to a reduced binding constant for Mg^{2+}, the involvement of O-11 in this reaction is further supported. Thus, the observed alterations of K_M as a result of substitutions in Tc are in agreement with the idea that metal binding occurs at the oxygens at positions 11 and 12.

TABLE 1. Association equilibrium constants of Tc derivatives with Mg^{2+} (K_M) and the Tet repressor (K_A)

Tc derivative	K_M (M^{-1} [10^3])	K_A (M^{-1} [10^9])
Tetracycline	2.7 ± 0.1	3.0 ± 0.1
7-chloro-Tc	1.3 ± 0.2	10.0 ± 5.0
4-Epi-7-chloro-Tc	1.3 ± 0.2	0.4 ± 0.2
Oxy-Tc	2.9 ± 0.1	3.0 ± 1.0
Doxycycline	3.3 ± 0.1	15.0 ± 5.0
6-Deoxy-6-demethyl-Tc	3.4 ± 0.1	11.0 ± 6.0
7-Azido-6-deoxy-6-demethyl-Tc	0.6 ± 0.1	5.0 ± 0.5
Anhydro-Tc	0.6 ± 0.01	100.0 ± 20.0

A typical example of a titration of Tc and the Tet repressor with Mg^{2+} is displayed in Fig. 3. Comparison with the titration shown in Fig. 2 reveals that fluorescence enhancement occurs at free Mg^{2+} concentrations that are about four orders of magnitude lower. This clearly confirms the coupled reaction scheme given above. The interaction of the two Tc molecules with the Tet repressor dimer seems to be noncooperative. In all of the calculations of Tc · Mg^{2+} binding to the Tet repressor, the best agreement between experimental and theoretical data was obtained with α values of between

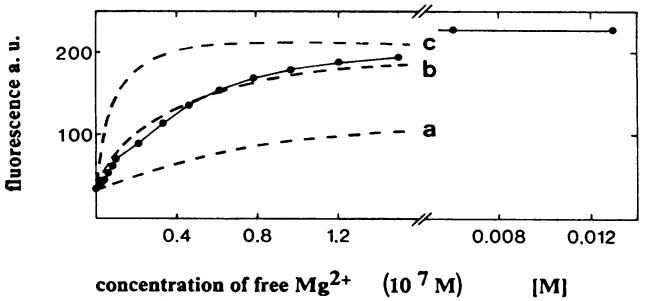

FIG. 3. Fluorescence titration of 1.12 μM anhydro-Tc and 0.63 μM Tet repressor dimer with Mg^{2+}. The fluorescence is given in arbitrary units (a.u.). The free Mg^{2+} concentration was defined by mixtures of $MgCl_2$ (total concentration varying from 0.1 to 20 mM) with EDTA as described previously (12). The buffer used was 0.15 M NaCl, 0.1 M Tris hydrochloride (pH 8.3), 0.02 M EDTA, and 1 mM dithiothreitol. The excitation and emission wavelengths were 460 and 550 nm, respectively. The solid line indicates the experimental result. The dotted lines show the calculated binding curves. They were derived by assuming K_A of values 10^{10} M^{-1} (a), 10^{11} M^{-1} (b), and 10^{12} M^{-1} (c).

1 (no cooperativity) and 2 (negligible cooperativity). However, the fits were not very sensitive to small changes in α. We therefore conclude that binding of Tc or its derivatives to the first site on the tet repressor dimer does not significantly influence binding to the second site. Considering the lack of apparent cooperativity, we redetermined the intrinsic association constants K_A with $\alpha = 1$ in all cases. Three theoretical binding curves are displayed in Fig. 3, in which K_A was varied 10-fold. It may be derived from the comparison of these theoretical curves with the experimental result in Fig. 3 that the K_A values can be determined with sufficient precision. The results for all Tc analogs are presented in Table 1. They were verified by changing the repressor (monomer) and Tc concentrations to between 0.15 and 10 μM. This led to identical results for K_A, indicating that the assumed reaction scheme is valid under these conditions. This is not surprising since it was shown previously that the Tet repressor dimer remains stable down to repressor concentrations of 10^{-7} M (5).

The binding constants of the Tc · Mg^{2+} complexes to the Tet repressor vary 250-fold between the weakest (4-epi-7-chloro-Tc) and strongest (anhydro-Tc) binders. Small substituents at position 7, like the chloro function, seem to support the interaction with the repressor slightly, as evidenced by chloro-Tc. However, the larger azido group at this position leads to a slight reduction in affinity for the Tet repressor. It was concluded that substituents in this size range show only minor interference with repressor binding, indicating that this position is not in contact with the protein. Minocycline carries a dimethylamino function at position 7 and showed a reduced binding to Tet repressor. Unfortunately, we were not able to determine the binding constant precisely because we did not obtain F_3 (see Materials and Methods) for this complex. Taken together, position 7 of Tc may be close to the protein but is not in contact with it.

Tc contains an OH and a methyl group at position 6 which are removed in 6-deoxy-6-demethyl-Tc. This increases binding to the Tet repressor only slightly. This result indicates that substituents at this position do not contribute a lot to the specific binding of the drug to this protein. The same holds true for oxy-Tc, which has an additional OH group at position five which does not affect the K_A in comparison with the K_A of Tc. These conclusions are supported by the only slightly increased affinity of doxycycline for the Tet repressor (Fig. 1). Doxycycline was included in a previous study that reported the inducing capacities of various Tc's (17). The result obtained in that study agrees well with the increased binding seen in this one. However, it should be considered that in vivo induction requires uptake of the drug in addition to binding to the Tet repressor. The affinity for 4-epi-7-chloro-Tc, in which the conformation of the substituents at position four was reversed, showed a roughly 30-fold reduction in repressor affinity compared with that for 7-chloro-Tc. This analog had the lowest affinity of the ones studied here except for that of minocycline. Considering the large change in structure brought about by moving the dimethylamino group from the α face side of the molecule to the β face, this 30-fold reduction in affinity indicates that position four is important for repressor recognition. The association rate constants of both Tc derivatives with the Tet repressor are the same, about $(1.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} (primary data not shown; determined as described previously [16]). This may indicate that the conformations of the Tc derivatives are similar. Thus, the reduction of the equilibrium association constant should result from an unfavorable interaction of the dimethylamino group in the 4-epi-Tc conformation with the Tet repressor. In conclusion, the substituents at positions seven, six, and five do not seem to be involved in specific interactions with the Tet repressor. Introduction of larger substituents at position seven or rearrangement of substituents at position four lead to clear reductions in repressor affinities, indicating that these positions are located near the protein surface in the complex. This conclusion agrees well with the interpretation of fluorescence data which indicated that Tc is bound mainly in the interior of the protein (16).

The largest change of affinity observed in this study was the 33-fold increase in K_A for anhydro-Tc compared with that for Tc. This modification involved the loss of the 6-OH group, which should not have had any effect (compare above), and an additional double bond in the C ring, which changed the keto-enol tautomerism at O-11 to the enol form, yielding an aromatic C ring. It is very likely that the structural change at C-11, O-11, caused the increase in K_A. This would indicate that the OH at position 11 interacts more favorably with the Tet repressor than the keto oxygen at position 11 does. On the basis of these results, we propose that recognition of Tc by the Tet repressor involves the oxygen at position 11. However, the planarization of the C^- ring and the increased hydrophobicity may also contribute to the change in K_A. In order to test this, the association rate constant for formation of the ternary complex from Tc · Mg^{2+} and anhydro-Tc has been determined and compared with that of Tc (16). It turned out to be $(3.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, about six times faster than the one determined for Tc (16). This may indicate that andydro-Tc must undergo fewer conformational changes in order to assume the structure bound by the Tet repressor. Thus, the planar conformation of anhydro-Tc may be more favorable for interaction with the Tet repressor. Taken together, the changes at O-11 and the planarization of the C ring probably contribute to the increased recognition of anhydro-Tc by the Tet repressor compared with recognition of Tc.

The results presented here indicate that the increased induction of Tc resistance seen by anhydro-Tc (3) results from the stronger binding of this drug to the Tet repressor, which is in contrast to the weak antibiotic activity of this compound (14). This may indicate that ribosomes and the Tet repressor use different recognition modes for the drug. The same observation has been made previously for doxycycline (17).

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