Engineered Tet Repressor Mutants with Single Tryptophan Residues as Fluorescent Probes

SOLVENT ACCESSIBILITIES OF DNA AND INDUCER BINDING SITES AND INTERACTION WITH TETRACYCLINE*

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Mutants of the Tn10-encoded Tet repressor containing single or no tryptophan residues were constructed by oligonucleotide-directed mutagenesis. The Trp-75 to Phe exchange reduces the dissociation rate of the complex with the inducer tetracycline by a factor of 2. The Trp-43 to Phe exchange has no effect on inducer binding. The fluorescence emission spectra of both tryptophan residues are quenched to a different extent by binding of tetracycline: Trp-75 is quenched to zero and Trp-43 to only 50%. It is concluded that Trp-75 is in the vicinity of the inducer binding site. The different fluorescence emission spectra of both tryptophan residues depend on the native structure of Tet repressor. Quenching studies with iodide indicate that the DNA binding motif is solvent exposed in free repressor and moves towards the interior of the protein upon inducer binding. The inducer binding site is in the interior of the protein. The fluorescence of tetracycline is enhanced upon binding to Tet repressor. The excitation at 280 nm results mainly from the change in environment and in part from energy transfer from tryptophan to the drug.

Furthermore, a new excitation band in the complex has been characterized which was interpreted as energy transfer from Trp to Tc in the complex (11).

A detailed interpretation of the fluorescence data with respect to functional domains of Tet repressor was hampered by the fact that the primary structure of the protein contains 2 tryptophans (12). One of them is located at position 43 forming part of the proposed DNA binding motif (13) and the other is at position 75. In order to distinguish between fluorescence effects of these 2 tryptophans upon ligand binding, we have constructed mutant Tet repressors with either one or both of the tryptophans replaced by phenylalanine. The mutant proteins were overexpressed, purified to homogeneity, and characterized with respect to tet operator binding. It was found that Trp-43 is involved in a sequence-specific contact to tet operator DNA while Trp-75 is not (14).

In this article we describe the solvent accessibilities of the operator and inducer binding motifs of Tet repressor, evaluate the effect of Trp to Phe exchanges on inducer binding, and quantify the fluorescence of each Trp in the respective repressor-inducer complexes.

MATERIALS AND METHODS

General Methods—All chemicals were of the highest purity available purchased from either Merck (Darmstadt), Serva (Heidelberg), Fluka (Buchs), Sigma, or Roth (Heidelberg). Tetracycline hydrochloride was from Fluka (Buchs) and was used without further purification. The concentration of tetracycline was determined in 0.1 M HCl using $\varepsilon = 13,920$ M$^{-1}$ cm$^{-1}$ (11). The wild type and mutant Tet repressor proteins were purified and characterized as described (14). The concentrations of Tet repressor solutions were determined by fluorescence titration with tetracycline as described (11). The reproducibility of this determination was 10% in repeat experiments. The concentrations of Tet repressor given in this article are based on this experiment and refer to the dimer. Absorption spectra were recorded using an Ulvon 810 spectrophotometer from Kontron operating at 2 nm band width.

Association Rate Determinations of Tet Repressor-Tetracycline Complexes—Association rate experiments of wild type and mutant Tet repressor-tetracycline complex formations were performed monitoring fluorescence changes as described (11). Equal volumes of solutions containing 0.09 $\mu$M Tet repressor or 0.2 $\mu$M tetracycline in 200 mM NaCl, 20 mM MgCl$_2$, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 7 mM $\beta$-mercaptoethanol were mixed at 27°C and the fluorescence emission at 510 nm excited at 370 nm was recorded for several minutes. The rate constants were determined assuming a bimolecular reaction as described (11).

Dissociation Rate Determinations of Wild Type and Mutant Tet Repressor-Tetracycline Complexes—Measurements of the dissociation rates of Tet repressor-tetracycline complexes were done by the nitrocellulose filter binding technique as described (10). 0.33 $\mu$M Tet repressor was incubated with 0.33 $\mu$M [$^3$H]tetracycline (specific activity 0.55 Ci/mmol) in a total volume of 1.35 ml containing 200 mM NaCl, 20 mM MgCl$_2$, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 7 mM

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1 The abbreviation used is: Tc, tetracycline.
β-mercaptoethanol, and 50 μg/ml bovine serum albumin for 15 min at 37°C. At the time t = 0, the reaction mixture was made 140 μM in nonlabeled tetracycline and incubated at 37°C for times indicated in Fig. 3. Then 100 μl were filtered over nitrocellulose as described (10).

Fluorescence Spectroscopy—All fluorescence measurements were done on an Aminco SPS500TM ratio fluorometer. No further correction for wavelength dependence of lamp intensities was done. The absorption of the samples was in all cases below 0.2. Therefore, no correction for the inner filter effect was done. For excitation at 280 nm a WG305 cutoff filter was used. Unless described otherwise all fluorescence measurements were performed in 5 mM MgCl₂, 5 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, and 0.1 mM dithiothreitol at 20°C. The fluorescence emission spectra of native Tet repressors were measured at a concentration of 0.1 μM. Excitation was at 280 nm in 1 x 1-cm quartz cuvettes. The slit width for excitation and emission was 8 nm. The fluorescence emission spectra of denatured Tet repressors were recorded at a concentration of 0.5 μM in 6 M guanidinium hydrochloride, pH 5.5. All other conditions were as described above. Fluorescence emission and excitation spectra of the Tet repressor-tetracycline complexes were measured at a concentration of 0.5 μM. Slit widths for excitation and emission were 1 and 20 nm, respectively, in excitation spectra and 4 and 8 nm, respectively, in emission spectra. Excitation spectra were not corrected to an external standard; however, fluctuations of the lamp intensity were internally corrected in the ratio mode. In order to compare the excitation spectra around 280 nm it was assumed that excitation quantum yield is identical for tetracycline in the complexes with the wild type and mutant Tet repressors. The measured fluorescence yields at 370 nm differed within 15% both in repeat experiments and among the mutant complexes. This is assumed to represent nonsystematic errors. The excitation spectra in Fig. 5B were normalized to the same quantum yield at 370 nm.

Quenching of Fluorescence Quantum Yield with Potassium Iodide—Fluorescence emission spectra in the presence of various concentrations of potassium iodide were measured with a slit width of 5 nm. Excitation was at 280 nm (slit width 8 nm) with a cuvette width of 0.4 cm. The length of the quartz cuvettes was 1 cm. Experiments were done at 20°C in the buffer described above with 200 mM potassium ions which were derived from variable amounts of potassium iodide as indicated in the respective figures and potassium chloride. The concentration of the native proteins was 0.5 μM. Addition of potassium iodide to F43 and F75 reduced fluorescence emission to the same extent at all wavelengths. Therefore, the amplitudes at the wavelengths of maximal emission were used to calculate the quench. To account for differences between the 2 tryptophans in wild type Tet repressor, the two wavelengths indicated in Fig. 24 were used for this calculation. The quench is expressed as the ratio of the fluorescence emission spectra of wild type, F75, and F43 Tet repressors are shown. The fluorescence spectra were recorded at identical concentrations of the three proteins. The fluorescence is given in arbitrary units.

RESULTS

Solvent Accessibilities of the Tryptophan Residues—The wild type Tet repressor contains 2 tryptophan residues at positions 43 and 75. To use these as fluorescent probes, each of them was replaced by phenylalanine. The respective mutants were called F43 and F75. The double mutant containing no tryptophan was constructed for control experiments and called F43F75 (14). It has been shown previously that the fluorescence emission spectra of the 2 tryptophans in Tet repressor are different. Analysis of the data suggested that the Trp-43 (DNA binding motif) is solvent-exposed, while the Trp-75 is buried in the hydrophobic interior of the protein (14). The tryptophan residues at the same position in the wild type and mutant proteins had identical spectra (14). This result motivated us to confirm the different local environments of both Trp residues experimentally by solvent accessibility studies.

Fig. 1A shows the fluorescence emission spectra of the wild type, F43, and F75 repressors. Fig. 1B shows the fluorescence spectra of the same proteins under denaturing conditions in 6 M guanidinium hydrochloride. While the spectra in Fig. 1A show different emission maxima, both tryptophans have their maximal emission at the identical wavelength of 355 nm under denaturing conditions. This observation indicates that the different spectral properties of Trp in the native proteins indeed result from the different local environments of the residues.

Fig. 2A shows the quench of fluorescence emission by increasing concentrations of iodide for the wild type and mutant proteins (15). The values of all Stern-Volmer constants are given in Table I. The absolute numbers of the constants in Table I depend on the evaluation of the spectral data (see “Materials and Methods”); however, the ratios of the constants do not. It is clear, that the Trp-43 (F75) is accessible for iodide, whereas the Trp-75 (F43) is not. The comparison with tryptophan accessibilities in other proteins was filtered over nitrocellulose as described (10).
Table 1

| Tet repressor | Native/wavelength | Denatured | Complexed with Tc |
|---------------|------------------|-----------|------------------|
|                | K<sub>q</sub>     | μ<sup>-1</sup>/m<sup>2</sup> | μ<sup>-1</sup>/m<sup>2</sup> |
| Wild type     | 10.2/360         | 4.1       | 7.5              |
| F43           | 1.9/320          |           |                  |
| F75           | 0.5/328          | 7.3       | nf<sup>6</sup>   |
| F75                                                     | 17.6/352        | 5.4       | 7.0              |

* Measured at the wavelength of maximal emission = 352 nm.

The excitation was at 280 nm in all cases.

**TABLE I**

**Stern-Volmer constants for potassium iodide-induced quenching of fluorescence emission**

**Fig. 2.** A, quench of fluorescence quantum yields of native repressors versus the concentration of KI. The ratio of fluorescence intensities without KI (F<sub>0</sub>) over the fluorescence at the indicated KI concentration (F) is plotted versus the KI concentration (see "Materials and Methods"). The effects on the proteins with single tryptophans (F43 and F75) were measured at the respective maximal emission wavelengths and compared with those on the wild type repressor at the two wavelengths indicated in the figure. The open circles indicate the KI concentrations used to measure fluorescence spectra. B, quench of fluorescence quantum yields of denatured repressors versus the concentration of KI. The wild type, F43, and F75 repressors were denatured in 5 M guanidinium hydrochloride prior to the measurement. The denotations are the same as in A.

reveals that Trp-43 belongs to the group of maximally solvent exposed residues while Trp-75 is completely buried (16).

The wild type repressor emission spectrum is the sum of both tryptophan spectra (see Fig. 1A). For comparison with the mutants, the Stern-Volmer plots shown in Fig. 2A were derived from the fluorescence at 320 and 360 nm. The former contains mostly contributions from Trp-75 and the latter mostly contributions from Trp-43. However, the fluorescence of the respective other Trp is not negligible at these wavelengths (see Table 1). However, it is quite clear that they exhibit very different accessibilities for iodide. The Trp residues at identical positions, on the other hand, are similarly accessible for iodide in the wild type and mutant proteins. Taken together these quenching studies support the conclusions that Trp-43 is maximally solvent-exposed, while Trp-75 is buried in the interior of the protein structure, and that the tryptophan residues at both positions have identical environments in the wild type and mutant Tet repressors.

Fig. 2B shows the Stern-Volmer plots for the denatured wild type and mutant Tet repressors. According to the results shown in Fig. 1B the same wavelength of maximal emission is used for this determination for all proteins. Fig. 2B indicates that the huge differences in accessibility of the Trp residues found in the native proteins have disappeared upon denaturation. Thus, they depend on the intact native structure of Tet repressor. The respective slopes are also given in Table 1. It may be noted that they are still different, which is within the experimental error.

The F75 Tet repressor contains the single Trp-43 located in the DNA binding motif (13, 14). This may serve as a fluorescent probe for possible structural alterations induced by binding of Tc. The accessibility of Trp in this mutant and in the wild type was measured in the complex with Tc. The results are shown in Fig. 2C. For both complexes the fluorescence at the wavelength of maximal Trp emission was used...
for determination of the Stern-Volmer constants given in Table I. The accessibility of Trp-43 in the complex with Tc is reduced to a great extent when compared to that in the noncomplexed proteins. This result suggests that Trp-43 in the DNA binding motif of the Tet repressor undergoes a conformational change upon binding of the inducer Tc. The same result is also obtained from the wild type Tet repressor complexed with Tc (see Fig. 2C). In this case, the fluorescence of Trp-75 is quenched to nearly zero (compare below). Therefore, the observed fluorescence should originate only from Trp-43. In agreement with this assumption, the slope of iodide quenching in Fig. 2C is nearly the same in the wild type and F75 repressors. This result suggests an identical structural environment for Trp-43 also in both protein-Tc complexes.

Interaction of Wild Type and Mutant Tet Repressors with Tc—It has been shown previously by gel mobility experiments that tet operator binding of the mutant Tet repressors is inhibited in the presence of Tc indicating that all mutants are able to recognize the inducer (14). In order to characterize inducer binding to the mutants quantitatively we determined the dissociation and association rate constants. The dissociation rate constants were measured as described previously by nitrocellulose filter binding (9). The results are summarized in Table II. The dissociation rates for the wild type and F43 repressor complexes with Tc are identical and agree with the values reported previously for the wild type (9). The dissociation rates of the F75 and F43F75 complexes with Tc are slower by a factor of 2. The difference between the latter two complexes is too small to be interpreted (see Fig. 3 and Table II). These results indicate that replacement of Trp-75 by Phe results in a kinetic stabilization of the Tet repressor-inducer complex.

The association rates of wild type and mutant Tet repressors with Tc have been measured as described (11). The results are also given in Table II. The wild type and mutant Tet repressors have identical association rate constants for complex formation with Tc. Assuming the simple reaction

\[ R + Tc \rightleftharpoons R \cdot Tc \]

where \( R \) is the concentration of free repressor, Tc the concentration of free Tc, and \( R \cdot Tc \) the concentration of the complex, the rate constants have been used to calculate the respective equilibrium constants given in Table II. The exchange of Trp-75 to Phe increases the affinity for Tc by a factor of 2. Although this is not a large change, it indicates that Trp-75 of Tet repressor is in the vicinity of the Tc binding site. Replacement of Trp-43 by Phe does not influence Tc binding at all, indicating that the DNA binding motif is not involved in inducer binding. We assume that the DNA and Tc binding sites of Tet repressor are located in different motifs.

Fluorescence Spectra of Wild Type and Mutant Tet Repressors with Tc—The fluorescence emission spectra of the wild type and mutant Tet repressors alone and complexed with Tc are shown in Fig. 4. The data for the wild type complex have been analyzed in detail previously (11) and were measured here for comparison. It should be restated that binding of Tc leads to about 60 to 70% quench of Tc fluorescence emission (see Fig. 4). The fluorescence of the F75 mutant is quenched by about 50% and that of the F43 mutant by 100% upon binding of Tc. The F43F75 double mutant defines the background of fluorescence resulting from contaminating proteins which are not affected by Tc. The fluorescence of the Trp-75 is twice as much affected as that of the Trp-43 by complex formation with Tc. This result also supports the assumption that Trp-75 is part of or nearby the Tc binding pocket.

The second effect of complex formation is the increase of Tc fluorescence at 510 nm. This fluorescence emission can be excited at 280 nm which has been interpreted as a possible energy transfer from Tc to Tc (11). The Tet repressor mutants were used to test for energy transfer. The fluorescence emission spectra of the repressor-Tc complexes in Fig. 4 indicate that Tc fluorescence at 510 nm is enhanced in all four complexes compared to that of free Tc (11). Since the F43F75 double mutant does not contain Trp, the Tc fluorescence of this complex must be entirely due to an increase in quantum yield resulting from the changed environment of Tc in the complex. Thus, the enhanced emission of Tc at 510 nm excited at 280 nm cannot be due to energy transfer alone. However, when the quantum yields of the 510 nm emission bands from the four complexes are compared, the wild type complex clearly shows the highest while the F43F75 complex shows the smallest one and the mutants with the single Trp residues fall in between. The quantum yield of Tc fluorescence in the F43 complex is higher than that in the F75 complex.

This result is confirmed by the excitation spectra of free Tc and the Tc-repressor complexes shown in Fig. 5A. Tc fluorescence can be excited around 280 and 370 nm in these complexes (11). The quantum yield of Tc excitation at 370 nm is identical for all four complexes. It is concluded that

![Fig. 3. Dissociation rates of wild type and mutant Tet repressor-tetracycline complexes.](image)

![Fig. 4. Fluorescence emission spectra of wild type and mutant Tet repressor-tetracycline complexes.](image)
**Trp Fluorescence of Tet Repressor-Inducer Complexes**

FIG. 5. A, absorption spectra of wild type and mutant Tet repressors. The molar extinction is shown as a function of the wavelength. The denotations are given in the figure. B, fluorescence excitation spectra of wild type and mutant Tet repressors complexed with Tc and of free Tc. The fluorescence emission was recorded at 510 nm. The concentrations of the protein-Tc complexes were identical. The spectra were normalized to quantum yield at 510 nm (see "Materials and Methods"). The denotations are given in the figure. Fluorescence is given in arbitrary units.

only the polarity change of the Tc environment in the complexes compared to free Tc causes this increase in quantum yield. The excitation around 280 nm, however, leads to quite different quantum yields for the emission at 510 nm (compare also Fig. 4). Since these spectra were recorded on the same day under identical conditions, their intensities may be compared. The Tc excitation at 280 nm is least efficient in the wild type Tet repressor-Tc complex. In the wild type repressor, both Trp residues contribute to the energy transfer. As a result, the Trp fluorescence is further enhanced by energy transfer from Trp to Tc. This process depends on the geometry of the Trp-Tc arrangement in the complex and is, therefore, different for the F43 and F75 complexes with Tc. The wild type repressor, both Trp residues contribute to the energy transfer. As a result, the increase of intensity of Tc excitation is the same obtained with both single Trp mutants (compare Fig. 5B). These results suggest that the excitation band around 280 nm in the wild type Tet repressor-Tc complex consists of contributions from enhancement of the intrinsic Tc fluorescence and energy transfer from both Trp residues to Tc.

Further support for the contribution of energy transfer to the excitation spectra around 280 nm is derived from a comparison of the excitation spectra with the absorption spectra of the proteins displayed in Fig. 5A. The F43F75 protein has the lowest absorption, the F43 and F75 mutants have roughly the same absorption, and the wild type containing 2 Trp residues has the highest absorption. The typical shoulder at 290 nm of the Trp absorption spectrum is clearly present in the wild type spectrum. To a lesser extent it is present in the mutants with single Trp residues and it is absent in the F43F75 complex. Comparison of the F43 and F75 spectra reveals that F43 has a more pronounced shoulder at 290 nm.

The same spectral properties are found in the fluorescence excitation spectra shown in Fig. 5B. The F43F75 complex shows no shoulder at 290 nm. The F43 complex has a more pronounced shoulder in excitation of Tc emission as the F75 complex. Furthermore, while the difference in absorption of the F43 and F75 proteins is small at 280 and 290 nm the excitation spectra of the F43 and F75 complexes with Tc show greater differences at these wavelengths. The Trp-75 has a greater efficiency of energy transfer compared to Trp-43. The wild type complex with Tc exhibits the most pronounced shoulder at 290 nm in the excitation spectrum.

It should be noted that the measured extinction coefficients displayed in Fig. 5A are greater than calculated from the content of aromatic amino acids. We attribute this to contaminating proteins.

**DISCUSSION**

The Tet repressor proteins from four classes of Tc resistance determinants contain 2 tryptophan residues at strictly conserved positions (17). In this article we demonstrate that the position-dependent differences of fluorescence spectra of the tryptophan residues result from their different environments by comparing the spectra of the native and denatured proteins. The results (Fig. 1) indicate clearly that the characteristic fluorescence emission of the tryptophan residues depends on the intact structure of the native protein and on the position on Trp in it. The spectral properties indicate that Trp-75 is in a hydrophobic and Trp-43 in a hydrophilic environment. The 2 tryptophan residues exhibit very distinct accessibilities for the low molecular weight fluorescence quencher iodide (see Fig. 2). The Trp-43 is as accessible for iodide as would be free tryptophan while the Trp-75 belongs to the class of tryptophans located in a maximally hydrophobic environment (see Table I) (16). Very similar results are found for the tryptophan residues in the wild type Tet repressor. Denaturing the Tet repressor proteins renders all tryptophans similarly accessible for iodide, confirming that the native structure determines the different environments for these residues.

Of particular interest is the behavior of the DNA binding motif in the presence or absence of inducer since this is the molecular switch for gene regulation. The single Trp-43 in F75 repressor is a fluorescent probe in the operator binding site. While this is easily accessible for iodide in the free repressor, the accessibility is greatly reduced in the repressor-inducer complexes formed from the wild type or F75 repressors (see Fig. 2C and Table I). Inspection of the emission spectra in Fig. 4 reveals that this is accompanied by a small blue shift of 5 nm of the tryptophan emission maximum in the F75-Tc complex. This may indicate a more hydrophobic environment of Trp-43 in the inducer complex. Therefore, we assume that the operator recognition site in the noncomplexed Tet repressor is maximally solvent-exposed and accessible for DNA on the surface of the tertiary structure. Binding of the inducer Tc leads to a change of that structure resulting in a reduced exposition of the DNA binding site. In this state it is not able to recognize the tet operator anymore (9).

Quantitative analyses of the mutant Tet repressors reveal that Trp to Phe exchanges do not influence Tc binding to a great extent. The association rate constants (Table II) are
Trp Fluorescence of Tet Repressor-Inducer Complexes

identical, indicating that Trp is not involved in the rate-limiting step of drug binding. Nevertheless, while the F43 and wild type repressors have identical dissociation rates of their complexes with Tc the substitution of Trp75 with Phe results in a slight stabilization of the complex. This indicates that Trp-75 is somehow part of the Tc binding motif of Tet repressor. This agrees well with the previous finding that Tc binds to the hydrophobic interior of the protein (11) because Trp-75 is located in a hydrophobic environment in the interior of Tet repressor. While Trp-43 is clearly essential for tet operator binding (14) it is not essential for or even involved in Tc binding. We conclude that operator and inducer bind to different motifs of Tet repressor.

In contrast to the small quantitative effects on Tc binding, the fluorescence emission quantum yield of the tryptophan residues is greatly affected by the drug. In accordance with their effect on binding the Trp-43 fluorescence is less quenched than that of Trp-75.

It has been assumed before that energy transfer from Trp to Tc contributes to the excitation of Tc at 280 nm (11). The enhanced quantum yield of fluorescence emission excited at 280 nm in the complex of F43F75 with Tc must result from an increase in quantum yield of the Tc fluorescence. This is possible because free Tc can be excited at 280 nm with a very low efficiency (see Fig. 5B). The results in Fig. 5B indicate that both increased quantum yield and energy transfer contribute to Tc excitation at 280 nm. In particular, the shoulder around 290 nm in the excitation spectra of the wild type, F43, and, to a lesser extent, F75 complexes with Tc lends support to this hypothesis. The energy transfer from Trp-75 seems to be more efficient than the one from Trp-43. This agrees well with their quantitative effects on binding of Tc.

In summary, the results presented in this article indicate that the fluorescence properties of Trp in Tet repressor depend on their position and the native structure of the protein. In functional Tet repressor Trp-43 is solvent-exposed and Trp-75 is buried. Binding of inducer leads to an allosteric change of the conformation resulting in a less exposed DNA binding motif. Trp-75 is part of or near the Tc binding site which is located in a different part of the protein compared to the DNA binding motif.

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