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A direct photo-activated affinity modification of tetracycline transcription repressor protein TetR(D) with tetracycline[1]

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Abstract Results of a first successful application of a direct photo-induced affinity modification of Tet repressor (TetR(D)) protein with tetracycline within a complex of known three-dimensional structure are described. The conditions of the modification have provided suitable yields of the modified complex and allowed characterization of the modified segments of the protein. The potential of tetracycline as a fine modifying reagent was established. In the complex of TetR(D) protein with tetracycline, the antibiotic modifies at least two segments, Ile59–Glu73 and Ala173–Glu183, which form a binding tunnel for the drug according to the X-ray analysis. These data open possibilities for the use of different tetracycline targets for structural studies in solution.

Key words: Tetracycline; Transcription repressor protein TetR(D); Photo-activated affinity modification; Protein sequencing; Binding site

1. Introduction

Tetracyclines (Tc) are a group of antimicrobial agents with a broad spectrum of activities. They have been widely used in the treatment of bacterial infections since their discovery at Lederle Laboratories in 1945 (cited from [1]). In recent years the emergence of Tc resistances among clinically important microorganisms have imposed severe limitations to the use of these antibiotics. Therefore the search for new antibiotics of that group remains a significant goal (for review [2–3]).

Bacterial sensitivity to Tc is mainly due to its inhibition of protein biosynthesis, the main target of Tc being the ribosome ([5] and references therein). The bacterial resistance to Tc is mainly determined by two mechanisms. In one, an active export (efflux) of Tc out of the cell by the membrane-intrinsic protein TetA decreases its intracellular concentration [2,3,6]. The other mechanism involves EF-G-like proteins that confer ribosome protection ([5] and references therein).

One of the key steps in new drug development is the determination or modeling of the three-dimensional structure of the drug–target complex. X-ray analysis and nuclear magnetic resonance are the major modern direct techniques to elucidate the structures of macromolecules and their complexes at atomic resolution. However, the application of these methods to different Tc–target complexes is not feasible at present as the structures of the ribosome or membrane-bound Tc transporter protein TetA are not known. In addition, the X-ray method cannot be applied to solutions. Therefore, complementary approaches have to be developed to study the active center of Tc targets in solution. Besides site-directed mutagenesis, photo-activated affinity modification is one of the most powerful techniques, which can be used to study a ligand binding center. The rapid development of high-sensitive mass spectrometry for partial protein sequencing provides a solid basis for achieving peptide level resolution by affinity modification [7–10].

To obtain relevant results by photo-induced affinity modification with a selected reagent, it is of great value to apply this approach to a reagent–target complex with already known three-dimensional structure. In the case of Tc, a repressor class D (TetR(D)) protein is the best choice to probe Tc as a direct photo-activating affinity reagent. TetR(D) is the prokaryotic transcription repressor protein, which regulates transcription of both its own gene and the gene for Tc transporter protein TetA [2,3]. The structures of the complexes of TetR(D) protein with several Tc derivatives have recently been published at high level resolution [11–15].

Due to the conjugated system of B–C–D rings (Fig. 1) the Tc molecule has an absorption band at about 365 nm [4,16]; neither proteins nor nucleic acids absorb at this wavelength. This is the main reason for the application of a direct photo-induced covalent modification of a protein with Tc to identify peptides in the vicinity of its binding center. Indeed, several attempts have already been made to apply this approach to the Tc–TetA protein complex [17], as well as to Tc–ribosome complexes [18–20]. However, under the irradiation conditions used, the yield of modification was just sufficient to reveal the sole fact of modification of the target, but not high enough to perform further analyses [17–19]. Using a 500 W high power Hg arc lamp for irradiation, Oehler et al. [20] achieved a good yield of modification in a reasonable time and identified some rRNA residues photo-modified with Tc within the ribosome.

This publication describes the results of the first successful

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[1] The paper is dedicated to Alexander A. Krayevsky.

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application of direct photo-induced affinity modification of TetR(D) protein with Tc within the complex of a known three-dimensional structure. The modification conditions provided a suitable yield of the modified protein that allowed the search for modified segments of the protein and exploration of the potential of Tc as a specific modifying reagent. In the complex of TetR(D) protein with Tc, the antibiotic modifies at least two polypeptide segments, which are part of the binding tunnel for the drug according to the X-ray data [14].

2. Materials and methods

2.1. Materials

TetR(D) protein was purified as already described [21]. The protein stock solution was 33 mg/ml in a buffer containing 20 mM Tris–HCl, pH 8.0; 200 mM NaCl. 7-[^{3}H]Tc, specific activity 18 GBq/mmol, was from New England Nuclear, USA.

Buffer A: 50 mM Tris–HCl, pH 8.0; 5 mM $MgCl_2$, 150 mM NaCl, 2 mM dithiothreitol [22].

2.2. Nitrocellulose filter-binding assay for [^{3}H]Tc binding with the protein TetR(D)

In one series of experiments, 120 pmol of TetR(D) protein was titrated with increasing amounts of [^{3}H]Tc (0–600 pmol) in 200 μl buffer A. The mixture was incubated for 10 min at 25°C and, after chilling on ice, applied onto a nitrocellulose membrane (Sartorius 113-06-N, 0.45 μm); the filtration rate was 1 ml/min. The membrane was washed three times with 0.5 ml buffer A and dried. The amount of bound Tc was heterogeneously counted as [^{3}H] radioactivity of the membrane in 5 ml toluene scintillation fluid (GS-106, Russia), Tracor Analitic scintillation counter (France).

In a series of 'reverse' experiments, 20 pmol [^{3}H]Tc were titrated with increasing amounts of TetR(D) protein (0–120 pmol) under conditions described above.

2.3. Measurements of Tc photolysis rate

Most experiments used a 250 W high power Hg arc lamp (DRSh-250, PhysPribor, Russia) with an emission maximum near 365 nm. Tc photolysis was performed in buffer A, using a 313 nm cut-off plastic cell with 10 mm optical path (Sarstedt, Germany). Samples had A_{365} in the range of 0.5–1.0 and were positioned 25 cm away from the lamp. During the course of irradiation, absorption spectra were recorded every 20 s using a Specord UV–VIS spectrophotometer (Germany).

In some experiments a 40 W electrical power Osram HQE Hg lamp (Germany) was used with emission maximum at 365 nm. The irradiation period was longer (2–3 h) and the distance between the lamp and the sample was 10 cm.

2.4. Direct photo-induced affinity modification of TetR(D) protein with [^{3}H]Tc

[^{3}H]Tc (0.85 μM) and 6.7 μM of TetR(D) protein were incubated in 2.16 ml of buffer A. The efficiency of complex formation was checked for 100 μl aliquots by the filter-binding assay. Two ml of the same solution were irradiated with the DRSh-250 lamp for 10 min at 0°C, as described above. The modified protein was isolated by reverse phase-high performance liquid chromatography (RP-HPLC) using a RP-300 BrownLee Labs C_8 column (Gilson 802-C chromatograph, France), flow rate 0.5 ml/min, elution with 10–75% acetonitrile gradient in 0.1% TFA. The eluate was monitored at 214 nm and the radioactivity of the collected fractions was measured by homogeneous scintillation counting after drying and re-dissolving in water as 100 μl aliquots in 5 ml dioxane scintillation fluid (GS-8, Russia).

2.5. Peptide sequence analysis

The fractions containing the modified protein were concentrated to dryness using a Speedvak concentrator (Savant, USA), dissolved in 200 μl 25 mM NH_4HCO_3, pH 7.8; and digested with protease V8 (Glu-C, kindly provided by A. Musolyamov, IMB, RAS) at the enzyme–substrate ratio 1:30 for 17 h at 25°C. The peptides were separated on the C_8 column by two steps of linear acetonitrile/0.1% TFA gradients: 0–40%, 90 min and 40–60%, 45 min. The eluate was monitored at 214 nm. Fractions with labeled peptides were concentrated to dryness and sequenced by Edman degradation, using a Knauer 816 automatic gas liquid phase micro-sequencer (Berlin, Germany) as described by the manufacturer with small modifications.

3. Results and discussion

Tc has already been used as a direct photo-activated affinity reagent for TetA protein [17] and ribosomes [18–20]. Under the irradiation conditions used, the yield of modification was just sufficient to reveal the modification of the target. Considering the applied conditions and the results of these experiments the affinity modification might not have been optimal for at least two reasons: (i) the Tc–target ratio might have been unfavorable and (ii) the power of the irradiation source might have been too small.

To achieve a maximal degree of complex formation for Tc with the ribosome, very high Tc to ribosome ratios have been used previously, namely a ratio of 20:1 [19], or 300:1 [20]. These ratios could yield artifacts because of the possibility of non-specific binding of Tc ([23] and our own results). In addition, large amounts of Tc strongly decrease an efficient photo-inducible modification, because almost all light quanta are absorbed by the free Tc, but not by the molecules within the complex.

For optimizing parameters essential for TetR(D)–Tc complex formation, we performed a careful study of pseudo-equilibrium binding, employing a nitrocellulose filter-binding assay. For an initial series of experiments a general protocol was applied based on the titration of a constant amount of TetR(D) protein with increasing amounts of [^{3}H]Tc [23]. It was shown that the molar excess of Tc over protein leads to additional non-specific binding of the drug, as the molar stoichiometry of the complex is 1:1 [11] (Fig. 2A). This had already been noticed by Hillen et al. [23], though their binding isotherm is different from ours, probably caused by small differences in the experimental conditions.

To avoid a large excess of Tc over the target, 'reverse binding' experiments have been performed. An exact amount of [^{3}H]Tc was titrated with increasing amounts of TetR(D) protein. In these experiments a rather high level ($\sim$60%) of binding was achieved. Saturation was reached at a Tc:TetR(D) ratio of 1:1, and at reasonable concentrations (Fig. 2B). As had already been noticed by Hillen et al. [23], the maximal level of binding of radioactive Tc did not yield 100%, probably because the concentration of active Tc in the radioactive sample able to bind to TetR(D) protein is only 60%.

As mentioned above, the yield of direct photo-activated

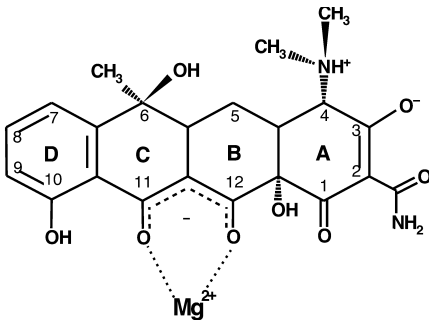

Fig. 1. Chemical structure of the $[MgTc]^+$ complex, which occurs under physiological conditions. A, B, C and D denote the four rings of Tc.

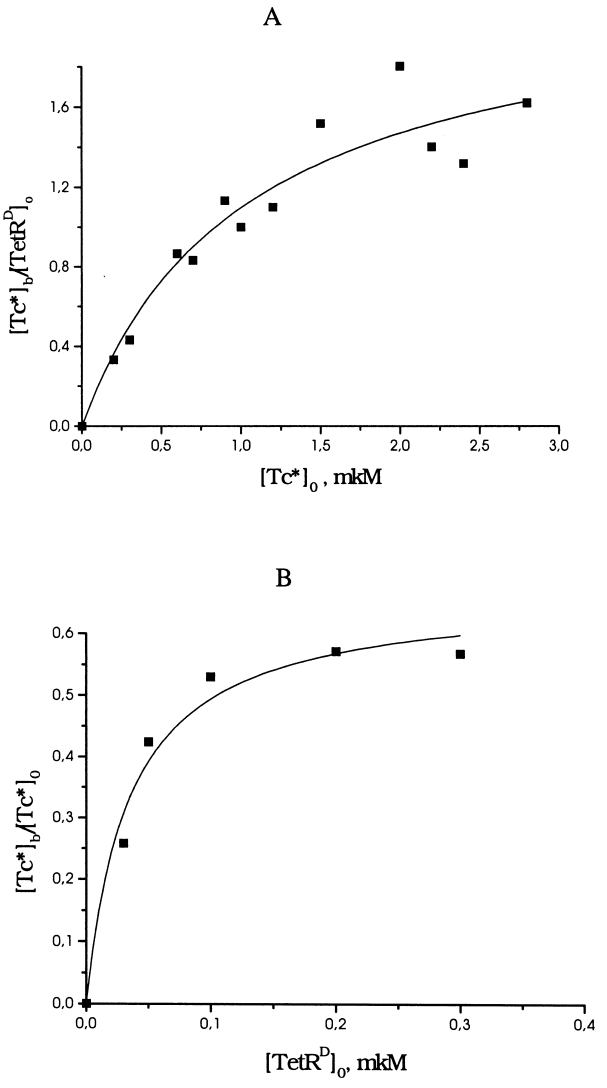

Fig. 2. Nitrocellulose filter-binding isotherms for 7-[^{3}H]Tc with TetR(D) protein. A: 0.6 μM protein is incubated with an increasing amount of Tc. B: 0.1 μM Tc is incubated with an increasing amount of the protein. $[Tc^*]_0$ and $[Tc^*]_b$ are initial and bound radioactive Tc concentrations, respectively. $[TetR^D]_0$ is the initial TetR(D) protein concentration.

affinity modification for the 30 W lamp is rather low [17–19]. To determine optimal irradiation conditions, the Tc photolysis rate was studied at different photon flow intensities. Two different irradiation sources, both with emission around 365 nm, have been used. The Tc photolysis rate was monitored by measuring the decrease of absorption at A_{365}. The widely used 40 W Osram lamp [18,19] was tested, yielding a

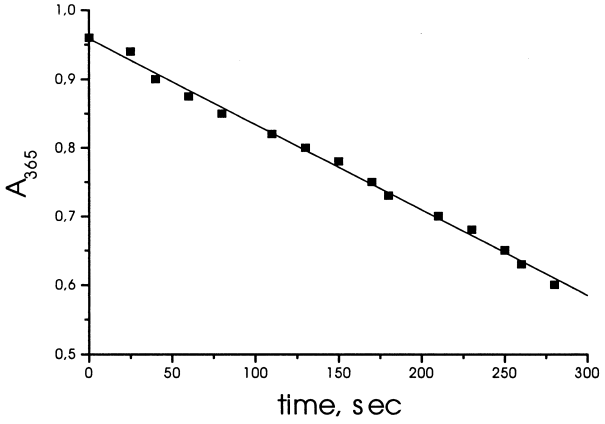

Fig. 3. Tc photolysis rate under 365 nm irradiation with DRSh-250 lamp.

rather low Tc photolysis rate. The absorption at 365 nm decreased to only 50% of the initial value after more than 200 min of irradiation (see also [19]). Applying the much more powerful Hg arc lamp DRSh-250, it was possible to increase the rate of Tc photolysis by about 30 times (Fig. 3). Oehler et al. [20] also reported a rather fast photo-activated modification of the 70S ribosome with Tc under irradiation with a 500 W high power arc lamp.

On this basis the following conditions were chosen for direct photo-induced affinity modification of TetR(D) protein with Tc in order to search for modified segments of the polypeptide chain. TetR(D) protein (14.5 nmol) was mixed with 1.8 nmol radioactive Tc (ratio 8:1) in buffer A and the mixture was irradiated with the DRSh-250 lamp as described in Section 2. Then the sample was applied onto a RP-HPLC column and the radioactive protein fractions were collected. The fractions contained about 1% of the bound Tc, providing a sufficient yield of modified material to pursue further protein structure analysis.

In order to cover most of the polypeptide chain for the analysis, we chose V8 protease, which cuts after Glu-C and, following the TetR(D) sequence, yields 17 peptides. According to the X-ray structure [11,12], 14 amino acids, which are in contact with Tc, belong to seven of these peptides (Table 1). Modified TetR(D) protein was digested under standard conditions with V8 protease and applied onto the C8 column to separate the obtained peptides. Examples of the separation and the distribution of Tc radioactivity are given in Fig. 4. In several experiments performed, most of the radioactivity corresponding to UV-absorbing fractions was found in just one fraction, fraction No. 7.

The labeled fraction No. 7 was subjected to automatic N-terminal microsequencing by Edman degradation using a

Table 1
Amino acid sequences of V8 peptides containing amino acids which are in contact with Tc (bold letters), according to the X-ray structure [11] and predicted from the primary structure of TetR(D) protein (AC P09164 [24])

ILARH**H**DYSLPAAGE	(59–73)
SWQSFLRN**N**AMS**F**RRALLRYRDGAKV**H**LGT**RP**DE	(74–107)
KQYDT**V**E	(108–114)
T**Q**LRFMTE	(115–122)
NGFSLRDG**L**YA**I**SAVSHFTLGAVL**E**	(123–147)
NLPPL**L**RE	(165–172)
A**L**QI**M**DSDDGE	(173–183)

Amino acid numbers are shown in parentheses.

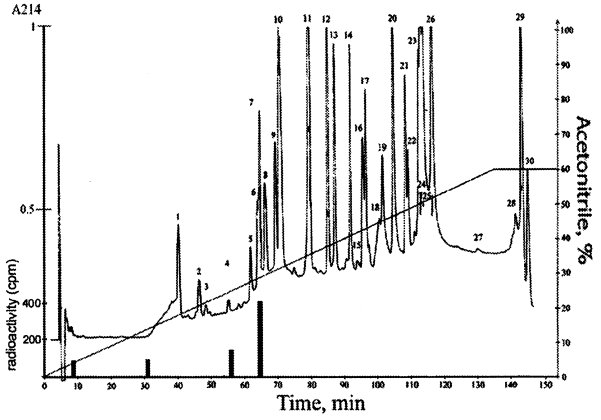

Fig. 4. An example of RP-HPLC separation of the protease V8 peptides of the modified TetR(D) protein on C8 column. Two steps of linear acetonitrile concentration gradients are shown on the right axis: 0–40%, 90 min; 40–60%, 45 min. The absorption at 214 nm was 1 at full scale and is shown on the left axis. [^{3}H]-Radioactivity (cpm per fraction) is shown on the left axis and depicted as black bars beneath the UV-profile, background is subtracted.

Knauer 816 gas–liquid sequencer. It turned out that this fraction contains two peptides: 90% of the major one and 10% of the minor one. Edman degradation revealed that the N-terminal sequences (6–9 amino acids) of these peptides unambiguously corresponded to the predicted N-terminal sequences of TetR(D) peptides (AC P09164 [24]): the major one was the undecapeptide Ala173 to Glu183 and the minor one was the pentadecapeptide Ile59 tp Glu73. Since the yield of modification was less than 10%, it was not possible to determine which of these peptides (or both) carry the modification. Complementary tryptic peptide analysis of the modified protein confirmed specific labeling of both peptides. They contain highly reactive amino acid residues able to be modified, which are in contact with the Tc molecule according to the X-ray structure, namely for example, Met177 which belongs to the major peptide and His64 located in the minor one (Fig. 5).

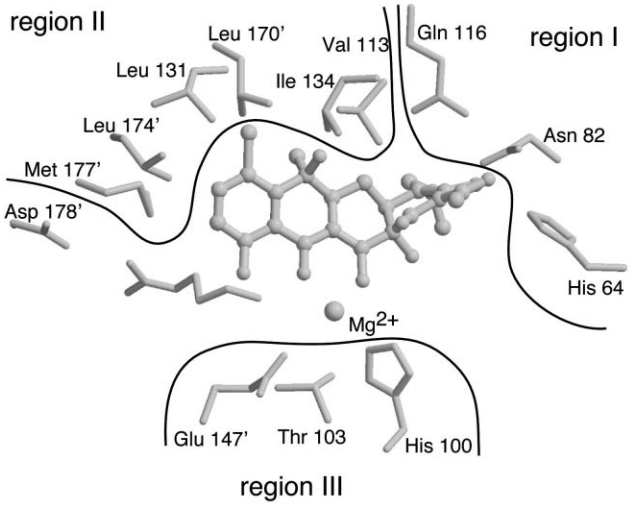

Fig. 5. A schematic illustration of the interactions between Tc and TetR(D) protein according to X-ray structure for the complex of 7-chloro-Tc with TetR(D) [12]. Amino acid residues in the two subunits are distinguished by dashes. The figure was produced using Molscript [25].

According to the X-ray analysis [11,12], TetR(D) protein is a homodimer. The monomer polypeptide chain contains 207 amino acids (out of 217; the C-terminal 10 amino acids are cleaved off during preparation) and is folded into 10 α-helices (α1–α10 for the first monomer and α1′–α10′ for the second one). The core domain harbors two identical, symmetry related, tunnel-like binding sites for Tc in complex with Mg^{2+} $[MgTc]^+$, formed by side chains of helices α5–α8, α8′ and α9′. The Tc binding tunnel has three regions, one hydrophobic region, region II, which fixes the B, C and D rings of Tc, and two hydrophilic regions, regions I and III, fixing ring A of Tc and the Mg^{2+} ion, respectively (Fig. 5). Our finding of two modified peptides containing amino acids which belong to regions I and II, respectively, indicates that the direct photo-activated affinity modification correlates well with the X-ray structure. No doubt Tc can be applied as a direct and highly specific photo-activated affinity modifying reagent.

The results of this study have revealed for the first time the potential of Tc for direct photo-induced affinity modification to identify segments of a protein target in the vicinity of the antibiotic binding site. This finding should open new avenues for the use of Tc and its variants as structural targets for TetR(D) and other related Tc binding proteins in solution.

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