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Fast large-scale purification of tetracycline repressor variants from overproducing *Escherichia coli* strains

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Abstract

We constructed a plasmid for overexpression of Tn*10* Tet repressor (TetR) by placing a synthetic *tetR* gene under control of the P_{tac} promoter. Active TetR is expressed up to 30% of the total soluble cell protein. A protocol containing anion-exchange, cation-exchange, and size-exclusion chromatography steps is described for the large-scale purification of milligram amounts of TetR in three days. Cation-exchange chromatography already yields almost homogenous TetR. Purification of about fifty TetR mutants demonstrates that this protocol is generally applicable. No correlation between net charge of TetR variants and elution behaviour was detected for the anion-exchange column. On the other hand, TetR mutants with increased negative charge in their DNA binding domain eluted at lower NaCl concentration from the cation-exchange column. The applicability of this purification protocol to the wide variety of TetR variants suggests that it can be used for the rapid purification of other DNA binding proteins as well.

Keywords: Escherichia coli; Proteins; Tetracycline; Tet repressor

1. Introduction

Tet repressor (TetR) is a DNA-binding protein of 207 amino acids in length. As a dimer, it regulates tetracycline (tc) resistance determinants common to Gram-negative bacteria which code for an active efflux system whereby tc is pumped out of the cell. These tc resistance determinants consist of two divergently oriented genes: *tetR* which encodes the repressor and *tetA*, which codes for the resistance protein, an integral membrane transporter which exports $[M-tc]^+$ in exchange for the uptake of a proton. In the absence of tc, TetR binds to two *tet* operators located in the intergenic region between *tetR* and *tetA*, thereby turning down their expression. In the presence of tc, a $[M-tc]^+$ complex binds to TetR, which looses its operator-binding specificity and expression of *tetR* and *tetA* is induced. Sequence variants of this genetic determinant are abundantly present in natural isolates and are designated by capital letters (for a recent review, see [1]). The crystal structure of TetR(D) in the complex with tc has been resolved at a resolution of 2.1 Å [2]. The structural interpretation of many non-inducible TetR mutants suggests a substantial reorganization of the

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TetR tertiary and quaternary structure as a consequence of tc binding [3]. Moreover, induction requires only nanomolar concentrations of tc. Therefore, TetR mediates the most sensitive effector-inducible transcriptional regulation known to date. This sensitivity is one reason for the successful application of TetR as a regulator of gene expression in cells as different as *Escherichia coli* [4], *Saccharomyces cerevisiae* [5], *Schizosaccharomyces pombe* [6], *Dictyostelium discoideum* [7], trypanosomes [8], transgenic animals [9–11], transgenic plants [12,13] and different cell cultures [14–17].

Despite this wide application as a regulator of gene expression and the continuing interest in the molecular mechanisms of the different functions of TetR, the described expression-purification protocol is time-consuming [18] and often leads to insoluble TetR variants [19]. In this article we describe an expression system yielding overproduction of soluble proteins and a protocol for a simple and efficient purification. The wide applicability of this protocol is demonstrated by overexpression and purification of about 50 different TetR variants.

2. Experimental

2.1. Materials and general methods

All chemicals were of the highest purity available from Merck (Darmstadt, Germany), Fluka (Buchs, Germany) or Roth (Karlsruhe, Germany). Trifluoroacetic acid (TFA) was obtained from Sigma (Munich, Germany). Milli-Q water was used for all buffers and solutions. Restriction enzymes and T4 DNA ligase were from Boehringer (Mannheim, Germany). Isolation, manipulation and sequencing of DNA was as described [20,21]. Oligodeoxynucleotides were purchased from MWG Biotech (München, Germany). A fast protein liquid chromatography (FPLC) system from Pharmacia (Freiburg, Germany) equipped with a conductometer from WTW (Weilheim, Germany) was used for protein chromatography. Sephadex G-25, Superose 12 prep grade and Superdex 75 columns were obtained from Pharmacia, and Fractogel EMD TMAE-650(s) and Fractogel EMD SO_3^--650(s) were from Merck. The analytical HPLC work was performed with a Hewlett–Packard (Waldbronn, Germany) 1090 Series II liquid chromatograph using a diode-array detector module. Samples were analyzed on a Vydac C_4 column (5 μm, 250×4.6 mm) and monitored at 214 and 280 nm simultaneously. Electrospray mass spectrometry (ES–MS) was performed on a Fisons (Danvers, MA, USA) VG–Quattro triple-quadrupole mass spectrometer equipped with a VG electrospray source and gas nebulizer probe. Desalted protein samples were dissolved in methanol–1% aqueous acetic acid (1:1) and delivered to the source at a flow-rate of 3 μl/min utilizing an Applied Biosystems Model 140B dual syringe pump. N-terminal sequences of the wild-type TetR(B) protein were determined by automated Edman degradation, using an Applied Biosystems 477A pulsed liquid sequencer. Protein SDS–PAGE [22] was run on a Pharmacia PhastSystem using precast 10–15% gradient PhastGels. The gradient gels were run for 60 Vh and stained either with silver or Coomassie blue in the development unit of the PhastSystem [23,24]. After denaturing electrophoresis, TetR was blotted onto a ProBlot PVDF membrane (Applied Biosystems, Weiterstadt, Germany) in a Pharmacia PhastTransfer unit and probed using an anti-TetR monoclonal antibody (kindly provided by Dr. H. Bujard). An anti-mouse alkaline phosphatase conjugate (Promega, Madison, WN, USA) was used as secondary antibody. The complex was stained by incubation with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Gibco–BRL, Eggenstein, Germany).

2.2. Cloning of tetR alleles in pWH1950

The pWH1950 derivatives (see Fig. 1) containing *tetR*(B) variants with single amino acid exchanges [*tetR*(B) variants will be named by the respective amino acid exchanged and its position, e.g., EK23 means the exchange of a glutamic acid (E) by a lysine (K) at position 23 of TetR(B)] have been described [3]. The respective *tetR* genes were amplified using PCR and inserted into pWH1950 via the *Xba*I and *Sph*I sites (see Fig. 1 for the locations of the relevant restriction sites). *tetR*(B) genes with deletions of residues T26–D53 (Δ26–53) and D164–M166 (Δ164–166) [25] were cloned as *Xba*I/*Sph*I fragments from the respective pWH520 [26] deriva-

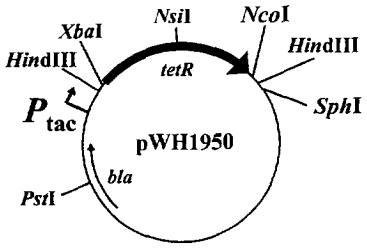

Fig. 1. Genetic organization of pWH1950. The plasmid contains the β-lactamase and the *tetR* gene. Overexpression of the latter is controlled by the P_{tac} promoter. The positions of relevant restriction sites are indicated.

tives into likewise digested pWH1950. The hybrid *tetR*(B) mutants s-cro27–47, s-lac27–47, s-cro37–45, s-lac37–46 in which the coding region of the *tetR*(B) α-Helix-turn-α-Helix (HTH) motif was replaced with the respective coding regions of bacteriophage 434*cro* or *lacI* from *E. coli* (Backes et al., submitted) were cloned as *Xba*I/*Nsi*I fragments from pWH1900 derivatives [27] into likewise digested pWH1950. Derivatives of pWH520 with *tetR*(B) genes containing (i) an insertion of a GGH motif at position 2 (iGGH), (ii) a substitution of the residues S2–D4 by a GGH motif (sGGH) and (iii) deletions of the residues R3–K6 (Δ3–6) and K6–K8 (Δ6–8) [26] were digested with *Hin*cII and ligated into pWH1950 which had been digested with *Hin*dIII and where the protruding ends had been filled-in with Klenow polymerase. A PCR protocol was used to amplify the *tetR* genes of the resistance classes C–E. The template DNA for *tetR*(C) was pSC101 [28], for *tetR*(D) pWH904 [19] and for *tetR*(E) pWH905 [19]. To facilitate cloning of these *tetR* genes into pWH1950, restriction sites for *Xba*I at codons 2 and 3 of the respective *tetR* genes and for *Nco*I immediately following the respective stop codons of *tetR* were introduced via the amplification primers. The presence of all mutations and the correct sequences of *tetR*(C–E) were verified by sequencing the respective pWH1950 derivatives.

2.3. Overexpression of tetR alleles

The overproducing *E. coli* strain RB791 [29] harboring the respective pWH1950 derivative was cultured in SB medium [30] either in a 2-l shaking flask or a 1.5-l Biostat M fermenter (Braun, Melsungen, Germany). The incubation temperature was 37°C except for the mutants WF75, iGGH and sGGH and for the TetR classes C–E where it was 28°C. Overexpression of TetR proteins was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1.5 m*M* at an A_{600} of about 1.0. The culture was incubated for another 2–4 h and harvested by centrifugation. For the preparation of perdeuterated TetR(B), cells were grown in M9 minimal medium [30] containing ^2H_2O, [$^2H_6$]-glucose and $N^2H_4^+$ salts at 37°C.

2.4. Isolation and sample preparation

Protein purification was performed at 4°C. *E. coli* cell pellets were resuspended in 20 ml of cell lysis buffer (200 m*M* NaCl, 5 m*M* DTT, and 20 m*M* Tris–HCl, pH 8.0) per g of wet cells. Cells were constantly cooled and lysed by sonication [3×3 min at 150 W using a Labsonic U sonicator (Braun, Melsungen, Germany)]. The cell debris was removed by centrifugation. Polyethyleneimine was added to a final concentration of 0.2% (v/v) to the cell-free supernatant. The slurry was stirred for 1 h and centrifuged again. A saturated solution of ammonium sulfate was added stepwise to give a final saturation of 60% (v/v). The solution was stirred for at least 2 h at 4°C, centrifuged, and the supernatant discarded.

2.5. Column chromatography

The pellet was dissolved in the minimal volume of buffer A (100 m*M* NaCl, 2 m*M* DTT, and 20 m*M* Tris–HCl, pH 8.0) and samples of 10 ml were desalted at a flow-rate of 2 ml/min through a Sephadex G-25 column (40×2.6 cm) equilibrated in the same buffer. Separation of proteins from contaminating salt was monitored by conductivity. Immediately after elution, desalted protein fractions were collected and passed through a tentacle-based Fractogel EMD TMAE-650(s) column (12×2.6 cm), previously equilibrated in the same buffer. The column was washed with buffer A until the UV absorption at 280 nm and the conductivity gave a stable baseline. Elution of bound proteins was car-

ried out with a 300-ml linear gradient between 0–60% buffer B (buffer B: buffer A with 1 *M* NaCl) at a flow-rate of 2 ml/min. Fractions of 10 ml column wash and 3 ml gradient eluate were collected and analyzed by SDS–PAGE. TetR containing samples were pooled and precipitated as above.

This pellet was dissolved in buffer C (50 m*M* NaCl, 2 m*M* DTT, 20 m*M* Na-PO_4^{3-}, pH 6.8) and desalted through Sephadex G-25 equilibrated in buffer C. A Fractogel EMD SO_3^--650(s) column (10×1.6 cm) was used for cation-exchange chromatography. The desalted sample was loaded onto the column equilibrated in buffer C at a flow-rate of 1 ml/min. TetR was eluted with a 110-ml linear gradient of 0–50% buffer D (buffer D: buffer C with 1 *M* NaCl) at the same flow-rate. TetR containing fractions were pooled and precipitated as before.

The ammonium sulfate precipitate was dissolved in the minimum volume of cell lysis buffer (typically 2 ml). After centrifugation in a bench top centrifuge at 12 000 *g* for 5 min, volumes of maximally 1.5 ml of the supernatant were applied to a Superose 12 prep grade (60×1.5 cm) or Superdex 75 column (60×1.6 cm) that had been equilibrated in the same buffer. Elution was performed isocratically at a flow-rate of 0.4 ml/min. Fractions of 1.5 ml were collected and analyzed for their TetR content. Those containing TetR were concentrated to about 20 mg/ml of protein by using Filtron centrifugal concentrators with a cutoff of M_r 30 000 (Filtron, Northborough, MA, USA). Purified TetR was stored at −20°C for several months in cell lysis buffer–glycerol (1:1) with no detectable decrease in activity.

2.6. Determination of protein concentration and activity

Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as standard. The concentration of purified TetR(B) was determined spectrophotometrically using an extinction coefficient of 38 000 cm^{-1} M^{-1} at 280 nm [21]. The binding activity of TetR variants to tc was determined by tc titration experiments [31]. A gel mobility shift assay demonstrated *tet* operator binding and induction by tc [32,33].

3. Results

3.1. Construction of TetR overexpressing plasmids

Genes under control of the P_{tac} promoter [34] allow inducible expression of the respective proteins to high amounts. The construction of TetR(B) overexpressing plasmids was achieved in three steps starting from pWH305 [18]. In the first step, the 1245 base pair (bp) *Pst*I/*Xba*I fragment from pWH305 was replaced by the 1015 bp *Pst*I/*Xba*I fragment from pWH1330 [35] containing the P_{tac} promoter. The resulting plasmid was named pWH1949. To facilitate construction, the *tetR*(B) gene encoded by pWH1949 was replaced by a synthetic *tetR* gene with four additional restriction sites (one for *Bst*XI between codons 31 and 35, *M*luI between 129 to 131, *Sau*I between 141 and 142, and *Nco*I situated 18 nucleotides downstream of the stop codon of the gene). In the final step, the ribosomal binding sequence (RBS) and the start codon of *tetR* (highlighted in bold face) were generated by insertion of the synthetic oligonucleotide duplex shown below into the *Xba*I site

```
                 HindIII          RBS          XbaI
5'-CTAGCTCGACAAGCTTAACAAAAATTAGGAATTAATGATGT
      GAGCTGTTCGAATTGTTTTTAATCCTTAATTACTACAGATC-'5
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The genetic organization and relevant restriction sites of the resulting plasmid pWH1950 are displayed in Fig. 1.

Mutant *tetR*(B) genes and those of *tetR* classes C–E were either amplified using PCR and inserted via suitable restriction sites (see above) or recloned from other *tetR* harboring plasmids (see Experimental).

3.2. Overexpression of TetR alleles

The *E. coli* strain RB791 served as the host strain for the isolation of TetR variants. Cultivation of 1 l SB medium in a shaking flask usually yielded 4–6 g of wet *E. coli* cells, whereas cultivation in a 1.5-l fermenter resulted in a cell mass of up to 20 g. Crude extracts showed TetR(B) expression of up to approximately 30% of the total amount of soluble protein

after induction with IPTG. Similar results were obtained for all single amino acid substitutions in TetR(B) and for the HTH variants. The expression levels for TetR(C–E) varied. While the amounts of soluble TetR(C) and TetR(D) protein were similar to TetR(B), the yield of TetR(E) was only about 5% of the total cell protein mass.

3.3. Purification of wild-type TetR(B)

Our newly developed purification protocol consists of three parts: (i) overexpression of the protein; (ii) isolation and sample preparation by cell lysis and removal of DNA; (iii) chromatography employing ion-exchange and size-exclusion chromatography columns. Fig. 2 shows a representative purification procedure for wild-type TetR(B) with typical yields. A typical purification started with 4.5 g of harvested wet cells. After sonication in 100 ml cell lysis buffer, about 200 mg of soluble protein were obtained in the cell lysate. Polyethylenimine and ammonium sulfate precipitation of the supernatant yielded approximately 140 mg protein mixture of which 38% interact with tc. The first step in the chromatographic purification was anion-exchange chromatography on Fractogel EMD TMAE. Column washing removed a large amount of contaminating proteins, whereas only a negligible amount of TetR was detected in the flow through. TetR(B) eluted in a sharp peak at a concentration of 230 m*M* NaCl. The elution profile is illustrated in Fig. 3A. About 55 mg protein were recovered from the crude protein applied to the column. Of these more than 50% bind tc.

The second chromatographic separation was carried out on Fractogel EMD SO_3^-. Almost no TetR(B) was found in the flow through, whereas a large portion of contaminating protein was washed from the column. During gradient elution more contami-

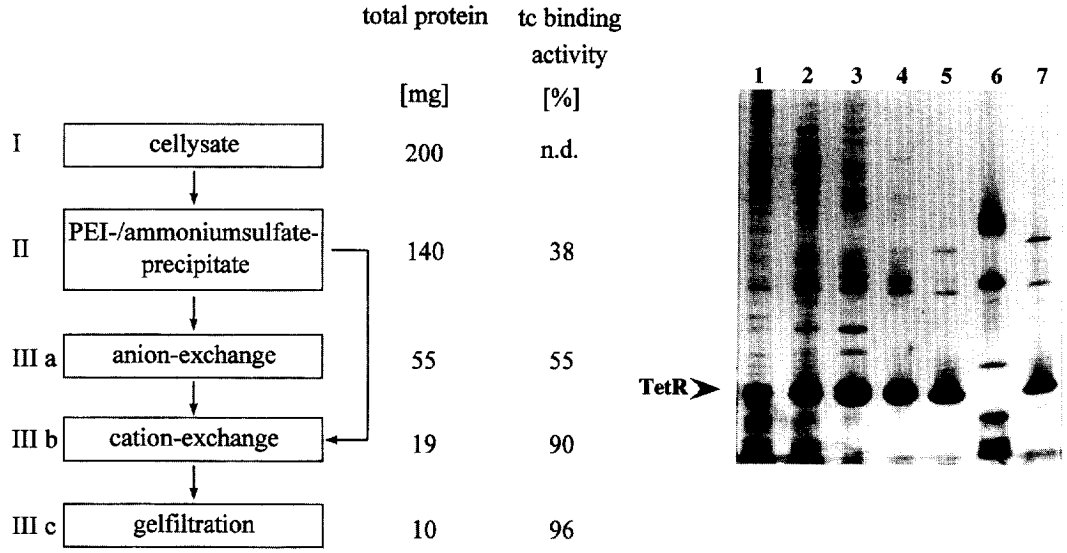

Fig. 2. Successive steps during purification of the TetR(B) protein. The left side shows the successive steps of TetR protein purification. Samples after sonication of the cells, after polyethylenimine- and ammoniumsulfate precipitation, after anion- and cation-exchange chromatography and after final size-exclusion chromatography were examined for total protein content and TetR content determined from tc binding given in [%] of total protein. At the right side a silver-stained polyacrylamide gel shows corresponding samples after denaturing electrophoresis. Lane 1: *E. coli* cell lysate after induction with IPTG, lane 2: protein sample after 60% (v/v) ammonium sulfate precipitation, lane 3: pooled TetR fractions after anion-exchange chromatography, lane 4: pooled TetR fractions after cation-exchange chromatography, lane 5: pooled TetR fractions after size-exclusion chromatography, lane 6: molecular mass marker and lane 7: TetR purified according to the previous protocol [18].

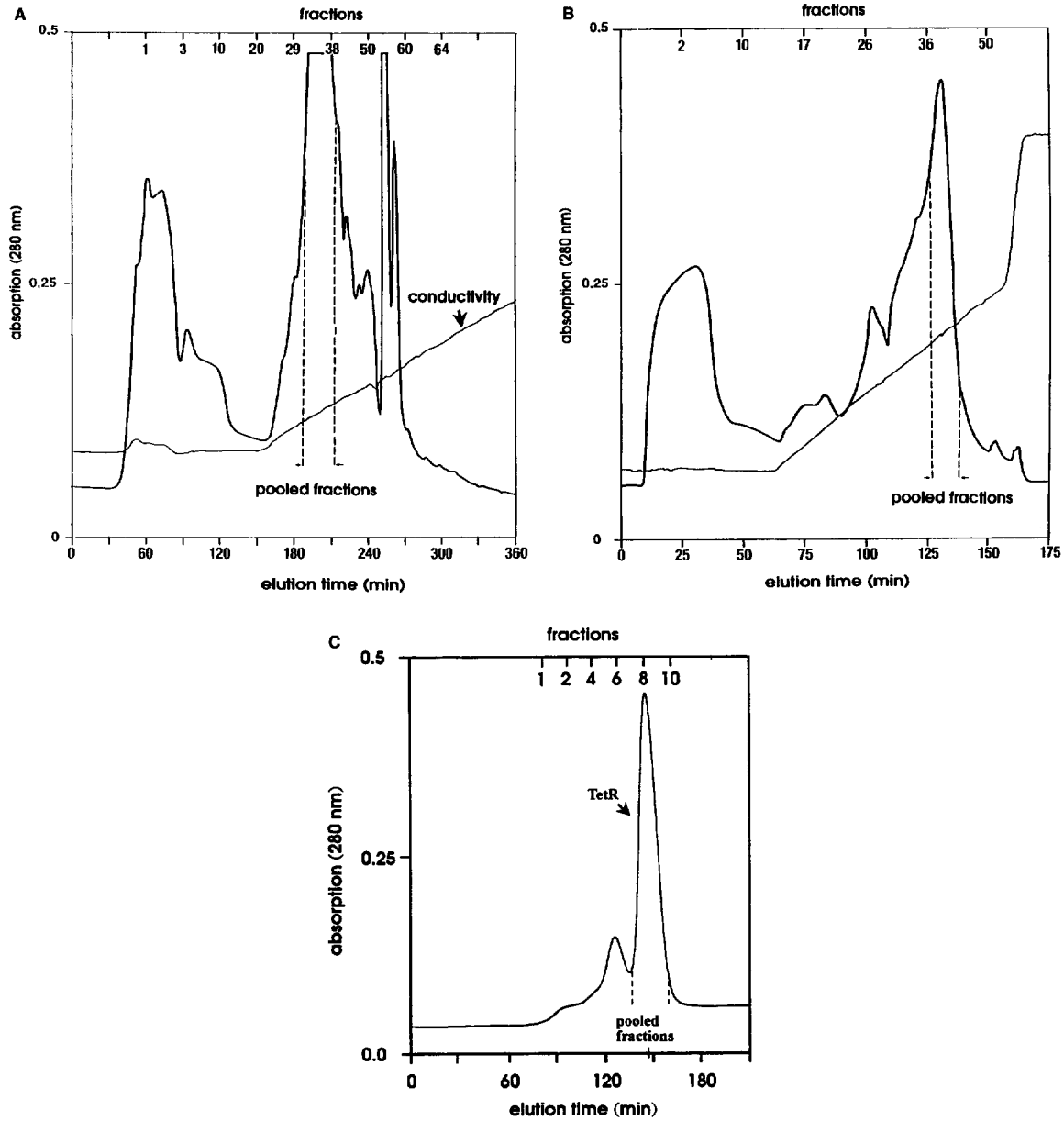

Fig. 3. Purification of TetR by anion-exchange, cation-exchange and size-exclusion chromatography. (A) Elution profile of anion-exchange chromatography of TetR on Fractogel EMD TMAE after 60% (v/v) ammonium sulfate fractionation and desalting. Elution was performed using a linear gradient between buffers A and B from 0–60% B in 150 min at a flow-rate of 2 ml/min. Buffer A: 100 m*M* NaCl, 2 m*M* DTT and 20 m*M* Tris–HCl, pH 8.0; buffer B: buffer A containing 1 *M* NaCl. (B) Cation-exchange chromatography of pooled fractions from the anion-exchange chromatography after precipitation and desalting. Elution was performed using a linear gradient between buffers C and D from 0–50% B in 110 min at a flow-rate of 1 ml/min. Buffer C: 50 m*M* NaCl, 2 m*M* DTT, 20 m*M* $Na\text{-}PO_4^{3-}$, pH 6.8; buffer D: buffer C containing 1 *M* NaCl. (C) Size-exclusion chromatography of TetR on Superose 12 prep grade. Pooled fractions containing TetR after the cation-exchange chromatography were precipitated and subjected to size-exclusion chromatography on Superose 12 prep grade at a flow-rate of 0.4 ml/min.

nants were separated from TetR, which eluted in a sharp peak at 450 m*M* NaCl. An amount of 19 mg of almost homogeneous TetR (90% tc binding activity) was found in the pooled fractions. The elution profile is depicted in Fig. 3B.

Repeat purifications with wild-type TetR(B) showed that anion-exchange chromatography can be omitted. The percentage of tc binding protein was identical after single or double ion-exchange chromatography. Additionally, similar purity of these samples was observed after Coomassie Blue staining of a SDS–polyacrylamide gel (data not shown). Therefore, this purification step can be omitted for TetR proteins which interact with the cation-exchange material. The resulting protein is pure enough, for example, for fluorescence titration experiments, DNA retardation assays or crystallisation of TetR(D) ([3]; Backes et al., submitted for publication; W. Hinrichs, personal communication).

Size-exclusion chromatography was always the final step of purification. After precipitation and solution in cell lysis buffer, TetR(B) was subjected to Superose 12 prep grade or Superdex 75 chromatography. An elution profile is shown in Fig. 3C. Native repressor eluted after 27 min and contained only traces of high-molecular mass impurities. The yield was 10 mg TetR(B) with a calculated purity of 96% according to titration with tc [31].

3.4. Criteria of purity and characterisation of TetR(B)

The homogeneity of TetR was confirmed by electrophoretic and chromatographic methods. When analyzed by SDS–PAGE and subsequent silver-staining (Fig. 2; lane 5), a major band at M_r 24 000 corresponding to a TetR(B) monomer and several faint bands were visible. Purified TetR was also checked on a C_4 reversed-phase column and eluted as a single symmetrical peak at 47% acetonitrile–aqueous TFA. The M_r was determined by ES–MS to be 23 228±2 which is in excellent agreement with the theoretical M_r of 23 223 derived from the amino acid sequence. N-terminal sequencing of the first 15 residues yielded a single sequence and showed that the N-terminal formyl methionine was completely removed [36].

3.5. Overexpression and purification of TetR(B) mutants and TetR(C–E) variants

All mutants were overexpressed and isolated in the manner described above. The mutants sGGH and iGGH resulted in a low level of overexpression (≈1% of total cell protein), but could still be isolated in milligram amounts. Protein expression of TetR(E) was also reduced to about 5% of the total cell protein. Therefore, cells of several cultivations were collected for further purification (see Table 1).

Table 2 lists all proteins purified by this protocol. They are all obtained in an active form as demonstrated by tc titration experiments and assays for binding either to non-specific or *tet* operator DNA. Only Δ26–53, s-cro27–47 and s-lac27–47 showed no DNA binding activity. Their DNA binding motif is either missing (Δ26–53) or completely exchanged (s-cro27–47, s-lac27–47). DNA binding of Δ3–6 and Δ6–8 was not checked.

Of 14 examined TetR mutants, only s-lac27–47 eluted from the anion-exchange chromatography material at higher NaCl concentration (310 m*M*). All other proteins eluted at about 230 m*M* NaCl as wild-type TetR(B). On the other hand, substantial differences were found in their properties in cation-exchange chromatography. Several repeats of purification with TetR(B) and mutants were done and NaCl concentrations for elution of the respective proteins differed maximally by 10% from one experiment to another (data not shown). Three mutants, namely Δ26–53, s-cro27–47 and s-lac27–47, did not bind to the cation-exchange material. Others dis-

Table 1
Purification of wild-type TetR(B), perdeuterated repressor and TetR variants

TetR variants	Wet cell mass (g)	Yield (mg)
TetR(B)	4.5[a]	10.0
TetR(B)-perdeuterated	4.0[a]	9.0
TetR(B) sGGH	12.0[b]	2.7
TetR(B) iGGH	14.0[b]	0.7
TetR(C)	6.0[a]	14.4
TetR(D)	5.6[a]	13.5
TetR(E)	40.0[a]	28.0

The respective yields of *E. coli* cells correspond to 1 l LB medium incubated in a shaking flask[a] or in a fermenter with 1.5 l SB medium[b].

Table 2
Elution behaviour of all purified TetR proteins after anion- and cation-exchange chromatography

TetR protein	Elution of the respective protein under NaCl (m*M*)		Net charge differences	TetR protein	Elution of the respective protein under NaCl [m*M*]		Net charge differences
	at anion-exchange material	at cation-exchange material			At anion-exchange material	At cation-exchange material	
TetR(B)	230	450	0	EV147	n.d.	400	+1
EK23	200	500[a]	+2	EG150	n.d.	400	+1
RQ49	220	390	−1	PL167	n.d.	450	0
HR64	n.d.	240	+1	LS176	n.d.	400	0
HY64	n.d.	420	0	FS177	n.d.	390	0
WF75	210	450	0	DG178	220	430	+1
NS82	210	360	0	sGGH	220	320[a]	−1
SN85	210	350	0	iGGH	n.d.	440[a]	0
FL86	n.d.	400	0	Δ3–6	n.d.	240	−1
KI98	n.d.	330	−1	Δ6–8	n.d.	230	−2
HR100	n.d.	340	+1	Δ26–53	210	–[b]	−4
HY100	n.d.	400	0	Δ164–166	220	500	+1
GR102	n.d.	520	+1	s-cro27–47	220	–[b]	−3
RQ104	210	350	−1	s-cro37–45	n.d.	450	+1
PT105	210	420	0	s-lac27–47	310	–[b]	−4
LH113	n.d.	440	0	s-lac37–46	n.d.	500[c]	+1
EG114	n.d.	440	+1				
QH116	n.d.	290	0	TetR(C)	n.d.	460	
LS117	n.d.	400	0	TetR(D)	n.d.	440	
LF146	n.d.	350	0	TetR(E)	n.d.	450	

TetR variants are indicated by their class designations, TetR(B) mutants by the respective mutation.
[a] The mutants EK23, sGGH and iGGH eluted over a broad range of salt. Therefore, only the maximum of the respective peak is indicated.
[b] Indicates that no interaction of the protein with the chromatography material was seen.
[c] The protein eluted from the column during the steep gradient from 50% to 100% buffer D.

played reduced or enhanced retention as seen with clearly reduced or elevated NaCl concentrations for elution. These were EK23 (500 m*M*), HR64 (240 m*M*), SN85 (350 m*M*), KI98 (330 m*M*), HR100 (340 m*M*), GR102 (520 m*M*), RQ104 (350 m*M*), QH116 (290 m*M*), LF146 (350 m*M*), sGGH (320 m*M*), Δ3–6 (240 m*M*), Δ6–8 (230 m*M*), Δ164–166 (500 m*M*) and s-lac27–47 (500 m*M*). The elution properties of the remaining mutants varied only slightly from those of the wild-type TetR(B) protein (450 m*M*). Another group, EK23, sGGH and iGGH, eluted in a broad peak.

4. Discussion

Mechanistic studies profit from the availability of large quanitities of the respective protein obtained in a minimal course of time. Moreover, the purification scheme should be applicable to mutant proteins. Some progress in protein purification has been introduced by tags which are localized at the N or C terminus of the protein [37]. These tags are specifically recognized by counterparts which are immobilized within chromatography material and thus select the interesting protein out of the majority of cellular proteins. Examples for these methods are histidine tags or immunologically active protein domains. Their disadvantage is a possible interference of the tag with protein functions. Therefore, after purification, proteins often have to be separated from the tags. Therefore, we have opted to optimize purification of non-tagged proteins.

4.1. Comparison of purification protocols

Our new method for the purification of TetR differs in two aspects, namely overexpression and

chromatographic separation, from previously reported protocols [18,38]. Although expression under the control of the λP_L promoter resulted in up to 13% TetR(B) of the total soluble cell protein [18], TetR(D) and (E) were insoluble after disruption of the cells and had to be denatured before purification [33,39]. These disadvantages may be due to the 42°C needed for temperature induction of λP_L. The *tetR* gene in the newly constructed pWH1950 is under control of the P_{tac} promoter. Induction of TetR expression with IPTG culminates in 30% soluble TetR(B) of the total cell protein. This is about twice as much as published earlier [18]. Furthermore, when TetR(D) and TetR(E) were overexpressed at 28°C using the P_{tac} based plasmids, no problem with insolubility of the expressed protein occurred. Because P_{tac} promoter expression is temperature independent, this fermentation parameter can be adjusted to the known temperature sensitivity of some TetR variants [19,26]. Since only TetR(D) crystallizes to a high resolution, but renaturation of the insoluble protein is inefficient and time consuming, overproduction of soluble TetR(D) greatly facilitates the structural analysis of TetR variants of interest for applicability [17] and regulatory mechanisms [3].

Newly developed chromatography materials are used in this scheme to shorten the time of protein purification by half. Desalting by size-exclusion chromatography on Sephadex G-25 was preferred to the time consuming step of dialysis. Because of the high capacity and porosity of the Fractogel ion-exchange supports, higher flow-rates (up to 3 ml/min) and reduced column dimensions were possible. This cuts the time for chromatography from 31 h in the former protocol (CM–Sephadex and Sephadex G-100) to nearly 5 h in the improved protocol (Fractogel SO_3^- and Superdex 75). The resulting TetR protein is at least as pure as TetR isolated according to the old method (compare lanes 5 and 7 in Fig. 2).

The main drawback of the previous protocol was the final purification step on CM–Sephadex, where significant amounts of TetR eluted during column washing with Tris buffer at pH 7.7. This deficiency was avoided by using a phosphate buffer at pH 6.8. TetR strongly binds to the cationic resin under these conditions and no repressor was detected in the flow through. The high NaCl concentration of 450 m*M* needed for elution of TetR(B) indicates a strong affinity of this material for the repressor protein. The flexibility of the ionic groups in this tentacle-based Fractogel resin may facilitate electrostatic interactions with the protein as indicated by their increased affinity [40]. The improved efficacy is demonstrated by comparing the yield of mutant WF75 overexpressed under the control of P_{tac} and purified with the new protocol with the one obtained after overexpression by λP_L and applying the previous procedure. About twice the amount of active protein was isolated from the same amount of wet cells in half the time using the newly developed method [41].

4.2. Chromatography of TetR variants

The new protocol was originally established for TetR(B). But it was successfully expanded for the purification of many TetR variants. These include not only single amino acid exchanges in TetR(B) [3,42], but also deletions [25,26], substitutions of secondary structure elements in the HTH motif (Backes et al., submitted) and different classes of TetR with amino acid identities ranging down to 48% [1].

Changes in the elution profile of a protein mutant should reflect weakened or enhanced interactions to the support. For ion-exchange materials, one might naively expect that changes in the net charge of a protein could lead to an altered elution behaviour. This assumption was not observed for the elution of the TetR variants from the anion-exchange column. Thirteen of the fourteen mutants tested eluted like the wild-type, even though they have net charge differences ranging from +2 to −4 compared to wild-type TetR [see Table 2 for the net charge differences of TetR(B) mutants to wild-type]. Only s-lac27–47, with a net charge difference of −4, eluted at a higher NaCl concentration. This would be consistent with our assumption. But Δ26–53 and s-cro27–47 with charge differences of −4 and −3, eluted like the wild-type at NaCl concentrations of 210 or 220 m*M*, respectively.

While there were only few differences in the elution behaviour of the TetR(B) mutants from the anion-exchange column, large variations were observed for elution from the cation-exchange column.

For three TetR mutants, Δ26–53, s-lac27–47, and s-cro27–47, cation-exchange chromatography could not be used because they did not bind to the column. Other mutants eluted at either markedly lower or higher NaCl concentrations. The eleven TetR(B) mutants with a higher net positive charge than the wild-type eluted at NaCl concentrations varying between 240 m*M* (HR64) and 520 m*M* (GR102). Of the sixteen mutants unchanged in their net charge, about half (nine) eluted at NaCl concentrations lower than the wild-type (290–400 m*M*), while the other half (seven) came off the column roughly like the wild-type (420–450 m*M* NaCl). On the other hand, a correlation to our proposal was observed for the nine mutants with a higher net negative charge than the wild-type. Mutants with a net charge difference of −1 and −2 always eluted at lower NaCl concentrations than wild-type TetR and mutants with a net charge difference of −3 and −4 did not bind to the cation-exchange column. This correlation is only qualitative because Δ6–8 with a charge difference of −2 interacts with the chromatography material like Δ3–6 which is only −1 in its charge difference. For Δ26–53, s-lac27–47 and s-cro27–47, the identical location of the deletion and the substitutions in the HTH motif, as well as the lack of mutants with identical charge differences in other locations makes their interpretation difficult. It is not possible to distinguish between the higher net negative charge or the location of the mutation within the HTH motif as the reason for their failure to bind to the cation-exchange support. Because the HTH motif in DNA-binding proteins is often positively charged [43] [in TetR(B) the net charge of the region between residues 27 and 49 is +5] this region might also be responsible for the interaction of Tet repressor with the negatively-charged cation-exchange material.

In conclusion, the purification protocol described here is simple, rapid, inexpensive, and yields milligram amounts of pure protein, even of perdeuterated TetR(B) for NMR studies, whose expensive preparation needs an efficient chromatographic separation. In addition, the protocol can be applied to a variety of different mutants with slight alterations.

5. Notation

E. coli	*Escherichia coli*
ES–MS	electrospray mass spectroscopy
HTH	α-Helix-Turn-α-Helix
$[M\text{-}tc]^+$	complex formed by a divalent metal ion and tc
RBS	ribosome binding sequence
tc	tetracycline
TetR	Tet repressor

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