Combinations of the α-Helix−Turn−α-Helix Motif of TetR with Respective Residues from LacI or 434Cro: DNA Recognition, Inducer Binding, and Urea-Dependent Denaturation†

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ABSTRACT: We constructed 10 different variants of TetR by substituting all or some of the residues in the α-helix−turn−α-helix (HTH) operator binding motif with the respective amino acids from LacI or 434Cro. The variants were soluble, negative transdominant over *tetR in vivo*, and as active as wild-type TetR in tetracycline binding *in vitro.* The urea-induced denaturation of the 10 variants occurs in single reversible transitions, which are centered around 4.3 M urea. Denaturation is concentration-dependent, supporting a simple two-state mechanism in which the folded dimeric protein is in equilibrium with unfolded monomers. An analysis according to the two-state model yields a Gibbs free energy of stabilization (at 0 M urea, 25 °C) of about 75 kJ/mol, typical for dimeric proteins of this size. Even a deletion of 24 residues from the reading head decreased the stability by only 2.7 kJ/mol. These results suggest that the DNA reading head of Tet repressor is a thermodynamically independent domain and that the thermodynamic stability of the Tet repressor dimer is determined by the association of the dimerization domains of the individual monomers. Variants containing replacements in the first α-helix of HTH did not show any DNA binding activity whatsoever. We attribute this to the alteration of the two N-terminal residues in this α-helix. TetR variants were active in nonspecific DNA binding, when either all or only the solvent-exposed residues in the recognition α-helix of HTH were exchanged to the respective LacI sequence. Replacement of the same residues by the respective amino acids from 434Cro yielded hybrid proteins that specifically recognize *tetO in vitro*. Taken together, these results establish that the similarity of operator recognition between 434Cro and TetR is greater than between TetR and LacI and confirm that prediction of the recognized DNA sequence is not obvious from the sequence of the respective HTH or recognition α-helix.

Many transcriptional regulatory proteins use only a few structurally well-defined DNA binding motifs to recognize target DNA sequences. The α-helix−turn−α-helix motif (HTH) has been intensively analyzed (Harrison & Aggarwal, 1990; Pabo et al., 1990; Baumeister et al., 1992b). The interactions between many HTH-containing proteins and their cognate DNA sequences have been studied in cocrystals (Otwinowski et al., 1988; Wolberger et al., 1988; Schultz et al., 1991; Beamer & Pabo, 1992; Shimon & Harrison, 1993; Feng et al., 1994; Schumacher et al., 1994) and by NMR (Chuprina et al., 1993; Zhang et al., 1994). Owing to the structural conservation of HTH there are similarities in the recognition patterns of these complexes (Helbl et al., 1993).

Altering the sequence specificity of a DNA binding protein by changing single amino acid residues (Wharton & Ptashne, 1987; Lehming et al., 1990; Baumeister et al., 1992a; Günes et al., 1995) has provided hints for specific protein−DNA interactions and led to the proposal of a generally applicable recognition code (Lehming et al., 1991). It should be possible to perform HTH swap experiments and predict the recognized sequence using this code. Chimeric proteins were indeed constructed by substituting amino acids of the 434cI HTH with the respective residues of 434Cro or P22 repressor. They bind DNA with the specificity of 434Cro or P22 repressor, respectively (Wharton et al., 1984; Wharton & Ptashne, 1985). The exchange of the entire HTH of LacI with the one of λCro, which both recognize similar DNA sequences (Lehming et al., 1988), resulted in a *lacO*-specific protein that was sensitive to proteolysis *in vivo* (Kolkhof et al., 1992). The partial exchange of 434Cro HTH against the one from 434cI resulted in a hybrid protein that was also rapidly degraded (Wharton et al., 1984), and the introduction of the λcI HTH into LacI did not yield a detectable protein (Kolkhof et al., 1992). These results support the idea that the exchange of base-contacting residues in structurally similar HTH proteins leads to predictable recognition specificites. But exchanges of HTH between unrelated proteins led to unstable hybrids and did not allow conclusions regarding a general recognition code.

TetR [for a review, see Hillen and Berens (1994)] contains a HTH (Hinrichs et al., 1994), whose interactions with base pairs in the *tetO* complex have been described in detail (Heuer & Hillen, 1988; Wissmann et al., 1988, 1991a; Sizemore et al., 1990; Baumeister et al., 1992a; Helbl et al.,

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1995). Recently, the deletion of the entire HTH motif yielded a transdominant truncated protein, indicating that the overall structure of the core remained intact (Berens et al., 1995). The crystal structures of two sequence variants of TetR demonstrate that it consists of two domains (Hinrichs et al., 1994; Kisker et al., 1995), a large C-terminal domain mediating dimerization and inducer binding and an N-terminal HTH DNA binding domain. The two domains are connected by the N-terminal domain. Functional crosstalk between the domains connects inducer binding to the loss of operator binding, an essential functional feature, which can, however, be offset by several point mutations (Hecht et al., 1993; Müller et al., 1995).

Dimerization of regulatory proteins occurs either by intertwining of the two subunits, e.g., as in CRP and TrpR (Weber & Steitz, 1987; Otwinowski et al., 1988) or by the binding of matching more or less flat surfaces, such as in PurR and LacI (Schumacher et al., 1994, Lewis et al., 1996). Dimerization of TetR is of the latter type, involving about 27% of the solvent-accessible surface of each monomer (Hinrichs et al., 1994). Dimers of TetR appear to be stable at 10^{-7} M concentrations (Hillen et al., 1989), suggesting a substantial binding energy between the monomers. We, therefore, anticipated that substitutions of the HTH in TetR by HTH from unrelated repressors may lead to stable chimeric proteins, which can be functionally characterized *in vivo* and *in vitro*. In this article we examine the properties of hybrid TetR proteins having their HTH entirely or partially substituted by the respective residues from LacI or 434Cro and describe reversible, urea-dependent denaturation of TetR and its variants.

MATERIALS AND METHODS

Materials and General Methods. Chemicals were of the highest purity available. Tetracycline (Tc) was from Lederle Laboratories (Pearl River, NY). The concentration of Tc was determined spectroscopically, using $\epsilon_{355} = 13\,320$ M^{-1} cm^{-1} in 0.1 M HCl (Takahashi et al., 1986). Oligonucleotides were obtained from MWG-Biotech (Ebersberg) and HPLC-purified. [^{32}P]-Radiolabeled nucleotides were purchased from Amersham (Braunschweig). Isolation and manipulation of DNA was as described (Sambrook et al., 1989). Sequencing was carried out according to the protocol provided by Pharmacia for use with T7 polymerase and [α-^{32}P]dATP. The buffer used in all spectroscopic measurements contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT. Urea was obtained from ICN Biochemicals, Eschwege, Germany. Urea stock solutions were prepared freshly each day.

Preparation of TetR Variants. Double-stranded oligonucleotide cassettes (see Table 1 for sequence) encoding the HTH variants were inserted in the *Stu*I and *Apa*I sites of *tetR* in pWH1900 (Baumeister et al., 1992b). Two complementary oligonucleotides were hybridized by heating to 70 °C and cooling to room temperature. Single-stranded ends were filled in with nucleoside triphosphates and Klenow fragment of DNA polymerase I, and the products were digested with *Apa*I and ligated with *Stu*I- and *Apa*I-digested pWH1900. All constructs were verified by DNA sequencing. Determination of the intracellular protein levels and overproduction and preparation of the proteins was done as described (Berens et al., 1995; Ettner et al, 1996). TetR concentrations were determined by Tc titration as described (Degenkolb et al., 1991) and are given for TetR monomer or dimer as indicated.

Determining Tc Binding Activity. Tc binding data were obtained by fluorescence titration measurements at limiting Mg^{2+} concentrations as described (Degenkolb et al., 1991; Takahashi et al., 1991). Free Mg^{2+} concentrations ranging from 10^{-11} to 10^{-2} M were adjusted using a buffer containing EDTA (Perrin & Dempsey, 1974) and diluted stock solutions of Mg^{2+}. Tc and TetR were provided at equimolar concentrations of 2 and 1 μM, respectively. An association constant K_m of 3100 M^{-1} for binding of Mg^{2+} to Tc was employed to analyze the titration curves (Takahashi et al., 1991).

DNA Fragments. PCR-generated DNA pools or end-labeled *tet* operator-containing fragments were used for *in vitro* DNA binding studies. Double-stranded randomized oligonucleotide cassettes were generated and labeled with [α-^{32}P]dATP by PCR with the 5′ primer (CGGCCGCG-CAAGCTTGTTTTTG) and the 3′ primer (CCGCGCGGC-GAGATCTAAAATA) and the templates *ran11x* (CGGC-CGCGCAAGCTTGTTTTTGTTGACACTXXXXCXXX-GXXXXAGTTATTTTAGATCTCGCCGCGCGG), *tetran* (CGGCCGCGCAAGCTTGTTTTTGTTGACACTCTATC-AXXXXXXXXGTTATTTTAGATCTCGCCGCGCGG) and *tetO* (CGGCCGCGCAAGCTTGTTTTTGTTGACACTC-TATCATTGATAGAGTTATTTTAGATCTCGCCGCGC-GG). Oligonucleotides were amplified with PCR, precipitated with ethanol, and dissolved in 25 μL of TE buffer. Optimal *lac* operator was generated by mixing two complementary oligonucleotides (CCGCGCAAGCTTGTTTTTGT-TGACATTGTGAGCGGC and CGGCGAGATCTAAAA-TAATTGTGAGCCGCTCACAAT) in equimolar amounts, heating to 70 °C, and cooling to room temperature. The overhanging ends were filled in using the Klenow fragment of DNA polymerase I, and the DNA was digested with *Hin*dIII and *Bgl*II and ligated into likewise-digested pWH1013 (Gülland & Hillen, 1992). Linear [^{32}P]-labeled DNA restriction fragments (135 bp) including *tetO*, *lacopt*, or *tetO* variants were generated by cleavage of pWH1013 or its derivatives with *Sal*I, labeling with [α-^{32}P]ATP, and cleavage with *Xba*I. The fragments were purified by polyacrylamide gel electrophoresis and elution with TE buffer.

DNA Binding Studies. The indicated amount of TetR variants was incubated with 8 μg of nonspecific plasmid DNA (pWH802; Unger et al., 1984) for 15 min in 40 μL of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM DTT, and 0.3% (v/v) Ficoll at ambient temperature. Then the indicated amounts of labeled DNA containing a binding site were added and the incubation was continued for 15 min. DNase I protection experiments were performed with these mixtures according to Galas and Schmitz (1978), or they were used for DNA retardation assays on nondenaturating 10% polyacrylamide gels (Sambrook et al., 1989).

Selection of Recognized DNA Binding Sites. Selection for recognized DNA binding sites was done according to Thiesen and Bach (1991). Preparation of randomized oligonucleotide cassettes, complex formation, and gel electrophoresis was done as described above, using 5 μL of the dissolved oligonucleotide mix and 100 ng of purified protein. The DNA from retarded bands was eluted, amplified by PCR, and subjected to further rounds of selection. Finally, the oligonucleotides were cloned into the *Hin*dIII and *Bgl*II sites of pWH1013.

β-Galactosidase Assays. DNA binding was assayed in *Escherichia coli* WH207(λ*tet*50) (Smith & Bertrand, 1988; Wissmann et al., 1991b). Transdominance of the mutant *tetR* alleles was determined in *E. coli* WH207(λ*tet*50)/pWH853. The plasmid pWH853 constitutively expresses a low level of TetR (Wissmann et al., 1991b). Cells were grown in LB medium supplemented with the appropriate antibiotics. β-Galactosidase activities were determined as described by Miller (1972). Three independent cultures were assayed for each strain and measurements were repeated at least twice.

Denaturation of TetR Variants. Equilibrium denaturation was performed by incubating protein samples for at least 1 h at the indicated urea concentration. Renaturation was measured by incubating the samples for 1 h at 8 M urea and then diluting them to the desired urea concentration. All reactions were performed at 25 °C. All Tet repressor concentrations are given as monomer. Protein solutions were desalted prior to denaturation experiments by gel filtration.

Fluorescence and CD Spectroscopy. Fluorescence intensities were measured with a Spex Fluorolog 1680 double spectrometer or a Hitachi F4010 spectrofluorometer. Excitation was at 280 nm (at protein concentrations of 0.4 μM) or at 295 nm (at protein concentrations of 5 μM). Emission intensity was recorded at 328 nm for 60 s and corrected by subtraction of the buffer fluorescence. The bandpass for excitation and emission was 8 nm for the Fluorolog spectrometer and 1 nm for excitation and 10 nm for emission for the Hitachi spectrofluorometer. Circular dichroism (CD) measurements were performed on a Jasco J600A spectropolarimeter at protein concentrations of 5 μM in 0.1-cm cells.

Thermodynamic Calculations. Denaturation curves were analyzed on the basis of a simple two-state model in which the folded dimeric protein N_2 exists in equilibrium with the unfolded monomers U (Bowie & Sauer, 1989; Grant et al., 1992):

$$N_2 \rightleftharpoons 2U \qquad (1)$$

An equilibrium constant K_U was calculated for each point in the denaturation transition curve according to the two-state model for denaturation of dimeric proteins:

$$K_U = [U]^2/[N_2] = 2P_t\,[f_U{}^2/(1 - f_U)] \qquad (2)$$

in which the measured quantities are f_U, the fraction of unfolded protein, and P_t, the total molar concentration of protein monomer. f_U is $[U]/P_t$ (Bowie & Sauer, 1989). The free energy of unfolding for the two-state denaturation model is assumed to be a linear function of the urea concentration (eq 3) (Pace, 1986), in which $\Delta G_U{}^0$ is the Gibbs free energy of unfolding at a 1 M concentration of all reactants and $\Delta G_U{}^0(H_2O)$ is the extrapolated free energy of unfolding in the absence of urea (Schellman, 1978; Pace, 1986). m is the slope of a plot of ΔG_U vs denaturant concentration. R and T are the gas constant and the absolute temperature, respectively.

$$\Delta G_U{}^0 = \Delta G_U{}^0(H_2O) - m[\text{urea}] = -RT \ln K_U \qquad (3)$$

Equations 2 and 3 were combined and rearranged to eq 4. By fitting of the urea denaturation curves to eq 4, values of $\Delta G_U{}^0(H_2O)$ can be directly obtained.

$$f_U = (\{\exp[-(\Delta G_U{}^0(H_2O) + m[\text{urea}])/RT]^2 + 8P_t \exp[-(\Delta G_U{}^0(H_2O) + m[\text{urea}])/RT]\}^{1/2} - \exp[-(\Delta G_U{}^0(H_2O) + m[\text{urea}])/RT])/4P_t \qquad (4)$$

Denaturation curves were fitted to eq 4 using the program GraFit 3.0 (Erithacus, England). Equation 4 can be rearranged and solved to determine $[\text{urea}]_{1/2}$, the urea concentration at which half of the protein is in the unfolded form ($f_U = 0.5$):

$$[\text{urea}]_{1/2} = -\{(\ln P_t)RT + \Delta G_U{}^0(H_2O)\}/m \qquad (5)$$

RESULTS

Design and Construction of Hybrid Tet Repressors with LacI- and 434Cro-Derived HTH Motifs. We selected the HTH motifs from LacI or 434Cro for engineering into the TetR background. The 434 Cro HTH motif and the recognition site of 434 O_{R3} are very similar to the respective TetR/*tetO* sequences. The orientation of the TetR HTH with respect to the palindromic center of *tetO* is identical to the orientation of LacI with *lacO* and opposite to that of the phage repressors (Boelens et al., 1987). The hybrid TetR constructed are shown in Figure 1 (top). In TetR/Lac1 and TetR/Cro1 (see Figure 1) residues 27−47 of TetR containing the entire HTH were exchanged. Since the HTH is three residues shorter in TetR than in LacI and 434Cro and since the turn between the recognition α-helix and the adjacent helix α4 in TetR may require structural flexibility of the respective residues (Hinrichs et al., 1994; Kisker et al., 1995), TetR/Lac2 and TetR/Cro2 contain exchanges limited to the length of the TetR recognition α-helix. Only one additional residue at the C-terminus of the LacI or 434Cro recognition α-helix was introduced (Figure 1, top), because it contributes to the architecture of the LacI and 434Cro HTH (Mondragon & Harrison, 1991; Chuprina et al., 1993). In the next set of constructs only the solvent-exposed amino acids of the respective HTH were exchanged (TetR/Lac3 and TetR/Cro3). TetR/Lac4 and TetR/Cro4 contain replacements of the last residue in the turn and the entire respective recognition α-helix, and in TetR/Lac5 and TetR/Cro5 only the solvent-exposed residues from the respective recognition α-helix are present. The genes encoding the chimeric proteins were constructed by cassette mutagenesis in the *tetR* gene of pWH1900 (Baumeister et al., 1992b).

In Vivo Activity of the Hybrid Repressors. The pWH1900 variants with the *tetR* mutants were transformed into *E. coli* WH207(λ*tet*50) to determine their binding to *tetO*. All *tetR* mutants were expressed to the same steady-state amount of soluble protein as wt *tetR*, as was confirmed by Western blot analysis of soluble cell extracts (data not shown) and by their transdominant phenotypes (see below). Repression of the *tetA*−*lacZ* transcriptional fusion on λ*tet*50 was determined at 28 and 37 °C. TetR gave about 100-fold repression but none of the hybrid TetR proteins repressed the *tetA*−*lacZ* fusion. Thus, no *tetO* binding of the chimeric proteins was detectable *in vivo*. Therefore, we performed a transdominance assay to test the TetR hybrids for their capacity to heterodimerize with wt TetR *in vivo*. pWH1900 derivatives encoding the *tetR* variants were transformed into *E. coli* WH207(λ*tet*50)/pWH853 (wt *tetR*) and assayed for β-galactosidase expression at 28 and 37 °C. The results are

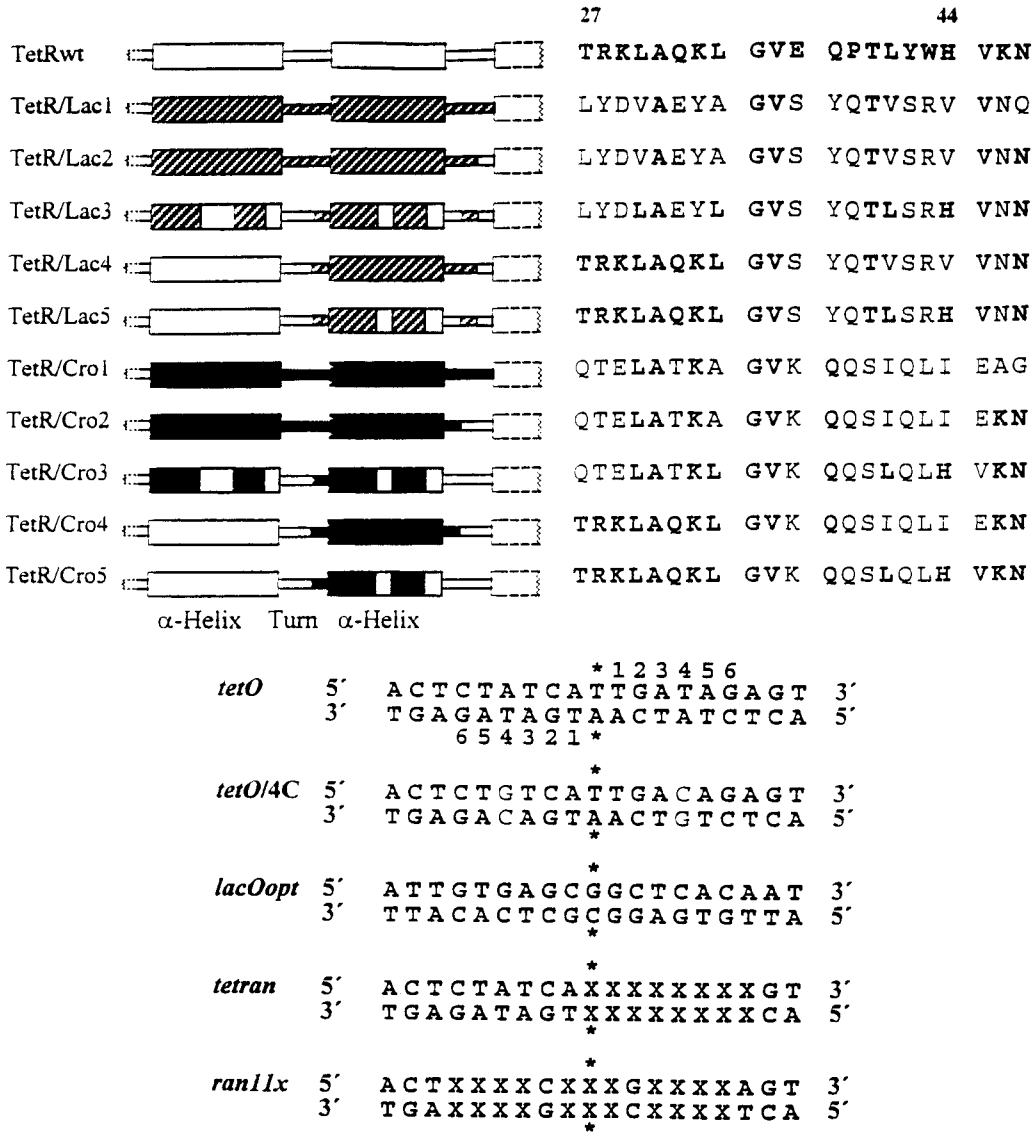

FIGURE 1: Specification of the substitutions constructed in HTH of TetR. (Top panel) A schematic presentation of the different substitutions is given on the left-hand side. The wt TetR amino acids are given in white, while substitutions against amino acids of LacI and 434Cro are indicated in gray or black, respectively. The amino acid sequences of the respective substitutions are shown on the right-hand side. Amino acids from TetR are shown in boldface type. (Bottom panel) Specification of DNA sequences used for binding experiments. The numbering of nucleotides is given in the top panel. The palindromic centers of the operators are indicated by asterisks. Randomized positions in *tetran* and *ran11x* are depicted as X.

presented in Table 1. All mutants were transdominant and led to similar derepression factors, indicating that they can form heterodimers with wt TetR. Derepression at 28 °C was between 4- and 10-fold, whereas 3−6-fold derepression was obtained at 37 °C. Ratios of derepression at both temperatures (see the 28 °C/37 °C ratio in the last column of Table 1) were between 1.4 and 1.9 and suggest that all TetR variants are only slightly temperature-sensitive. We conclude that they are stable and soluble in the cell.

Probes of Conformational Stability. Conformational changes in purified TetR proteins during unfolding were followed employing two spectroscopic methods. The intrinsic tryptophan fluorescence was used to observe changes in the tertiary structure of TetR. The fluorescence of TetR originates largely from Trp 43 in the HTH and from Trp 75 in the dimerization domain. It decreases upon denaturation (Figure 2a) and the emission maximum is shifted from 340 to 350 nm. Both changes are typical for protein unfolding (Pace et al., 1989). The largest fluorescence intensity change occurs at about 328 nm, and this wavelength was used to follow the changes in the tertiary structure of TetR upon denaturation.

The circular dichroism spectra above 220 nm of TetR in the absence and in the presence of 8 M urea are shown in Figure 2b. Measurements below 220 nm were precluded by the strong absorption of urea. The spectrum of folded TetR in the absence of urea shows a maximum of negative ellipticity around 222 nm. This band reflects the high content of α-helical secondary structure of TetR (Hinrichs et al., 1994). It is virtually absent in 8 M urea. The ellipticity at 222 nm was used to follow the changes in secondary structure in the course of TetR unfolding.

Two-State Model for Unfolding of TetR by Urea. The urea-induced unfolding of TetR proteins follows a monophasic, sigmoidal decrease in fluorescence, and identical transitions were observed when the unfolding of TetR was monitored by fluorescence or by CD (Figure 3a). As expected for a bimolecular reaction, the midpoint of the unfolding transition depends on protein concentration (not shown). It increases from 4.3 to 5 M urea when the protein

Table 1: Transdominance of TetR Variants[a]

	β-galactosidase activity (%)				
tetR allele	28 °C	derepression factor	37°C	derepression factor	ratio 28 °C/37 °C
none	2.2 ± 0.3	1.0	2.5 ± 0.2	1.0	1.0
WT	1.0 ± 0.1	0.5	1.2 ± 0.1	0.5	1.0
Lac1	14.3 ± 1.4	6.5	8.7 ± 0.7	3.5	1.9
Lac2	11.4 ± 1.4	5.2	8.1 ± 0.1	3.2	1.6
Lac3	9.3 ± 0.2	4.2	7.5 ± 0.1	3.0	1.4
Lac4	11.1 ± 0.1	5.0	8.7 ± 0.5	3.5	1.4
Lac5	10.2 ± 1.0	4.6	7.5 ± 0.1	3.0	1.5
Cro1	21.2 ± 1.3	9.6	15.1 ± 1.1	6.0	1.6
Cro2	22.2 ± 2.1	10.1	13.0 ± 1.1	5.2	1.9
Cro3	13.1 ± 1.4	6.0	7.9 ± 0.5	3.2	1.9
Cro4	14.2 ± 1.1	6.5	8.8 ± 0.4	3.5	1.9
Cro5	13.7 ± 2.9	6.2	8.4 ± 0.5	3.4	1.8

[a] β-Galactosidase activities are given as percent of the values determined in strain WH207(λ*tet*50)/pWH806 cotransformed with pWH1401. Both plasmids do not encode *tetR* genes. One hundred percent corresponds to 7550 units (Miller, 1972) at 28 °C and to 5000 units at 37 °C. The derepression factors are the activity ratios for the mutant over row "none". The ratios 28 °C/37 °C were calculated by dividing the respective derepression factors.

Table 2: Gibbs Free Energy of Unfolding of Wild-Type TetR[a]

	from fluorescence		from circular dichroism	
[protein] (μM)	$\Delta G_U^0(H_2O)$ (kJ/mol)	$[urea]_{1/2}$ (M)	$\Delta G_U^0(H_2O)$ (kJ/mol)	$[urea]_{1/2}$ (M)
0.4	76 ± 4.6	4.3	nd	nd
5.0	78 ± 3.8	5.0	71 ± 4.3	5.0

[a] Data were obtained at 25 °C. $\Delta G_U^0(H_2O)$ values were obtained from fits to eq 4 and values of $[urea]_{1/2}$ from fits to eq 5.

concentration is increased by 12.5-fold, suggesting that the dissociation of native dimeric TetR and the subsequent unfolding of the monomers are tightly coupled reactions and that folded monomers do not accumulate to a significant extent at equilibrium. Taken together, the results are well explained by a two-state model as in eq 1, in which only folded dimers and unfolded monomers exist at equilibrium in significant concentrations.

The unfolding of wild-type TetR is a reversible reaction. After unfolding in 8 M urea and refolding by a subsequent 200-fold dilution with urea-free buffer, the fluorescence emission spectrum of TetR was identical with that of a native control (Figure 2a). The functionality of the refolded TetR protein was examined by titration with Tc. Over 95% of the original Tc binding activity was recovered after the unfolding/refolding cycle (data not shown). We therefore conclude that denaturation under these conditions is a reversible reaction.

The kinetics of unfolding and refolding of wild-type TetR were also investigated by fluorescence. At a protein concentration of 5 μM both unfolding in the region of the unfolded baseline (e.g., at 8.0 M urea; cf. Figure 3) and refolding in the region of the native baseline (0−2 M urea) were found to be very rapid reactions, which were complete within the time required for manual mixing (5 s). Folding reactions are usually slowest in the transition region. Therefore, the unfolding and refolding kinetics of 5 μM TetR were repeated near the midpoint of the unfolding transition at 5.0 M urea. In these experiments (Figure 2c) similar half-times of about 50 s were found for the equilibration of the unfolded and folded molecules irrespective of the initial conditions. The final fluorescence values observed in unfolding and refolding were very similar, confirming that unfolding of TetR is a reversible reaction.

Thermodynamic Stability of TetR. Unfolding of TetR was assumed to follow the simple two-state model (Bowie & Sauer, 1989; Grant et al., 1992) in eq 1, and eq 4 was used to calculate the Gibbs free energy of unfolding $\Delta G_U^0(H_2O)$ of TetR from the data obtained at various protein concentrations and with different spectral probes. The results are summarized in Table 2. The $\Delta G_U^0(H_2O)$ values determined at two different protein concentrations and from the fluorescence- and the CD-detected transitions are identical within experimental error. In all cases values between 71 and 76 kJ/mol were obtained for $\Delta G_U^0(H_2O)$. This good coincidence further supports the assumption that unfolding of TetR is a two-state transition.

The stability of the 10 variants was also determined by urea denaturation experiments. All variants showed coincident transitions when followed by fluorescence and by circular dichroism, and the transition curves depended on protein concentration (not shown). Denaturation curves for three of the TetR variants are shown in Figure 3b. All transition curves are sigmoidal and monophasic, suggesting that denaturation also follows the same simple two-state mechanism. Moreover, these transitions are nearly coincident, suggesting that all the variants show very similar, if not identical, stabilities. This was confirmed by the thermodynamic analyses of these transitions. Table 3 lists the thermodynamic parameters for denaturation of all TetR mutants. To avoid errors that are easily introduced by the long extrapolation of the ΔG_U^0 values to 0 M urea (Johnson & Fersht, 1995), we compared the ΔG_U^0 values of the HTH variants and the wild-type protein of TetR at the midpoint of the transition of the wild-type protein. As is evident from the data in Table 3, the conformational stability of TetR is indeed almost unaffected by all the extensive substitutions in its HTH. All ΔG_U^0 values fell into the narrow range of 36.5 ± 2.5 kJ/mol. The conformational stability of the TetR that lacks the entire HTH motif (Berens et al., 1995), is not significantly different from that of the wild-type protein (Table 3). Figure 3b includes the denaturation curve of the deletion mutant for comparison with other TetR proteins.

Binding Constants of $[Mg\text{-}Tc]^+$ to the TetR Variants. The influence of alterations in HTH on the affinity of the TetR variants for $[Mg\text{-}Tc]^+$ was determined. All mutants and wt TetR showed nearly the same binding constants ranging from $(0.6 \pm 0.2) \times 10^9$ M^{-1} to $(3.5 \pm 0.3) \times 10^9$ M^{-1}. Thus,

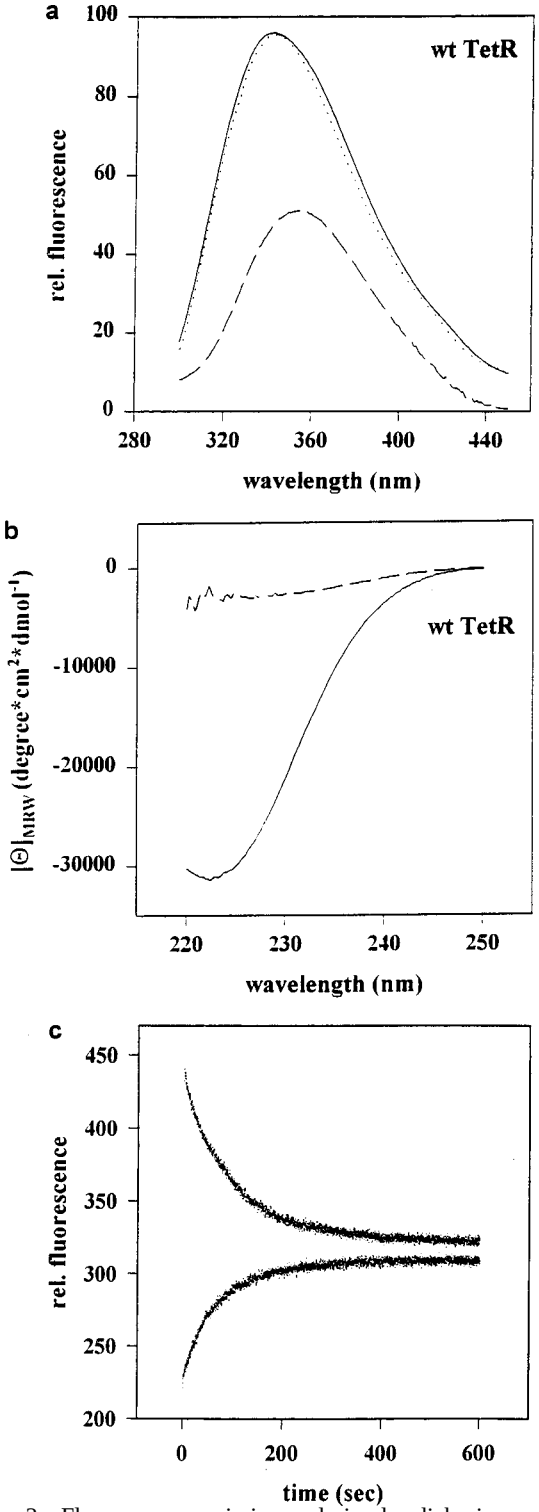

FIGURE 2: Fluorescence emission and circular dichroism spectra of TetR. Conditions were as described in Materials and Methods. (a) Fluorescence emission spectra for native, denatured, and renatured states of TetR. Spectra were obtained with 0.4 μM wt TetR equilibrated at 0 M urea (solid line), 8 M urea (dashed line) and with wt TetR equilibrated in 8 M urea and then diluted 200-fold to a final protein concentration of 0.4 μM and an urea concentration of 0.04 M (dotted line). (b) CD spectra of 5 μM TetR between 220 and 250 nm at 0 M (solid line) and 8 M urea (dashed line). (c) Unfolding and refolding rates of TetR to the midpoint of transition as monitored by fluorescence emission following a jump from 0 to 5 M urea and from 8 to 5 M urea, respectively. Protein concentration was 5 μM monomer. The measurements were done as described in Materials and Methods.

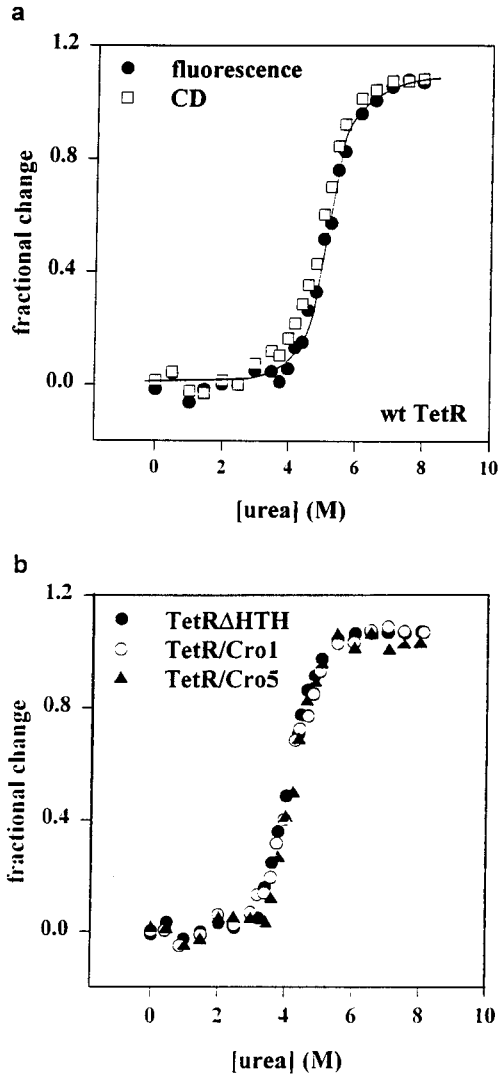

FIGURE 3: Urea denaturation curves of TetR variants. (a) The unfolding reaction of wild-type TetR was monitored by changes in relative fluorescence at an emission wavelength of 328 nm and by CD. The fractions of unfolded protein (f_U) were obtained as described in Materials and Methods. The protein concentration was 0.4 μM monomer. The curve drawn through the experimental points was calculated by nonlinear least-squares analysis of the data according to eq 3. (b) Denaturation curves of TetRΔHTH, TetR/Cro1, and TetR/Cro5. The data points were determined by changes in fluorescence intensity at 25 °C. The fractions of unfolded protein (f_U) were obtained as described in Materials and Methods. Protein concentration was 0.4 μM monomer. Conformational stability and midpoints of urea unfolding curves derived from these data are listed in Table 3.

complete or partial exchange of the HTH has no significant influence on $[Mg\text{-}Tc]^+$ binding. This result supports the notion that the structure of the protein core in these mutants is similar to that of wt TetR.

In Vitro DNA Binding. We determined the *in vitro* DNA binding activity of purified TetR variants. Different DNA fragments, containing nonspecific sequences, wt *tetO*, *tetO* variants, *lacO*, and a pool of randomized sequences were used for this purpose. An overview of these DNA fragments is shown in Figure 1 (bottom). The mutants TetR/Lac1, TetR/Lac2, TetR/Lac3, TetR/Cro1, TetR/Cro2, and TetR/Cro3 did not yield detectable retardation of unspecific DNA in PAGE as shown in Figure 4 for the DNA pool *ran11x*. The mutants TetR/Lac4, TetR/Lac5, TetR/Cro4, and TetR/Cro5 exhibited retardation of this DNA at high concentrations, as does wt TetR. DNA retardation experiments with fragments containing $tetO_1$ and $tetO_2$ showed specific binding

Table 3: Thermodynamic Parameters of wt TetR and TetR Variants[a]

	ΔG_U^0(4.3 M urea)[b] (kJ/mol)	$\Delta\Delta G_U^0$(4.3 M urea)[c] (kJ/mol)	$[urea]_{1/2}$[d] (M)
wt TetR	36.7 ± 0.9	0.0	4.3
TetRΔHTH	34.0 ± 0.5	−2.7	4.0
TetR/Lac1	34.9 ± 0.2	−1.8	4.1
TetR/Lac2	37.9 ± 0.9	1.2	4.4
TetR/Lac3	38.9 ± 0.5	2.2	4.4
TetR/Lac4	37.0 ± 0.0	0.3	4.4
TetR/Lac5	38.9 ± 1.2	2.2	4.4
TetR/Cro1	34.5 ± 0.6	−2.2	4.1
TetR/Cro2	34.8 ± 0.1	−1.9	4.1
TetR/Cro3	36.7 ± 0.9	0.0	4.3
TetR/Cro4	35.5 ± 0.8	−1.2	4.2
TetR/Cro5	37.4 ± 0.9	0.7	4.4

[a] Data were obtained at 25 °C as described under Materials and Methods. TetR protein concentration was 0.4 μM monomer. [b] Values of ΔG_U^0(4.3 M urea) were obtained by fitting the data from urea denaturation curves to eq 4 at the midpoint of denaturation of wt TetR. [c] $\Delta\Delta G_U^0$(4.3 M urea) is the ΔG_U^0(4.3 M urea) of the mutant minus the ΔG_U^0(4.3 M urea) of wt TetR. [d] Values of urea concentration at the midpoint of the unfolding transition were calculated from eq 5.

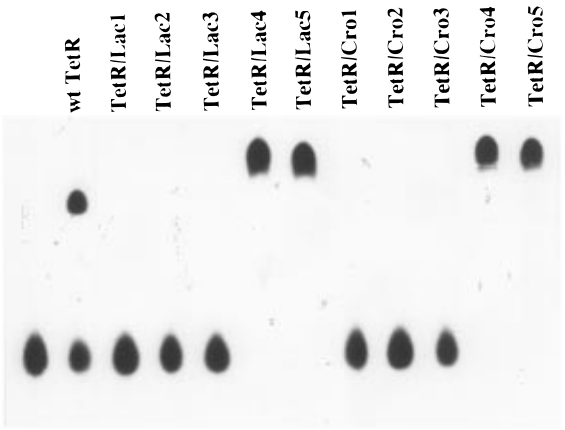

FIGURE 4: Electrophoretic mobility shift assays with the randomized oligonucleotide pool (*ran11x*) and purified TetR variants. About 10 pmol of PCR-generated double-stranded oligonucleotides (68 bp) were incubated with 2.5 μg of purified protein.

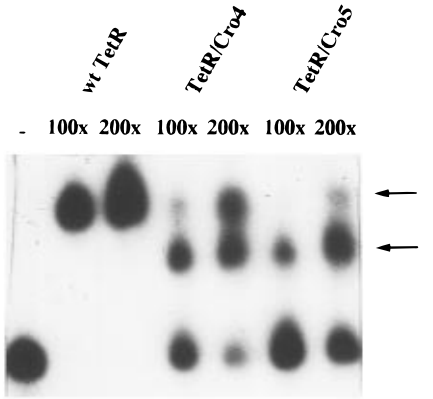

FIGURE 5: Electrophoretic mobility shift assays with [α-^{32}P]-end-labeled fragment containing $tetO_1$ and $tetO_2$. Operator fragment (0.5 pmol) was incubated with a molar excess of purified protein as indicated above the respective lane.

of TetR/Cro4 and TetR/Cro5 (Figure 5). Compared to wt TetR, higher concentrations of both TetR variants were required.

Specific *tetO* binding was verified by DNase I protection experiments. Both TetR/Cro4 and TetR/Cro5 show the same DNase I protection pattern as wt TetR (Figure 6), but an about 1000-fold higher ratio of protein over DNA was necessary to obtain binding. Thus, TetR mutants with 434Cro amino acids in the TetR recognition α-helix bind weakly but specifically to *tetO*. These complexes are inducible with Tc (Figure 6). TetR/Lac4 and TetR/Lac5 led only to smearing of the *tetO* fragment in retardation experiments, indicating nonspecific binding (data not shown).

Symmetrical base pair exchanges have been generated in both half-sites of *tetO* (Figure 1, bottom; Sizemore et al., 1990). The TA → CG exchange at position 4 generates a recognition site similar to 434 O_{R3} [see Figures 1 (bottom) and 9]. In DNA retardation experiments with this set of *tetO* variants, TetR/Cro4 and TetR/Cro5 only gave rise to specific complexes with AT → CG exchange at position 5 of *tetO* but not with *tetO*-4C (data not shown). TetR/Lac4 and TetR/Lac5 led to similar smearing of DNA with the operator variants as with wt *tetO*, indicating only nonspecific binding. In order to test the capacity of TetR/Lac4 and TetR/Lac5 to bind *lacO*, we constructed *lacOopt* (see Figure 1, bottom) by adding a central base pair to ideal *lacO* (Lehming et al., 1987). This alteration adjusts the *lacO* half-sites to the *tetO* spacing shown to be crucial for TetR binding (Wissmann et al., 1988). No specific binding to *lacOopt* was detected in either DNA retardation experiments or DNase I footprinting (data not shown). A smearing of DNA in the retardation experiments indicated nonspecific binding. In contrast to the 434Cro-based TetR variants, the introduction of amino acids responsible in LacI for specific interactions with DNA in the context of TetR does not yield a hybrid protein able to form a specific complex with DNA.

Binding Site Selection. We used a modified target detection assay (Thiesen & Bach, 1991) to select optimal binding sequences for the TetR variants TetR/Cro4, TetR/Cro5, TetR/Lac4, and TetR/Lac5. These HTH substitutions contain Arg as the second residue in the positioning α-helix of HTH. An essential sequence-specific interaction with bp 2 of *tetO* was demonstrated in the TetR−*tetO* complex for this residue (Baumeister et al., 1992a). Therefore, *ran11x* was designed to bear a G residue at operator position +2 as defined in Figure 1 (bottom). We also designed an oligonucleotide (*tetran*) containing one half-site of *tetO* combined with one randomized half-site to serve as a positive control with wt TetR for the target detection assay. After eight rounds of selection with *tetran* and wt TetR, we found an enrichment of the *tetO* sequence (Figure 7, left-hand panel). The same result was obtained after twelve rounds with *ran11x* (Figure 7, right-hand panel). Under the same conditions, neither TetR/Cro4, TetR/Cro5, TetR/Lac4, nor TetR/Lac5 showed an enrichment of nucleotides at any position. This indicates that no high-affinity binding site, comparable to *tetO* for wt TetR, exists for the TetR variants.

DISCUSSION

Urea-induced unfolding of TetR is a reversible reaction and fully functional protein is obtained after an unfolding/refolding cycle. We could not find evidence for unfolding intermediates, such as in the case of the Trp repressor, which is also a dimeric protein (Eftink et al., 1994). This confirms that the TetR dimer dissociates and unfolds in a concerted reaction and that the folded monomers are unstable. They either revert back to the native state or unfold to the

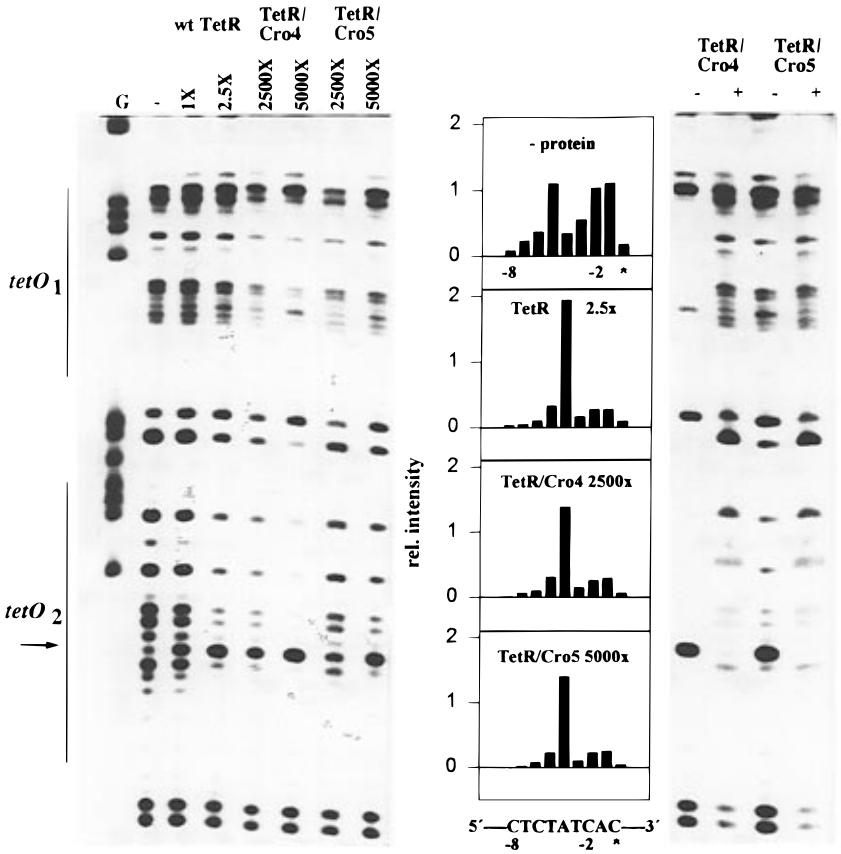

FIGURE 6: DNase I protection experiments. (a) Binding of wt TetR, TetR/Cro4, and TetR/Cro5 to $tetO_1$ and $tetO_2$. [^{32}P]-End-labeled operator fragments (0.5 pmol) were incubated with the indicated excess of protein over DNA. Maxam−Gilbert G reaction products of the same DNA were used as a marker (lane G). The boundaries of the two binding sites $tetO_1$ and $tetO_2$ are indicated on the left-hand side of the figure. A DNase I hypersensitive site is indicated by an arrow. (b) Densitometric analysis of DNase I protection of $tetO_2$ bound to wt TetR, TetR/Cro4, and TetR/Cro5 and the free fragment. The respective nucleotides of the left half-site of $tetO_2$ are indicated at the bottom. The intensity of the bands was normalized to the intensity of a band outside of the operator. (c) Binding of TetR/Cro4 and TetR/Cro5 to $tetO_1$ and $tetO_2$ in the presence (+) and absence (−) of Tc. The same fragment as in panel a was incubated with a 5000-fold molar excess of purified protein and a 100-fold molar excess of Tc over protein monomer.

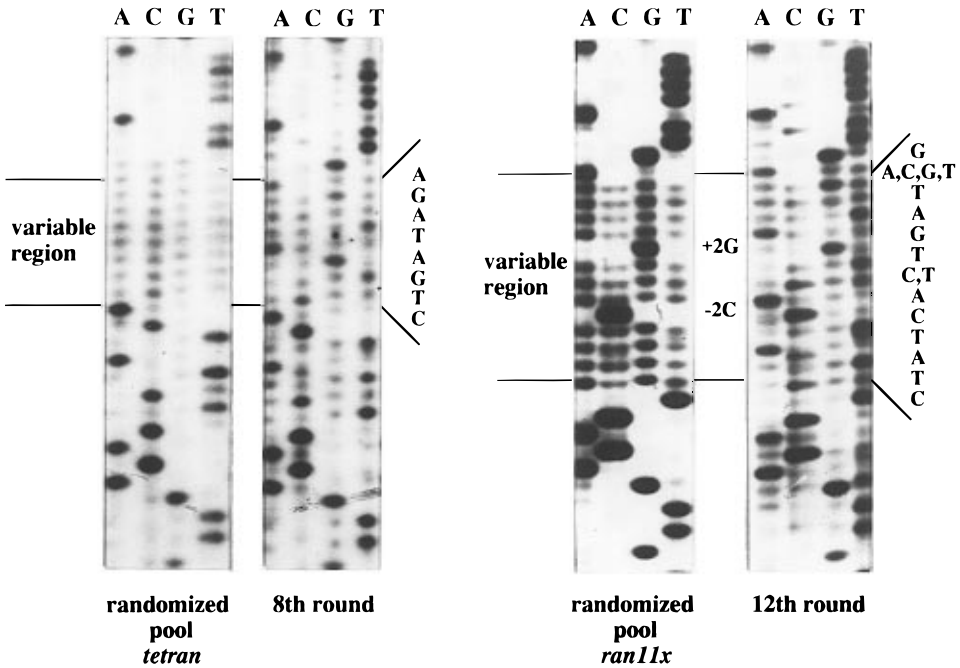

FIGURE 7: Nucleotide sequences of selected binding sites. Comparison between the starting randomized oligonucleotide pool and the sequences after eight (left-hand side) and 12 (right-hand side) rounds of selection with wt TetR. The variable region and the sequence preferences are shown. Binding site selection was performed as described in Materials and Methods.

completely unfolded state. It is conceivable that the exposition of 27% mainly hydrophobic surface to the solvent upon dissociation renders the folded monomers unstable. The K_{diss} of smaller than 10^{-7} M^{-1} measured previously for the TetR

dimer (Hillen et al., 1983) agrees with these results. Arc repressor, another small dimeric DNA binding protein (Breg et al., 1990; Raumann et al., 1994), also dissociates and unfolds in a concerted reaction (Bowie & Sauer, 1989; Milla & Sauer, 1994). At low protein concentrations, Arc repressor is predominantly monomeric and unfolded.

The unfolding and refolding kinetics of TetR are fast. They could be followed after manual mixing only in the transition region. It should be noted that TetR does not contain cis prolines or disulfide bonds, which often lead to slow steps in protein folding (Fischer & Schmid, 1990; Creighton, 1994).

The $\Delta G_U^0(H_2O)$ values reported for small dimeric proteins seem generally to be higher than those of monomeric proteins (Neet & Timm, 1994; Pace et al., 1989). This enhanced stability of dimeric proteins was assumed to be due mainly to interactions between both subunits (Neet & Timm, 1994). The stability of TetR is 77 kJ/mol, in the range of values reported for other protein dimers of similar size (Neet & Timm, 1994). CAP is of comparable size and exhibits a $\Delta G_U^0(H_2O)$ of 80 kJ/mol (Cheng et al., 1993). It is important, however, to keep in mind that the Gibbs free energies of association of dimeric molecules refer to a standard concentration of 1 M of all components. This is a very unrealistic reference state for proteins. At a concentration of, e.g., 0.1 μM (which might be closer to the cellular concentrations of repressor proteins) the Gibbs free energy is reduced by RT ln 10^{-7} to about 40 kJ/mol. Values of similar magnitude are often observed for the stability of small, monomeric proteins.

Alterations in the amino acid sequence can have strongly different consequences for the stability of a protein. It can increase or decrease, depending on the structural context of the site of mutation and on the chemical properties of both the original residue and its substitute (Cupo & Pace, 1983; Matthews, 1987; Milla et al., 1994). Even extensive mutations in the HTH motif of TetR did not decrease the thermodynamic stability of the respective mutant protein to a significant extent. At the transition midpoint of the wild-type protein (4.3 M urea), all ΔG_U^0 values are in the same range as observed for the wild-type protein. Even the deletion of the entire HTH motif (Berens et al., 1995) led to a destabilization by only about 2.7 kJ/mol. This is remarkable because even hydrophobic residues were exchanged, which are thought to contribute most to the stability of a protein (Matthews, 1993). Often, such alterations result in dramatic destabilizations (Fontana, 1991; Hecht et al., 1984; Lim & Sauer, 1989), whereas substitutions of solvent-exposed residues usually have small effects on stability (Fontana, 1991; Heinz et al., 1992). Substitutions of hydrophobic residues in λ repressor had also small effects when the hydrophobic character of the residue was maintained (Lim & Sauer, 1989; 1991). This may be due to the fact that HTH motifs have a tendency to be unstable, as some of these motifs are apparently unstable in the uncomplexed protein and assume their structure only when complexed with their cognate DNA (Pabo & Sauer, 1992).

Mutations in the HTH have only local effects on TetR conformation. This conclusion is in agreement with the crystal structure of the TetR−[Mg-Tc]$^+$ complex, in which the DNA binding domain is spatially very well separated from the protein core. The connecting helix α4 between the two entities was not affected by the mutations studied here, and hence, the structural changes that occurred in the DNA binding domain were probably not transferred into the protein core. The fact that this is even true for the mutant containing a deletion of 24 residues in the DNA binding domain clearly underscores the structural separation of that domain from the core.

The existence of a recognition code allowing the prediction of DNA binding specificity based on the amino acid sequence of a HTH protein is controversial (Matthews, 1988; Lehming et al., 1991; Anderson, 1992; Brennan, 1992; Helbl et al., 1993; Suzuki & Yagi, 1994). We address this question by HTH, recognition α-helix, or solvent-exposed residue swap experiments moving the DNA-contacting regions of LacI and 434Cro into the sequence context of TetR. The resulting TetR hybrids are stable in the cytoplasm, as demonstrated by their similar negative transdominance over wt TetR. Even when overproduced, all of the hybrid TetR proteins were stable and soluble *in vivo*. Furthermore, their similar [Mg-Tc]$^+$ binding constants indicate that activity, and hence the core structure of the hybrid proteins, is not affected by the sequence changes in the DNA binding domain. This agrees very well with the stabilities of the TetR variants and implies that their DNA binding properties are not obscured by stability problems of the mutated proteins.

The TetR variants show major differences in their DNA binding activities. No DNA binding was detected for TetR/Lac1, TetR/Lac2, TetR/Lac3, TetR/Cro1, TetR/Cro2, and TetR/Cro3. Whereas structural distortions of the HTH motifs in TetR/Lac1, TetR/Lac2, TetR/Cro1, and TetR/Cro2 are likely because substitutions of amino acids in the core of the motif and beyond the motif were made (see Figure 1, top), only solvent-exposed residues were exchanged in TetR/Lac3 and TetR/Cro3. Therefore, the failure of TetR/Lac3 and TetR/Cro3 to bind DNA should not be due to local structural distortions in the motif.

It is surprising that TetR/Lac3 and TetR/Cro3 do not even bind nonspecifically to DNA. This may be due to changes of the net charge on the surface of the DNA reading heads. Two positively charged amino acids in wt TetR were exchanged for neutral and negatively charged side chains in the variants (see Figure 1, top). This could reduce their affinity to the negatively charged DNA. The TetR variants TetR/Lac4, TetR/Lac5, TetR/Cro4, and TetR/Cro5 contain the wt positioning α-helix, including the two positively charged residues, and bind at least nonspecifically to DNA. Furthermore, the exchanges in TetR variants incapable of DNA binding involve the two N-terminal amino acids (T27 and R28) in the positioning α-helix that are crucial for *tetO* binding (Wissmann et al., 1991a; Baumeister et al., 1992a). We assume that this causes the loss of DNA binding activity. Taken together, the exchange of the recognition α-helix of wt TetR leads to proteins capable of binding DNA at least nonspecifically, whereas TetR variants with additional substitutions in the positioning α-helix do not even bind nonspecifically to DNA.

While the wt positioning α-helix is necessary for nonspecific DNA binding, it is not sufficient for sequence-specific DNA binding, since TetR/Lac4 and TetR/Lac5 show only nonspecific DNA binding. This may be due to structural distortions in the HTH of these variants. But residues obviously determining the structural integrity of the HTH were not changed in TetR/Lac5. This leads to the hypothesis that the DNA-contacting amino acids in the two reading

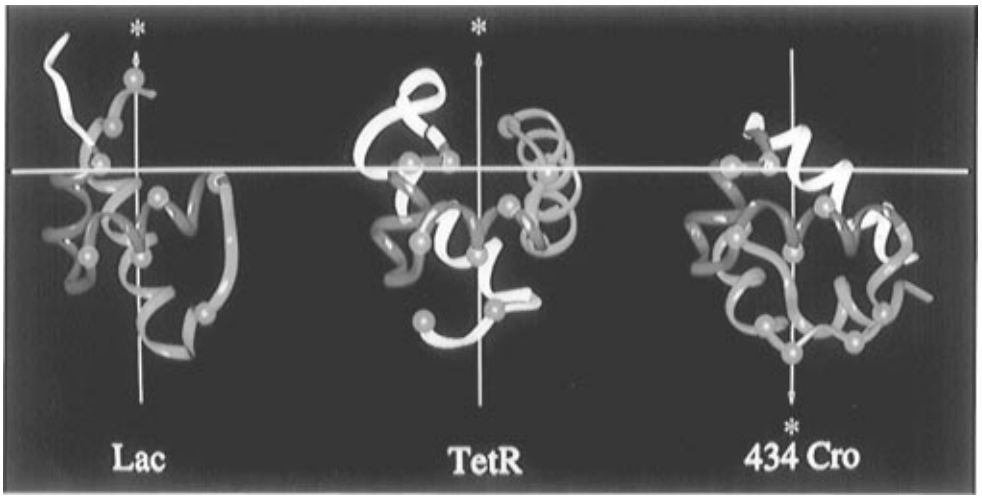

FIGURE 8: Comparison of the orientations of the DNA reading heads from LacI, TetR, and 434Cro with respect to the recognized operator half-sites. The HTH are shown in red, residues located N-terminal of the HTH are in white, and residues C-terminal of the HTH are in green. The horizontal line defines the location of the respective base pair (operator position 3) recognized by the first residue in the recognition α-helix. The vertical arrows represent the axes of the DNA with the asterisks indicating the palindromic center of the respective operator. The blue balls indicate contacts between side chains or the backbone with the DNA phosphates.

heads are not oriented such that they could discriminate DNA sequences. In contrast, TetR/Cro4 and TetR/Cro5 carry exchanges of the same residues as TetR/Lac4 and TetR/Lac5 and are able to recognize *tetO*. This indicates that LacI and TetR have their respective HTH at drastically different locations with respect to the core of the proteins and also to the recognized DNA, as is confirmed by the LacI−*lacO* and PurR−*purF* cocrystal structures, which revealed the presence of a so-called hinge α-helix making crucial contacts to the operator by bending the DNA away from the protein (Chuprina et al., 1993; Schumacher et al., 1994; Lewis et al., 1996). This DNA deforming element is missing in the TetR−LacI hybrids. The orientations of the LacI, TetR, and 434Cro HTH with respect to each recognized operator half-site shown in Figure 8 reveal large differences between LacI and the proposed orientation of TetR. The results obtained here are in agreement with such differences. In particular, the other location of residues outside of HTH providing phosphate contacts may explain the lack of nonspecific DNA binding for TetR/Lac1,2. The lack of specific DNA binding of the TetR/Lac chimera agrees with the different angles of both recognition α-helices with respect to the major groove of DNA.

Despite the fact that the N-termini of the recognition α-helices point to different sides of the operators in TetR and 434Cro, the positioning of the motifs and many of the phosphate contacts are very similar (see Figure 8). Thus, the result that TetR/Cro4 and TetR/Cro5 specifically bind to *tetO* supports the TetR−*tetO* recognition model and can be explained by similarities between the residues contacting DNA in TetR and 434Cro and their recognized sequences. The respective operators bound by TetR and 434Cro, *tetO* and 434 O_{R3} (see Figure 9), share the most important base pairs for binding, except for the one at position 4 (Wissmann et al., 1988; Sizemore et al., 1990; Koudelka & Lam, 1993). Arg at position 2 of TetR HTH forms a specific contact with G at bp2 in *tetO* (Baumeister et al., 1992a) and is also present in TetR/Cro4 and TetR/Cro5. Furthermore, the first three amino acids of the recognition α-helices of TetR and 434Cro contribute to DNA binding specificity (Mondragon & Harrison, 1991; Baumeister et al., 1992a). The first of these is

		2	3	4	5	6	
tetO	*	G	A	T	A	G	
croO$_{R3}$			A	C	A	G	*

	28	37	38	39	40	41	42	43	44	45
TetR	R	E	**Q**	**P**	**T**	**L**	**Y**	**W**	**H**	V
434Cro	T	K	**Q**	**Q**	**S**	**I**	**Q**	**L**	**I**	E
TetR/Cro4	R	K	**Q**	**Q**	**S**	**I**	**Q**	**L**	**I**	E
TetR/Cro5	R	K	**Q**	**Q**	**S**	**L**	**Q**	**L**	**H**	V
	2	11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	19

FIGURE 9: (Top) Comparison of the contacted nucleotides of *tetO* and *croO*$_{R3}$. The sequences are aligned at base pair 3, which is contacted by the first residue of the respective recognition helix in TetR and 434Cro, and are designated as indicated. The asterisk indicate the location of the palindromic center in the respective operator. (Bottom) Alignment of the amino acid sequences of TetR, 434Cro, TetR/Cro4, and TetR/Cro5. The numbering is given according to the respective position in wt TetR at the top. The numbering starting with the first residue in the HTH is given at the bottom. The residues corresponding to the recognition α-helix of wt TetR are shown in boldface type.

identical in TetR and 434Cro and, hence, in TetR/Cro4 and TetR/Cro5. The second residue is Gln in 434Cro and Pro in TetR. The Gln side chain in 434Cro adopts a Pro-like conformation (Mondragon & Harrison, 1991). The third residue in the 434Cro recognition α-helix is Ser and that of TetR is Thr, which can be substituted by Ser without affecting *tetO* binding (Baumeister et al., 1992a). Consequently, it seems reasonable that TetR/Cro4 and TetR/Cro5 are able to bind specifically to *tetO*, albeit with reduced affinity compared to TetR.

The *tetO*-4C variant (Figure 1, bottom) resembles the 434 operator O_{R3} when the opposite orientation of the HTH motifs with respect to the operators is taken into account. The failure of TetR/Cro4 and TetR/Cro5 to bind to *tetO*-4C is remarkable, since the TetR mutant P39Q recognizes *tetO*-4C better than wt *tetO* (Baumeister et al., 1992a). The lack of binding of TetR/Cro4 and TetR/Cro5 to *tetO*-4C must, therefore, be due to other amino acid differences, presumably E37K, Y42Q, and W43L. The assumption that these amino acids do not contribute to sequence-specific binding of *tetO*

supports the view that recognition of DNA requires not only the residues chemically able to form specific contacts. Even when the motifs are so similarly located, a fine-tuned specific location of the recognition α-helix in the major groove of DNA may be necessary to allow the formation of these contacts. According to the results presented here, HTH from TetR and 434Cro differ in this respect.

The results presented here lead to the conclusion that a chemical correspondence between amino acids side chains and base pairs is not sufficient for recognition of a specific sequence. It seems that TetR chimera allowing the original TetR phosphate contacts show at least nonspecific DNA binding, whereas residues inside and outside the HTH contribute to the formation of the specific protein−DNA complex by positioning the recognition α-helix. For this reason a simple general recognition code between HTH proteins with extensive sequence differences outside their respective HTH is not applicable.

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