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Determinants of protein–protein recognition by four helix bundles: changing the dimerization specificity of Tet repressor

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Homo- and heterodimerization is essential for the activity of many proteins, particularly transcription factors. One widely distributed structural motif for protein recognition is the four helix bundle. To understand the molecular details determining specificity of subunit recognition in a dimer formed by a four helix bundle, we investigated Tet repressor (TetR) sequence variants TetR(B) and TetR(D), which do not form heterodimers. We used molecular modeling to identify residues with the potential to determine recognition of subunits. Directed mutagenesis of these residues in TetR(B) by the TetR(D) sequence resulted in chimeric TetR(B/D) repressors with new subunit recognition specificities. The single LS192 exchange in TetR(B/D)192 in the center of the helix bundle leads to a relaxed specificity since this variant dimerizes with TetR(B) and (D). To construct a variant with a new specificity it was not sufficient to mutate the contacting residue, F197, in the other subunit. Instead, it was necessary to exchange two more residues in the vicinity of F197 and S192. The resulting TetR(B/D)188, 192,193,197 forms dimers with TetR(D) but not with TetR(B), indicating that four amino acid exchanges are sufficient to change subunit recognition. These results establish that targeted alterations in the structural complementarity of protein–protein interaction surfaces can be used to construct new recognition specificities. However, it is not sufficient to adjust the complementary residues since the surrounding amino acids contribute essentially to protein–protein recognition.

Keywords: dimerization/protein–protein interaction/Tet repressor

Introduction

Highly specific interactions between macromolecules are crucial for almost every biological process. The sequence specific recognition of DNA by proteins, for example, has been intensively studied in the past (Pabo and Sauer, 1992). The processes involved in transcription, replication and repair of DNA not only depend on protein–DNA complexes but also on specific protein–protein complexes, which have only recently been studied intensively (Greenblatt, 1992; Nossal, 1992; Sancar 1994; Ptashne and Gann, 1997). Much information on the interactions of heterologous pairs of proteins has been gained from complexes that contain antibodies and antigens (Davies and Cohen, 1996), enzymes and inhibitors (Schreiber and Fersht, 1995) and receptors and hormones (Wells, 1996). Similar molecular recognition principles seem to govern the formation of transcription factor complexes (Kallipolitis *et al.*, 1997). Most DNA-binding proteins form both heterologous and homologous protein–protein complexes resulting in dimers or oligomers. The interaction between subunits is also important for the folding of many oligomers, since in contrast to the proteins of heterologous complexes, the isolated subunits are seldomly stable after dissociation (Neet and Timm, 1994; Jones and Thornton, 1996). In addition, heterodimerization of transcription factor variants is important for regulating their activity. The formation of heterodimers can increase (Kouzarides and Ziff, 1988) or decrease (Garrel and Modolell, 1990) the capacity to bind DNA, modify the sequence specificity of DNA-binding (Hai and Curran, 1991; Zechel *et al.*, 1994), or alter the ability to activate or repress the transcription of a target gene (Nakabeppu and Nathans, 1991; Hsu *et al.*, 1994). Therefore, controlled formation of heterodimers is an important step in many signal transduction pathways. Protein–protein recognition is achieved by a limited set of structural motifs including leucine zippers (Landschulz *et al.*, 1988) and four helix bundles (Lin *et al.*, 1995). Residues that determine the specificity of subunit interaction in leucine zippers have been thoroughly characterized (for a review see Lupas, 1996). In contrast, the mechanisms specifying subunit recognition by commonly found four helix bundles are far less well understood. Therefore, we performed a systematic mutagenesis study of residues in Tet repressor (TetR) which lead to the construction of variants with altered specificities of heterodimer formation.

Tet repressors regulate the expression of tetracycline resistance genes in Gram negative bacteria in response to the presence of tetracycline (tc) (for a review see Hillen and Berens, 1994). Based on sequence comparisons they have been grouped into seven classes, TetR(A–E), TetR(G) and TetR(H), which share between 45 and 80% sequence identity. Recently, the structure of TetR(D) in complex with tetracycline revealed that a four helix bundle mediates dimerization in this Tet repressor (Figure 1) (Hinrichs *et al.*, 1994; Kisker *et al.* 1995). We investigated and altered the specificity of dimerization between two similar TetR variants, TetR(B) and TetR(D) (Figure 2A), *in vivo*. The results identify specificity determinants in the four helix bundle and suggest a strategy for designing specificity of protein–protein interactions in general.

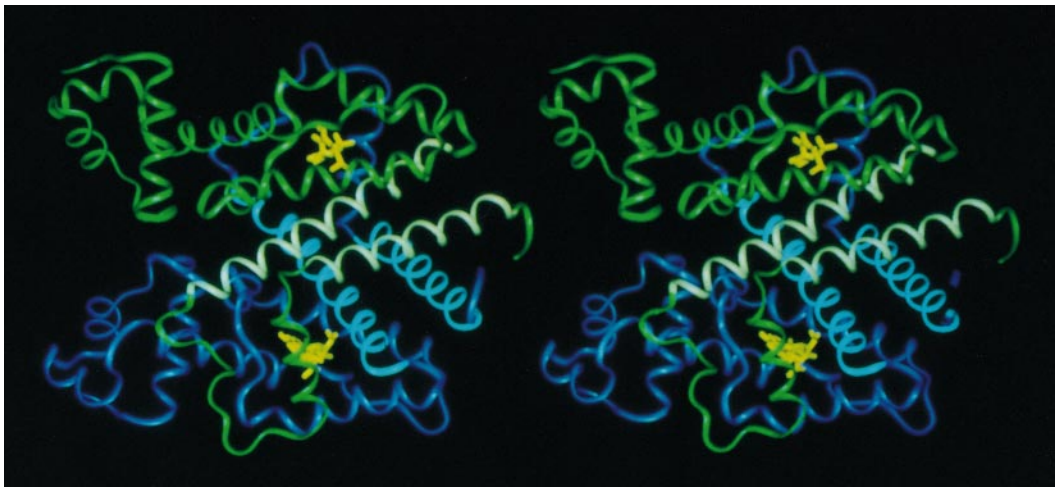

Fig. 1. Stereo view of TetR(D)/[(Mg–tc)$^+$]$_2$ structure, with α helices depicted as ribbons. The two monomers are colored green and blue. The helices of the four helix bundle are shown in light green and light blue. Tetracycline is shown in yellow.

Results and discussion

TetR(B) and TetR(D) do not form heterodimers in vivo

Protein–protein interactions can be analyzed *in vivo* by transdominance of negative mutants over wild type (Herskowitz, 1987). In the case of DNA-binding proteins, the repression of a reporter gene is quantified in the presence and absence of a mutant that is unable to bind DNA. Repression decreases in the presence of excess DNA-binding mutant if heterodimers are formed. An *in vivo* transdominance assay for TetR(B) has been established previously (Wissmann *et al.*, 1991). The indicator strain *Escherichia coli* WH207(λ*tet50*) bears a chromosomal *tetA–lacZ* fusion and two compatible expression plasmids constitutively producing TetR. The DNA-binding Tet repressor variant is expressed at a low level from pWH853 and the negative dominant TetR mutant is expressed at a higher level from a pWH520-derivative. We have cloned *tetR*(D) into pWH853 to examine dimerization between TetR(B) and TetR(D) using the transdominance assay. TetR(D) is a weak repressor because it shows a 7-fold increase in β-galactosidase expression under repressing conditions compared with TetR(B) (Table I, column 2). Since efficient repression by the DNA-binding protein is important for the transdominance assay, we increased TetR(D) repression by constructing TetR(B/D)51–208 bearing the DNA reading head of TetR(B) fused to the core of TetR(D) (see Figure 2A). This repressor variant shows 11-fold more efficient repression than TetR(D) (Table I, column 2). The corresponding chimera TetR(B/D)1–50, which contains the TetR(B) protein core and the TetR(D) DNA reading head shows decreased repression (Table I, column 2).

The TetR(B)Δ26–53 deletion mutant was tested for negative transdominance since it is strongly negatively transdominant over TetR(B) (Berens *et al.*, 1995) and the thermodynamic stability of this mutant is only slightly decreased compared with wild-type (wt) TetR(B) (Backes *et al.*, 1997). The results are also shown in Table I (columns 3 and 4). The deletion mutant is strongly transdominant over TetR(B) and TetR(B/D)1–50, but not over TetR(D) and TetR(B/D)51–208. Identical results were obtained for other TetR(B) mutants (data not shown). The lack of transdominance indicates that TetR(B) does not form dimers with TetR(D) *in vivo*. To confirm this result, we established a pulldown assay for TetR dimers formed *in vivo*. A TetR(B)–SG_4H_6 fusion protein which bears the C-terminal extension Ser(Gly)$_4$(His)$_6$ was constructed. This TetR(B) variant can be precipitated by Ni–NTA agarose and exhibits a lower mobility in SDS–PAGE than TetR(B) and TetR(D) (Figure 3A). *In vivo* dimerization of any TetR variant with TetR(B)–SG_4H_6 is indicated by co-precipitation with Ni–NTA agarose from *E.coli* lysates. *In vivo* repression efficiency and inducibility, as well as the thermodynamic stability of TetR(B)–SG_4H_6, are identical to wt TetR(B) (data not shown). Dimers of TetR(B)–SG_4H_6 with TetR(B) and TetR(B/D)1–50 are found in the pulldown assay. No dimerization is detected between TetR(B)–SG_4H_6 and TetR(D) or TetR(B/D)51–208 (Figure 3A). Therefore, the transdominance assay with TetR(B)Δ26–53 and the pulldown assay yield mutually confirming results. In order to investigate dimerization with TetR(D), *tetR*(D)Δ26–53 was constructed. This deletion mutant is negatively transdominant over TetR variants with a TetR(D) protein core, but not over those with a TetR(B) protein core (Table I, columns 5 and 6).

Taken together these results establish that TetR(B) and TetR(D) do not form heterodimers *in vivo* and that negative transdominance of the deletion mutants is a valid indication and screen for heterodimer formation.

Residues in helix α10 of TetR(B) and TetR(D) determine dimerization specificity

TetR(B) and TetR(D) share 63% identical amino acids and a common fold (Hinrichs *et al.*, 1994). Despite this pronounced similarity, they do not form heterodimers *in vivo*. This indicates that some of the different amino acids must destabilize a TetR(B)/TetR(D) dimer, determining the altered specificity of recognition. To identify such residues, we remodeled the interaction surface by exchanging amino acids in one TetR(D) monomer with the corresponding TetR(B) residues in the crystal structure. The minimal distances separating the two monomers in this model of a TetR(B/D) hybrid were calculated for every

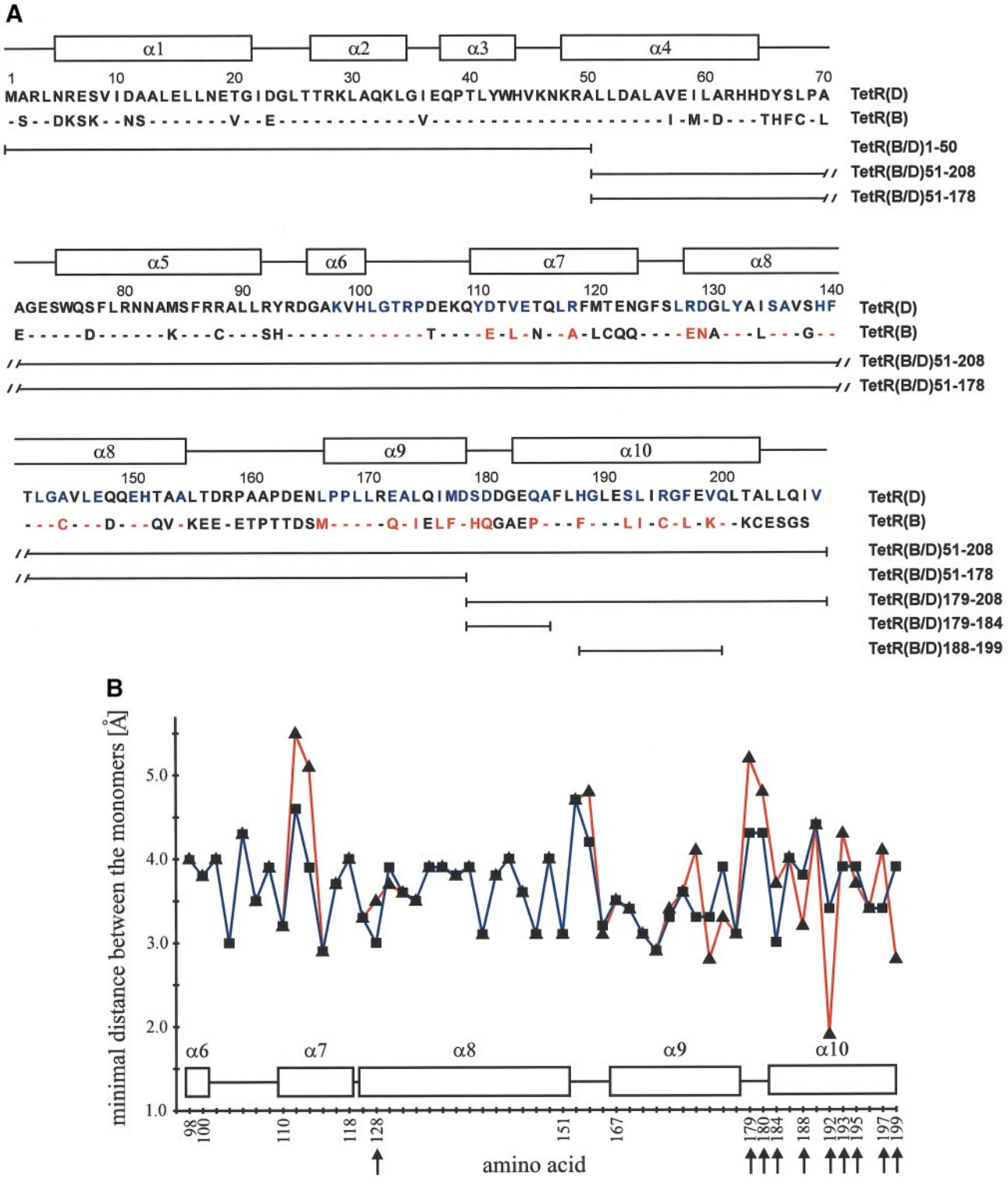

Fig. 2. (**A**) Comparison of the TetR(B) and TetR(D) amino acid sequence. The TetR(D) residues, which build the dimerization surface in the TetR(D)/[(Mg–tc)$^+$]$_2$ crystal structure are shown in blue and the corresponding TetR(B) residues in red. Amino acids of TetR(B) which are identical to the corresponding TetR(D) residue are indicated by dashes ('–'). The lines below the sequence alignment indicate those parts of the TetR(B/D) chimera that are encoded by *tetR*(D). α-Helices of the TetR(D)/[(Mg–tc)$^+$]$_2$ crystal structure are shown as rectangles above the sequence alignment. (**B**) Minimal distance analysis of the TetR(D) amino acids that form the dimerization surface. The minimal distance between one atom of a TetR(D) dimerization amino acid and the second monomer is shown by the blue curve. The red curve represents those distances that result from a replacement with TetR(B) residues. Those regions of the dimerization surface that are α-helical are indicated by open rectangles.

Table I. *In vivo* repression of TetR variants and negative transdominance of deletion mutants

Tet repressor	% β-gal	Deletion mutant			
		TetR(B)Δ26–53		TetR(D)Δ26–53	
		% β-gal	derepression factor	% β-gal	derepression factor
(B)	1.5 ± 0.1	22 ± 0.9	15	1.7 ± 0.1	1.1
(D)	10 ± 0.2	16 ± 1.0	1.6	102 ± 2.7	10
(B/D)51–208	0.9 ± 0.1	1.0 ± 0.0	1.1	16 ± 0.5	18
(B/D)1–50	21 ± 1.8	113 ± 3.6	5.4	34 ± 1.3	1.6
(B/D)51–178	1.0 ± 0.1	4.5 ± 0.5	4.5	3.7 ± 0.2	3.7
(B/D)179–208	1.0 ± 0.1	1.1 ± 0.0	1.1	5.3 ± 0.4	5.3
(B/D)179–184	1.1 ± 0.0	12 ± 2.8	11	1.1 ± 0.0	1.0
(B/D)188–199	1.1 ± 0.1	1.1 ± 0.0	1.0	11 ± 0.1	10
(B/D)192	1.2 ± 0.1	6.9 ± 0.6	5.8	8.5 ± 0.2	7.1
(B/D)197	1.8 ± 0.1	34 ± 0.4	19	2.9 ± 0.1	1.6
(B/D)192,197	1.1 ± 0.0	5.4 ± 0.6	4.9	26 ± 1.4	24
(B/D)188,192,193,197	1.1 ± 0.0	1.1 ± 0.0	1.0	4.7 ± 0.1	4.3
(B/D)192,193,197	1.2 ± 0.0	1.6 ± 0.1	1.3	12 ± 0.2	10
(B/D)188,192,193	1.1 ± 0.0	5.8 ± 0.5	5.3	6.2 ± 0.5	5.6
(B/D)188,192,197	1.0 ± 0.0	1.1 ± 0.1	1.1	2.7 ± 0.0	2.7
(B/D)188,193,197	1.9 ± 0.0	30 ± 1.0	16	2.3 ± 0.1	1.2
(B/D)192,193	1.1 ± 0.1	8.2 ± 1.5	7.5	14 ± 1.1	13
(B/D)193,195	1.3 ± 0.0	28 ± 2.3	22	1.5 ± 0.1	1.2
(B/D)193,197	1.3 ± 0.0	19 ± 0.9	15	2.6 ± 0.0	2.0
(B/D)188,192	1.2 ± 0.0	7.8 ± 0.1	6.5	2.8 ± 0.0	2.3
(B/D)188,193	3.8 ± 0.0	45 ± 2.4	12	4.2 ± 0.2	1.1
(B/D)188,197	1.8 ± 0.0	44 ± 0.4	24	2.1 ± 0.1	1.2
(B/D)188	2.8 ± 0.1	28 ± 0.3	10	2.3 ± 0.2	0.8
(B/D)193	1.4 ± 0.0	26 ± 1.0	19	1.8 ± 0.1	1.3

amino acid in the dimerization interface and compared with the ones in the TetR(D) structure. Residues 155–164 were not included because this part of the protein is disordered in the crystal structure. The results of that calculation are shown in Figure 2B. The blue curve represents the distances in the TetR(D) structure and the red curve represents the distances in the TetR(B)/TetR(D) model. Residues showing altered distances between the monomers in the TetR(B)/TetR(D) model might destabilize the heterodimer. Such residues are located in the region spanning helices α7 to α10. Helix α7 interacts with α9. The loop connecting α8 and α9 is flexible (Hinrichs *et al.*, 1994; Berens *et al.*, 1997) and positioning of helix α9 is critical for the induced but not for the *tet* operator (*tetO*)-binding conformation (Müller *et al.*, 1995). Therefore the residues located in α7, α9 and the loop connecting α8 and α9 may not contribute to dimerization specificity. The remaining residues are marked by arrows in Figure 2B. One is located in α8 and nine are found before and in α10 beginning at position 179. We assumed that these residues cause the different dimerization specificities of TetR(B) and TetR(D).

A TetR(B) variant containing the TetR(D) residues 188–199 does not dimerize with TetR(B)

We constructed TetR(B/D)51–178 and TetR(B/D)179–208 (see Figure 2A) to test which part of the TetR(D) protein core inhibits dimerization with TetR(B). The *in vivo* repression efficiencies of the chimeric Tet(B/D) repressors are similar to those of TetR(B) (Table I, column 2). TetR(B)Δ26–53 is negatively transdominant over TetR(B/D)51–178 which contains the TetR(D) protein core, except for the C-terminal portion from position 179 to 208. In contrast, it is not negatively transdominant over TetR(B/D)179–208. This demonstrates that replacing the TetR(B) amino acids located C-terminal of residue 179 by the TetR(D) sequence is sufficient to inhibit dimerization with TetR(B), confirming our structural predictions. TetR(B/D)179–208 contains the TetR(D) residues forming the β-turn prior to helix α10, α10 and the residues C-terminal of this helix (Figure 4A). Residues 179–185 interact with α8′ and the amino acids 192–199 contact α10′. Residue 188 interacts with α8′ and α10′. Residue 208 contacts α10′ via the C-terminal carboxylate oxygen atom and residues 201–207 are not involved in subunit contacts. The largest alteration of the distance separating the monomers in the TetR(B/D) model and the TetR(D) structure occurs at residue 192 (Figure 2B), which is part of the interaction surface of α10 and α10′ formed by residues 188–199 of both subunits (Figure 4A). Therefore, we constructed TetR(B/D)188–199. It shows no dimerization with TetR(B)Δ26–53 in the transdominance assay underlining the importance of the contacts between helices α10 and α10′. TetR(B/D)179–184 contains only those TetR(D) amino acids which interact with α8′. It efficiently forms dimers with TetR(B) as evidenced by negative transdominance of the TetR(B) deletion mutant over TetR(B/D)179–184. Thus, only the contacts between helices α10 and α10′, and not the ones between α10 and α8′, are sufficient to inhibit dimerization of TetR(B/D) chimera with TetR(B).

TetR(B/D)192 forms dimers with TetR(D)Δ26–53

The TetR(B)/TetR(D) dimerization model predicts the largest alteration in the monomer distance at residue 192, where S192 interacts with F197′ in the TetR(D)/[(Mg–tc)$^+$]$_2$ structure. F197′ is completely buried in the dimerization interface and is not accessible for solvent, whereas S192 is partially solvent accessible. The replacement of

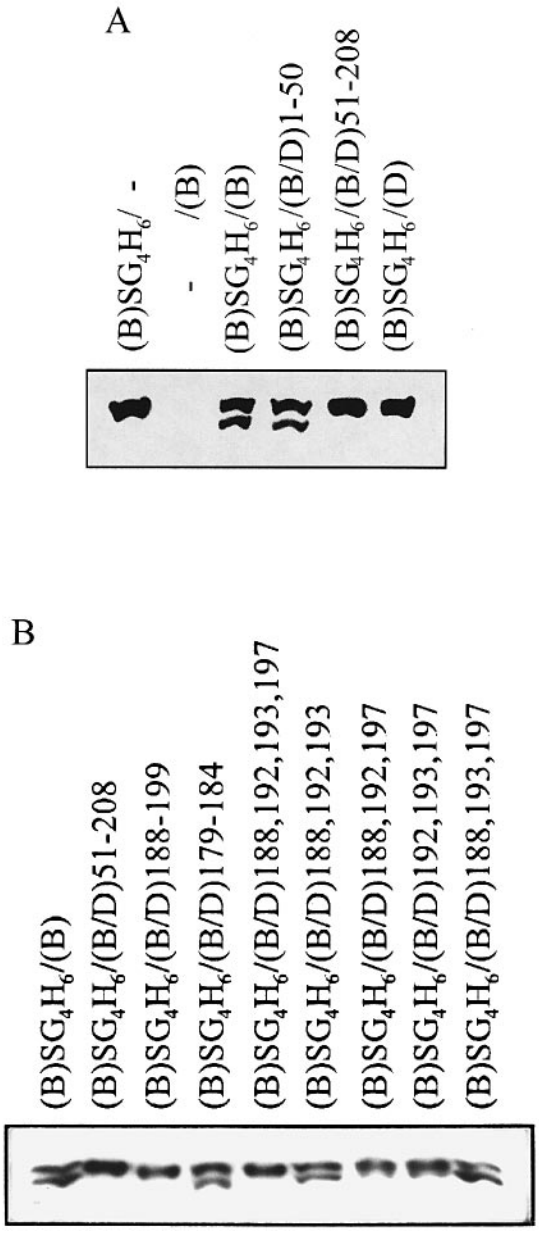

Fig. 3. Immunoblot analysis of the Ni–NTA-agarose pulldowns. TetR variants indicated above the lanes were co-expressed in *E.coli* WH207λ*tet*50. Proteins precipitated with Ni–NTA-agarose were separated by 15% SDS–PAGE and analyzed by immunoblotting using antibodies which bind TetR(B) and TetR(D). (**A**) shows an immunoblot of proteins precipitated after singular expression of TetR(B)–SG_4H_6 or TetR(B) as well as proteins precipitated from *E.coli* co-expressing TetR(B)–SG_4H_6 and TetR(B), TetR(D), TetR(B/D)51–208 or TetR(B/D)1–50. (**B**) shows an immunoblot of proteins precipitated from *E.coli* co-expressing TetR(B)–SG_4H_6 and wt TetR(B), TetR(B/D)51–208, TetR(B/D)188–199, TetR(B/D)179–184, TetR(B/D)188,192,193,197 or the TetR(B/D) threefold mutants.

S192 with the TetR(B) amino acid, L192, leads to a sterical clash with F197′ (Figure 4B), which should strongly destabilize a TetR(B)/TetR(D) dimer. Therefore L192 might be responsible for the fact that TetR(B) does not form dimers with TetR(D). If this is true, the mutation LS192 will lead to dimerization of this TetR(B) mutant with TetR(D). We constructed TetR(B/D)192, which bears the LS192 exchange. As predicted, TetR(D)Δ26–53 is negatively transdominant over TetR(B/D)192 (Table I, columns 5 and 6). TetR(B)Δ26–53 is also negatively transdominant over TetR(B/D)192, although the derepression factor is reduced compared with TetR(B). This indicates that the single mutation LS192 is sufficient to gain negative transdominance for TetR(D)Δ26–53, whereas dimerization with the TetR(B) mutants remains largely unaffected. Thus, LS192 leads to a loss of specificity in protein recognition of TetR(B).

The 4-fold exchange FH188, LS192, IL193 and LF197 creates a new dimerization specificity of TetR(B)

The data presented so far indicate that L192 of TetR(B) does not fit into the dimerization surface of TetR(D) because of a sterical interference with F197′ of TetR(B). Correspondingly, we assumed that it might be possible to alter the dimerization specificity of TetR(B) using the mutation LF197. We constructed TetR(B/D)197 and TetR(B/D)192,197 and tested them for dimerization with TetR(B) (Table I). The single mutation LF197 does not affect negative transdominance efficiency of TetR(B)Δ26–53, whereas negative transdominance over the double mutant is reduced but still detectable. This indicates that additional mutations are necessary to abolish dimerization of TetR(B/D)192,197 with TetR(B). H188 and I193′ are located next to the residues S192 and F197 in the TetR(D) structure (Figure 4C) and, therefore, might influence the conformation assumed by residues 192 and 197′. The combination of FH188 and IL193 with LS192 and LF197 in TetR(B/D)188,192,193,197 abolishes dimerization with TetR(B) (Table I). To further investigate the effect of H188 and L193 we constructed each single mutant and all possible combinations of triple and double mutants involving H188, S192, L193 or F197. The results obtained in the transdominance assay with these variants are also shown in Table I. Negative transdominance efficiencies of the TetR(B) deletion mutant are nearly unaffected by the single mutations FH188 or IL193. Thus, these mutations alone do not alter the dimerization specificity of TetR(B). None of the double mutants affect negative transdominance efficiencies of TetR(B)Δ26–53 more than 2.3-fold. Negative transdominance efficiency of the TetR(B) probe is strongly reduced for TetR(B/D)192, 193,197 and no negative transdominance is detectable for TetR(B/D)188,192,197. This indicates that mutating F188 to H188 or I193 to L193 in TetR(B/D)192,197 further reduces dimerization with TetR(B). To confirm these results, we analyzed dimerization for all triple mutants, TetR(B/D)188,192,193,197, TetR(B/D)179–184 and TetR (B/D)188–199 with TetR(B)–SG_4H_6 using the pulldown assay (Figure 3B). TetR(B/D)188,192,197 and TetR(B/D)192,193,197 gave strongly reduced negative transdominance efficiencies and also show reduced co-precipitation with TetR(B)–SG_4H_6. No dimerization with TetR(B)–SG_4H_6 is detectable for TetR(B/D)188–199 and TetR(B/D)188,192,193,197. Tet Repressors (B/D)179–184, (B/D)188,193,197 and (B/D)188,192,193 dimerize with TetR(B)–SG_4H_6 as shown in Figure 3B. These results are in agreement with the transdominance assay. In conclusion, genetic and biochemical evidence demonstrate that the combination of FH188, LS192, IL193 and LF197 can be used to construct a TetR(B) variant that does not form dimers with wt TetR(B) *in vivo*. LS192 and LF197

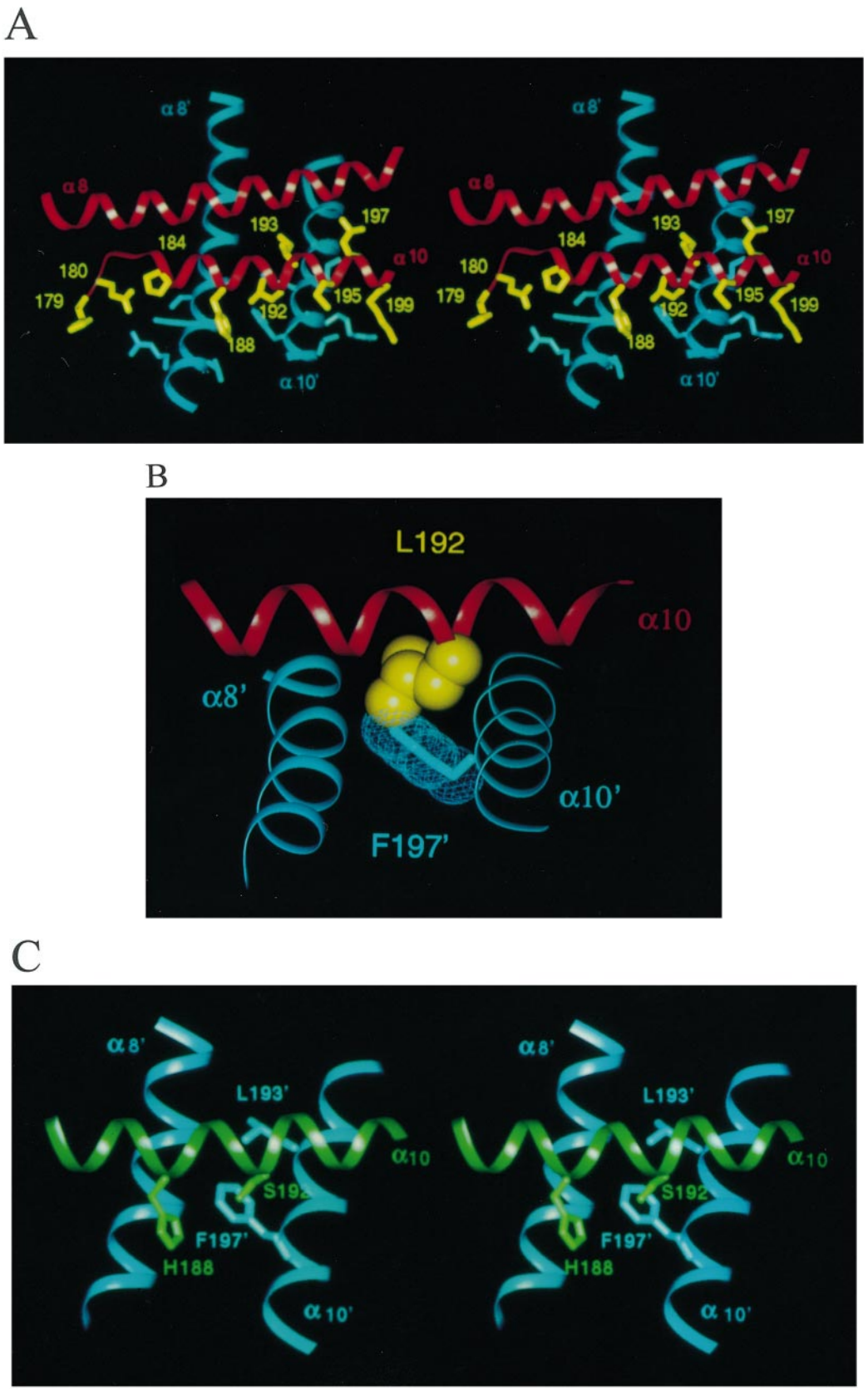

Fig. 4. (**A**) Stereo view of the four helix bundle of the TetR(B)/TetR(D) model. Helices of the TetR(B) monomers are shown as red ribbons and those of TetR(D) as blue ribbons. Dimerization residues of the TetR(B) monomer that are different from TetR(D) are shown in yellow. The side chains of TetR(D), which build the surface that interacts with α10, are colored in light blue. (**B**) Sterical interference of TetR(D) F197′ and TetR(B) L192. L192 is shown as a space filling model. The light blue lines indicate the Van der Waal's surface of F197′. (**C**) Stereo view of the interaction of H188, S192, L193′ and F197′ in the TetR(D) structure. Different monomers are distinguished by green and blue color.

Table II. Temperature-dependent activity of TetR variants

Tet repressor	Growth temperature		Ratio
	42/42°C β-gal (%)	28/42°C β-gal (%)	
–	100 ± 3.3	100 ± 1.6	1.0
(B)	3.6 ± 0.1	2.2 ± 0.1	1.6
(B/D)188,192,193,197	3.2 ± 0.0	1.7 ± 0.0	1.9
(B/D)192,193,197	3.7 ± 0.2	1.4 ± 0.0	2.6
(B/D)188,192,193	4.1 ± 0.2	1.6 ± 0.0	2.6
(B/D)188,192,197	10 ± 0.5	2.3 ± 0.0	4.3
(B/D)188,193,197	69 ± 1.3	21 ± 0.3	3.3
(B/D)192,193	2.8 ± 0.3	1.6 ± 0.1	1.8
(B/D)192,197	12 ± 0.7	2.2 ± 0.1	5.5
(B/D)193,197	29 ± 1.9	9.0 ± 0.8	3.2
(B/D)188,192	3.6 ± 0.1	2.1 ± 0.0	1.7
(B/D)188,193	2.8 ± 0.1	3.8 ± 0.2	0.7
(B/D)188,197	81 ± 2.5	34 ± 1.0	2.4
(B/D)188	2.6 ± 0.0	3.7 ± 0.2	0.7
(B/D)192	2.2 ± 0.1	1.7 ± 0.0	1.3
(B/D)193	2.0 ± 0.0	1.7 ± 0.0	1.2
(B/D)197	38 ± 2.4	9.3 ± 1.0	4.1

are necessary to alter the dimerization specificity, since TetR(B)Δ26–53 is negatively transdominant over all Tet(B/D) repressors which do not contain LS192 and LF197. FH188 and IL193 decrease dimerization with TetR(B) when combined with LS192 and LF197. They do not influence the dimerization specificity as single mutants and show only a small effect as double mutants.

Negative transdominance measurements with the TetR(D)Δ26–53 probe demonstrate that other residues in addition to S192 affect dimerization of a Tet(B/D) repressor with TetR(D). TetR(D)Δ26–53 only shows negative transdominance over all Tet repressors bearing S192 (Table I, columns 5 and 6). However, the derepression factors vary from 2.3-fold for TetR(B/D)188,192 to 24-fold for TetR(B/D)192,197. Even TetR(B/D)179–208 dimerizes less efficiently with TetR(D)Δ26–53 than TetR(B/D)51–208, as evidenced by the decreased derepression factors of TetR(D)Δ26–53. This indicates that residues outside of helix α10 also influence dimer formation. Further experiments are necessary to identify those residues and their mechanism of action.

Temperature sensitivity of Tet(B/D) repressors

In vivo stability of TetR variants was tested by determining their activity at 42°C (Table II, column 2). Repression efficiencies of Tet(B/D) repressors not bearing the LF197 exchange differ only <2-fold from wt TetR(B). Mutants containing LF197 are temperature sensitive (ts), showing reduced repression at 42°C, as seen in the single mutant TetR(B/D)197, the respective double mutants and the two triple mutants TetR(B/D)188,192,197 and TetR(B/D)188,193,197. A comparison of TetR(B/D)188,197 with TetR(B/D)197 reveals that FH188 reduces repression. A similar influence of the FH188 mutation is detected in TetR(B/D)188,193,197, which is also a worse repressor than TetR(B/D)193,197, but not in the single mutant TetR(B/D)188, which shows wt-like activity. Repression efficiencies are increased for TetR(B/D)192,197 and TetR(B/D)188,192,197 as compared with TetR(B/D)197 and TetR(B/D)188,192,197 respectively, but still lower than wt TetR(B). In contrast, TetR(B/D)192,193,197, TetR(B/D)188,192,193,197 and wt TetR(B) show nearly identical activities at 42°C. Thus, the combination of LS192 and IL193 is necessary to restore wt stability. These results establish that TetR(B) is destabilized by a mutation introducing the bulky F residue at position 197 in the dimerization surface. Residues contacting F197 from the same monomer, L193, and the second monomers, S192 and H188, determine *in vivo* stability of the homodimers and dimerization with TetR(B). This is consistent with the observation that folding of the TetR dimer occurs in a single, concentration-dependent transition (Backes *et al.*, 1997).

All Tet repressors with a ts phenotype show increased repression efficiencies when shifted to 42°C after synthesis has taken place at 28°C (Table II, columns 3 and 4), establishing their temperature sensitive folding (tsf) phenotype. The extent by which repression at 42°C increases when the proteins are folded at 28°C in comparison with 42°C varies from 2.4-fold in the case of TetR(B/D)188,197 to 5.5-fold in the case of TetR(B/D)192,197. In fact, the *in vivo* repression efficiency of TetR(B/D)192,197 at 42°C is identical to that of TetR(B) when folded at 28°C. Tsf phenotypes indicate destabilization of a folding intermediate, rather than the completely folded protein as was shown, for example, for mutants of the P22 tailspike and coat proteins (Yu and King, 1984; Gordon and King, 1993). Therefore, decreased repression of TetR(B/D)192,197, detectable after synthesis at 42°C, is caused by a temperature-dependent folding defect and not by a destabilization of the completely folded structure. Stabilization of folding is accomplished by introducing the IL193 mutation.

Inducibility of the repressors by tetracycline

Mutations in the dimerization surface of TetR(B) influence inducibility of TetR (Müller *et al.*, 1995). The *in vivo* inducibility of all Tet(B/D) repressors was quantified at a low expression level in the presence of 0.2 μg/ml tetracycline. As shown in Table III, none of the mutations leads to reduced inducibility under these conditions. TetR(B/D)51–208, TetR(B/D)188–199, TetR(B/D)179–184 and TetR(B/D)188,192,193,197, as well as all TetR(B/D) triple mutants, were also tested for inducibility at a high expression level. These conditions permit detection of even slightly reduced inducibilities (Berens *et al.*, 1997). The induction efficiencies of all TetR variants were identical to that of wt TetR(B) (data not shown). This indicates that the TetR(B/D) mutations do not interfere with inducibility of TetR.

Surface complementarity determines specificity of protein–protein interactions

We have identified residues which distinguish dimerization between TetR(B) and TetR(D) and used these residues to construct TetR(B/D)188,192,193,197, a variant unable to form dimers with wt TetR(B) *in vivo*. The dimerization specificity is the only *in vivo* property changed, since repression efficiency, temperature dependence of repression and inducibility are not impaired. The functions of LF197 and LS192 are readily explained from the TetR(B)/TetR(D) molecular model. F197 destabilizes Tet repressors containing L192 since it clashes into L192′. No other TetR(B/D) mutation leads to overlapping Van der Waal's radii in the dimerization surface of the TetR(B)/TetR(D)

Table III. *In vivo* inducibility with tetracycline

TetR	% β-gal	TetR	% β-gal	TetR	% β-gal
(B)	95 ± 3.8	(B/D)188–197	99 ± 3.4	(B/D)188,197	102 ± 1.1
(D)	93 ± 5.6	(B/D)188,192,193,197	102 ± 2.8	(B/D)192,193	100 ± 2.2
(B/D)1–50	105 ± 5.4	(B/D)188,192,193	96 ± 1.5	(B/D)192,197	103 ± 2.8
(B/D)51–208	97 ± 2.6	(B/D)188,192,197	95 ± 3.3	(B/D)193,197	110 ± 1.4
(B/D)51–178	105 ± 1.4	(B/D)188,193,197	99 ± 2.3	(B/D)188	107 ± 7.0
(B/D)179–208	97 ± 4.2	(B/D)192,193,197	95 ± 2.8	(B/D)192	101 ± 6.5
(B/D)179–184	99 ± 1.6	(B/D)188,192	97 ± 2.6	(B/D)193	107 ± 3.0
(B/D)188–199	106 ± 2.1	(B/D)188,193	104 ± 3.8	(B/D)197	108 ± 2.0

model. Therefore, LF197 is necessary to abolish dimerization of a Tet(B/D) repressor with TetR(B). The sterical interference of L192 and F197 is crucial for the different dimerization specificities of TetR(B) and TetR(D). This is evidenced by the relaxed specificity of protein recognition observed for TetR(B/D)192 which contains S192 instead of L192. Mutating L192 to S192 in TetR(B/D)192 resolves the sterical interference of F197 with L192′ in the homodimer but not in the TetR(B/D)/TetR(B) heterodimer. Therefore, LS192 is necessary to selectively stabilize the TetR(B/D) homodimer. However, the mutation LS192 alone is neither sufficient to create a TetR(B/D) repressor of wt stability *in vivo*, nor is it sufficient to abolish dimerization with TetR(B). It takes the additional mutations at positions 188 and 193, which are both located in the vicinity of positions 192 and 197 in the TetR(D)/$[(Mg–tc)^+]_2$ structure to achieve that goal. Since FH188 destabilizes TetR(B/D) variants that contain F197 and L192, this mutation presumably reduces the stability of TetR(B/D)/TetR(B) dimers. L193 stabilizes a folding intermediate of TetR(B/D)188,192,193,197 and is necessary to abolish dimerization of a Tet(B/D) repressor with TetR(B). Thus, the folding intermediate stabilized by L193 might contribute to the dimerization specificity of TetR. In conclusion, the dimerization specificity of TetR(B/D)188, 192,193,197 is determined by the packing of mostly buried, nonpolar residues in the four helix bundle and an additional amino acid which stabilizes protein folding. This mechanism is quite different from that dictating the dimerization specificity of leucine zippers, which mainly depends on electrostatic interactions (Vinson *et al.* 1993; Lavigne *et al.* 1995).

Structural complementarity is a common property of protein–protein interaction surfaces (Jones and Thornton, 1996). Mutations changing this complementarity might, therefore, be valuable to alter the specificity of protein–protein recognition in general. The data presented here emphasize, in addition, the importance of amino acids, which interact with the residues forming the altered complementarity. These amino acids clearly stabilize the complex and are, therefore, important determinants of the new specificity.

Materials and Methods

General methods

Chemicals were obtained from Merck (Darmstadt), Serva (Heidelberg), Sigma (München) or Roth (Karlsruhe) and of the highest purity available. Tetracycline was purchased from Fluka (Buchs). Enzymes for DNA restriction and modification were obtained from Boehringer (Mannheim), Gibco-BRL (Eggenstein), New England Biolabs (Schwalbach) or Pharmacia (Freiburg). Isolation and manipulation of DNA were carried out as described (Sambrook *et al.*, 1989). Sequencing was carried out according to the protocol provided by Perkin Elmer for cycle sequencing.

Bacterial strains, plasmids and phage

All bacterial strains were derived from *E.coli* K12. Strain DH5α [*hsd*R17($r_K m_K^+$), *rec*A1, *end*A1, *gyr*A96, *thi*, *rel*A1, *sup*E44, ϕ80d*lac*ZM15, (λ*acZYA-argF*)U169] was used for general cloning procedures. Strain WH207 (*lacX*74, *galK*2, *rpsL*, *recA*13) (Wissmann *et al.*, 1991) served as host strain for β-galactosidase assay and the pulldowns. The plasmids pWH1200 (Altschmied *et al.*, 1988), pWH806, pWH853 (Wissmann *et al.*, 1991), pWH520 (Berens *et al.*, 1992), pWH520Δ26–53 (Berens *et al.*, 1995) pWH620 (Berens *et al.*, 1997) and phage *tet*50 (Smith and Bertrand, 1988; Wissmann *et al.*, 1991) which were used in the *in vivo* studies have been described.

Construction of tetR variants

tetR variants were constructed by PCR (Landt *et al.*, 1990) and cloned in pWH853. *tetR*(D), *tetR*(B/D)1–50, *tetR*(B/D)51–208, *tetR*(B/D)188–199, *tetR*(B/D)178–184, *tetR*(B/D)188,192,193, *tetR*(B/D)188,192,197, *tetR*(B/D)188,193,197 and *tetR*(B/D)192,193,197 were also cloned in pWH520. For construction of pWH853(D), pWH520(D) and all *tetR*(B/D) chimera, a *tetR*(D) variant with a deletion of the sequence encoding the nonfunctional amino acids 209–218 was used. DNA of positive candidates was analyzed by digestion with restriction enzymes and sequencing of *tetR*.

β-Galactosidase assays

Repression, temperature dependence of repression, transdominance and inducibility was assayed in *E.coli* WH207(λ*tet*50). The phage λ*tet*50 contains a *tetA–lacZ* transcriptional fusion integrated as single copy into the WH207 genome. Bacteria were grown in LB medium supplemented with the appropriate antibiotics. For the quantification of induction efficiency, 0.2 μg/ml tc were added to overnight and log phase cultures. To test tsf phenotypes, overnight cultures were grown at 28°C and log phase cultures at 42°C. β-Galactosidase activities were determined as described by Miller (1992). Three independent cultures were assayed for each strain and measurements repeated at least twice.

Ni–NTA pulldown

Strain WH207λ*tet*50 was transformed with pWH853(B)SG_4H_6 and the respective pWH520 derivative and grown to an OD_{600} of 0.8–1.0 at 28°C in 1 l LB medium. The pulldown was performed at 4°C. Cell pellets were resuspended in W-buffer (200 mM NaCl, 7 mM β-mercaptoethanol, 20 mM Tris-HCl pH 8.0, 30 mM imidazol, 250 μg/ml BSA), sonicated and cleared by centrifugation. Ni–NTA agarose (Qiagen, Heidelberg) was washed with W-buffer. 800 μl Ni–NTA agarose were added to the protein solution and pelleted by centrifugation. The pellet was washed five times with 50 ml volumes of W-buffer. Protein bound to the agarose was eluted with 200 μl E-buffer (500 mM imidazol, 7 mM β-mercaptoethanol, 20 mM Tris-HCl pH 8.0). Samples were analyzed on 20% SDS–PAGE[30] and transferred to a Fluorotrans membrane (Pall) in a Mini V8.10 electrophoresis and blotting apparatus (Gibco-BRL). TetR was visualized using the ECL-detection system (Amersham) and monoclonal antibodies which bind to TetR(B) and TetR(D) to a similar extent.

Molecular modeling

All structural analysis was performed with InsightII 95.0 (Biosym). The TetR(B)/TetR(D) dimerization model was build with the biopolymer module. In the case of TetR(B) amino acids, those rotamers which gave the lowest non-bond energy were used for the distance calculations.

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