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Conformational changes necessary for gene regulation by Tet repressor assayed by reversible disulfide bond formation

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We constructed and characterized four Tet repressor (TetR) variants with engineered cysteine residues which can form disulfide bonds and are located in regions where conformational changes during induction by tetracycline (tc) might occur. All TetR mutants show nearly wild-type activities *in vivo*, and the reduced proteins also show wild-type activities *in vitro*. Complete and reversible disulfide bond formation was achieved *in vitro* for all four mutants. The disulfide bond in NC18RC94 immobilizes the DNA reading head with respect to the protein core and prevents operator binding. Formation of this disulfide bond is possible only in the tc-bound, but not in the operator-bound conformation. Thus, these residues must have different conformations when bound to these ligands. The disulfide bonds in DC106PC159′ and EC107NC165′ immobilize the variable loop between α-helices 8 and 9 located near the tc-binding pocket. A faster rate of disulfide formation in the operator-bound conformation and a lack of induction after disulfide formation show that the variable loop is located closer to the protein core in the operator-bound conformation and that a movement is necessary for induction. The disulfide bond in RC195VC199′ connects α-helices 10 and 10′ of the two subunits in the dimer and is only formed in the tc-bound conformation. The oxidized protein shows reduced operator binding. Thus, this bond prevents formation of the operator-bound conformation. The detection of conformational changes in three different regions is the first biochemical evidence for induction-associated global internal movements in TetR.

Keywords: induction/redox-dependent activity/ tetracycline/transcriptional regulatory protein

Introduction

The understanding of protein mechanics is of fundamental importance because changes in conformations often lead to different functional states. They are in many cases linked to modifications or effector binding. Thus, they form the basis for regulated activities of many proteins, e.g. receptor proteins often make use of defined conformational changes to transmit information as part of a signal transduction chain (Careaga *et al*., 1995; Chervitz and Falke, 1995; Hughson *et al*., 1997). DNA-binding proteins active in regulation of transcription often switch between DNA-binding and non-binding states dependent on effector binding. The conformational changes underlying these different activities are the mechanistic basis of gene regulation. They have been postulated for many bacterial regulatory proteins, but only little detailed information about the nature and magnitude of these motions is available. Presently there is no common pattern of structural changes resulting in altered DNA binding. CRP and TrpR belong to a group of regulatory proteins where effector binding leads to another orientation of the DNA-binding domain with respect to the ligand-binding domains (Zhang *et al*., 1987; Kolb *et al*., 1993), whereas parts of the core protein are also involved in structural changes in the Lac repressor (Lewis *et al*., 1996). No structural changes at all were found in MetJ (Cooper *et al*., 1994).

Most of the information about structural changes is deduced from crystallographic analyses. The redox-dependent activity of proteins with suitably located cysteines is a useful tool to study protein conformations in solution, e.g. formation of disulfides has resulted in enhanced thermostability in proteins (for a review and references, see Fontana, 1991) and was used to study dimerization of Hin recombinase (Lim, 1994) and the topology of membrane proteins (for references, see Hughson *et al*., 1997). Intermolecular disulfide bond formation was applied to trap ligand-induced structural changes for the Tar receptor (Falke and Koshland, 1987; Chervitz and Falke, 1995) and Trg receptor (Lee *et al*., 1995) during transmembrane signaling. Motions of surface helices in globular proteins have been investigated by measuring intramolecular disulfide bond formation in the D-galactose chemosensory receptor (Careaga and Falke, 1992).

We have employed the tetracycline (tc)-inducible Tet repressor (TetR) to study the importance of dynamics for induction (Beck *et al*., 1982; Hillen and Berens, 1994; Gossen *et al*., 1995). Changes of TetR upon tc binding have been suggested from the crystal structure of the TetR–$([Mg\text{-}tc]^{+})_2$ complex (Hinrichs *et al*., 1994; Kisker *et al*., 1995) and functional characterization of non-inducible mutants (Hecht *et al*., 1993; Müller *et al*., 1995). The DNA recognition helices in the induced TetR dimer are 39 Å apart and tilted by 110° with respect to each other, and they cannot bind to successive major grooves in B-type DNA. Fluorescence quenching studies have indeed indicated a movement of the α-helix–turn–α-helix motif (HTH) during induction (Hansen *et al*., 1987). However, no data are presently available to describe internal movements in the protein associated with induction by tc binding. The goal of the study is to identify directly the importance of such movements by introducing cysteine residues at suitable positions and comparing the activities of the mutant TetRs in their reduced and oxidized states.

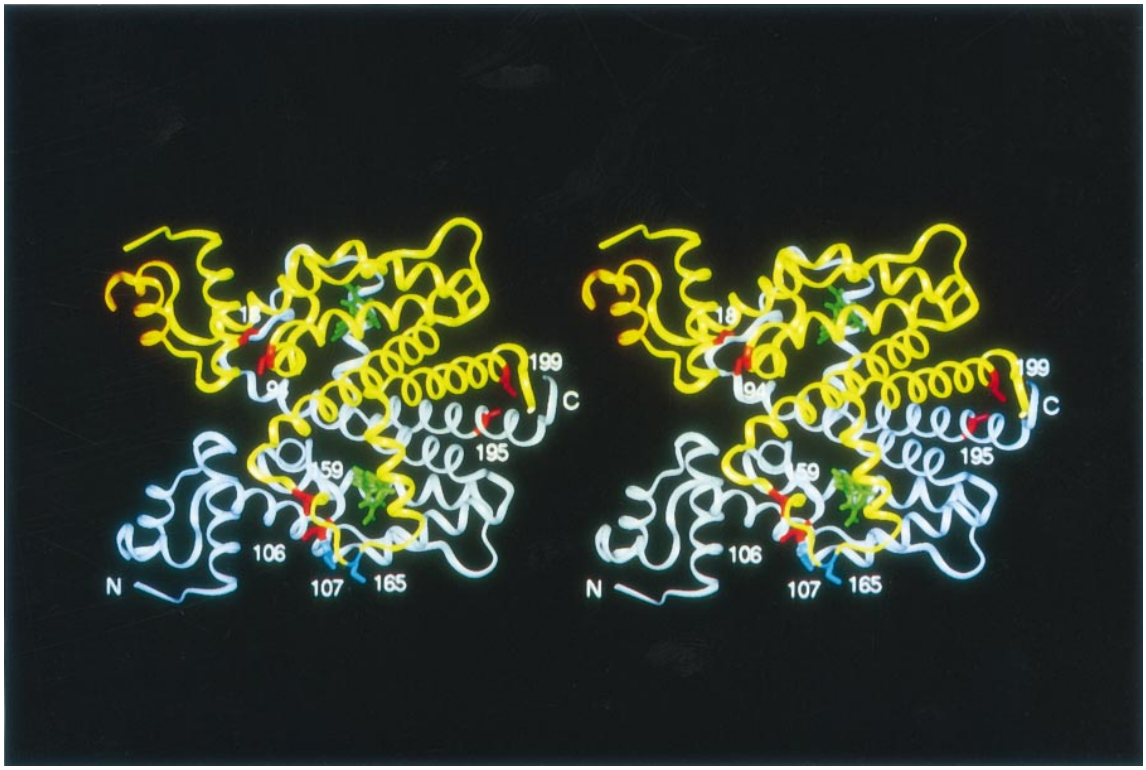

Fig. 1. Stereo view of the crystal structure of TetR(D)–$([Mg\text{-}tc]^+)_2$ with the indicated locations of engineered cysteines. The two TetR monomers are shown as yellow and gray ribbons, respectively, and tc as a green stick model. The cysteine exchange NC18 (red) is located in the α-helix 1 in the DNA reading head, and RC94 (red) is in the loop between α5 and α6. The cysteine exchanges PC159′ (red) and NC165′ (blue) are located in the variable loop between α8 and α9, and the opposite positions DC106 (red) and EC107 (blue) are located in the loop between α6′ and α7′ of the other monomer. The cysteine pair RC195VC199′ (red) is located at the crosspoint of the C-terminal helices α10 and α10′. The HTH (α2 and α3, orange in the yellow subunit) is located near the N-terminus.

Results and discussion

Sites for engineered disulfides

Two models have been proposed for the conformational changes of TetR during induction. In a first model, a movement of α4 connecting the DNA reading head with the protein core adjusts the distance between the two DNA reading heads (see Figure 1; Hinrichs *et al*., 1994; Kisker *et al*., 1995). Non-inducible mutants located in the contacting area between the DNA reading head and the protein core suggest such movements during signal transduction (Müller *et al*., 1995). However, a lot of non-inducible mutants are located at the dimer interface and are not explained by this model. They have led to the second induction model (Müller *et al*., 1995) in which a tc-induced reorientation of the four-helix bundle formed by α8 and α10 of both monomers is accompanied by a shift of α9, which holds the four-helix bundle in the induced conformation. We attempted to identify regions which need to be flexible for TetR induction by placing cysteine pairs at critical positions in TetR as defined by these models. The TetR(D) allele as it appears in the crystal structure does not contain an endogenous cysteine residue.

We searched the crystal structure of the TetR–$([Mg\text{-}tc]^+)_2$ complex (Hinrichs *et al*., 1994; Kisker *et al*., 1995) for pairs of amino acids which are at a favorable distance and orientation for disulfide formation upon mutation to cysteine residues (Cα–Cα′ 4.6–7.4 Å; Thornton, 1981) and are in regions where the induction models assume conformational changes. Furthermore, the desired positions must not have given rise previously to mutations with a non-inducible phenotype (Hecht *et al*., 1993; Müller *et al*., 1995). These considerations yielded four pairs of amino acids, which are depicted in the crystal structure of TetR in Figure 1. A disulfide bond between NC18 and RC94 should prevent a movement of the DNA reading head relative to the protein core as proposed in the first induction model. The pairs DC106PC159′ and EC107NC165′ (amino acids from the second monomer are indicated by a prime) immobilize the variable loop between α8 and α9 with respect to the protein core. The RC195VC199′ pair cross-links the dimerization surface at the crosspoint of the helices α10 and α10′. The distances between the respective amino acids and their structural context are presented in Table I. The described mutations

Table I. Predicted distances of engineered disulfides

TetR variant	ΔCα (Å)	ΔCβ (Å)	Structural context	Type of disulfide bond
NC18RC94	4.79	5.0	helix–loop	intrachain
DC106PC159′	6.12[a]	4.72[a]	loop–loop	interchain
EC107NC165′	8.3	7.25	loop–loop	interchain
RC195VC199′	7.87	5.23	helix–helix	interchain

Indicated distances were determined from the 2.3 Å resolution crystal structure of the induced Tet repressor (Kisker *et al*., 1995).
[a]The loop segment between 156 and 164 is not resolved in the crystal structure. The distances between the amino acids 106 and 159′ were estimated using a modeled loop segment.

Table II. *In vivo* DNA binding and inducibility of TetR variants by atc

tetR variant	pWH624 derivative	
	no atc	0.4 μM atc
Wild type	0.0 ± 0.17	100.0 ± 0.1
NC18	0.0 ± 0.11	84.3 ± 1.9
RC94	0.0 ± 0.05	62.4 ± 3.9
NC18RC94	0.0 ± 0.14	19.4 ± 0.9
DC106	0.0 ± 0.12	87.3 ± 2.9
PC159	0.0 ± 0.08	92.5 ± 4.7
DC106PC159′	0.0 ± 0.03	85.3 ± 7.2
EC107	0.0 ± 0.11	96.2 ± 2.6
NC165	0.0 ± 0.16	98.0 ± 1.9
EC107NC165′	0.0 ± 0.05	97.5 ± 2.6
RC195VC199′	0.0 ± 0.06	75.0 ± 1.4

The host strain was *E.coli* WH207pWH1012 transformed with pWH624 variants. The results are given as β-galactosidase activities in percentages, determined in units according to Miller (1972). Expression in the absence of *tetR* was set to 100% and corresponds to 237 ± 7 units. TetR wild-type represses the activity to 0.04 units. The final concentration of atc was 0.4 μM for the overnight and log cultures.

were introduced, and the mutant TetR were analyzed *in vivo* and *in vitro*.

In vivo repression and induction of the TetR mutants

The *tetR* variants were cloned into pWH624 and the resulting plasmids transformed into *Escherichia coli* WH207 pWH1012 where they are constitutively expressed at a high level (Berens *et al*., 1997). Repression of the *tetA–lacZ* transcriptional fusion located on plasmid pWH1012 and induction were determined at 28°C in the absence and presence of 0.4 μM anhydrotetracycline (atc). The results are shown in Table II. All TetR mutants exhibit the same repression efficiency as wild-type TetR in this assay. The induction efficiencies are also the same, except for TetR NC18RC94, which shows 5-fold reduced inducibility. This indicates that the contact area between the DNA reading head and protein core is critical for signal transduction.

The nearly wild-type activities of the four TetR double mutants *in vivo*, where they reside most likely in the reduced form, demonstrate that the mutants are functional. Thus, they can be used for the analysis of conformational changes.

Assaying disulfide formation

The mutant TetRs were purified to homogeneity for *in vitro* analysis. The formation of disulfide bonds was achieved by oxidation with ambient oxygen and copper phenanthroline (Cu-ph). We then analyzed the mobility of the oxidized proteins in non-reducing SDS–PAGE. The results shown in Figure 2 demonstrate that the reduced and oxidized forms of the TetR mutants show distinctly different mobilities. Thus, their mobility in gel electrophoresis can be employed to assay the formation of disulfide bonds. The oxidized forms of TetR DC106PC159′, TetR EC107NC165′ and TetR RC195VC199′ migrate like a TetR dimer, in accordance with an interchain disulfide bond. During the course of the oxidation reaction of TetR DC106PC159′ and TetR EC107NC165′, a minor band

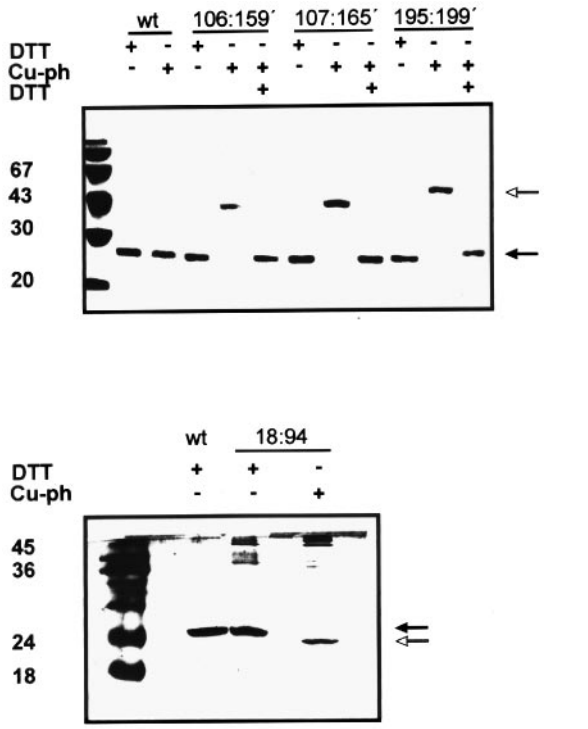

Fig. 2. Non-reducing SDS–PAGE of reduced and oxidized TetR variants. Filled and open arrows on the right sides of the gels indicate the reduced and oxidized forms, respectively. The upper 10% SDS–PAGE contains, in the leftmost lane, a molecular weight standard followed by the reduced (DTT +) and oxidized (Cu-ph +) wild-type TetR. The next lanes show TetR variants DC106PC159′, EC107NC165′ and RC195VC199′ in the reduced (DTT +) and oxidized (Cu-ph +) forms, and treated with DTT after previous oxidation (Cu-ph +, DTT +). The lower 20% SDS–PAGE contains, in the leftmost lane, a molecular weight standard; in the next lane, the reduced wild-type TetR is followed by the reduced (DTT +) and oxidized (Cu-ph +) forms of TetR NC18RC94.

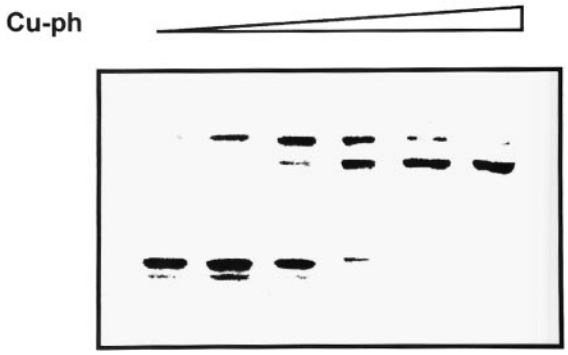

Fig. 3. Mobility of TetR EC107NC165′ in non-reducing SDS–PAGE is dependent on the copper phenanthroline concentration. TetR EC107NC165′ (1 μM) is shown oxidized with increasing concentrations (0, 1, 2, 3, 4 and 5 μM) of copper phenanthroline.

migrating more slowly than the dimer is also observed (Figure 3). The intensities of the two differently migrating forms depend on the concentration of copper phenanthroline present in the oxidation reaction. We therefore suspect that the slower migrating protein corresponds to an intermediate where only one disulfide bond is formed. A dimer connected by two covalent bonds should migrate faster in a non-reducing SDS–PAGE because the unfolding of the protein by SDS is more inhibited than in the form with

Table III. Equilibrium association constants $K_{\alpha=1}$ and association rate constants k_{ass} of TetR variants with $[Mg\text{-}tc]^+$

TetR variant		$K_{\alpha=1}$ (/M×10^9)	k_{ass} (/M/s×10^5)
Wild-type	reduced	2.1 ± 0.50	3.65 ± 0.81
	oxidized	2.0 ± 0.1	3.43 ± 1.05
NC18RC94	reduced	0.50 ± 0.08	0.50 ± 0.07
	oxidized	0.80 ± 0.10	1.00 ± 0.07
DC106PC159′	reduced	2.00 ± 0.51	1.89 ± 0.29
	oxidized	2.80 ± 0.57	3.06 ± 0.69
EC107NC165′	reduced	3.50 ± 0.95	3.66 ± 0.28
	oxidized	0.87 ± 0.10	3.34 ± 0.63
RC195VC199′	reduced	2.6 ± 0.1	n.d.
	oxidized	2.6 ± 0.1	n.d.

one disulfide bond. The two disulfide bonds in TetR RC195VC199′ are too close together to detect migration differences. TetR NC18RC94 migrates faster than wild-type (Figure 2), presumably because the intrachain disulfide bond reduces the radius of gyration. Since the oxidized TetR mutants appear to be mostly homogeneous, disulfide bond formation is complete in the four double mutants of TetR. The treatment of the oxidized proteins with the reducing reagent dithiothreitol (DTT) leads to complete reduction of the disulfide bonds as judged from the gel mobility (Figure 2). This demonstrates that the oxidation is fully reversible.

To verify the results obtained in the gel mobility assays, the formation of disulfide bonds was also chemically identified by titration of free thiols with the Ellman reagent DTNB (5,5′-dithiobis-2-nitrobenzoic acid). No free thiol was detected after oxidation of either of the four TetR mutants (data not shown). Thus, two independent assays confirm that the cysteine residues were completely oxidized in all TetR variants.

Taken together, the formation of disulfide bonds upon oxidation of the purified TetR mutants was determined unambiguously by two different methods, and the reversibility of the reaction was demonstrated. Thus, the correlation of the *in vitro* activities of the proteins to their oxidation state is possible.

Influence of cysteine exchange and disulfide formation on in vitro activities of the TetR mutants

Binding of $[Mg\text{-}tc]^+$ to reduced and oxidized TetR mutants. The binding constants of $[Mg\text{-}tc]^+$ to TetR variants were determined under equilibrium binding conditions by fluorescence titration with limiting Mg^{2+} concentrations (Takahashi *et al*., 1986). The results are displayed in Table III. The equilibrium association constant $K_{\alpha=1}$ of wild-type TetR (2.1×10^9/M) is nearly the same as that of reduced and oxidized TetR DC106PC159′ and TetR RC195VC199′ and reduced TetR EC107NC165′. The oxidized TetR EC107NC165′ and the reduced and oxidized TetR NC18RC94 exhibit a 2- to 4-fold lower affinity for $[Mg\text{-}tc]^+$.

The association rate constants k_{ass} of $[Mg\text{-}tc]^+$ with the TetR variants were determined from the time-dependent increase of tc fluorescence after addition of TetR (Takahashi *et al*., 1986) and are also shown in Table III. All TetR mutants, except TetR NC18RC94, show the same association rate constant as wild-type. The oxidized and reduced TetR NC18RC94 show an ~4- and 7-fold reduced association rate constant, respectively.

Taken together, the mutations affect *in vitro* tc binding only marginally. These results agree with the *in vivo* data and reconfirm that the cysteine mutations do not lead to altered activities of the respective TetR mutants.

Different operator binding of reduced and oxidized TetR mutants. The influence of the cysteine residues and disulfide bond formation on *in vitro* operator binding of the TetR mutants was assayed by DNA retardation analyses. A synthetic 42 bp DNA fragment carrying *tet* operator *O1* was incubated with increasing amounts of reduced and oxidized TetR variants and complex formation was analyzed by PAGE. The results are shown in Figure 4. All reduced double mutants and the oxidized TetR DC106PC159′ and TetR EC107NC165′ show no impaired operator affinities compared with wild-type TetR. The oxidized TetR RC195VC199′ shows a slightly reduced affinity for *O1*, while the oxidized form of TetR NC18RC94 shows a drastically reduced affinity. After a cycle of oxidation and subsequent reduction by DTT, the same operator affinities as wild-type are restored for all TetR variants (data not shown).

This demonstrates that the amino acid exchanges to cysteine and the immobilization of the variable loop by formation of disulfide bonds do not reduce operator binding. In contrast, fixing the DNA reading head to the protein core or the two monomers against each other near the crosspoint of α10 leads to reduced operator binding.

Disulfide bond formation affects the inducibility of the TetR mutants. The *in vitro* inducibility of the TetR variants was demonstrated employing the DNA retardation assay (Figure 5). The *O1*–TetR complex was treated with 1 μM tc and analyzed by native PAGE. TetR binds less well to operator DNA in the presence of tc, as indicated by increased amounts of free operator DNA. Oxidation of wild-type TetR does not influence the results (data not shown). The reduced TetR DC106PC159′ and TetR EC107NC165′ proteins are inducible with about wild-type efficiency in this experiment. In contrast, the oxidized proteins are not inducible under these conditions. The fixation of the variable loop to the core of the protein apparently prevents induction by tc. After reduction by DTT, full inducibility is restored, demonstrating that the observed influence on induction must be attributed to the presence of the disulfide bond. TetR NC18RC94 and TetR RC195VC199′ were not included in this study, because the oxidized forms show reduced operator binding.

Taken together, these results establish that the cysteine exchanges have no influence on the *in vitro* activities of the reduced TetR variants, but operator binding and inducibility are affected by disulfide bond formation between the variable loop and the protein core.

Different rates of disulfide bond formation of operator- and inducer-bound TetR correspond to in vitro activities

Interface between DNA reading head and protein core. The efficiencies of disulfide bond formation in TetR NC18RC94 were compared in the free, operator- and tc-bound forms after 15 min incubation. Formation of disul-

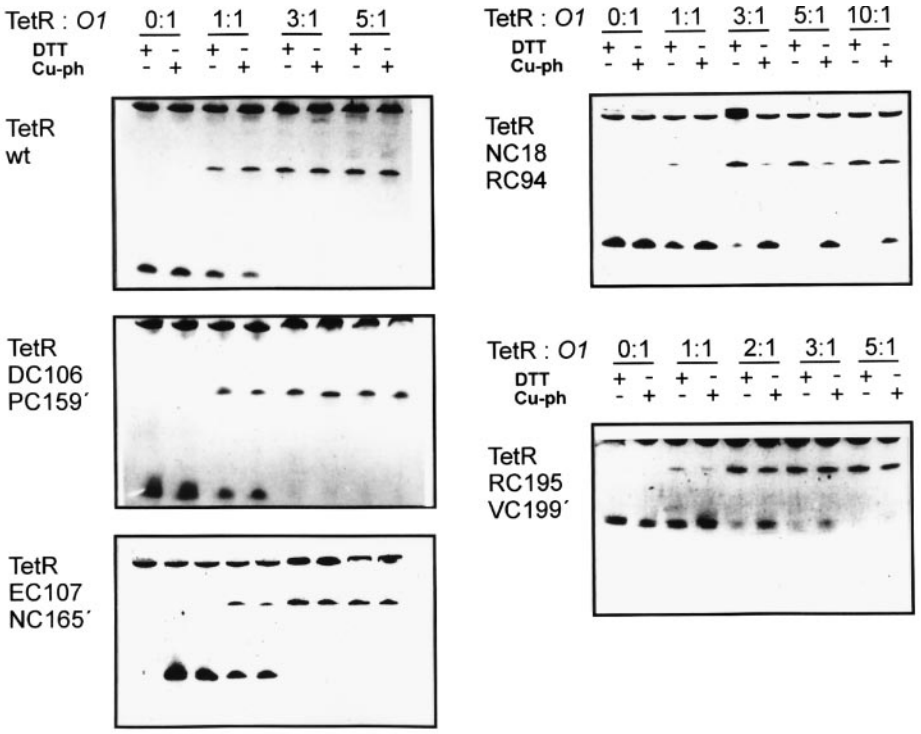

Fig. 4. Operator binding of reduced and oxidized TetR variants analyzed by DNA retardation. DNA retardation analyses on 10% polyacrylamide gels are shown for wild-type and each TetR variant as indicated on the left side of each photograph. The molar ratios of TetR to operator DNA *O1* are indicated on the top of each photograph. A 2.5 pmol aliquot of operator DNA (*O1*) was incubated with different amounts of reduced (DTT +) and oxidized (Cu-ph +) TetR variants (0, 2.5, 7.5 and 12.5 pmol for TetR wild-type, DC106PC159′ and EC107NC165′; 0, 2.5, 7.5, 12.5 and 25 pmol for TetR NC18RC94; 0, 2.5, 5, 7.5 and 12.5 pmol for TetR RC195VC199′).

fide bonds was assayed by Ellman titration. The results are shown in Table IV. Disulfide bond formation is slower in the operator-bound form than in the tc-bound or free forms, since we detect a higher portion of free thiol groups in the operator-bound protein. This indicates that the cysteines in the DNA-bound conformation are oriented less favorably for disulfide formation than in the other two forms. The migration analysis of this mutant in non-reducing SDS–PAGE (Figure 6) shows that intermolecular disulfide bonds are formed in the DNA-bound and free repressor. This indicates that one of the cysteines must be solvent exposed to such an extent as to be able to form a disulfide bond with another dimer. This is not the case in the tc-bound form of this TetR mutant. Thus, these experiments define three different conformations: (i) a disulfide bond cannot be formed in the DNA-bound protein; (ii) complete formation is observed in the tc-bound conformation, but no intermolecular disulfide bond can be formed; and (iii) the free protein contains no thiol groups but forms intermolecular disulfide bonds, thus it must assume a conformation which is different from that of the other two. The results are in excellent agreement with the reduced affinity of the oxidized form for DNA. Disulfide bond formation is fastest in the tc-bound conformation and impairs operator binding, indicating that the cysteine cross-link fixes a conformation which is different from the DNA-bound conformation of TetR. For the first time, these results directly indicate a movement of the DNA reading head with respect to the protein core.

The DNA reading head–protein core contact area cross-linked by oxidation of TetR NC18RC94 is stabilized in the wild-type TetR crystal structure by many interactions

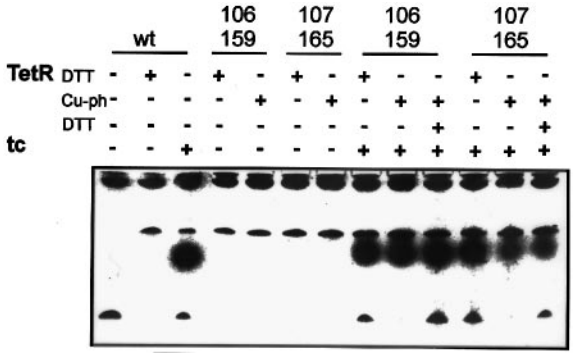

Fig. 5. Induction analysis of reduced and oxidized TetR variants. DNA retardation analyses in the absence and presence of the inducer tc are shown on 10% polyacrylamide gels for wild-type and the TetR variants DC106PC159′ and EC107NC165′. The leftmost three lanes display the induction assay of wild-type TetR, the first lane shows free operator DNA (*O1*), followed by the *O1*–TetR complex and the *O1*–TetR complex induced with tc. The next four lanes show the *O1*–TetR complex of the reduced (DTT +) and oxidized (Cu-ph +) forms of the TetR variants DC106PC159′ and EC107NC165′. The six lanes on the right side show these *O1*–TetR complexes induced with tc. The reduced (DTT +), oxidized (Cu-ph +) and DTT-treated after previous oxidation (Cu-ph +, DTT +) forms of the TetR variants DC106PC159′ and EC107NC165′ are shown. The molar ratio *O1*:TetR:tc of 1:3:10 was used (2.5 pmol of *O1*:7.5 pmol of TetR:25 pmol of tc).

(hydrogen bonds, salt bridges and contacts to tc; Müller *et al.*, 1995). Three hydrogen bonds exist between Asn18 and Arg94 ($O^{\delta 1}$...N, O...N^{ε}, O...$N^{\eta 2}$; Kisker *et al.*, 1995). These H-bonds are replaced in TetR NC18RC94 by a disulfide bond, thus immobilizing the DNA reading head which results in reduced operator binding. We conclude

Table IV. Thiol contents of TetR NC18RC94 determined in free, operator- and tc-bound forms

TetR variant		Moles of SH/molecule
Wild-type		0 ± 0.2
NC18RC94 free	reduced	4
	oxidized	–0.7 ± 0.8
NC18RC94 operator-bound	reduced	3.8 ± 0.4
	oxidized	2.3 ± 0.1
NC18RC94 tc-bound	reduced	3.3 ± 0.1
	oxidized	–0.2 ± 0.4

Operator DNA was used in 3-fold, tc in 10-fold excess over TetR. Protein concentrations were determined by UV spectroscopy.

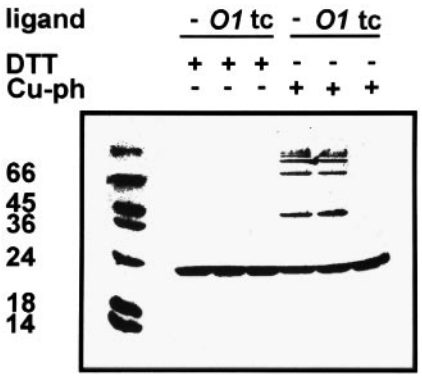

Fig. 6. Disulfide bond formation of TetR NC18RC94 determined in the free, operator- and tc-bound forms. Disulfide bond formation was monitored by non-reducing 10% SDS–PAGE. The first lane shows a molecular weight standard, the next three lanes show free (–), operator-bound (*O1*) and tc-bound (tc) repressor under reducing conditions (DTT +), whereas the three lanes on the right side show the same analysis for oxidized repressor (Cu-ph +, 1 μM).

that induction of TetR leads to the formation of these H-bonds, which may contribute to fixing the positions of the DNA reading head to the protein core, so that operator DNA cannot be bound.

Variable loop between α-helices 8 and 9. Oxidation of TetR EC107NC165′ and TetR DC106PC159′ was analyzed at different concentrations of copper phenanthroline. Representative results are shown for EC107NC165′ in Figure 7. At 1 mM copper phenanthroline, all proteins are cross-linked irrespective of the bound ligand. The cross-linking efficiency at 1 μM copper phenanthroline, however, is ligand dependent. DNA-bound protein is cross-linked rapidly, whereas tc-bound repressor is not cross-linked at all. These results indicate different conformations of TetR in these complexes. Since disulfide bond formation requires collision of two sulfhydryls, this rate provides information about the proximity of the reacting cysteines (Careaga and Falke, 1992).

We conclude that cysteines 106 and 159′, and 107 and 165′ are closer together in the operator-bound than in the induced form. Hence, the variable loop must be nearer to the protein core in the operator-bound form. As indicated by the lack of induction seen with the oxidized forms of TetR DC106PC159′ and TetR EC107NC165′ and the faster rate of disulfide bond formation in the operator-bound TetR, the variable loop between α-helices 8 and 9 needs to be flexible, and movement of the variable loop is necessary for induction. This conclusion explains the

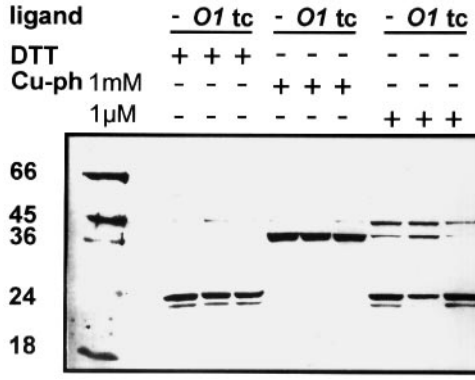

Fig. 7. Disulfide bond formation of TetR EC107NC165′ determined in the free, operator- and tc-bound forms. Disulfide bond formation was monitored by non-reducing 10% SDS–PAGE. The first lane shows a molecular weight standard, the next three lanes show free (–), operator-bound (*O1*) and tc-bound (tc) repressor, respectively, under reducing conditions (DTT +), the following three lanes show the same analysis under oxidation conditions with 1 mM Cu-ph, and the three rightmost lanes under oxidation conditions with 1 μM Cu-ph.

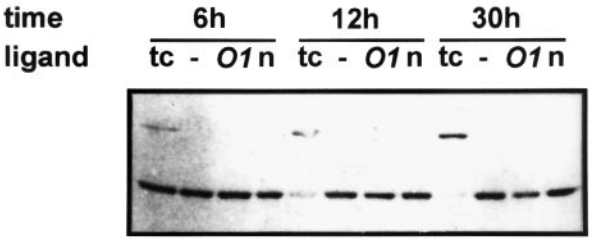

Fig. 8. Disulfide bond formation of TetR RC195VC199′ determined in the free, operator- and tc-bound forms. Disulfide bond formation was monitored by non-reducing 10% SDS–PAGE. The leftmost four lanes show free (–), operator-bound (O1), tc-bound (tc) TetR and TetR with non-specific DNA (n) incubated at 22°C in the presence of ambient oxygen without a catalyst for 6 h. The following four lanes show the same analysis after 12 h incubation and the rightmost four lanes after 30 h incubation.

previous observation that this region must be flexible in functional TetR (Berens *et al*., 1997).

Dimerization surface. The third region we have investigated is the dimerization surface. TetR RC195VC199′ shows disulfide bond formation in the presence of ambient dissolved oxygen without a catalyst (Figure 8). Different efficiencies were observed depending on the ligand. Tc-bound TetR RC195VC199′ shows complete formation of disulfide after a 30 h incubation period at 22°C, whereas the DNA-bound form does not (Figure 8). This experiment establishes the presence of different conformations also for this region. The preferred disulfide bond formation in the tc-bound form is in agreement with the fact that the distances between the Cα atoms taken from the crystal structure of the induced complex are favorable for a disulfide bond (see Table I). Since the rate of disulfide bond formation is slower in the DNA-bound conformation, the cysteines should be further apart in that structure. This explains why immobilizing the two helices results in a reduced affinity for operator (Figure 4).

A tc-induced reorientation of the four-helix bundle formed by α8 and α10 from both monomers has been proposed on the basis of non-inducible TetR mutants (Müller *et al*., 1995). Such a movement of α-helices was observed in the subunit interface of glycogen phosphoryl-

ase, where a ligand-induced conformational change reduces the angle of the α-helices in the four-helix bundle by 60°, leading to activation of the enzyme (Barford and Johnson, 1989). The data presented here would be in agreement with such a movement in TetR, which would render cysteines 195 and 199′ too far from each other to form a disulfide bond in the DNA-bound conformation. Since this conformation would locate α9 and the variable loop closer to the β-turns between residues 101 and 109, the faster rate of disulfide bond formation between cysteines 106 and 107, and 159′ and 165′, respectively, could also be explained. Thus, binding of tc might trigger a motion of the four-helix bundle coupled with a movement of α9 and the variable loop. The disulfide bonds in TetR DC106PC159′ and EC107NC165′ would prevent such a movement, explaining the observed lack of induction.

Internal movements correlated to induction of Tet repressor

We have investigated three regions of TetR where conformational changes during induction have been proposed (Müller *et al*., 1995). The movements in the interface between the DNA reading head and the protein core required by the first induction model (Hinrichs *et al*., 1994) are demonstrated by the data presented here. In addition, the results support a reorientation of the four-helix bundle and an active role of the variable loop, as proposed in the second induction model. No information about the nature or the magnitude of these local movements can be deduced from the data presented here.

Our results show that binding of the effector to TetR triggers defined and large conformational changes involving the entire protein. This distinguishes it clearly from TrpR, CRP and LacI, where only portions of the polypeptide are involved in conformational changes.

Materials and methods

Materials and general methods

Chemicals of the highest purity available were obtained from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (München, Germany) or Roth (Karlsruhe, Germany). Tc and atc were purchased from Fluka (Buchs, Switzerland). Enzymes for DNA restriction and modification were obtained from Boehringer Mannheim (Mannheim, Germany), New England Biolabs (Schwalbach, Germany) or Pharmacia (Freiburg, Germany). Isolation and manipulation of DNA was done as described (Sambrook *et al*., 1989). Sequencing was carried out according to the protocol provided by Pharmacia for use with T7 DNA polymerase with [α-^{32}P]dATP from Amersham (Braunschweig, Germany).

Bacterial strains and plasmids

All bacterial strains are derived from *E.coli* K12. DH5α was used for general cloning, WH207 served for β-galactosidase assays and RB791 was used for overexpression of TetR variants. The plasmids pWH1012, pWH1201 and pWH620 were used for determining TetR activity *in vivo*, and the plasmid pWH1950D for overexpression of TetR variants (for references, see Berens *et al*., 1997).

Construction of tetR mutants

Mutations were introduced into the cysteine-free *tetR(D)* allele by PCR according to the three primer method (Landt *et al*., 1990). The conditions for PCR were chosen as described (Berens *et al*., 1997). After mutagenesis, the entire *tetR* gene was sequenced to verify the desired mutations and to exclude secondary mutations. *tetR(D)* was cloned in pWH620 (Berens *et al*., 1997) using *Xba*I and *Nco*I. The resulting plasmid was named pWH620D. Additional single restriction sites were introduced by silent mutations. The pWH620D derivative containing restriction sites for *Bgl*II, *Mlu*I, *Bss*HI and *Bsu*36I was named pWH624. Ten different *tetR* mutants bearing cysteine residues were constructed: Asn18→Cys, denoted NC18, Arg94→Cys, denoted RC94, the double mutant NC18RC94, Asp106→Cys, denoted DC106, Pro159→Cys, denoted PC159, the double mutant DC106PC159, Glu107→Cys, denoted EC107, Asn165→Cys, denoted NC165, the double mutant EC107NC165 and the double mutant Arg195→Cys Val199→Cys, denoted RC195VC199. For construction of overexpressing plasmids, the respective pWH624 derivatives were digested with *Xba*I and *Sph*I, and the 714 bp *tetR* fragments ligated into similarly digested pWH1950D to place the mutant *tetR* allele under *tac* promoter control.

β-Galactosidase assays

Repression and induction by atc were determined in *E.coli* WH207 pWH1012. The plasmid pWH1012 contains a *tetA–lacZ* transcriptional fusion. Cells were grown in LB supplemented with appropriate antibiotics at 28°C. β-Galactosidase activities were determined as described (Miller, 1972). Three independent cultures were assayed for each mutant, and measurements were repeated at least twice.

Protein purification

Escherichia coli RB791 transformed with pWH1950D derivatives was used for expression of TetR mutants. Purification of the proteins to homogeneity was done as described (Ettner *et al*., 1996). Protein concentrations were determined by UV spectroscopy using an extinction coefficient of ε_{280} = 35 800/M/cm and by saturating titration with tc observing the change of fluorescence.

Fluorescence measurements

The binding activity of tc to Tet repressor was determined by tc and protein fluorescence using a Spex fluorimeter (Spex industries, Edison, NJ) equipped with double monochromators by measuring fluorescence emission of the drug at 515 nm and excitation at 370 nm as described (Takahashi *et al*., 1986). Association constants $K_{\alpha=1}$ and association rate constants k_{ass} were determined at 28°C. All measurements were repeated at least twice.

DNA mobility shift analysis

Purified protein (2.5–25 pmol) was incubated with 0.2 μg of non-specific DNA and 2.5 pmol of synthetic 42 bp DNA carrying *tet* operator *O1* in 20 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 40 mM KCl for 10 min at ambient temperatures. The mobility of the DNA was analyzed by electrophoresis on 10% polyacrylamide gels.

Disulfide bond formation

During the purification and storage of di-cysteine repressors, cysteine residues were kept in the presence of 5 mM DTT. Oxidation of cysteine-containing TetR was carried out in 20 mM NaH_2PO_4, 50 mM KCl, 50 mM NaCl, adjusted to pH 7.0 with NaOH (Careaga and Falke, 1992). The oxidation reaction by oxygen was accelerated by addition of copper (II) (1.10-phenanthroline)$_3$ to final concentrations as stated in the respective Results sections. The presence of disulfide bonds was identified by non-reducing SDS–PAGE or by assaying the remaining free thiols by Ellman titration (Matsumura and Matthews, 1991). For non-reducing SDS–PAGE, samples were incubated for 10 min at 20°C and then mixed with an equal volume of 100 mM EDTA, 130 mM Tris–HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM iodoacetamide, 0.05% (w/v) bromophenol blue before electrophoresis. For Ellman titration, a 1 ml aliquot of each sample was adjusted to pH 7.0, and 50 μl of 4 mg/ml solution of DTNB was added. After 15 min, the absorbance at 410 nm was measured and a reagent blank was subtracted from the apparent absorbance to give the net absorbance (Matsumura and Matthews, 1991). The value obtained for the reduced form of the ligand-free form of TetR 18NC94RC was set to 4 mol of sulfhydryl per TetR dimer.

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