The induction of folding cooperativity by ligand binding drives the allosteric response of tetracycline repressor

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Tetracycline (Tc) repressor (TetR) undergoes an allosteric transition upon interaction with the antibiotic, Tc, that abrogates its ability to specifically bind its operator DNA. In this work, by performing equilibrium protein unfolding experiments on wild-type TetR and mutants displaying altered allosteric responses, we have delineated a model to explain TetR allostery. In the absence of Tc, we show that the DNA-binding domains of this homodimeric protein are relatively flexible and unfold independently of the Tc binding/dimerization (TBD) domains. Once Tc is bound, however, the unfolding of the DNA binding domains becomes coupled to the TBD domains, leading to a large increase in DNA-binding domain stability. Noninducible TetR mutants display considerably less interdomain folding cooperativity upon binding to Tc. We conclude that the thermodynamic coupling of the TetR domains caused by Tc binding and the resulting rigidification of the DNA-binding domains into a conformation that is incompatible with DNA binding are the fundamental factors leading to the allosteric response in TetR. This allosteric mechanism can account for properties of the whole TetR family of repressors and may explain the functioning and evolution of other allosteric systems. Our model contrasts with the prevalent view that TetR populates two distinct conformations and that Tc causes a switch between these defined conformations.

allostery | DNA binding | drug binding

Protein allostery, whereby binding of a ligand at one site in a protein alters the function of a distant site in the protein, is imperative for the regulation of most biological processes (1–3). Comparison of crystal structures of free and ligand-bound allosteric proteins has led to the commonly held view that allosteric proteins have the ability to assume two distinct conformations with different activities. Ligand binding is seen to act as the switch causing interconversion between these two conformational states. However, recent work indicates that ligand-induced changes in protein stability and dynamics that cannot be observed by classical structure determination may play a fundamental role in mediating allostery (1, 4, 5). To address the potential importance of ligand-induced protein stabilization in allosteric mechanisms, we have investigated the relationship of folding thermodynamics and allosteric mechanism in one of the best-characterized allosteric systems, the tetracycline (Tc) repressor (TetR) (6, 7).

TetR is a homodimeric protein in which each monomer is comprised of an N-terminal DNA-binding domain and a C-terminal tetracycline-binding and dimerization (TBD) domain (Fig. 1). Interaction with the antibiotic, Tc, causes a large decrease in the DNA-binding affinity of TetR (8). Because the Tc-binding sites in TetR are >30 Å from the DNA-binding helices, an allosteric mechanism is responsible for this effect. The model to explain TetR allostery was constructed by comparing the X-ray crystal structures of TetR complexed with either Tc or its DNA operator. Tc binding was seen to induce a pendulum like movement of α -helix 4 that results in an increase of the distance between the DNA recognition helices of the two monomers (α -helices 3 and 3'). This change in position of the recognition helices would prevent them from

Fig. 1. The structure of TetR. (*Left*) TetR consists of two monomers (light and dark blue) that interact through a four-helix bundle. Tc (green) binds in a hydrophobic pocket in the core of each monomer. The position of residues mutated in this study, I22 (cyan), G96 (red), and L146 (orange) are displayed. (*Right*) A close-up of the boxed region in *Left*, showing an Arg residue substituted at position 96, which is the revTetR substitution. Introducing the large Arg side chain is expected to disrupt the packing of the TBD/DNA-binding domain interface residues (yellow).

binding DNA because they could no longer fit into successive major grooves (9–13).

Despite intensive study of this system, certain aspects of TetR function remain unexplained. If Tc binding were responsible for inducing a conformation of TetR that is incompatible with DNA binding, then the apo form of TetR would be expected to resemble its DNA-bound form. However, the distance between the DNA recognition helices in unliganded TetR is actually greater than that seen in the Tc-bound form of the protein, suggesting that the DNA-binding domains of apo-TetR must possess considerable flexibility to allow them to access the DNA-bound conformation (11). A variety of noninducible TetR mutants (ninTetR) are not subject to Tc-induced transcriptional derepression even though they still bind to Tc with WT affinity (14, 15). Another group of mutants, called reverse TetRs (revTetRs), bind DNA more tightly in the presence of Tc derivatives than in their absence (16–19). A unified explanation for these phenotypes has been difficult to formulate because many of the amino acid substitutions causing them lie at positions that are not directly involved in drug binding or the conformational changes seen upon drug binding. Furthermore, some mutants display divergent allosteric effects depending the particular Tc derivative that is used in the assay (20, 21).

Shortcomings in the current structural model of TetR allostery motivated us to investigate the thermodynamic ramifications of drug binding to TetR. In a previous study, we demonstrated that

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Fig. 2. Urea denaturation profiles of WT and mutant TetRs in the absence of drug. (Lower) A blow-up of the pretransition region of these profiles. This region is boxed in Upper. Protein unfolding was monitored by measuring the change in CD ellipticity at increasing concentrations of urea. The data were normalized by overlaying the main-transition region as described in *Materials and Methods*.

binding of Tc can significantly stabilize the DNA-binding domain (22). This result showed that binding of Tc to TetR imparts a thermodynamic stabilization signal that is conveyed from the TBD domain to the DNA-binding domain. We hypothesized that this thermodynamic relay might play an important role in the TetR allosteric mechanism. In the current study, we have pursued this hypothesis by characterizing the unfolding behavior of WT TetR and mutants displaying the *ninTetR* or *revTetR* phenotypes. Through these investigations, we have been able to put forward a comprehensive explanation for TetR allostery that primarily involves a Tc-induced coupling of folding between the DNA-binding and TBD domains. We believe that this mechanism applies to the whole family of TetR-like repressors and may be relevant to other allosteric systems.

Results

The DNA-Binding Domain Unfolds Independently of the TBD Domain in Apo-TetR. To address the question of how Tc binding might affect the stability of the DNA-binding domain, it was necessary to investigate the equilibrium unfolding mechanism of TetR. For this purpose, circular dichroism (CD) spectroscopy was used to monitor the helical content of TetR at increasing concentrations of the denaturant, urea. As can be seen in Fig. 2, WT TetR displayed a major unfolding transition between 4 and 6 M urea. This transition has been shown to be a reversible two-state reaction that primarily reflects the concerted and reversible unfolding of the TBD domain from a folded dimer to two unfolded monomers (23–25). Although a TetR mutant completely lacking its DNA-binding domain (Δ DNB mutant) showed a major unfolding transition at a similar position as WT TetR, striking differences were observed in the shape of the unfolding curves of WT TetR and the Δ DNB mutant at urea concentrations <4 M, which we refer to as the pretransition region (Fig. 2). Whereas WT TetR displayed a straight but positively sloped line in its pretransition region, the Δ DNB mutant displayed a pretransition line with a slope close to 0 (Fig. 2). The I22D mutant, which possesses an almost completely unfolded DNA-binding domain (22), displayed an unfolding profile that was

almost indistinguishable from the Δ DNB mutant featuring a flat pretransition region. These data indicate that the sloped pretransition behavior observed for WT TetR is caused by unfolding of the DNA-binding domain over this range of urea concentrations. This unfolding reaction was not seen in mutants either lacking the DNA-binding domain or possessing an unfolded version of it. It should be noted that the difference in the absolute molar ellipticity values between the Δ DNB domain mutant and WT TetR corresponds very closely to helical content of the DNA-binding domain seen in the crystal structure (22).

To further clarify the unfolding mechanism of the DNA-binding domain, we sought to characterize a series of incrementally destabilizing substitutions in this domain. For this purpose, residues expected to be less destabilizing than Asp at a buried hydrophobic position, Ala, Thr and Asn, were introduced at the Ile-22 position. Interestingly, denaturation of the I22N mutant resulted in a sloped line until a urea concentration of ≈ 1 M urea that then flattened out to resemble the I22D and Δ DNB domain mutants. The I22T and I22A mutants exhibited a similar behavior, but their denaturation curves remained sloped to higher urea concentrations. The intermediate behaviors of these Ile-22 mutants are likely a result of partial destabilization of the DNA-binding domain, a notion that is also supported by the reduced total ellipticity of these mutants compared with WT even in the absence of urea.

Overall, these experiments provide two important results. First, the DNA-binding domain unfolds independently of the TBD domain in the absence of drug; and second, the shape of the pretransition region of these denaturation curves reflects the stability of the DNA-binding domain. These conclusions are strengthened by a quantitative analysis of the urea denaturation curves (*SI Text, Figs. S1 and S2*). We have shown that the unfolding of the DNA-binding domain can be modeled as a simple two-state transition independent from the unfolding of the TBD domain. This simple model is able to provide excellent quantitative fits for the curves shown in Fig. 2 (Fig. S3 and Table S1).

Tc Binding Induces Folding Cooperativity Between the DNA-Binding and TBD Domains in WT TetR, but Not in *ninTetR*. To directly investigate the relationship between TetR inducibility and thermodynamic stability, we measured the effects of Tc and a stronger binding derivative of Tc, anhydrotetracycline (Atc), on the urea denaturation profiles of WT TetR and a *ninTetR* mutant. The *ninTetR* chosen for study was the L146F mutant, which was shown to possess WT Tc-binding affinity but reduced inducibility (15). Leu-146 is located in the dimerization interface, and this residue does not change conformation upon Tc binding (Fig. 1).

As can be seen in Fig. 3A, the presence of Tc or Atc substantially shifted the midpoint of the major urea-induced unfolding transition of WT TetR toward higher concentrations of urea, indicating an increase in thermodynamic stability. Strikingly, both Tc and Atc also significantly reduced the slope of the pretransition region, implying that ligand binding also stabilized the DNA-binding domain against denaturant induced unfolding. This stabilization was strong enough that DNA-binding domain unfolding could no longer be distinguished from the unfolding transition of the TBD domain, suggesting that the two domains were unfolding in a cooperative manner in the presence of Tc or Atc.

The unfolding curve of the *ninTetR* mutant in its apo form was similar to WT TetR, displaying an equally sloped pretransition region (Fig. 3B). In contrast with WT, however, the slope of the pretransition region of the *ninTetR* mutant at low urea concentration did not change at all upon addition of Tc, indicating that Tc addition did not elicit stabilization of the DNA-binding domain. The flattening of the pretransition region observed at concentrations of urea >2 M urea was likely caused by the completion of DNB domain unfolding before unfolding of the TBD domain had commenced. Even though stabilization of the DNA-binding domain was not observed, the *ninTetR* mutant was clearly binding Tc

Fig. 3. Protein unfolding of WT TetR and the ninTetR mutant in the presence of absence Tc derivatives. Urea denaturation profiles of WT (A) and ninTetR (B) in their unliganded form or in the presence of Tc or Atc were monitored by measuring CD ellipticity at increasing concentrations of urea. In B a close-up of the pretransition region is shown and the lines denoting the WT profiles from A are shown for comparison.

as indicated by the substantial shift of its major unfolding transition. The denaturation of ninTetR mutant in the presence of Atc resulted in two detectable unfolding transitions, indicating that Atc binding partially stabilized the DNB domain, but it still unfolded before the TBD domain (Fig. 3B). Thus, even in the presence of Atc, the ninTetR DNA-binding domain did not unfold in a highly cooperative manner with respect to the TBD domain.

It is evident that the L146F substitution slightly decreased the overall stability of TetR as indicated by its major unfolding transition occurring at a lower concentration of urea (Fig. 3B). However, this reduced stability is unlikely to be the cause of the noninducibility of this mutant as it has been shown that changes in overall stability of TetR resulting from monomer–monomer interface alterations do not lead to significant changes in inducibility (24). It can also be seen that the steepness of the major transition of the ninTetR mutant varied from WT in the presence of Tc and was more similar to WT in the presence of Atc. We believe that these phenomena are caused by the dissociation of ligand from folded dimers at high urea concentration as is explained in detail in *SI Text*.

Atc Binding Induces Folding of the revTetR DNA-Binding Domain, but Cooperativity with the TBD Domain Is Weak. To obtain further insight into the TetR allosteric process we investigated the G96R substitution, which had been shown to cause a revTetR phenotype (i.e., it bound DNA only in the presence of Tc or other Tc derivatives) (19). The Gly-96 position lies in α -helix 6 and an Arg residue at this position would likely interact and potentially clash with residues in α -helix 1 of the DNA-binding domain (Fig. 1). In examining the far-UV CD spectrum of this revTetR mutant, we discovered that it was greatly reduced in ellipticity as compared with the WT (Fig. 4A), indicating its possession of significantly less helical content. Because the level of ellipticity seen for the revTetR mutant was the same as that seen for the I22D mutant (Fig. 4A), which possesses a completely unfolded DNA-binding domain (22), we concluded that the revTetR mutant also possesses an unfolded DNA-binding domain. Remarkably, addition of Atc to the revTetR mutant induced it to gain ellipticity to a level similar to the WT domain (Fig. 4A), implying that Atc-binding induces the folding of the DNA-binding domain. As observed (22), the I22D mutant shows a similar increase in ellipticity upon the addition of Atc (Fig. 4A).

As would be expected for a TetR mutant lacking a folded DNB domain, revTetR unfolded with a nonsloped pretransition in the absence of ligand in a manner identical to the I22D mutant (Fig. 4B). However, the unfolding behavior of revTetR in the presence of Atc was distinct from the I22D mutant. Whereas the I22D mutant unfolded with an unsloped pretransition region and showed only a single unfolding transition in the presence of Atc, revTetR clearly displayed two unfolding transitions, implying that its DNA-binding domain, although stabilized by drug, still completely unfolded before the onset of TBD domain unfolding. Although the revTetR mutant binds to Tc ≈ 300 -fold more weakly than I22D (Table S2), the difference in behavior between these mutants in the pretransition region of the melts was not likely the result of drug dissociation from revTetR. This conclusion is based on our failure to observe a significant change in the revTetR melting profile in this

region even when the experiment was carried out in a 5-fold higher concentration of Atc (Fig. S4).

Partial Proteolysis Experiments Confirm the Deficiency in DNA-Binding Domain Stabilization Induced by Tc and Atc in the ninTetR and revTetR Mutants. To support the results of the urea denaturation experiments described above, we performed partial proteolysis experiments in which samples were incubated with trypsin for varying periods of time in the absence or presence of Tc or Atc. The prominent digestion products observed on SDS/PAGE gels were shown by MALDI-TOF mass spectrometry to be the result of cleavage at sites within the DNB domain and α -helix 4, which lies at the interface between the DNB and TBD domains (22). Thus, the accessibility of these sites to protease reflects the stability of the DNA-binding domain.

The DNA-binding domain of WT TetR, although susceptible to proteolysis in its apo form, was almost completely protected from proteolysis upon addition of either Tc or Atc (Fig. 5). In contrast, the proteolytic cleavage pattern of ninTetR, which was similar to WT in the absence of drug, changed little whether Tc or Atc was added. RevTetR was highly susceptible to proteolysis in the absence of drug, as was expected because its DNB domain is unfolded. While addition of Tc slightly stabilized the DNA-binding domain of this mutant, Atc provided a much greater degree of stabilization. However, even in Atc, revTetR was not stabilized nearly as much as WT and much more closely resembled ninTetR proteolyzed under the same conditions. Overall, the proteolysis results confirmed that for both ninTetR and revTetR ligand binding failed to

Fig. 4. Folding behavior of the revTetR mutant. (A) Far UV CD scans of WT TetR and mutants in the presence or absence of Atc. (B) Urea-induced unfolding profiles for the revTetR and I22D mutants in the presence or absence of Atc.

fully stabilize the DNA-binding domain. This experiment also emphasized that even in WT TetR the DNA-binding domain is less stable in the absence of drug.

The I22D Mutant Can Bind DNA In Spite of Its Unfolded DNA-Binding Domain. We performed electrophoretic mobility-shift assays to confirm the DNA-binding phenotypes of the mutants used in this study. In these assays, a labeled DNA fragment containing the TetR operator (*tetO*) was mixed with TetR in the presence or absence of Atc. It can be seen that the binding of WT TetR to the *tetO* fragment led to formation of a complex with retarded mobility, and, as expected, the addition of Atc caused its disappearance (Fig. 6A). In contrast, Atc caused only a partial dissociation of the ninTetR:tetO complex. The revTetR mutant was able to bind to *tetO* only in the presence of Atc, and, as reported for other revTetRs (18, 21), the binding affinity of this mutant for DNA was considerably lower than WT even in the presence of drug (note that 10-fold more protein was added to these reactions and that Atc was required in these assays because Tc does not induce DNA binding by this revTetR).

Surprisingly, the I22D mutant, which possesses a completely unfolded DNB domain (Fig. 4A), was still able to bind to *tetO* (Fig. 6A). To investigate the mechanism of this DNA binding, we assessed the helical content of the I22D mutant in the presence of

Fig. 6. In vitro operator binding by WT and mutant TetRs. (A) Electrophoretic mobility-shift assay on WT, ninTetR, I22D, and revTetR in the absence or presence of Atc. Atc was added to WT and ninTetR at a concentration of 1 μ M, and assays with I22D and revTetR contained 10 μ M Atc. (B) Far UV CD scans of WT and I22D TetRs in the presence or absence of *tetO*.

Fig. 7. Model of ligand induced folding cooperativity in TetR. (A) The conformation of the DNA-binding domains of WT TetR in the absence of ligand is flexible as indicated by the gray blurred domains. Drug binding induces folding cooperativity between the DNA-binding and TBD domains as indicated by the black domain. Mutations (X) in the hydrophobic core of the TBD domain reduces the induced folding cooperativity between the two domains, resulting in a ninTetR. Mutations at the interface between the two domains causes destabilization of the DNA-binding domain and reduction in ligand induced folding cooperativity, creating a revTetR phenotype. Folding states that are able to bind *tetO* are surrounded by a red box. (B) The continuous core of TetR. The backbone of TetR is represented as a ribbon model with hydrophobic core residues (80% burial) represented in space-filling mode. The core of the DNA-binding domain is shown in yellow, the buried interface between the DNA-binding and TBD domains is shown in orange, the buried intermonomer contact region is shown in blue, and the TBD domain core is shown in red. Tc is shown in green.

access the conformation required for DNA binding. In the Tc-bound state, the DNA-binding domains become strongly linked to the TBD domains, which leads to cooperative unfolding of both domains. This strengthened interdomain attachment rigidifies the DNB domains into a conformation in which the DNA-binding helices are too far apart to interact with successive major grooves of the DNA. In our model, thermodynamic stabilization resulting from Tc binding and the consequent reduction of DNA-binding domain flexibility are the fundamental factors leading to the allosteric response.

The behaviors of the ninTetR and revTetR mutants can be well accounted for by our allosteric model. In the case of the ninTetR mutant, binding of Tc or Atc failed to induce cooperative folding of the DNA-binding domain with respect to the TBD domain (Fig. 3B); thus, the DNA-binding domains of the dimer remained flexible enough for DNA binding to be possible. The persistent flexibility of the ninTetR DNA-binding domains was clearly illustrated by the minimal alteration observed in its digestion by protease in the presence of Tc or Atc (Fig. 5). Like ninTetR, the DNA-binding domain of revTetR also unfolded independently of the TBD domain in the presence of Atc (Fig. 4B). Consistent with this finding, the degree of protease digestion of the revTetR mutant in Atc was similar to that of ninTetR under the same conditions. We conclude that revTetR mutants are simply noninducible mutants that also possess DNA-binding domains that are unfolded and cannot bind DNA in the absence of drug. Drug binding is able to stabilize the revTetR DNA-binding domain to some extent, but flexibility with respect to the TBD domain is maintained so that DNA binding is still possible. The mechanistic similarity between ninTetR and revTetR mutants explains why the random mutagenesis screens performed to isolate these mutants revealed extensive overlap in the positions of mutations that caused each phenotype (14, 15, 19). Interestingly, the I22D mutant still binds DNA in the absence of drug and is induced by Atc (Fig. 6A) even though it possesses an unfolded DNA binding in its apo form. This phenomenon can be explained by our observation that the DNA-binding domain of the I22D mutant unfolds cooperatively with the TBD domain upon addition of Atc (Fig. 4B). Although we have provided only a qualitative analysis of our urea denaturation curves here, our conclusions are also supported by quantitative analysis (see *SI Text*). We have, for example, estimated that the stability of the DNA-binding domain of WT TetR is increased by at least 2 kcal/mol upon addition of Tc and that no increase in stability is imparted by Tc binding to ninTetR.

Our data on the G96R revTetR mutant agree with a recent study showing that a different revTetR mutant possesses a partially unfolded DNA-binding domain that becomes more helical and stable to proteolysis after Atc addition (26). The X-ray crystal structure of this mutant in the presence of Atc showed that the DNA-binding helices were not oriented in a conformation capable of binding DNA, and Resch et al. (26) concluded that the DNA-binding domains must be able to adjust their conformation in the presence of DNA, an idea that is entirely in agreement with our allosteric model. However, Resch et al. concluded that revTetR operates through a different allosteric mechanism from WT TetR. By contrast, we explain the revTetR phenotype within a single allosteric mechanism that applies to WT and all TetR mutants investigated here.

In the X-ray crystal structure of apo-TetR, the DNA-binding helices are not positioned in a conformation compatible with DNA binding, and this has been difficult to explain using the structural model of allostery. However, in our allosteric model, the main role of drug binding is to rigidify the DNA-binding domains not alter their average conformation; thus, there is no need for the apo form of TetR to resemble the DNA-bound form of the protein. We believe that the DNA-binding domain in apo-TetR is flexible enough to assume the conformation required for DNA binding some of the time, even though this is likely not the predominantly

populated conformation. The potential for the TetR DNA-binding domain to be both malleable and yet still able to adopt a DNA-binding conformation is emphasized by the I22D mutant. This mutant possesses a predominantly unfolded DNA-binding domain, yet this domain can still access the conformation required for DNA binding and predominantly assume this conformation when *tetO* DNA is present (Fig. 6B). Malleability of the DNA-binding domain appears to be generally prevalent in the TetR family of repressors. In the only other example where a repressor of the TetR family was solved in the apo form and in a form bound to a biologically relevant ligand, the apo form and ligand-bound form also showed no significant structural differences, and the DNA recognition helices lay at positions incompatible with DNA binding in both structures (27). We have examined >30 different structures of TetR family repressors in their apo forms and found that the distances between DNA-binding helices range widely from 33 to 63 Å. In the majority of cases where we could determine the DNA-binding sites for these proteins, the distance between the DNA helices was too large to fit into consecutive major grooves of the DNA. These crystallographic data strongly support our conclusion that the DNA-binding domains are flexible in the TetR-family apo-proteins and that the role of drug binding cannot be simply to shift them from a DNA-binding competent conformation to one that is not.

A better understanding of TetR allostery can be gained by considering the Tc-bound state as the true “native state” of this protein. Tc-bound TetR can be seen to actually possess a single hydrophobic core that encompasses the drug-binding site and the DNB domain (Fig. 7B). Because Tc and its derivatives are hydrophobic molecules and bind within a hydrophobic cavity in TetR, removal of Tc from TetR is similar to making a hydrophobic core substitution (e.g., exchanging a hydrophobic residue for a polar residue). Hydrophobic core substitutions are known to cause a variety of effects on protein conformation and stability, which include large changes in stability and dynamics, and loss of folding cooperativity (28–31). Furthermore, the effects of core substitutions can easily propagate to any part of the protein because of the cooperative network of core residue interactions. Thus, creation of a cavity in one part of this core by drug removal could cause a generalized loosening of core residue packing, leading to a partial loss of folding cooperativity, which particularly affects folding of the DNA-binding domain. In the same way, substitutions to any residue within the core packing network (e.g., L146F) could lead to a similar loss in folding cooperativity and result in a noninducible phenotype. The variety of locations within the TetR structure of substitutions causing the noninducible phenotype (14, 15) emphasizes the extensive core network that maintains Tc-induced folding cooperativity. Following the same reasoning, the differences in the specific packing interactions of different Tc derivatives within the binding cavity is a likely origin of their varying allosteric effects (20, 21). For example, Atc lacks a hydroxyl group, which likely enhances its packing to a specific hydrophobic surface in the binding cavity and causes it to elicit a stronger stabilization signal (Fig. 3). However, because of the well-established context-specific nature of the effects of hydrophobic core substitutions, it can be expected that the effect of a given Tc derivative, which is acting in essence like a hydrophobic core residue, may change as the surrounding hydrophobic core residues are altered, a phenomenon that has been seen in a previous study (21).

The view of TetR function that we have presented here not only accounts for the allosteric properties of TetR, but also provides a mechanism for the function and evolution of this whole family of repressors. One of the remarkable aspects of the TetR family is the ability of its members to bind a diverse array of small ligands with some individual proteins, such as QacR and TtgR, each binding many different unrelated molecules (32). This property is difficult to rationalize whether allostery requires very specific ligand–protein interactions as would be needed to induce a specific conformational change. However, if induction only requires a

molecule to interact with any part of the binding cavity well enough to elicit some degree of generalized core stabilization, then proteins with multiple diverse inducers can be much more easily understood. Given that single amino acid substitutions in the hydrophobic core of T4 lysozyme can create internal ligand-binding cavities (33), we speculate that allosteric systems such as TetR could have evolved from nonallosteric systems through destabilizing hydrophobic core substitutions that created cavities for ligand binding. In such cases, ligand binding to the cavity could cause a generalized stabilization of the protein that would have the potential to be communicated from the point of binding across the whole protein because of the inherently cooperative nature of hydrophobic cores (i.e., stabilization of one part of the core will lead to stabilization of the whole core). Allosterism could naturally arise out of this process.

In conclusion, an analysis of the WT and three mutant forms of TetR using denaturant-induced unfolding analyses has provided insight into the allosteric mechanism of TetR. We have found that the key role of ligand is to strengthen the interactions between the DNA-binding domain and TBD domain and consequently reduce the flexibility of the DNA-binding domain, and we provide evidence that this process is mediated through hydrophobic core packing interactions. Our model clearly explains how allosterism can act even when no significant ligand-induced shift in conformation can be detected by X-ray crystallography. This model is consistent with other theoretical and experimental studies showing the importance of dynamic changes in allosterism (34–36), and we believe that the mechanism described here could apply to many other allosteric systems.

Materials and Methods

Protein Expression and Purification. The WT TetR protein used was TetR(B) with a C-terminal 6-His tag. The plasmid constructs and protein purification methods

have been described (22). All studies were performed with protein in 50 mM sodium phosphate, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT solution (pH 7.0). Site-directed mutants were generated as described (22). All mutations were verified by DNA sequence analysis.

Protein Thermodynamic Analysis. CD measurements were performed in an Aviv 62A DS circular dichroism spectrometer as described (22). Detailed methods of urea denaturation experiments and a description of data fitting error can be found in *SI Text*. All trypsin-limited proteolysis measurements were carried out at a protein concentration of 20 μ M at 298 K as described (22). Tc derivatives were added at a saturating concentration of 40 μ M. Partially digested samples were analyzed by MALDI mass spectrometry using an Applied Biosystem Qstar XL Q-TOF mass spectrometer. All proteolysis experiments were repeated at least twice.

Electrophoretic Mobility-Shift Assays. Complementary oligonucleotides bearing the tetO sequence (5'-cgttgacactctatcatgatagagttatttacc3') and (5'-tggtataaataactctaatgatagagtgtaacgggtac-3') were biotinylated at the 3' end using the Biotin 3' End DNA Labeling Kit (Pierce), and then annealed. tetO (1 nM) was incubated for 30 min at room temperature with the indicated amounts of TetR and drugs in a total volume of 10 μ L. Assays were performed in 50 mM NaCl, 50 mM Tris (pH 7.5), 2.5% glycerol, 1 mM EDTA, 1 mM DTT, 8 mM MgCl₂, 200 ng/ μ L BSA, and 20 ng/ μ L poly(dI:dC). Reaction mixes were loaded onto 6% polyacrylamide gels and run in 0.5% TBE buffer at 60 V for 2 h. The DNA was then electro-transferred to Hybond N+ nylon membrane (GE Healthcare), UV-cross-linked using Stratilinker (Stratagene), and detected with the Phototope-Star Detection Kit (New England Biolabs). Assays were repeated twice.

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