The Multidrug Efflux Regulator TtgV Recognizes a Wide Range of Structurally Different Effectors in Solution and Complexed with Target DNA

EVIDENCE FROM ISOTHERMAL TITRATION CALORIMETRY*

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TtgV modulates the expression of the ttgGHI operon, which encodes an efflux pump that extrudes a wide variety of chemicals including mono- and binuclear aromatic hydrocarbons, aliphatic alcohols, and antibiotics of dissimilar chemical structure. Using a lacZ fusion to the ttgG promoter, we show that the most efficient in vivo inducers were 1-naphthol, 2,3-dihydroxynaphthalene, 4-nitrotoluene, benzonitrile, and indole. The thermodynamic parameters for the binding of different effector molecules to purified TtgV were determined by isothermal titration calorimetry. For the majority of effectors, the interaction was enthalpy-driven and counterbalance by unfavorable entropy changes. The TtgV-effector dissociation constants were found to vary between 2 and 890 μM. There was a relationship between TtgV affinity for the different effectors and their potential to induce gene expression in vivo, indicating that the effector binding constant is a major determinant for efficient efflux pump gene expression. Equilibrium dialysis and isothermal titration calorimetry studies indicated that a TtgV dimer binds one effector molecule. No evidence for the simultaneous binding of multiple effectors to TtgV was obtained. The binding of TtgV to a 63-bp DNA fragment containing its cognate operator was tight and entropy-driven (KD = 2.4 ± 0.35 nM, ΔH = 5.5 ± 0.04 kcal/mol). The TtgV-DNA complex was shown to bind 1-naphthol with an affinity comparable with the free soluble TtgV protein, KD = 4.8 ± 0.19 and 3.0 ± 0.15 μM, respectively. The biological relevance of this finding is discussed.

Pseudomonas putida DOT-T1E is a paradigm of solvent-tolerant microorganisms because it can grow in the presence of high concentrations of extremely toxic and harmful compounds such as aromatic hydrocarbons (1, 2). These compounds preferentially partition in the cell membrane, disorganizing it and leading to cell death (3). Efflux pumps have been shown to play a critical role in the removal of toxic compounds such as biotics, biocides, dyes, detergents, fatty acids, and organic solvents from the cell membranes (2, 4–13). In P. putida DOT-T1E, the cooperative action of up to three efflux pumps, TtgABC, TtgDEF, and TtgGHI, is needed to achieve maximal tolerance against toluene, one of the most toxic aromatic hydrocarbons. TtgGHI appears to be the most important efflux element since, in contrast to the other two efflux pumps, a knock-out mutant in which this efflux pump is not functional was not able to withstand a sudden 0.3% (v/v) toluene shock regardless of the growth conditions (14). TtgGHI, like other multidrug-resistant pumps, possesses a broad substrate specificity reflected in its capacity to extrude not only aromatic hydrocarbons such as toluene, xylene, or styrene but also aliphatic alcohols such as octanol, nonanol, and decanol, as well as antibiotics of different chemical structure, e.g. ampicillin, tetracycline, and nalidixic acid (14, 15).

The expression of the ttgGHI operon is regulated by the TtgV protein (16). The TtgV gene is transcribed divergently from the ttgGHI operon, and the corresponding promoters, called PttgV and PttgG, overlap each other. The two start codons are separated by only 210 bp, 40 bp of which constitute the TtgV operator so that TtgV covers the −10 region of ttgG promoter and the −35 region of ttgG promoter (16, 17). Basal expression from the ttgG and ttgV promoters occurs, but expression has been shown to increase in response to the presence of some, but not all, of the pump substrates in the culture medium. Direct evidence of in vitro TtgV binding to drugs has only been obtained with 1-hexanol; Guazzaroni et al. (17) showed in EMSA† that this aliphatic alcohol released TtgV from its target operator. The present study was undertaken to determine the effector profile of TtgV, elucidate the TtgV-effector stoichiometry, and determine the thermodynamic parameters for the binding of the most potent effectors. Furthermore, the binding of 1-naphthol, one of the most potent effectors, by free TtgV and the protein complexed to its operator DNA has been compared using ITC.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Medium—The bacterial strains and plasmids used in this study are shown in Table I. Bacterial strains were grown in LB medium at 30 °C as described before (14) or in 2×YT for the production of the TtgV protein (18). Liquid cultures were shaken on an orbital platform operating at 200 rpm. When re-

† The abbreviations used are: EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry; Km, kanamycin; LB, Luria-Bertani culture medium.
required, the following antibiotics were added to the cultures: Km, 50 μg/ml; rifampicin, 20 μg/ml; and tetracycline, 20 μg/ml.

β-Galactosidase Assays—Plasmid pRNA96 carries a transcriptional fusion of the P_{ttgG} promoter region to the lacZ gene in the low copy pMP220 promoter probe vector. F. putida DOT-T1E (pRNA96) was grown on LB medium with tetracycline. Cultures were diluted to an initial OD_{600} of 0.05 in the same medium supplemented or not with the chemicals under study at 1 mM. These compounds were dissolved in Me2SO when needed (note that the later did not interfere with the induction assays performed in this study). When cultures reached an OD_{600} of 0.9–1.0, β-galactosidase activity was determined in triplicate in permeabilized cells (19).

TtgV Expression and Purification—Plasmid pTGF2 was constructed by cloning a 784-bp NdeI-BamHI fragment bearing the ttgV open reading frame in the Km’ pET29a (+) plasmid (Novagen) digested with the same enzymes to allow the expression of the native TtgV protein. Plasmid pTGF2 was transformed in Escherichia coli B834 (DE3) cells. The cells were grown in two-liter conical flasks containing 500 ml of S-cation column (16/10, Amersham Biosciences) and eluted with a sodium chloride gradient. The fraction containing TtgV was then dialyzed into the buffer used for ITC measurements were performed on a VP-Micropcalorimeter (MicroCal, Northampton, MA) at 30 °C. The protein was thoroughly dialyzed against 25 mM Tris-acetate, pH 8.0, 8 mM magnesium acetate, 10 mM KCl, and 1 mM dithiothreitol. The protein concentration was determined using the Bradford assay. Stock solutions of 1-naphthol, 2,3-dihydroxynaphthalene, indole, and 4-nitrotoluene at a concentration of 500 mM were prepared in Me2SO and subsequently diluted with dialysis buffer to a final concentration of 0.3 mM (1-naphthol and 2,3-dihydroxynaphthalene), 1.5 mM (indole), and 1 mM (4-nitrotoluene). The appropriate amount of Me2SO (0.1%) was added to the protein sample in each assay. Solutions of benzonitrile (4 mM) and hexanol and toluene (5 mM) were directly prepared in dialysis buffer. All chemicals were manipulated in glass vessels, and effector samples were neither degassed nor filtered, to avoid evaporation or nonspecific binding. Each titration involved a single 2-μl injection and a series of 4-μl injections of effector molecules into the protein solution. For DNA binding studies, oligonucleotides corresponding to both strands of the TtgV promoter were synthesized (5′-GAATTC/CAAGGTT-ATACAAAAAGCTA/CATCCACGAGTCTGGTA/CAGACACTGGA-3′ and its corresponding complementary oligonucleotide). Annealing was carried out by mixing equal molar amounts (at a concentration of 60 μM) of each complementary oligonucleotide in 0.5 mM Tris-HCl, pH 8.0, 0.5 mM MgCl2. The mixture was incubated 95 °C for 5 min and then chilled on ice and dialyzed in the buffer used for ITC studies. The mean enthalpies measured from injection of the ligand in the buffer were subtracted from raw titration data before data analysis using ORIGIN software (MicroCal). Titration curves were fitted by a nonlinear least squares method to a function for the binding of a ligand to a macromolecule (21). From the curve thus fitted, the parameters ΔH (reaction enthalpy), K\textsubscript{d} (binding constant, K\textsubscript{d} = 1/K\textsubscript{d}), and n (reaction stoichiometry) were determined. From the values of K\textsubscript{d} and ΔH, the change in free energy (ΔG) and in entropy (ΔS) were calculated with the following equations:

\[ \Delta G = -RT \ln K\textsubscript{d} \]
\[ \Delta S = \frac{\Delta H - \Delta G}{T} \]

where T is the absolute temperature.

Measurements of TtgV Ligand Binding Ratio Using Equilibrium Dialysis Assays—Four samples of TtgV with different protein concentrations were dialyzed against protein buffer containing effectors using Slide-a-Lyzer (Pierce) equipment for 5 days at 4 °C with stirring to ensure equilibrium. The proteins inside the cassette were then denatured by incubating at 100 °C for 5 min to release the bound effectors into the buffer. The denatured proteins were then centrifuged for 2 min at 13,000 × g. Ultraviolet light absorption at the appropriate wavelength was then measured in the supernatants that contained the effectors, and the effector concentrations determined using the corresponding extinction coefficients. The concentration of protein-bound effectors was obtained after correction for the effector concentration in the buffer. Protein concentrations were determined with the Bradford assay (Pierce). The binding ratio and dissociation constant were obtained with the following equation: binding ratio = [protein bound]/[effectors bound], K\textsubscript{d} = [effectors free]/[protein free]/[effectors bound].

RESULTS

In Vivo Effector Profile of TtgV—As an initial approach to the identification of the effectors recognized by TtgV, we used a P_{ttgG}:lacZ fusion (pRNA96) to measure β-galactosidase activity in P. putida DOT-T1E cells grown in the absence or in the presence of 1 mM compounds (Fig. 1). The basal level of expression from the ttgG promoter was 438 ± 34 Miller units, and expression increased up to 5-fold in response to 1 mM tested.
chemicals. The effectors that yielded the highest induction levels (4–5-fold increase) were two-ring aromatic compounds such as 1-naphthol, 2,3-dihydroxynaphthalene, and indole and one-ring aromatic compounds such as benzonitrile and 4-nitrotoluene (Fig. 1). Other compounds such as alkylphenols, halogenated aromatic rings, and aliphatic and aromatic alcohols also behaved as effectors and increased expression from the \( P_{\text{ttgG}} \) promoter by at least 2-fold.

In Vitro TtgV-Effector Interactions—Equilibrium dialysis experiments were carried out to shed light into the effector-TtgV stoichiometry. The results for the binding of 1-naphthol and 2,3-dihydroxynaphthalene to TtgV, as shown in Tables II and III, indicated that one effector molecule binds to the TtgV dimer. The apparent affinity for these molecules was in the low micromolar range. The thermodynamic parameters for the interaction of these two effectors as well as those of other effectors (biaromatic, monoaromatic compounds, and aliphatic alcohols) were subsequently determined by ITC at 30 °C. These effectors were chosen to cover the spectrum of chemically different compounds that behaved as inducers in vivo.

The dissociation constants for the different effectors span the micromolar range (Table IV). Although benzonitrile and indole were shown to be efficient effectors in vivo, the affinity of TtgV for these two chemicals was lower (in the range of 50–70 \( \mu \)M) than for 1-naphthol. Substantially lower affinities were determined for toluene (\( K_\text{D} = 118 \mu \text{M} \)) and 1-hexanol (\( K_\text{D} = 892 \mu \text{M} \)), which may account for the relatively modest activity of these effectors in vivo. The nitro substitution of toluene at position 4 resulted in a substantial increase in \( \Delta H = 6.3 \) to \(-14.9\) kcal/mol and an 8-fold increase in affinity (Table IV), which is in agreement with 4-nitrotoluene being a more efficient effector than toluene in vivo. This is compatible with a potential direct recognition of the nitro group at position 4 by TtgV since a nitro group at position 2 or 3 resulted in a less efficient effector.

Different effectors have been shown to bind to different sites in the large binding pocket of the QacR protein (22–25). This raises the question whether the effector binding pocket of TtgV can accommodate different molecules at a time. We carried out a series of sequential ITC experiments that involved the initial saturation of TtgV with a first effector followed by the titration with a second effector. In a first series of experiments, TtgV (6.4 \( \mu \text{M} \)) was saturated with an aliquot of 300 \( \mu \text{M} \) 1-naphthol. This complex was titrated with 300 \( \mu \text{M} \) 2,3-dihydroxynaphthalene. In a second series of experiments, TtgV (6.7

![Fig. 1. Expression from the ttgGHI promoter in the presence of different compounds. \( \beta \)-galactosidase activity was determined from the \( P. \text{putida} \) DOT-T1E \( ttgGHI \) promoter (pANA96 plasmid) in cultures grown in the absence and in the presence of 1 mM indicated chemicals. Results are the mean and standard error of eight different experiments. DMSO, \text{Me}_2\text{SO}; \text{Miller } U, \text{Miller units.}]](http://www.jbc.org/content/20889)
Ligand Recognition by TtgV

Characterization of 1-naphthol binding to TtgV determined by equilibrium dialysis

TtgV at the different concentrations given below were dialyzed against buffers containing effectors for 5 days at 4 °C with stirring to ensure equilibrium. Other experimental conditions are given in “Experimental Procedures.”

| [1-naphthol-bound] | [1-naphthol-free] | [TtgV-bound] | [TtgV-free] | Binding ratio (effector/dimer TtgV) | $K_d$ |
|---------------------|-------------------|--------------|-------------|------------------------------------|------|
| $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ |
| 3.3 | 23.6 | 3.7 | 0.4 | 0.89 | 2.9 |
| 3.8 | 23.3 | 4.1 | 0.3 | 0.93 | 1.7 |
| 10.4 | 22.9 | 11.7 | 1.3 | 0.89 | 2.9 |
| 11.8 | 22.6 | 12.5 | 0.7 | 0.94 | 1.3 |
| average | 0.91 ± 0.03 | 2.2 ± 0.8 |

Characterization of 2,3-dihydroxynaphthalene binding to TtgV determined by equilibrium dialysis

TtgV at the different concentrations given below were dialyzed against buffers containing effectors for 5 days at 4 °C with stirring to ensure equilibrium. Other experimental conditions are given in “Experimental Procedures.”

| [2,3-dihydroxynaphthalene-bound] | [2,3-dihydroxynaphthalene-free] | [TtgV-bound] | [TtgV-free] | Binding ratio (effector/dimer TtgV) | $K_d$ |
|----------------------------------|----------------------------------|--------------|-------------|------------------------------------|------|
| $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ | $\mu M$ |
| 15.9 | 19.1 | 16.2 | 0.3 | 0.98 | 0.8 |
| 16.7 | 18.3 | 17.4 | 0.8 | 0.96 | 2.1 |
| 19.8 | 19.7 | 21.9 | 2.2 | 0.90 | 1.0 |
| 20.4 | 19.2 | 21.5 | 1.1 | 0.95 | 0.7 |
| average | 0.95 ± 0.03 | 1.2 ± 0.6 |

**Table IV**

Thermodynamic parameters derived from the calorimetric titration of TtgV with effector molecules

TtgV solutions at 6–8 µM in 25 mM Tris-acetate, 8 mM magnesium acetate, 10 mM KCl, 1 mM dithiothreitol, pH 8.0, were titrated with 0.3–5 mM solutions of effectors. Experiments were carried out at 30 °C. Further assay conditions are given under “Experimental Procedures.”

| Effector | Ligand | $K_D$ | $K_A$ | $\Delta H$ | $T\Delta S$ | $\Delta G$ |
|----------|--------|-------|-------|------------|------------|-----------|
| 1-Naphthol | TtgV | $3.0 \pm 0.15$ | $(3.4 \pm 0.17) \times 10^3$ | $-8.6 \pm 0.16$ | $-1.1 \pm 0.15$ | $-7.5 \pm 0.03$ |
| 1-Naphthol | TtgV-DNA complex | $4.8 \pm 0.19$ | $(2.1 \pm 0.08) \times 10^3$ | $-5.2 \pm 0.05$ | $2.1 \pm 0.09$ | $7.3 \pm 0.02$ |
| 2,3-Dihydroxynaphthalene | TtgV | $2.3 \pm 0.42$ | $(4.4 \pm 0.80) \times 10^4$ | $-6.8 \pm 0.06$ | $6.9 \pm 0.10$ | $5.7 \pm 0.10$ |
| 4-Nitrotoluene | TtgV | $16.9 \pm 0.43$ | $(5.9 \pm 0.15) \times 10^4$ | $-14.9 \pm 0.18$ | $-8.5 \pm 0.18$ | $-6.4 \pm 0.01$ |
| Benzonitrile | TtgV | $51.3 \pm 1.26$ | $(1.9 \pm 0.48) \times 10^4$ | $-12.5 \pm 0.14$ | $-6.7 \pm 0.15$ | $-5.8 \pm 0.02$ |
| Indole | TtgV | $67.1 \pm 1.58$ | $(1.5 \pm 0.04) \times 10^4$ | $-13.0 \pm 0.17$ | $-7.3 \pm 0.18$ | $-5.7 \pm 0.01$ |
| Toluene | TtgV | $118.0 \pm 2.64$ | $(8.5 \pm 0.19) \times 10^4$ | $-6.3 \pm 0.07$ | $-0.9 \pm 0.09$ | $-5.4 \pm 0.01$ |
| 1-Hexanol | TtgV | $892.8 \pm 135$ | $(1.1 \pm 0.17) \times 10^5$ | $-14.9 \pm 0.15$ | $-10.8 \pm 1.56$ | $-4.1 \pm 0.09$ |

$\mu M$ was saturated with 2,3-dihydroxynaphthalene and subsequently titrated with 1 mM benzonitrile. In both cases, heat changes were very small and corresponded to the competition of two effectors to a single site and not to the simultaneous binding of both effectors to the protein with a physiological relevant affinity (data not shown). We were thus unable to provide evidence for the simultaneous binding of multiple effectors to TtgV.

**ITC Binding Studies of 1-n-Naphthol to the TtgV-DNA Complex—**As stated above, TtgV exerts its biological function by an up-regulation of gene expression as a result of the effector-
mediated dissociation of the regulatory protein from its operator. ITC experiments were carried out to study the interaction of 1-naphthol with the TtgV-DNA complex. Synthetic 63-bp oligonucleotides corresponding to both strands of the operator sequence protected by DNaseI footprint experiments were synthesized and annealed (see “Experimental Procedures”) (17). In a first series of ITC assays, 8.1 \( \mu M \) TtgV was titrated with 10-\( \mu M \) DNA at 30 °C in STA buffer. Binding was entropy-driven (\( \Delta H = -5.5 \pm 0.04 \) kcal/mol, \( T\Delta S = 17.5 \pm 0.09 \) kcal/mol) and very tight (\( K_D = 2.4 \pm 0.35 \) nM). Experiments were designed so that the protein concentration after saturation with DNA corresponded exactly to the protein concentration used for the titration of unliganded protein with 1-naphthol. After saturation, the TtgV-DNA complex was titrated with 1-naphthol in a similar fashion as the titration of the unliganded protein (Fig. 2, right-hand panel). The resulting heat changes corresponded to the binding of the effector to the protein and to the dissociation of the protein from DNA. Peaks were narrow, indicating that operator/TtgV dissociation occurred immediately upon effector binding by TtgV. After titration, the sample was subjected to EMSA, which demonstrated that protein has dissociated quantitatively from its target operator DNA (data not shown). In a control assay, free DNA at the same concentration as in the titration of the DNA-protein complex was titrated with 1-naphthol. Resulting peaks were small and uniform, indicative of that dilution. ITC data for the titration of the DNA-TtgV complex with 1-naphthol were analyzed assuming that one effector molecule was bound per dimer, and derived thermodynamic parameters are given in Table IV. 1-Naphthol binds with a \( K_D \) of 4.8 \( \pm 0.19 \) \( \mu M \) to the TtgV/operator complex, which is comparable with the affinity of this effector for the TtgV protein free in solution (3.0 \( \pm 0.15 \), Table IV). Most interestingly, binding was less exothermic as compared with the binding to the TtgV protein in solution, which was unexpected.

Effectors That Increased ttgGHI Expression Released TtgV from Its Target Operator and Allowed Transcription from Pert—We previously showed that TtgV was released from its operator upon binding of 1-hexanol (17). The above in vivo and in vitro results suggested that the different effectors should
release TtgV from its target site. To test this hypothesis, we used EMSA assays with TtgV at 50 nM, a concentration that shifted about 50% of the DNA (Fig. 3), and the most efficient inducers of TtgG in vivo were tested at different concentrations. EMSA studies revealed that there was a direct correlation between the concentration of the tested compound and the amount of TtgV that was released from its target DNA (see Fig. 3 for 1-naphthol, 2,3-dihydroxynaphthalene, benzonitrile, naphthalene, indole, 4-ethylphenol, and 4-nitrotoluene). In fact, with 0.5 mM 1-naphthol, 2,3-dihydroxynaphthalene, 3-ethylphenol, and 4-nitrotoluene, 90% of TtgV was released from its operator DNA (Fig. 3B). Indole and benzonitrile seemed to be similarly recognized by TtgV (around 70–80% of the DNA was freed from TtgV at a concentration of 1 mM compound (Fig. 3B)). These results are, in general, in agreement with the in vivo measurements of β-galactosidase activity since the compounds that yielded the highest induction level of the PttgG promoter in vivo also released more TtgV from its operator site in the in vitro experiments. Furthermore, compounds such as p-isopropylbenzoate, tetracycline, and chloramphenicol, which did not induce in vivo (not shown), failed to release TtgV from its target operator in vitro.

From a mechanistic point of view, TtgV was suggested to prevent RNA polymerase from accessing the promoter region (17). This suggestion was based on the observation that transcription inhibition was more effective when TtgV was added before the formation of the open complex by RNA polymerase. It was further reasoned that upon ligand binding, TtgV was released from its operator site and ttgG transcription occurred. To test whether the chemicals capable of releasing TtgV from its operator allowed expression from the PttgG promoter, we performed in vitro transcription assays in which TtgV was added before RNA polymerase in the absence and in the presence of some of the most efficient effectors (Fig. 4). As expected, expression from the ttgG promoter was inhibited (lane 2), whereas the presence of 100 μM benzonitrile, 2,3-dihydroxynaphthalene, or 1-naphthol enabled transcription from PttgG at a level similar to that observed in the absence of TtgV. These results support a mechanism by which TtgV represses ttgG expression by blocking the RNA polymerase binding site. Nevertheless, in the presence of ligand molecules of different structures and substituents, TtgV dissociates from its operator site, allowing RNA polymerase to start transcription from PttgG.

DISCUSSION

EMSA, equilibrium dialysis, and ITC experiments (Figs. 2 and 3 and Tables II–IV) suggested that TtgV is able to bind a large number of structurally different compounds and that this interaction leads to the induction of ttgGHI expression. This is the first case in which a regulator belonging to the IclR family is shown to interact directly with different chemicals. Our findings contrast with the relatively narrow effector specificity found for other regulators of the IclR family that recognize a single aromatic compound. For example, PobR and PcaU of Acinetobacter calcoaceticus interact only with 4-hydroxybenzoate and 3,4-dihydroxybenzoate, respectively (26–28). However, the ability of TtgV to recognize various effectors of dissimilar structure seems to be a particular feature of the regulators that control the expression of multidrug efflux pumps. This is consistent with a mechanism based on the direct recognition of structurally dissimilar compounds rather than the involvement of a secondary messenger, which has also been shown for a limited number of other multidrug pump regulators such as QacR (29) and BmrR (30, 31).

Our data indicate that one effector molecule binds to a TtgV dimer. This stoichiometry has also been seen for the QacR regulator (25) but is different from TetR, where two molecules of tetracycline bind to the protein dimer (32, 33). QacR accommodates structurally diverse ligands in different parts of the large binding pocket (25). However, signal transduction for all ligands is mediated by an identical induction mechanism, and QacR-drug complexes undergo the same transition (25, 33).

Our results support that TtgV, a member of the IclR family of repressors, may function similarly to QacR since there is a relation between the in vitro affinity of TtgV for its effectors and their in vivo efficiency. However, details of TtgV-effector interactions await the resolution of the three-dimensional structure of TtgV bound to these chemicals.

The affinity of TtgV for its effectors spans the micromolar range, and the binding constants are similar to those of other repressor proteins such as TrpR (34) or QacR (35), the latter having a Kd around 1 μM for rhodamine 6G (25). Although there is no strict correlation (R2 = 0.76) for the linear fit of the plot of lnKd against β-galactosidase activity, a clear relation was observed between the affinity of TtgV for different effectors and their potential to induce gene expression (measured by β-galactosidase assays). Effectors with the highest affinity, such as 1-naphthol and 2,3-dihydroxynaphthalene, were shown to be more efficient in vivo. Effectors with slightly lower in vivo induction activities, such as indole, 4-nitrotoluene, and benzonitrile, were also found to bind less strongly to the protein in vitro, whereas effectors characterized by moderate in vivo activity (toluene and hexanol) showed significantly reduced affinity. The lack of a strict correlation between in vitro-determined Kd values and β-galactosidase levels induced in vivo is mainly due to differential cell extrusion of the tested compounds, which is mediated by the TtgGHI pump and other multidrug extrusion elements rather than to the existence of secondary layers of regulators influenced by these chemicals. Furthermore, it should be taken into account that the positive correlation between the affinity of TtgV for an effector molecule and the release of the protein from the promoter is not always found in transcriptional regulators. This is exemplified by FadR, for which the effector palmitoyl-CoA is around 50-fold more efficient than myristoyl-CoA in inhibiting FadR from

\[ \text{2 M. E. Guazzaroni, M. T. Gallegos, and J. L. Ramos, unpublished results.} \]
DNA binding. However, ITC assays showed that palmityl-CoA binds around one-sixth as strongly to FadR as does myristoyl-CoA (36). The binding affinity of the effector to TtgV can thus be considered the major determinant of gene expression.

In general, the binding modes of structurally similar ligands to proteins with narrow substrate specificity are comparable. This was not the case for the binding of TtgV to 1-naphthol and 2,3-dihydroxynaphthalene (Table IV), which were enthalpy- and entropy-driven, respectively. It remains to be explored whether the different binding modes observed here for structurally similar effectors is a general feature of multidrug recognition. TtgV is a dimer in solution and binds to its corresponding target, DNA covering four potential direct repeats within the tggG promoter (17). In the absence of effectors, TtgV binds tightly to its target promoter ($K_D = 2.4 \pm 0.35 \text{nM}$).

The effector 1-naphthol was shown to bind with high affinity to the protein-DNA complex ($K_D = 4.8 \mu\text{M}$), an affinity similar to that determined for the binding to free TtgV ($K_D = 3.0 \mu\text{M}$). Furthermore, TtgV bound to 1-naphthol (or other effectors) does not interact with its target DNA promoter. This set of observations is of physiological importance and can be critical whether the different binding modes observed here for structurally similar effectors is a general feature of multidrug regulation of bacterial multidrug transporters.

The finding that the binding of the TtgV-DNA complex to 1-naphthol was less exothermal than the binding to free TtgV ($K_D = 5.5 \mu\text{M}$) is of interest. In general, very little is known about the molecular mechanisms of protein-DNA association and dissociation differ. Further studies are necessary to elucidate the conformational changes caused by effector binding to free and TtgV-bound DNA.

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