Novel Effector Control through Modulation of a Preexisting Binding Site of the Aromatic-responsive $\sigma^{54}$-Dependent Regulator DmpR*

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Eric O'Neill, Chun Chau Sze, and Victoria Shingler‡

From the Department of Cell and Molecular Biology, Umeå University, S-901 87 Umeå, Sweden

The Pseudomonas derived $\sigma^{54}$-dependent DmpR activator regulates transcription of the (methyl)phenol catabolic dmp-operon. DmpR is constitutively expressed, but its transcriptional promoting activity is positively controlled in direct response to the presence of multiple aromatic effectors. Previous work has led to a model in which effector binding by the amino-terminal region of the protein relieves repression of an intrinsic ATPase activity essential for its transcriptional promoting property. Here, we address whether the observed differences in the potencies of the multiple effectors (i) reside at the level of different aromatic binding sites, or (ii) are mediated through differential binding affinities; furthermore, we address whether binding of distinct aromatic effectors has different functional consequences for DmpR activity. These questions were addressed by comparing wild type and an effector specificity mutant of DmpR with respect to effector binding characteristics and the ability of aromaticities to elicit ATPase activity and transcription. The results demonstrate that six test aromatics all share a common binding site on DmpR and that binding affinities determine the concentration at which DmpR responds to the presence of the effector, but not the magnitude of the responses. Interestingly, this analysis reveals that the novel abilities of the effector specificity mutant are not primarily due to acquisition of new binding abilities, but rather, they reside in being able to productively couple ATPase activity to transcriptional activation. The mechanistic implications of these findings in terms of aromatic control of DmpR activity are discussed.

DmpR is the specific regulator of the pVI150 plasmid encoded dmp-operon, which encodes the enzymes of the (methyl)phenol catabolic pathway of Pseudomonas sp. CF600 (1). This pathway confers the ability to utilize phenol, mono-methylated phenols, and 3,4-dimethylphenol as sole carbon and energy sources (reviewed in Refs. 2 and 3). DmpR tightly regulates transcription from the operon promoter, Po, resulting in expression of the specialized catabolic enzymes only in the presence of pathway substrates or some structural analogs (4).

DmpR belongs to the prokaryotic family of enhancer binding proteins that exert their action on transcription mediated by RNA polymerase utilizing the alternative sigma factor, $\sigma^{54}$ ($\sigma^N$) that recognizes −24 GG, −12 GC promoters (reviewed in Ref. 5). DmpR, like other family members, has a distinct domain structure consisting of an amino-terminal signal reception A-domain linked to a central activation C-domain by a short B-domain, and a carboxyl-terminal D-domain that contains a helix-turn-helix DNA binding motif.

The central C-domains are the hallmark of the family and seven (C1 to C7) highly conserved regions within this domain have been identified (5, 6). The C-domain contains a nucleotide binding motif and mediates ATP binding and hydrolysis essential for transcriptional activation of, and open complex formation by, $\sigma^{54}$-RNA polymerase (7, 8). Mutational and cross-linking studies have also implicated the conserved C3 region of this domain in direct interaction with the transcriptional apparatus through the $\beta$- and $\sigma^{54}$-subunits of the holoenzyme (9, 10).

Most $\sigma^{54}$-dependent activators are constitutively produced, but their activity is controlled in response to environmental signals. Distinct activation mechanisms are mediated through modulation of the A-domains of the regulators and include phosphorylation cascades, ligand binding, and signal-responsive protein-protein interactions (reviewed in Ref. 11). DmpR belongs to a new extensive subgroup of ligand-responsive regulators that also includes the aromatic-responsive regulators XylR (12, 13), PhhR (14), ThuT (15), and MopR (16); the formate-responsive PhLA (17); and the ornithine/citrulline-responsive RocR (18).

In the cases of DmpR and XylR, effector specificity mutants and hybrid proteins have genetically demonstrated that the effector response is mediated via the A-domain (4, 13, 19). Most recently, the A-domain of DmpR has been shown to be both necessary and sufficient to bind its ligand phenol (20). Binding of effectors to DmpR releases its intrinsic ATPase activity required for transcriptional activation, a process that is mimicked by removal of the A-domain of DmpR, which results in a derivative that has full ATPase and transcriptional promoting activities in the absence of effectors (19). These observations, together with genetic and biochemical evidence for specific interactions between the A- and C-domains of DmpR (19, 20, 21), have led to a model in which effector binding to the A-domain alleviates interdomain repression, resulting in release of the C-domain-mediated ATPase activity and thus promotion of transcription.

In addition to the specific regulation mechanism described above, the DmpR-mediated regulatory circuit is also subject to physiological regulation leading to silencing of the system in rich media until the exponential to stationary phase transition. Recently, the global signaling molecule (p)ppGpp has been shown to be essential for efficient DmpR-mediated transcription from the operon promoter and to be a molecule that links this specific regulatory circuit to the physiological status of the cell (22).

DmpR is activated by a wide range of phenolic compounds, including substrates of the pathway it controls and some, but

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‡ To whom correspondence should be addressed. Tel.: 4-6-90-7852534; Fax: 46-90-771420; E-mail: victoria.shingler@cmb.umu.se.
not all, structurally related compounds (4, 19). However, the magnitude of the DmpR-mediated transcriptional response from the Po promoter varies, depending on the position and nature of substrates on the aromatic ring. Using a DmpR mutant derivative, DmpR-E135K, altered in its ability to respond to pathway effectors, it has been shown that these differences can have a profound effect on the efficiency with which the bacteria can degrade compounds that are poor effectors of DmpR (23). In addition to novel effector specificity, other amino acid substitutions of residue 135 result in protein derivatives that are still effector-responsive but can mediate varying levels of transcriptional activation even in the absence of effectors (19). Hence, residue 135 is located in a position that can influence both effector specificity and the A-domain-mediated repression of C-domain function.

Activation of DmpR can be divided into at least two interrelated steps: (i) binding of the effector to a specific site(s) on the A-domain, and (ii) alleviation of repression of the C-domain-mediated activities. Here, we use in vivo and in vitro assays of wild type DmpR and DmpR-E135K to determine how these two processes are coupled for different effectors and whether different aromatic effectors act through a single binding site on DmpR.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Plasmids used for in vivo assays, expressing DmpR (pVI401) and DmpR-E135K (pVI428) from the natural promoter of dmpR on a broad host range RSF1010 replicon, have previously been described (23). Plasmid pVI453 expressing the constitutively active ΔA2-DmpR derivative from the Pm promoter of the broad host range RSF1010-based vector, has also been previously described (19). T7 promoter plasmids were used for high level expression of DmpR derivatives harboring a carboxyl-terminal fusion of the eight amino acid flag epitope tag (24). Plasmids for the expression DmpR-flag (pVI456) and ΔA2-DmpR-flag (pVI457) have previously been generated (19). Analogous plasmids, pVI538 and pVI539, for expression of DmpR-E135K-flag and DmpR-G268S-flag, respectively, were constructed by replacing the Ndel to Stul fragment of pVI457 (spanning the ATG initiation codon to within the C-domain) with the Ndel to Stul fragments from plasmids expressing these mutant proteins from Pbac promoter vectors (21, 23).

**Assays—**Cells were grown in LB broth to early mid-log phase (OD600 = 1.5) containing 100 μg/ml ampicillin. Cells were grown through the exponential phase in Luria broth (LB) (25) containing appropriate antibiotics for plasmid selection. Because the DmpR-mediated transcriptional response in LB is not observed until the exponential to stationary phase transition (22), cultures were diluted and grown to A600 = 2.5 prior to exposure to the indicated concentrations of effectors for 3 h. Luciferase assays of the luxAB gene product were performed on whole cells, using a 1:2000 dilution of bead-containing crude extract, as described previously (28).

**Western Blot Analysis—**Crude extracts of cytosolic proteins, SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose filters, and Western blot analysis with polyclonal rabbit anti-DmpR sera were as described previously (19). Antibody decorated bands were revealed using the chemiluminescence reagents of Amersham Pharmacia Biotech as directed by the supplier. Differences in expression level were assessed by comparison of dilution series of the samples and quantification using a Molecular Dynamics densitometer.

**Affinity Purification of DmpR-flag Derivatives—**Protein expression, crude extract preparation, and immunoprecipitation of tag-protected proteins using Flag-M2 affinity gel (Sigma) were as described previously (19), except for a modification in the washing procedure. Here, after incubation with the crude extracts, bead-bound proteins were washed three times with lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol) in the presence of 0.5 mM NaCl and protease inhibitors (Complete™, Roche Molecular Biochemicals), followed by one wash with lysis buffer containing 1% Triton X-100 and three washes with 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol. Unlike the previously reported washing condition (19), these modified conditions resulted in bead preparations that, when treated with crude extract from bacteria harboring a vector control plasmid, are devoid of detectable ATPase activity. However, the previously reported background level of ATPase activity of DmpR-flag in the absence of effectors still persists. A control preparation of a mutant protein harboring a single amino acid substitution in the Walker A motif (DmpR-G268S-flag) possesses no detectable ATPase activity in the presence or absence of 2-methylphenol (Ref. 21 and data not shown). Hence, the high background ATPase activity (approximately 20% of fully activated levels) is an intrinsic property of DmpR-flag and not due to contaminating ATPase competent proteins.

The bead-bound proteins generated as described above were resuspended as a slurry in assay buffer (55 mM Tris-Ac, pH 7.9, 5 mM MgAc, 70 mM KAc, 20 mM NH4Ac, 1 mM dithiothreitol) and kept on ice prior to their use in ATPase or [14C]phenol binding assays. The concentration of bead-bound proteins was determined by SDS-polyacrylamide gel electrophoresis comparison of a serial dilution of each sample against standards of known concentration and quantification using a Molecular Dynamics densitometer.

**Luciferase Assays—**ATPase assays were performed at 30 °C in 60 μl of 1× assay buffer as described previously (19). Reactions contained 1 μl of bead-bound protein and the indicated concentrations of aromatic compound(s). Assays were initiated by the addition of radiolabeled [γ-32P]ATP (Amersham Pharmacia Biotech) that had been diluted (1:9) with unlabeled ATP (Amersham Pharmacia Biotech) and added to a final concentration of 3 mM. Rates of ATP hydrolysis at each concentration of effector was determined by removing aliquots at 10-min intervals over 1 h, during which the reaction was linear. Samples were adjusted to 0.1% SDS, boiled for 1 min, spotted onto polyethyleneimine cellulose plates (Merck), and dried, and the free phosphate was separated from ATP by ascending chromatography in 0.75 M potassium hydrogen phosphate buffer, pH 5.5. Radioactivity was quantified using a Molecular Dynamics PhosphorImager. The value for phosphate released was calculated as a percentage of the total amount of radioactivity per sample. Effector-stimulated ATPase hydrolysis was calculated by subtraction of a basal ATPase activity.

**[14C]Phenol Binding Assays—**Unlabeled [14C]phenol (5.22 Gbq/mmol) was custom synthesized by Amersham Pharmacia Biotech. Binding assays were performed with 10 μl of bead-bound affinity-purified flag-tagged protein (0.95 μg of protein per μl) in a total of 200 μl of assay buffer prepared as described above. To determine the [14C]phenol binding capacity of DmpR-flag and DmpR-E135K-flag, proteins were exposed to increasing concentrations of [14C]phenol in the presence or absence of 500 μM unlabeled phenol, essentially as described previously (20). In brief, samples were allowed to reach equilibrium (<5 min under these conditions) prior to separation of protein-bound phenol from free phenol. Separation was achieved by centrifugation through a gradient composed of 200 μl of assay buffer layered onto 300 μl of a 40% sucrose cushion, using a bench microcentrifuge at 14,000 rpm. The radioactivity in the upper layer, containing the free phenol, and the lower layer, containing the protein-bound phenol, was independently recovered and analyzed by scintillation counting. Specific binding of phenol was calculated by subtraction of nonspecific binding observed in the presence of 500 μM unlabeled phenol. For the [14C]phenol binding competition assays, unlabeled aromatic compounds were incubated with DmpR-flag at room temperature for 5 min prior to addition of [14C]phenol to a final concentration of 16 μM.

**RESULTS**

**Effector Dose-dependent Activation of Transcription by DmpR—**As a first step to address the question of what mechanism(s) underlies the specific levels of DmpR-mediated transcription from the Po operon promoter in response to different effectors, we made use of a previously constructed in vivo luciferase reporter system consisting of a single copy of Po promoter fused to the luxAB gene product of the luxAB gene product of the Pseudomonas putida host (KT2440::Po-luxAB) and a plasmid (pVI401) expressing wild type DmpR from its native promoter (23). The range of effectors chosen for testing included the two best DmpR effectors (phenol and 2-methylphenol), the two poorest DmpR effectors (4-methylphenol and 3,4-dimethylphenol), and noneffectors of DmpR to which the mutant DmpR-E135K had gained the ability to respond (2,4-dimethylphenol and 4-ethylphenol). Cultures were grown and assayed for luciferase activity in response to up to 3 mM concentration of the different effectors; higher concentrations in all cases resulted in varying degrees of inhibition (data not shown). The results in Fig. 1 (closed symbols) show that activation of DmpR differed both in the minimum concentration of effector required to elicit a detectable response (approximately a 100-fold difference: 3 μM for 2-methylphenol to 300 μM for 3,4-
The luciferase transcriptional response of *P. putida* and sion levels of DmpR and DmpR-E135K. Western analysis of 30 (closed symbols) DmpR (Fig. 1, A–F, in four independent experiments; shown are the averages of triplicate determinations in each of two to concentrations of effector as described under “Experimental Procedures.” (pVI428) was measured in the absence or presence of increasing concentrations of the indicated aromatic compound. Conditions were as under Fig. 1, except that 0.5 mM isopropyl-β-D-thiogalactopyranoside was included to induce transcription from the plasmid-located *P. putida* luxAB promoter. Shown are the averages of triplicate determinations in each of two independent experiments; bars indicate standard error. Dim, dimethylbenzoate; dmp, dimethylphenol; ep, ethylphenol; mp, methylphenol.

![Effector Binding and Transcriptional Activation by DmpR](image)

**Fig. 1. In vivo mediated transcriptional response of Po by DmpR (closed symbols) and DmpR-E135K (open symbols).** A–F, the luciferase transcriptional response of *P. putida* KT2440::Po-luxAB harboring plasmids expressing DmpR (pVI401) or DmpR-E135K (pVI428) was measured in the absence or presence of increasing concentrations of effector as described under “Experimental Procedures.” Shown are the averages of triplicate determinations in each of two to four independent experiments; bars indicate standard error. G, expression levels of DmpR and DmpR-E135K. Western analysis of 30 (lanes 1 and 3) and 15 (lanes 2 and 4) µg of crude extract derived from cells used in A–F were separated by 11% SDS-polyacrylamide gel electrophoresis and treated with anti-DmpR as described under “Experimental Procedures.”

The two best effectors (phenol and 2-methylphenol) (Fig. 1, A and B) showed a biphasic response curve. In the case of the poor effectors, 4-methylphenol (Fig. 1C) shows a monophasic response curve, whereas for 3,4-dimethylphenol (Fig. 1D), a plateau was not discernible before inhibitory effects were observed. The responses to 4-methylphenol and 3,4-dimethylphenol and the first phase of the responses to phenol and 2-methylphenol appear to be like typical saturating binding curves, which is consistent with the previous in vitro data of a single affinity binding site for phenol (20). The second phase of the response to high concentrations of phenol and 2-methylphenol could be either due to a second very low affinity binding site or, more likely, due to a cellular response to these high concentrations of the effectors. To directly test the latter possibility, we made use of the A-domain-deleted derivative ΔA2-DmpR. This derivative is constitutively active both *in vivo* and *in vitro* and thus bypasses the need for the aromatic activation step (19). Plasmid pVI453 carries ΔΔ2-dmpR downstream from the isopropyl-β-D-thiogalactopyranoside-inducible promoter of the vector. This plasmid was introduced into the reporter strain *P. putida* KT2440::Po-luxAB and cultured in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside to produce ΔΔ2-DmpR at approximately the same level as wild type DmpR produced from its native promoter (19). The level of transcription achieved in the presence of aromatic compounds was monitored as luciferase activity. As shown in Fig. 2B, low levels of 2-methylphenol did not affect the level of transcription mediated by this constitutively active derivative, whereas exposure to higher concentrations (1–3 mM) resulted in higher levels of transcription. The level of transcription mediated by ΔΔ2-DmpR in the presence of 3 mM concentrations of the six test effectors (and a control compound, 3,4-dimethylbenzoate, that does not elicit a response from wild type DmpR) is shown in Fig. 2B. The various aromatic compounds had different effects on the transcriptional activity ranging from essentially no effect (4-methylphenol) to a 40% enhancement of luciferase activity (3,4-dimethylphenol). Most importantly, 3 mM concentrations of the two effectors that showed a second phase in the dose-dependent response curves (phenol and 2-methylphenol, Fig. 1, A and B), showed marked enhancement of the ΔΔ2-DmpR-mediated transcription, whereas a 3 mM concentration of 4-methylphenol that resulted in a monophasic dose-response curve (Fig. 1C) had little or no effect on ΔΔ2-DmpR activity (Fig. 2B). Hence, the effect of the different compounds on global cellular response primarily accounts for the second phase of the response seen with phenol and 2-methylphenol but not 4-methylphenol. Recently, (p)ppGpp has been shown to be required for, and to enhance, maximal levels of DmpR-mediated transcription from Po (22). Therefore, elevation of cellular concentrations of (p)ppGpp in response to high concentrations of phenol or 2-methylphenol is a likely mechanism underlying the data shown in Fig. 2.

Given the above results, the data in Fig. 1 are consistent with a single class of binding sites for each compound. However, the data clearly dissociate sensitivity, as measured by the dose response, and the functional output of binding, as measured by the magnitude of the response. Phenol and 2-methylphenol elicit a maximum plateau level of Po transcription of...
about 6000–7000 luciferase units/A600 (Fig. 1, A and B). The maximum activation level achieved using the poorest effector (3,4-dimethylphenol, Fig. 1D) was approximately equal to those of the plateau levels for phenol and 2-methylphenol. Notably, however, the activation profile obtained with 4-methylphenol reached a maximal plateau level of only 3000–4000 luciferase units/A600 (Fig. 1C), which is approximately 50% of the levels for phenol/2-methylphenol and the maximum response with 3,4-dimethylphenol. This observation suggests that the level of effector activation of DmpR involves more than a simple direct correlation with binding affinity of the effectors, a conclusion further substantiated by in vitro assays shown below.

Dose-dependent Activation Profiles of the Effector Specificity Mutant DmpR-E135K—The effector specificity mutant DmpR-E135K was independently isolated during a genetic selection for mutants that respond to either 2,4-dimethylphenol or 4-ethylphenol (19, 23). To shed further light on the mechanism of effector activation of DmpR, the dose-dependent effector profiles of this mutant were compared with those of wild type DmpR using the luciferase reporter described above and a plasmid, pV428, expressing DmpR-E135K. Western analysis revealed that DmpR-E135K is present at levels that were slightly lower than (approximately 75%) but still comparable to those of wild type DmpR in the cells (Fig. 1G). At high effector concentrations, DmpR-E135K was similar to wild type DmpR in its activation to phenol and 2-methylphenol, had an enhanced ability to respond to 4-methylphenol and 3,4-dimethylphenol, and had gained the novel ability to respond to 2,4-dimethylphenol and 4-ethylphenol (Fig. 1, open symbols). The dose-dependent effector profiles of this mutant show that 2,4-dimethylphenol and 4-ethylphenol are almost as potent activators of DmpR-E135K as 3,4-dimethylphenol is of the wild type protein (Fig. 1, compare D to F). In the case of phenol and 2-methylphenol, although high concentrations of effector elicit a similar response from both the wild type and mutant DmpR, the shift in dose responses at lower concentrations of effector suggests that the E135K mutation may also influence binding affinity of these compounds.

\[ ^{14}C \text{phenol binding by DmpR and DmpR-E135K} \]

To directly test the prediction from the in vitro experiments described above, namely that DmpR-E135K has a lower binding affinity for phenol than the wild type protein, we used flag epitope-tagged derivatives of DmpR. The presence of the eight amino acid carboxyl-terminal flag epitope has previously been epitope-tagged derivatives of DmpR. The presence of the eight amino acid carboxyl-terminal flag epitope has previously been shown to have no effect on the protein stability or effector-mediating transcriptional responses of DmpR in vivo (19). A system employing \([^{14}C]\)phenol and affinity-purified bead-bound DmpR-flag and DmpR-E135K-flag was used to analyze phenol binding (see under “Experimental Procedures”). The data shown in Fig. 3 demonstrate specific and saturable binding of phenol by these proteins. Evaluation of the binding data by Scatchard analysis gives a dissociation constant of approximately 16 \(\mu M\) for DmpR-flag (as found previously, Ref. 20), and a dissociation constant of 40–65 \(\mu M\) for DmpR-E135K-flag. Hence, the E135K mutant does indeed have a decreased affinity for phenol in vitro but retains the ability to achieve maximal activation of Po in vivo.

In Vitro Effector Binding and Response Properties of DmpR—The in vivo experiments outlined in Fig. 1 provide a comparison of the responses of different protein derivatives to a given compound and give a true reflection of the sensitivity of the response to these compounds by the bacteria as a whole. However, interpretation of the results with respect to binding affinities and comparison of the direct influence of different effectors on the regulator can potentially be complicated by factors such as (i) differential permeability of the membrane to the different compounds, (ii) preferential uptake of some compounds by putative active uptake systems, and (iii) as illustrated in Fig. 2, differing global cellular responses to the different compounds. Therefore, we employed two distinct in vitro assays that utilize a flag epitope-tagged affinity-purified derivative of DmpR. The first in vitro assay measures the effector-stimulated ATPase activity of DmpR-flag to the test compounds. The second compares the ability of the test effectors to compete for binding of \([^{14}C]\)phenol at its \(K_d\) concentration of 16 \(\mu M\), to evaluate whether all effectors bind through the same site and, if this is the case, to provide apparent binding affinities for the different compounds.

The dose-dependent ATPase stimulation profiles of DmpR-flag to the test effectors are shown in Fig. 4, top panels. A high basal level of ATPase activity observed in the absence of effectors was subtracted to obtain these values (see under “Experimental Procedures”). Surprisingly, all the effectors, including the two compounds that were not able to provoke an in vivo transcriptional response, were found both to elicit an ATPase activity and to be able to compete \([^{14}C]\)phenol binding. As a specificity control, 3,4-dimethylbenzoate was also employed in these assays and found to be unable to elicit an ATPase activity or compete \([^{14}C]\)phenol binding (data not shown). Based on the ability of all six test effectors to cross compete \([^{14}C]\)phenol binding by wild type DmpR, it can be inferred that they must all share a common binding site. The pathway substrate effectors (phenol, 2- and 4-methylphenol, and 3,4-dimethylphenol) elicit saturable activity profiles, compatible with an effector binding event being coupled to ATPase activity stimulation (Fig. 4, top panels). The concentrations of each effector required to elicit 50% of the plateau ATPase activity correspond well with the concentrations required to inhibit 50% of \([^{14}C]\)phenol binding (Fig. 4, bottom panels), i.e., binding affinity and elicitation of an ATPase activity are commensurate. However, importantly, binding affinity of the effector did not correlate with the magnitude of the ATPase activity response it elicited. For example, 4-methylphenol, to which DmpR has comparatively high affinity, elicited slightly lower maximal ATPase activity than 3,4-dimethylphenol, to which DmpR has at least a 10-fold lower apparent binding affinity (see Fig. 4, C, D, I, and J). As a control for these experiments, 3 mM concentrations of all the test compounds were found to have no effect on the ATPase activity.
activity of \( \Delta A2\text{-DmpR-flag} \) (data not shown), ruling out complications of potential toxic effects of specific compounds on ATPase activity per se. Hence, the binding affinity directly correlates to the sensitivity with which DmpR responds with ATPase activity to the presence of the effector, but it can be dissociated from the magnitude of this response.

The magnitude of the effector-stimulated ATPase activities of DmpR-flag produced with phenol, 2- and 4-methylphenol, and 3,4-dimethylphenol correspond in each case to the magnitude of the transcriptional response observed in the \textit{in vivo} transcriptional activation assay shown in Fig. 1; i.e. phenol and 2-methylphenol elicit similar high maximal levels, 4-methylphenol elicits a lower approximately 50% maximal level, and 3,4-dimethylphenol requires markedly higher concentrations to elicit a response but eventually elicits a higher level than 4-methylphenol. Thus, the level of ATPase activity elicited by effector binding in these cases correlates with the level of the transcriptional response, suggesting that effector binding to release the ATPase activity and functional coupling of the ATPase activity to the transcriptional apparatus are fully coordinated. However, the nonpathway substrate compounds 2,4-dimethylphenol and 4-ethylphenol can elicit a low but significant ATPase activity (Fig. 4, E and F), but not a transcriptional response (Fig. 1, E and F). Hence, it appears in these cases that the ATPase activity elicited by binding of these compounds is functionally uncoupled from transcriptional activation.

\textit{In Vitro ATPase Activity of DmpR-E135K-flag}—The ATPase stimulation profiles in response to the test compounds were also determined for DmpR-E135K. However, with this derivative, binding competition assays could not be performed with any degree of confidence due to its reduced capacity to bind \(^{14}\text{C}\)phenol (see Fig. B). Fig. 5 shows a comparison of the effector-stimulated ATPase activities of DmpR-flag and DmpR-E135K-flag. The dose-dependent ATPase stimulation profiles of DmpR-E135K-flag for phenol and 2- and 4-methylphenol show profiles that correlate with values from the \textit{in vivo} transcriptional assays; i.e. a slightly reduced ability to respond at low concentrations of phenol and 2-methylphenol but the same maximum values as wild type reached at high concentrations, and for 4-methylphenol, an enhanced ability to respond in terms of the magnitude of ATPase activity was achieved (compare Fig. 5, A–C, with Fig. 1, A–C). In the cases of 3,4-dimethylphenol, 2,4-dimethylphenol, and 4-ethylphenol, the ATPase stimulation profiles suggest both an increased binding affinity of DmpR-E135K (note shifts in the curves by a 10-fold difference in effector concentration) and an increased ability to respond as indicated by the higher ATPase activity achieved. The increased ability to respond in terms of elicitation of the ATPase activity by the three effectors is also observed in the \textit{in vivo} transcriptional response (see Fig. 1, D–E). Assessment of the effect of the increased sensitivity of the ATPase activity of DmpR-E135K to these three effectors can only be made in the case of 3,4-dimethylphenol, because wild type DmpR lacks an \textit{in vivo} response to 2,4-dimethylphenol and 4-ethylphenol. Comparison of the curves in Fig. 5D and Fig. 1D show that the increased sensitivity to 3,4-dimethylphenol is not observed as a concomitant shift in the \textit{in vivo} transcription response profile. These results argue against a high threshold level of ATPase activity being a rate-limiting step for transcriptional activation \textit{in vivo}. Rather, they support the idea that other properties of DmpR-E135K bound to these effectors, such as conformation, reduces effective coupling of the ATPase activity to a transcriptional response.

\textbf{Competitive Inhibition of Phenol Activation of DmpR in Vivo}—The novel response abilities of DmpR-E135K could be envisioned to occur by two mechanisms: (i) acquisition of a novel ability to bind the effectors, or (ii) modulation of a preexisting but nonproductive ability to bind the compounds. The above finding, namely that the E135K mutation influences to varying degrees the response to all six effectors tested, suggested that in this derivative, all the effectors may mediate their action through the same binding site. The \(^{14}\text{C}\)phenol binding competition results shown in Fig. 4 demonstrate that 2,4-dimethylphenol and 4-ethylphenol can compete phenol binding, albeit inefficiently, on wild type DmpR. These results therefore, support the idea that the novel effector specificity of DmpR-E135K has evolved through modulation of the functional outcome of binding these compounds through its phenol-binding site. Such a mechanism would predict that 2,4-dimethylphenol and 4-ethylphenol should act as competitive inhibitors of phenol-stimulated DmpR in functional activity assays. To test this prediction, we performed competition assays for both
response is seen by DmpR-E135K (Figs. 1 and 4, compare E32430 32430 

Both compounds were able to inhibit phenol-mediated activa-

tion when present in the 0.1–3 mM range, 

in vitro wild type DmpR

The aromatic-responsive regulator DmpR and its close rela-
tives are unusual in that they have to directly respond to the presence of the multiple compounds that serve as substrates for the pathways that they control. As outlined in the Introduction, the consequences of interaction of DmpR with the aromatic effectors in terms of the level of transcription and expression of the specialized catabolic enzymes can be a major limiting factor for growth at the expense of some compounds (23). Here, we dissect the different steps by which phenolic compounds control DmpR-driven transcription.

The A-domain of DmpR is both necessary and sufficient to bind phenol (20). Based on cross competition of [14C]phenol binding and phenol-stimulated functional assays (Figs. 4 and 6), all six effectors tested here share a common binding site. Because these effectors span the range from the best to the poorest effector of DmpR and also include two compounds that are unable to elicit a transcriptional response, this result strongly supports the idea that all (>20 known) DmpR effectors mediate their action through a single binding site within the A-domain of DmpR. Comparison of the binding affinities with the ATPase activity elicited by the different effectors (Fig. 4) revealed that the binding affinity of DmpR for the effectors determined the approximate 100-fold difference in sensitivity of the response of DmpR to the different compounds. However, the magnitude of the ATPase activity and transcriptional response elicited by the different effectors was independent of binding affinity (Fig. 1 and 4). Hence, another factor(s) must play a role in determining the functional outcome of binding in terms of its regulation of the levels of these activities.

**DISCUSSION**

true competitive inhibition through the same binding site as phenol. Given that these two compounds compete both [14C]phenol binding and phenol-stimulated activity on wild type DmpR, the results further argue that the E135K mutation recouples the ATPase response to 2,4-dimethylphenol and 4-ethylphenol binding to transcriptional activation that is functionally uncoupled in wild type DmpR.
Residue 135 has been shown to be intimately involved in effector response of both DmpR (19, 23) and its close relative XylR (13, 27). The E135K mutation of DmpR imparts the ability to mediate a transcriptional activation in response to 2,4-dimethylphenol and 4-ethylphenol. Direct assessment of the binding ability of DmpR-E135K for the novel effectors was not possible. However, the dose-dependent ATPase activity profiles indicated both an increased binding affinity for the two compounds and an increased ability for these compounds to elicit an ATPase activity (Fig. 5). As outlined in the Introduction, other substitutions of Glu-135 (E135A and E135D), in addition to conferring novel effector specificity, also weaken the A/C-domain repressive interaction, resulting in a semiconstitutive phenotype in the absence of effectors. Therefore, although the E135K substitution does not result in a detectable semiconstitutive phenotype, the E135K mutation may also modulate the A/C-domain interaction and thus enhance the maximal response to the novel compounds. Both DmpR and DmpR-E135K can bind both 2,4-dimethylphenol and 4-ethylphenol. However, unlike wild type DmpR, DmpR-E135K is able to couple at least part of the ATPase activity elicited to an in vivo transcriptional response (Figs. 1 and 4). Hence, the novel effector specificity of DmpR-E135K appears to be derived primarily by modulation of functional consequences of binding the compounds, rather than acquisition of a novel binding capacity.

Deletion of the regulatory A-domain of DmpR and many other $\sigma^4$-dependent regulators results in signal-independent derivatives possessing high intrinsic ATPase activity and the ability to promote transcription (19, 28–33). These observations suggest that, with the exception of NtrC, the regulatory A-domains do not serve any activating function per se (reviewed in Ref. 11). Indeed, the A-domain-deleted derivative of DmpR possesses ATPase and transcriptional promoting activities comparable to those of DmpR fully activated by its best effector (Fig. 2) (19). Thus, binding of the different effectors by the A-domain controls the activity of DmpR by alleviating repression of both its C-domain-mediated activities, namely its intrinsic ATPase activity and productive interaction with the transcriptional apparatus. Previous analysis using only the best effectors of DmpR had suggested a model whereby binding of phenol by the A-domain of DmpR controls hydrolysis of ATP, which, because it is a prerequisite for transcriptional activation, in turn controls the level of the transcriptional promoting property of DmpR (19, 20). However, whether this control is exerted directly, by blocking access of ATP, or indirectly by some other mechanism, such as control of the oligomeric state of the protein, remains to be elucidated (11). The finding that purified DmpR has high basal ATPase activity even in the absence of effector suggests that ATP has a degree of access to its binding site on DmpR under all conditions. The results shown here with the novel effectors of DmpR-E135K, and the poorer effectors of DmpR, suggest that binding of different aromatic effectors may result in either complete or only partial alleviation of the inhibitory action of the A-domain. Furthermore, they also suggest that partial alleviation has the potential to result in differential effects on ATPase activity and its productive coupling to the transcriptional apparatus, i.e. that regulation of these two processes can under some circumstances be dissociated. With pathway substrate effectors, the different levels of ATPase activity elicited from DmpR in vitro correlated well with the levels of the DmpR-mediated transcriptional response in vivo (Figs. 1 and 4). This is exemplified by 4-methylphenol to which DmpR has relatively high affinity but that is only capable of eliciting approximately half maximal ATPase and transcriptional response (Figs. 1 and 4). This correlation suggests that in some cases, the levels of ATPase activity directly determine the level of transcriptional initiation, and control of ATPase activity and productive interaction with the transcriptional apparatus by effector binding is coordinated. However, in the case of 2,4-dimethylphenol and 4-ethylphenol, elicitation of an ATPase activity is uncoupled from transcriptional activation. The in vitro ATPase stimulation assays are performed in the absence of the transcriptional apparatus, and it is not known how much of the ATPase activity elicited would be productively coupled to transcriptional activation in vivo. The mechanism by which ATPase activity of the C-domain is coupled to transcriptional activation is complex and as yet not fully understood. ATP binding per se has been shown to mediate conformational changes that promote oligomerization required prior to ATP hydrolysis (34–37). Mutations within the C3 region, required for productive interactions with $\sigma^4$-holoenzyme, that uncouple ATPase activity from transcriptional activation have been found for NtrC (38), DetD (10), and DmpR. Similarly, a mutation in a different location of the central domain of NtrC, within the proposed ATP binding pocket, has also been shown to uncouple ATP hydrolysis from transcriptional activation (39). Thus, exposure and correct presentation of both the ATP binding site and the transcriptional apparatus interactive surface must be important for productive coupling of the catalytic activity via protein-protein interactions. Thus, for DmpR, it would appear plausible that A-domain binding of certain aromatic compounds may mediate conformational changes that allow access (and thus hydrolysis) of a small ATP molecule but still mask regions of the C-domain necessary for productive protein-protein interaction with the transcriptional apparatus. Hence, we would speculate that for productive effectors, binding of the aromatic compound to the A-domain unmasks C-domain regions involved in both these processes and that the defect in coupling 2,4-dimethylphenol and 4-ethylphenol stimulated ATPase activity with transcriptional activation resides in the conformation/oligomeric state adopted by DmpR upon binding of these compounds. Testing the possible mechanisms outlined above using purified components and in vitro transcription assays is a future focus of our laboratory.

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