An active role for a structured B-linker in effector control of the σ^{54} -dependent regulator DmpR

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The activities of many prokaryotic σ^{54} -dependent transcriptional activators are controlled by the N-terminal A-domain of the protein, which is linked to the central transcriptional activation domain via a short B-linker. It used to be thought that these B-linkers simply serve as flexible tethers. Here we show that the B-linker of the aromatic-responsive regulator DmpR and many other regulators of the family contain signature heptad repeats with regularly spaced hydrophobic amino acids. Mutant analysis of this region of DmpR demonstrates that B-linker function is dependent on the heptad repeats and is critical for activation of the protein by aromatic effectors. The phenotypes of DmpR mutants refute the existing model that the level of ATPase activity directly controls the level of transcription it promotes. The mutant analysis also shows that the B-linker is involved in repression of ATPase activity and that allosteric changes upon effector binding are transduced to alleviate both B-linker repression of ATP hydrolysis and A-domain repression of transcriptional activation. The mechanistic implications of these findings for DmpR and other family members are discussed.

Keywords: coiled coil/DmpR/linker/ σ^{54} /transcriptional activation

Introduction

The σ^{54} -dependent activator DmpR regulates transcription of the *dmp* operon of *Pseudomonas* CF600, which encodes enzymes for catabolism of (methyl)phenols (Shingler *et al.*, 1993). σ^{54} -dependent regulators control transcription from $-24/-12$ promoters through binding to upstream activator sites and are brought into contact with σ^{54} -RNA polymerase to provide ATP-dependent initiation of transcription (Kustu *et al.*, 1991). Regulators of this family typically have a domain structure (see Figure 1) in which the highly conserved central C-domain is thought to determine the essential transcriptional activation properties of the regulators, namely ATP hydrolysis (Weiss *et al.*, 1991; Austin and Dixon, 1992) and interaction with σ^{54} -RNA polymerase (Lee and Hoover, 1995; Wang *et al.*, 1997). The N-terminal A-domains define different subgroups and, where present, are involved in controlling the activity of the regulator in response to environmental

signals (Shingler, 1996). For the archetypal members NtrC and DctD, signal perception is mediated through sensory protein kinases resulting in phosphorylation of the A-domain of the cognate regulator (Ninfa *et al.*, 1987; Keener and Kustu, 1988; Huala *et al.*, 1992; Klose *et al.*, 1993; Gu *et al.*, 1994). In the case of NifA and PspF (which lacks an A-domain), signal-responsive control is mediated through regulatory protein–protein interactions (Austin *et al.*, 1994; Berger *et al.*, 1994; Dworkin *et al.*, 2000). DmpR, however, belongs to the distinct mechanistic subgroup that directly senses and responds to small effector ligands. This group comprises many aromatic-responsive regulators (see Figure 1) typified by the (methyl)phenol-responsive DmpR (Shingler and Moore, 1994; Shingler and Pavel, 1995) and xylene/toluene-responsive XylR (Delgado and Ramos, 1994), but also includes the mechanistically related formate-responsive FhlA (Hopper *et al.*, 1994).

The various substrates of the *dmp* pathway, and some structural analogues, can serve as aromatic effectors of DmpR (Shingler and Moore, 1994; Shingler and Pavel, 1995). We have previously shown that the multiple effectors of DmpR mediate their action through a single common binding site on the A-domain (O'Neill *et al.*, 1998, 1999). During productive activation by aromatic effectors, the binding of aromatic compounds alleviates inhibitory interactions between the A- and C-domains, with concomitant release of its catalytic ATPase activity and transcriptional activating property (Fernández *et al.*, 1995; Pérez-Martín and de Lorenzo, 1995; Shingler and Pavel, 1995; Ng *et al.*, 1996). Hence the A-domain of DmpR mediates two distinct properties: binding of effectors and repression of the C-domain-mediated activities; it can perform both activities autonomously *in vitro* as an independently expressed domain. However, release of its inter-domain repression of the C-domain in response to binding of effectors is completely dependent on physical continuity, which is normally provided by the short B-linker (O'Neill *et al.*, 1998). Based on mutational evidence that various insertions in the Q-rich B-linker of both NtrC and NifA do not affect their function, the B-linker has generally been considered to serve simply as a flexible linker (Wootton and Drummond, 1989). However, proline substitutions in the B-linkers of both DmpR and its close relative XylR result in semi-constitutive regulators that can promote some degree of transcription in the absence of their activating signals (Fernández *et al.*, 1995; Shingler and Pavel, 1995). Residues that link sensory and output domains of many regulators commonly serve more active functions than that of a simple tether (Spronk *et al.*, 1999). The dissimilar effects of B-linker insertions and substitutions suggest functional differences between the B-linkers of different σ^{54} -dependent activators and point towards an active role

Analysis of the B-linker region of σ^{54} -dependent transcriptional regulators using the Coils program (Lupas *et al.*, 1991) revealed two potential classes of this type of regulator (Figure 1). The B-linkers of DmpR, XylR and some but not all ligand-responsive members of the family are predicted to form short α -helical coiled coils, as is the phosphorylation-responsive DctD. Significantly, the B-linkers of NtrC and NifA, which are tolerant of amino acid insertions in this region (Wootton and Drummond, 1989), are not predicted to form a coiled-coil structure. To

Fig. 1. Coils analysis of the linker region of DmpR and other σ^{54} -dependent transcriptional regulators. Above, schematic representation of *dmpR* indicating the location of restriction sites used in generating DmpR derivatives. The domain structure of DmpR, discussed in the text, is superimposed on the restriction map. The shaded box indicates the location of the ATP-binding motif of the family (Morett and Segovia, 1993) and the flag represents a C-terminal fusion of an eight amino acid epitope tag. Below, alignment of the linker region of selected members of the σ^{54} -dependent family of transcriptional activators. Alignment was made relative to two highly conserved residues, a hydrophobic residue followed by glycine (indicated by &&), which mark the start of the C-domain (Jaspers *et al.*, 2000). The a- and d-positions within heptad (a-b-c-d-e-f-g) repeats of proteins predicted to form a coiled coil by the Coils program (Lupas *et al.*, 1991) are shown in bold and underlined. Arrows indicate residues of DmpR targeted in specific mutants used in this study. 'CP' indicates the propensity prediction to form a coiled coil of >90% (++), >50% (+) or <50% (-). The linker regions of the different family members are grouped according to known or suspected activation mechanisms with * indicating small-ligand responsive regulators, # indicating phosphorylation-responsive regulators, and $\square$ indicating those lacking an A-domain and/or controlled by protein-protein interactions. Underlined sequence indicates the Q-linker regions of NtrC and NifA referred to in the text. Sequences are: AphR from *Comamonas testosteroni* TA441 (X68033; Arai *et al.*, 1998); BphR encoded on plasmid pNL1 from *Sphingomonas aromaticivorans* F199 (AF079317; Romine *et al.*, 1999); DctD from *Rhizobium meliloti* (P13632; Watson, 1990); DmpR encoded on plasmid pV1150 from *Pseudomonas* sp. strain CF600 (A47078; Shingler *et al.*, 1993); FhlA from *E. coli* K-12 (P19323; Schlenz and Bock, 1990); HbpR from *Pseudomonas azelaica* HBP1 (U73900; Jaspers *et al.*, 2000); HrpS from *Pseudomonas syringae* (P26316; Xiao *et al.*, 1994); MopR from *Acinetobacter calcoaceticus* NCIB8250 (Z69251; Schirmer *et al.*, 1997); NifA from *Klebsiella pneumoniae* (P03027; Arnold *et al.*, 1988); NtrC from *K. pneumoniae* (P03029; Drummond *et al.*, 1983); PheR from *Pseudomonas putida* BH (D63814; M. Takeo, unpublished); PhhR from *P. putida* P35X (X79599; Ng *et al.*, 1995); PhIR encoded on plasmid pPGH1 from *P. putida* H (Müller *et al.*, 1996) indicated in the figure as PhIR(P); PhIR from *Ralstonia eutropha* JMP134 (AF065891; Ayoubi and Harker, 1998) indicated in the figure as PhIR(R); PhnR from *Burkholderia* sp. strain RP007 (AF061751; Laurie and Lloyd-Jones, 1999); PilR from *Pseudomonas aeruginosa* PA01 (Q00934; Ishimoto and Lory, 1992); PoxR from *R. eutropha* E2 (GAF026065; Hino *et al.*, 1998); PspF from *E. coli* K-12 (P37344; Jovanovic *et al.*, 1997); RocR from *Bacillus subtilis* (P38022; Calogero *et al.*, 1994); RtcR from *E. coli* K-12 (P38035; Blattner *et al.*, 1997); TbuT from *Ralstonia pickettii* PK01 (U72645; Byrne and Olsen, 1996); TmbR from *P. putida* TMB (U41301; Favaro *et al.*, 1996); TouR from *P. putida* (AJ005663; Bertoni *et al.*, 1998); and XylR encoded on plasmid pWW0 from *P. putida* mt-2 (M10143; Inouye *et al.*, 1985).

elucidate the function of the B-linker region and to dissect the potential role of the identified heptad repeats in control of DmpR by aromatic effectors, we took a mutagenesis approach. This approach used a derivative of *dmpR* in which unique *Cla*I and *Sna*BI sites had been introduced (see Figure 1). Substitution of the *Cla*I–*Sna*BI fragment with specifically designed oligonucleotides allows the 12 core residues of the B-linker (211-EL_YEL_QS_QLL_SL₂₂₂, see Figure 1), which span the centre of the heptad repeats, to be replaced with any desired sequence of amino acids. The resulting DmpR derivatives all contain a C-terminal fusion of an eight amino acid 'Flag tag'. This tag does not alter the magnitude or specificity of the response of DmpR to aromatic effectors *in vivo*, and allows affinity purification of the proteins for *in vitro* analysis of ATPase and aromatic effector binding properties (Shingler and Pavel, 1995; O'Neill *et al.*, 1998).

As outlined in the Introduction, the B-linker of DmpR is involved in signal transduction upon aromatic effector binding. To interpret the phenotypes of linker mutants and dissect the structural contribution of this region, it was important, therefore, that the mutant derivatives could still bind aromatic effectors. Hence, the [¹⁴C]phenol-binding capacities of all the DmpR-Flag B-linker mutants, including one in which the order of the B-linker residues was 'scrambled' and five with specific amino acid substitutions, were determined at a phenol concentration of 16 μ M, corresponding to the K_d for phenol binding of wild-type DmpR-Flag. As shown in Figure 2, all derivatives retained >35% of the ability of the wild-type protein to bind the effector at this low concentration. We have previously shown, using an A-domain mutant (DmpR-E135K-Flag), that this level of binding is more than adequate for wild-type levels of *in vivo* transcriptional activation when assessed with effector concentrations of >1 mM (O'Neill *et al.*, 1999). As will be shown below, the most affected derivative (L215,219,222A, Figure 2) had essentially wild-type ability to respond to aromatic effectors *in vivo*. These results allow us to attribute defects in the ATPase and transcription-promoting properties of DmpR mutants to the effects of specific amino acid substitutions on B-linker function.

Effector activation requires ordered sequence of heptad repeats

To determine whether the ordered array of amino acid residues within the heptad repeats is critical for effector activation, we generated a mutant designated DmpR-scramble-Flag, which would be expected to generate a loss-of-function phenotype. In this mutant, the central 12 residues of the motif are shuffled to disrupt the ordered sequence, but retain the wild-type amino acid composition and thus innate flexibility and overall hydrophobicity. The ability of DmpR-scramble-Flag to respond to aromatics was compared with that of DmpR-Flag using a previously constructed luciferase reporter system in which the DmpR-controlled *Po* promoter is fused to *luxAB* on the host *P. putida* chromosome. Plasmids expressing DmpR derivatives complete the experimental system. *In vivo* transcription in response to the presence of the best (2-methylphenol) and poorest (4-methylphenol) natural effectors of DmpR were measured. The DmpR-Flag and DmpR-scramble-Flag proteins are expressed at the same

Fig. 2. [¹⁴C]phenol binding of Flag-tagged DmpR derivatives. (A) [¹⁴C]phenol binding of DmpR-Flag derivatives was measured at a phenol concentration of 16 μ M (the K_d for wild-type DmpR-Flag). The value for DmpR-Flag was set at 100% and the data represent the average of triplicate determinations. (B) Coomassie Blue stain of proteins separated by 11% SDS-PAGE after release from 3 μ l of anti-Flag beads treated with the indicated extracts, which were used for both [¹⁴C]phenol binding and ATPase analysis.

level (Figure 3A) but the DmpR-scramble-Flag derivative is totally incapable of responding to the aromatic effectors *in vivo* (compare histograms in Figure 3B and C). A second linker scramble mutant, in which the residues in the d-positions were left intact (LELSLQLQSEYL), also showed severe defects in response to aromatic effectors, with <10% wild-type levels of response to 2-methylphenol and an inability to respond to 4-methylphenol (data not shown). We conclude that the ordered sequence of the amino acids within the B-linker is critical for responsiveness of DmpR to aromatic effectors.

To test whether the scrambling of the B-linker also results in failure to produce a productive rearrangement that releases the ATPase activity of DmpR, we tested the ability of DmpR-scramble-Flag to hydrolyse ATP *in vitro* in response to 2-methylphenol. The results, shown in Figure 3C (line graph), demonstrate that DmpR-scramble-Flag is not stimulated by effectors to hydrolyse ATP. The B-linker scramble mutation did not abolish the background level of ATP hydrolysis observed with wild-type DmpR-Flag in the absence of effectors (Figure 3B). This background ATP hydrolysis by DmpR is an innate and specific property of the protein, since introduction of a single amino acid substitution in the ATP binding site (DmpR-G268S-Flag) abolishes even basal ATPase activity (O'Neill *et al.*, 1999). As DmpR-scramble-Flag did not respond to effectors and behaved like DmpR-Flag in the absence of effectors in terms of both its ATPase activity and its inability to promote transcription, it appears that the disordered linker amino acid sequence of DmpR-scramble-Flag locks DmpR in an inactive conformation that cannot respond to the effector-binding signal.

The B-linker of DmpR contains five leucine residues located at the a- and d-positions within the heptad repeats, with those at positions 215, 219 and 222 marking the centre of the whole motif (Figure 1). The hydrophobicity of the a- and d-position residues underlies the coiled-coil structure, and substitutions of these residues by amino acids with different properties would be expected to have differential effects. The Coils program (Lupas *et al.*, 1991) predicts that substitutions of L215, L219 and L222 by the

Fig. 3. Comparison of *in vivo* and *in vitro* activities of DmpR-Flag derivatives. (A) Western analysis of expression levels of DmpR-Flag (lane 1), DmpR-scramble-Flag (lane 2), DmpR-L215,219,222E-Flag (lane 3) and DmpR-L215,219,222A-Flag (lane 4) in 30 µg of total protein derived from cultures used to determine *in vivo* transcriptional responses. (B–E) Histograms show the *in vivo* luciferase transcriptional response of *P. putida* KT2440::Po-luxAB harbouring plasmids expressing DmpR-Flag (B), DmpR-scramble-Flag (C), DmpR-L215,219,222E-Flag (D) or DmpR-L215,219,222A-Flag (E) measured in the absence or presence of 2 mM 2-methylphenol (2-mp) or 4-methylphenol (4-mp). Values are the averages of triplicate determinations from each of two independent experiments. Line graphs show the level of *in vitro* ATP hydrolysis mediated by 1 µl of bead-bound Flag-tagged protein, in the absence (open symbols) and presence (closed symbols) of 1 mM 2-methylphenol. Data are the average of duplicate experiments and expressed as the percentage of total ATP (180 nM) hydrolysed.

less hydrophobic amino acid alanine would be tolerated since these substitutions only reduce the propensity prediction from 99.8 to 79.0%. Substitutions by the negatively charged glutamic acid, on the other hand, give a much lower predicted propensity value, 15.1%, and thus might be expected to impart at least a partial loss-of-function phenotype. To test these predictions, DmpR-L215,219,222A-Flag and DmpR-L215,219,222E-Flag were generated and subjected to the assays and controls described above for DmpR-Flag and DmpR-scramble-Flag. Both proteins were present at wild-type levels in cells (Figure 3A). However, while DmpR-L215,219,222A-Flag had wild-type ability to promote transcription in response to 2-methylphenol and had only a slightly impaired response to 4-methylphenol, DmpR-L215,219,222E-Flag was much more severely affected (see histograms in Figure 3D and E). These results are consistent with the idea that the B-linker forms a coiled coil that is involved in control of DmpR by aromatic effectors.

The *in vitro* ATPase activity of DmpR-L215, 219,222E-Flag and DmpR-L215,219,222A-Flag was not repressed in the absence of effectors (Figure 3D and E, line graphs), so substitutions within the B-linker of DmpR can uncouple aromatic effector control of ATPase activity without loss of aromatic effector control of transcription. As expanded upon below, these findings have repercussions for the prevalent model for aromatic effector control of the XylR/DmpR family of transcriptional activators.

Isoleucine substitutions within the B-linker affect response to aromatic effectors

The results described above show the importance of the regular spacing and hydrophobic nature of residues in the a- and d-positions in the heptad repeats. However, it should be noted that analysis of the periodicity of hydrophobic residues cannot distinguish very short coiled-coil structures (such as that predicted for DmpR) from short amphipathic helices. Moreover, the phenotypes of DmpR-L215,219,222A-Flag and DmpR-L215,219,222E-Flag would also be compatible with the predicted effects of alanine and glutamic acid substitutions on a hydrophobic interaction between two amphipathic helices. One major property that distinguishes coiled coils from other amphipathic helices is their distinctive 'knobs-into-holes' packaging interaction. Physical constraints imposed by the side chains of residues at the a- and d-positions dictate the number and orientation of the α-helices that can be accommodated (Lovejoy *et al.*, 1993; Harbury *et al.*, 1994). Leucine and isoleucine differ very little in their hydrophobicity but the side-chain packing preference of these amino acids in the a- and/or d-positions lead to dramatic differences in the resulting coiled-coil interactions in peptides. Peptides carrying the four possible combinations of leucine and isoleucine at the a- and d-positions differentially form coiled coils with two, three, four and six chains (Harbury *et al.*, 1993). We reasoned, therefore, that leucine-to-isoleucine substitutions would probably affect a coiled-coil structure but leave a hydrophobic amphipathic helix interaction essentially intact. Hence, we generated a series of derivatives in which the a- and/or d-leucines of the core 12 residues of the B-linker were substituted for isoleucine. All the leucine-to-isoleucine substitution mutants were expressed at wild-type levels in cells (Figure 4A). As shown in Figure 4, DmpR-L212,219I-Flag (a-position substitutions; dimer in peptides) and DmpR-L215,222I-Flag (d-position substitutions; tetramer in peptides) had impaired ability to promote transcription in response to aromatic effectors, while DmpR-L212,215,219,222I-Flag (a- and d-position substitutions; trimer in peptides) was only marginally affected in this respect. The location of the leucine-to-isoleucine substitutions had a graded effect on the control of ATPase activity by aromatic effectors, ranging from no effect in the case of a-position substitutions (Figure 4B), through partial uncoupling in the case of d-position substitutions (Figure 4C) to complete uncoupling with isoleucine substitutions in both a- and d-positions (Figure 4D). These results strongly favour the interpretation that the heptad repeats of the B-linker of DmpR specify a coiled-coil structure rather than an amphipathic helix. They also demonstrate that ATPase activity is

Fig. 4. *In vivo* and *in vitro* responses of DmpR-Flag derivatives with leucine to isoleucine substitutions within the linker. (A) Western blot and (B–D) *in vivo* transcriptional responses (histograms) and *in vitro* ATPase activities (line graphs) are as in Figure 3. DmpR-Flag [lane A1], DmpR-L212,219I-Flag [lane A2 and (B)], DmpR-L215,222I-Flag [lane A3 and (C)] and DmpR-L212,215,219,222I-Flag [lane A4 and (D)]. The predicted coiled-coil propensity values for these mutants are: DmpR-L212,219I-Flag, 78%; DmpR-L215,222I-Flag, 41%; and DmpR-L212,215,219,222I-Flag, 21%.

uncoupled from aromatic-effector control and from the resulting level of transcriptional activation (Figure 4D).

While deductions from peptide analysis cannot always be translated to behaviour within intact proteins (see Lupas, 1996), it is interesting to note that di-, tetra- and hexameric coiled coils are observed in peptides containing Leu in both a- and d-positions, as in the B-linker of wild-type DmpR (Harbury *et al.*, 1993). The data above suggest, though this is highly speculative, that stimulated ATPase activity might be compatible with dimeric/tetrameric arrangement of the B-linker, while efficient transcriptional activation may be compatible with trimeric/hexameric arrangements.

Effector-stimulated ATPase activity is not required for efficient transcriptional activation *in vivo*

A striking feature of the mutant analysis described above is the ability of DmpR-L212,215,219,222I-Flag to promote efficient transcriptional activation *in vivo* in the absence of effector-stimulated ATPase activity. Irrespective of the presence of aromatic effectors, this derivative had only basal ATPase levels equivalent to that of wild-type DmpR-Flag in the absence of effectors (compare Figures 3B and 4D). This suggests either that the conformation of DmpR-L212,215,219,222I-Flag renders

Fig. 5. *In vivo* transcriptional responses of DmpR-Flag derivatives with ATP binding and/or hydrolysis defects and leucine-to-isoleucine substitutions within the linker. (A) *In vivo* transcriptional responses of the indicated DmpR-Flag derivatives in response to 2-methylphenol and (B) western analysis of expression levels in the cells used, as described in the legend to Figure 3.

it capable of promoting efficient transcription with only basal ATPase activity or that it may be able to promote transcription in the absence of ATP binding and/or hydrolysis. To distinguish between the two possibilities, we used three single amino acid substitutions that are critical for ATP binding or hydrolysis. DmpR with a G268S substitution (within the ATP-binding motif) is unable to bind ATP, while DmpR with an E335L or L356P substitution can bind ATP but cannot hydrolyse it. All these single amino acid substitutions completely abolish ATP hydrolysis by A-domain-deleted derivatives of DmpR (Ng *et al.*, 1996; P.Wikström, E.O'Neill, L.C.Ng and V.Shingler, in preparation). These single amino acid substitutions were combined with the leucine-to-isoleucine substitutions in DmpR-L212,215,219,222I-Flag. As shown in Figure 5, none of the resulting derivatives promoted transcription *in vivo*. Taken together, these results suggest that the leucine-to-isoleucine substitutions of DmpR-L212,215,219,222I-Flag relieve some structural constraint that allows full transcriptional levels *in vivo* with only basal ATP activity of DmpR.

B-linker controls ATPase activity of DmpR

In addition to uncoupling ATP hydrolysis from proficiency in transcriptional activation, the B-linker mutants also uncouple ATPase activity from aromatic effector control. This begs the questions of what determines the level of ATPase activity in DmpR and how the ATPase activity is normally controlled in response to aromatic effectors. To address these questions, we generated and purified a series of C-terminal His-tagged DmpR derivatives (Figure 6A and B). The epitope tag of DmpR-His, like that of DmpR-Flag, did not alter the regulatory properties of DmpR *in vivo* (data not shown). Moreover, DmpR-His mediated *in vitro* transcription in response to increasing concentrations of aromatic effectors (Figure 6C) faithfully reproduced both the dose-response and maximal relative levels with which 2- and 4-methylphenol promote transcription by DmpR *in vivo* (O'Neill *et al.*, 1999). As with DmpR-Flag bound to beads, the basal level of *in vitro* ATP hydrolysis by DmpR-His in the presence of its specific

Fig. 6. Purification and *in vitro* activities of DmpR-His derivatives. (A) The linker sequence and (B) Coomassie Blue stain of proteins separated by 11% SDS-PAGE: lane 1, DmpR-His; lane 2, ΔA1-DmpR-His; lane 3, ΔA2-DmpR-His; lane 4, ΔA3-DmpR-His; lane 5, standards. (C) *In vitro* transcription mediated by DmpR-His in response to increasing concentrations of the effectors 2-methylphenol (2-mp) and 4-methylphenol (4-mp). (D and E) *In vitro* ATPase activity (D) and transcription (E) mediated by the indicated derivatives in a coupled assay in the absence or presence of 0.5 mM 2-methylphenol. The results are the average of duplicate determinations.

DNA-binding sites and RNA polymerase was ~20% of fully effector-activated levels (Figure 6D).

The uncoupling of ATPase activity from transcription-promoting ability observed with the linker mutants suggests that in the native protein the B-linker keeps the ATPase activity in check and that allosteric changes upon effector binding relieve the inhibitory effect of the linker. If this were the case, then deletion of the A-domain and B-linker of DmpR should result in a derivative with full ATPase activity. Deletion of just the A-domain, on the other hand, might be expected to leave B-linker-mediated repression of the ATPase activity intact and therefore result in a derivative with just basal ATPase levels. The previous finding that deletion of the A-domain and the N-terminal half of the B-linker results in a derivative, ΔA2-DmpR, which has constitutive ATPase activity and transcriptional activation properties equivalent to that of fully activated wild-type protein (Shingler and Pavel, 1995; O'Neill *et al.*, 1999) is consistent with this idea. However, to test the hypothesis rigorously, we generated, in addition to ΔA2-DmpR-His, A-domain-truncated DmpR derivatives that differed in possession of the B-linker: ΔA1-DmpR-His completely lacks both the A-domain and B-linker while ΔA3-DmpR-His lacks just

the A-domain (Figure 6A and B). The ability of these derivatives to hydrolyse ATP and promote transcription *in vitro* was determined from the same reaction mixtures and compared with that of DmpR-His in the presence and absence of 2-methylphenol (Figure 6D and E). The data show that the predictions hold true since the B-linker-containing derivative (ΔA3) mediated only basal ATPase activity, while the B-linker-deleted (ΔA1 and ΔA2) derivatives both mediated high ATPase activities similar to that of effector-activated wild-type DmpR-His (Figure 6D). Irrespective of the level of ATPase activity, ΔA1-, ΔA2- and ΔA3-DmpR-His were equally proficient at promoting transcription *in vitro* to a level equivalent to that of effector-activated DmpR-His (Figure 6E). Thus, as with the B-linker mutants, ATPase activity and the ability to promote transcription can be uncoupled.

Two plausible mechanisms, which are compatible with both the deletion analysis and the B-linker mutant data, can explain the B-linker-mediated control of the ATPase activity of DmpR. In both cases, allosteric changes upon A-domain binding of effectors alleviate B-linker-mediated constraints on the ATP-hydrolysing C-domain. In the first case, and by analogy with the mechanism of the metal-binding ArsA-dependent ATPase pump (Li and Rosen, 2000), relief of B-linker-mediated constraints may result in optimal contacts between two or more ATP-binding domains and thus facilitate catalysis. Alternatively, allosteric changes may modify the ATP-binding affinities. In either case, effector binding would coordinately result in alleviation of both B-linker repression of the catalytic ATPase activity and A-domain inhibition of interaction with σ^{54} -RNA polymerase.

Integrated model for effector activation of DmpR

The prevailing model for aromatic effector control of the DmpR/XylR class of regulators has been that the A-domain controls transcription-promoting activity simply by controlling the level of essential ATPase activity. This model was primarily based on the findings that aromatic effectors control ATP hydrolysis by DmpR *in vitro* (Shingler and Pavel, 1995) and that deletion of the A-domains of DmpR and XylR mimics the state activated by aromatic effectors (Fernández *et al.*, 1995; Shingler and Pavel, 1995). However, the observation that some phenolic compounds can be bound by DmpR and elicit an ATPase activity but do not promote transcriptional activation is difficult to explain using this simple model (O'Neill *et al.*, 1999). Here we demonstrate uncoupling of high aromatic effector-induced levels of ATPase activity from transcription-promoting ability of DmpR in the linker mutants (e.g. DmpR-L212,215,219,222I-Flag; Figure 4D) and with truncation derivatives that differentially possess the B-linker residues (Figure 6D and E). These results provide sufficient evidence to refute the previous model. Binding of aromatic effectors via the A-domain undoubtedly does alleviate repression of the ATPase activity of DmpR. However, since possession of the B-linker results in basal ATPase activity while its deletion results in high ATPase activity (Figure 6D), the data strongly suggest that the B-linker controls this event. Furthermore, since the levels of transcription mediated by derivatives lacking the A-domain are indistinguishable irrespective of basal or high ATPase activity (Figure 6E),

the primary regulatory role of the A-domain is likely to be control of transcriptional activation by hindering productive interaction with the σ^{54} -transcriptional apparatus in the absence of effectors. Although stimulation of the ATPase and transcription-promoting activities by effectors can be genetically uncoupled, control of these two events is coordinated in the wild-type protein (Figure 6C and D). Taken together, the data support a model in which allosteric changes upon effector binding to the A-domain coordinately alleviate both B-linker repression of the catalytic ATPase activity and A-domain inhibition of interaction with σ^{54} -RNA polymerase.

The analysis of DmpR-L212,215,219,222I-Flag and Δ A1-DmpR-His demonstrates that, in the absence of other constraints, the basal ATPase activity of DmpR is sufficient for full transcription activation (Figures 4D and 6E). This raises the question of the role, if any, of effector-stimulated ATPase activity. One possibility is that it helps to counteract inhibitory A/C-domain interactions that silence DmpR in the absence of aromatic effectors and are presumably relatively positioned by the B-linker. In this respect, it is interesting that, for the mechanistically related FhlA regulator, effector (formate)-dependent transcription *in vitro* is only observed at low ATP concentrations, while at high ATP concentrations transcription becomes completely independent of effector (Hopper *et al.*, 1996).

The B-linker mutant analysis demonstrates that the heptad repeats of this region are critical for the response of DmpR to its aromatic effector-binding signal. Whether short sequences of heptad repeats take up a coiled-coil conformation or not can be context dependent, being dictated by binding of a protein to DNA or the context provided by the flanking regions (Lupas, 1996). Scrambling of the B-linker residues totally abolishes the ability to respond to effectors both in terms of high ATPase activity and ability to promote transcription (Figure 3C), while concomitant deletion of both the A-domain and the B-linker results in constitutive high ATPase activity (Figure 6D). These results suggest either that a structured B-linker is required for relief of repression of ATPase activity upon effector activation or that, in the scrambled mutant, the A-domain is aligned and represses the C-domain in a different manner than in the wild-type protein.

Concluding remarks

The mutant and deletion analysis presented here unequivocally demonstrates that the B-linker possesses a structure that is actively involved in and critical to the response of DmpR to its aromatic effector-binding signal. The observation of similar signature heptad repeats within both effector-responsive and phosphorylation-responsive σ^{54} -dependent regulators suggests that an active role of the B-linkers is probably a common mechanistic feature of a whole subgroup of the family. It is plausible that possession of the heptad repeats may underlie significant differences in the outcome of deletions of the N-terminal regulatory domains, such as those observed between the phosphorylation-responsive regulators NtrC and DctD. In this respect, it is interesting to note that DctD possesses heptad repeats and, similar to DmpR, N-terminal truncation of the regulatory domain results in a signal-independ-

ent constitutively active derivative (Lee *et al.*, 1994). Conversely, N-terminal truncation of NtrC, which does not possess heptad repeats in the B-linker, results in a derivative incapable of promoting transcriptional activation (Porter *et al.*, 1995).

Materials and methods

DNA manipulations and general procedures

Construction of broad-host-range plasmids expressing *dmpR* (pVI401) or *dmpR*-Flag (pVI455) with a fusion of an eight amino acid epitope, Flag tag (Hopp *et al.*, 1988), from the native promoter for *dmpR* has previously been described (Shingler and Moore, 1994; Shingler and Pavel, 1995). A similar plasmid, pVI545, contains a *Clal* site overlapping codon 210 of *dmpR*-Flag (which results in an E210D substitution) and a silent *SnaBI* site overlapping codons 221 and 222 (see Figure 1; Skärfstad *et al.*, 2000). DmpR-Flag linker mutants were generated by replacing the *Clal*-*SnaBI* fragment of pVI545 with synthetic oligonucleotides encoding the desired sequence and that regenerated a glutamic acid codon for E210. This strategy thus resulted in plasmids expressing the derivatives from the native *dmpR* promoter. Replacement of the *NdeI*-*ScaI* fragment of pVI401-derived plasmids carrying ATP-binding or hydrolysis mutants of DmpR by that of *dmpR*-L212,215,219,222I generated DmpR derivatives with the L212,215,219,222I linker mutation in the context of different ATP defects. T7 expression plasmids for DmpR-Flag linker mutations were constructed by cloning *NdeI*-*HindIII* fragments spanning the entire regulator and Flag tag into pET3H as previously described for DmpR-Flag (pVI456; Shingler and Pavel, 1995). For expression of His-tagged proteins, DmpR-His, Δ A1-DmpR-His and Δ A2-DmpR-His, analogous T7 expression plasmids were constructed by replacing the *BamHI*-*HindIII* Flag tag fragment (Shingler and Pavel, 1995) with oligonucleotides that introduced a serine codon and six histidine codons followed by a stop codon. For Δ A3-DmpR-His, a T7 expression plasmid was constructed using the same PCR-based strategy as described for Δ A1-DmpR (Shingler and Pavel, 1995). The DNA sequence integrity of all oligonucleotides and PCR-generated DNA was confirmed. Plasmids expressing DmpR-Flag derivatives were introduced into the luciferase reporter *P. putida* KT2440::Po-*luxAB* and assayed as previously described (Sze *et al.*, 1996). Protein levels of DmpR-Flag derivatives were determined by western blot analysis using an M2 mouse monoclonal antibody against Flag (Sigma) as previously described (Shingler and Pavel, 1995).

Purified protein

Core RNA polymerase was purchased from Epicentre Technology; *Escherichia coli* σ^{54} and IHF (integration host factor) were purified essentially as previously described (Hunt and Magasanik, 1985; Vorgias and Wilson, 1991). T7 promoter-driven expression of DmpR derivatives and crude extract preparation were as previously described (Shingler and Pavel, 1995). DmpR-Flag derivatives were affinity purified using Flag-M2 affinity gel (Sigma) as described previously (Shingler and Pavel, 1995). The bead-bound proteins were resuspended as a slurry in ATPase assay buffer [35 mM Tris-acetate pH 7.9, 5 mM Mg(OAc)₂, 70 mM KAc, 20 mM NH₄Ac, 1 mM dithiothreitol (DTT)] and kept on ice until their use in ATPase or [¹⁴C]phenol-binding assays. The concentration of bead-bound proteins was determined by SDS-PAGE comparison of a serial dilution of each sample against standards of known concentration in the linear 0.5–3 μ g range and quantification by densitometry. His-tagged derivatives were purified using 1 ml nickel chelating columns (Pierce) equilibrated with buffer A (20 mM NaPO₄ pH 7.2, 0.5 M NaCl, 5 mM imidazole, 10% glycerol, 0.1% ultrapure Triton X-100). Protein-loaded columns were extensively washed with buffer A containing 100 mM imidazole and the His-tagged DmpR derivatives recovered by stepwise elution using buffer A containing 0.2–1 M imidazole. The most concentrated fractions were clarified through gel filtration Bio-Spin P30 columns (Bio-Rad) equilibrated with storage buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 2 mM β -mercaptoethanol, 30% glycerol, 0.25% ultrapure Triton X-100) and stored at -80°C . Protein concentrations were determined by a BCA (bicinchoninic acid) assay (Pierce) with bovine serum albumin (BSA) as a standard. This purification procedure, when used with a DmpR His-tagged derivative with the ATP-binding mutation G268S, yielded a preparation with no detectable ATPase activity (data not shown).

[¹⁴C]phenol-binding assays

Flag-tagged proteins were tested for the ability to bind custom-synthesized universally labelled [¹⁴C]phenol (5.22 Gbq/mmol; Amersham Pharmacia Biotech). Experiments were performed at a final concentration of 16 µM phenol (the *K_d* of wild-type DmpR-Flag) using bead-bound proteins, with background levels bound to a ΔA-NifA-Flag derivative subtracted to determine specific binding, as previously described (O'Neill *et al.*, 1998). Values are expressed as a percentage of [¹⁴C]phenol bound by wild-type DmpR-Flag.

ATPase assays with Flag-tagged proteins

ATPase assays were performed at 30°C in 1× ATPase assay buffer (see above) and 1 mM 2-methylphenol where indicated, as previously described (Shingler and Pavel, 1995). Reactions (60 µl) contained 1 µl of bead-bound protein (0.3 µg). Assays were initiated by the addition of [γ-³²P]ATP (Amersham Pharmacia Biotech) diluted (1:9) with unlabelled ATP and added to a final concentration of 3 mM.

Coupled *in vitro* transcription and ATPase assays

Plasmid pTE-Po, which carries the binding sites for DmpR and the *dmp* operon promoter and directs formation of a 311 base RNA (Carmona *et al.*, 2000), was used as the DNA template. Supercoiled plasmid DNA was prepared by CsCl gradients, extensively dialysed and clarified through Micro Bio-Spin P30 columns (Bio-Rad) equilibrated with sterile water. Single-round transcription assays were performed at 37°C by a method adapted from Claverie-Martin and Magasanik (1992). Core enzyme (20 nM) and 80 nM σ⁵⁴ were mixed in transcription buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.275 mg/ml BSA) containing [γ-³²P]ATP (3 mM, diluted 1:9) for 5 min to allow holoenzyme formation. Template (5 nM), IHF (10 nM), His-tagged DmpR derivatives (100 nM) and the indicated concentration of effector were then added to initiate the reaction. For measurement of ATPase hydrolysis, samples were removed at 10 min intervals and treated as described above. For transcripts, after 15 min incubation at 37°C to allow open complex formation, an aliquot of the same mix was placed in a separate tube and a single round of transcription was initiated by adding a mixture of ATP, GTP, CTP (final concentration 0.4 mM each), UTP (final concentration 0.06 mM), [α-³²P]UTP (5 µCi at >3000/mmol) and heparin (0.1 mg/ml, to prevent re-initiation). After an additional 10 min at 37°C, the reactions were terminated and analysed on a 7 M urea/4% polyacrylamide sequencing gel; the density of bands was quantified using a Molecular Dynamics PhosphorImager.

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