Characterisation of the Putative Effector Interaction Site of the Regulatory HbpR Protein from *Pseudomonas azelaica* by Site-Directed Mutagenesis

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Abstract

Bacterial transcription activators of the XylR/DmpR subfamily of σ^54^-dependent regulatory proteins play pivotal roles in controlling gene expression in bacterial aromatic compound catabolism [1,2]. Classical and very well characterized examples include XylR, the primary activator for the *xyl* genes of the TOL plasmid for toluene and xylene degradation in *Pseudomonas putida* mt-2 [3], DmpR, the sole transcription activator of the *dmp* genes for phenol and o-cresol metabolism in *Pseudomonas* sp. strain CF600 [4], and TouR, from *Pseudomonas stutzeri* OX1 [5]. A large number of more diverse members of the same subfamily have been identified in the course of the recent years, among which are PhnR from members of the same subfamily have been identified in the course of the recent years, among which are PhnR from *Pseudomonas sp.* strain CF600 [4], and TouR, from *Pseudomonas stutzeri* OX1 [5]. A large number of more diverse members of the same subfamily have been identified in the course of the recent years, among which are PhnR from *Pseudomonas* sp. strain PKO1 [8].

Despite extensive genetic and biochemical data on XylR and DmpR, there is still no clear picture on the A-domain residues implicated in effector interaction, neither does a clear hypothesis exists on the mechanism of effector-mediated triggering of the activation process. Most information so far comes from the analysis of XylR and DmpR, from screening of spontaneous

Introduction

Transcription activators of the XylR/DmpR subfamily of σ^54^-dependent regulatory proteins play pivotal roles in controlling gene expression in bacterial aromatic compound catabolism [1,2]. Classical and very well characterized examples include XylR, the primary activator for the *xyl* genes of the TOL plasmid for toluene and xylene degradation in *Pseudomonas putida* mt-2 [3], DmpR, the sole transcription activator of the *dmp* genes for phenol and o-cresol metabolism in *Pseudomonas* sp. strain CF600 [4], and TouR, from *Pseudomonas stutzeri* OX1 [5]. A large number of more diverse members of the same subfamily have been identified in the course of the recent years, among which are PhnR from *Burkholderia satissoli* RP007 (regulating phenanthrene metabolism) [6], HbpR from *Pseudomonas azelaica* (activating 2-hydroxybiphenyl metabolism) [7] and ThiT from *Burkholderia picketti* PKO1 [8].

XylR/DmpR subfamily members belong to the even larger class of NirC-type transcription regulators, which are involved in a variety of physiological processes in response to diverse environmental signals [9]. Generally, these transcriptional activators act at a distance of 100 to 200 bp from the actual promoter by binding to what are called enhancer-like elements or upstream activating sequences (UAS) [10]. In addition, they specifically interact with σ^54^-dependent RNA polymerase [1]. A further hallmark of proteins from this family is the presence of two conserved domains, one of which is called the central C-domain and contains a triple-AAA ATPase motif [11]. The C-domain is supposed to interact with σ^54^-dependent RNA polymerase and hydrolyzes ATP, perhaps to facilitate open transcriptional complex formation. The second conserved feature of these proteins is a carboxy terminal D-domain, which contains a typical helix-turn-helix DNA binding motif and is implicated in interaction with the UAS-DNA [12]. In contrast to NtrC, members of the XylR/DmpR subfamily have a distinct N-terminal or A-domain necessary for recognition of chemical effector molecules that unleashes activity of the transcription activator [13]. A further small region called the B-domain or Q-linker because of its abundance in glutamine residues, connects the A- and the C-domain. It is supposed to act as a flexible molecular hinge, releasing intramolecular repression by the A-domain and exposing the ATP-ase activity of the C-domain upon recognition of the effector [14]. Indeed, XylR and DmpR mutants devoid of their A-domain act as constitutive transcription activators on their cognate promoters [15,16]. Importantly, however, an A-domain deletion of the distantly related HbpR protein (see below) is constitutively repressed [17].
mutants [18], of mutants obtained by directed evolution [19] or by DNA family shuffling [13,20], and of mutants obtained by site-directed mutagenesis [21]. Attempts to obtain direct structural information on the proteins of this subclass have been frustrated by the difficulty to purify and stabilize the full protein. Nevertheless, a structural model for the XylR A-domain was proposed based on low but pertinent similarity to the A-chain of eukaryotic catechol O-methyl transferase [22]. This model, however, consists of only a single XylR A-domain protomer whereas our current hypothesis predicts that proteins from this class undergo an activation cycle of multimerization and multimer disassembly [23].

The goal of the current work was to identify the residues critical for effector-mediated triggering in the HbpR protein from P. azelaiaca [7,17]. In its native host, the hbpR gene product regulates expression from two promoters, called the Pc and Pd promoters, which are located in front of two small operons (hbpCA and hbpD) encoding the enzymes for initial steps of 2-hydroxybiphenyl (2-HBP) degradation (Fig. 1A) [24]. The hbpR gene is located directly upstream of and is divergently oriented from the hbpCA and hbpR genes. HbpR displays only 37% amino acid sequence identity with XylR, and in contrast to XylR and DmpR, is responsive to biaromatic compounds such as 2-HBP, 2,2’-dihydroxybiphenyl, 2-aminobiphenyl and 2-hydroxydiphenylmethane [7]. XylR and HbpR display detectable but little crossbinding to each other’s DNA binding sites although hybrid promoters can be produced that are activated by both XylR and HbpR in the same cell [25]. In contrast to XylR and DmpR, the Q-linker of HbpR is shorter and A-domain deletions of HbpR result in a constitutive repressor protein [17]. Since such A-domain deletions are made without any protein structure basis, it is possible that they accidentally produce different effects in HbpR and XylR or DmpR.

In order to decipher possible determinants in the A-domain of HbpR for 2-HBP-mediated triggering, we assumed that 2-HBP would interact with specific residues exposed to the A-domain surface. To make a more rational guess on the choice of residues to investigate, we expanded the modeling approach previously developed for the A-domain of XylR [22] to predict a tertiary structure for that of HbpR, and predicted the regions of possible 2-HBP interaction using small effector binding domain of HbpR or close relative has been established XylR model as template. (A) Organization of the hbp regulatory system and tertiary structure modeling of the HbpR A-domain using a previously described XylR model as template. (A) Organization of the hbp genes and the location of the HbpR binding sites (UAS, upstream activating sequences) in front of the Pc and Pd promoters. HbpR domains are depicted to scale according to the predictions by Jaspers et al [7], (B) to (E) Fitting used SWISS-MODEL and was performed on XylR A-domain PDB coordinates as calculated by Devos et al [22]. (B) Ribbon model of HbpR A-domain residues 11–209, with predicted coils, alpha-helices and beta-sheets indicated. (C) Superposition of the predicted HbpR and XylR A-domains in the same conformation as A. (D) Tertiary structure model of HbpR A-domain with calculated molecular surface at 1.4Å and 40% transparency, in order to see the helical, coil and sheets. Model turned into a position which enables visualization of the proposed tunnel entry (b). C-terminal end of coil ending the A-domain indicated with an arrow at (a). Pinkish region in the centre of the A-domain illustrates a predicted cavity within the A-domain. (E), as C but now for the XylR A-domain, with exception of the ten most C-terminal residues, which otherwise are predicted to occlude the tunnel.

doi:10.1371/journal.pone.0016539.g001

Results

Prediction of A-domain folding and the 2-HBP interaction site on HbpR

To make a rational prediction of which amino acids in the HbpR A-domain could be implicated in effector interaction, its tertiary structure was modeled. Because no crystal structure of the effector binding domain of HbpR or close relative has been determined, the domain was modeled using a bioinformatics approach similar as proposed earlier for XylR [22]. Directly fitting a tertiary structure model for the HbpR A-domain failed because of too low homology to any existing structures in the PDB database [26]. The first 218 amino acids of HbpR were thus structurally aligned to the XylR and DmpR A-domain computational models [22] as templates using the program SWISS-MODEL [26,27]. The computed HbpR A-domain model for the amino acids 9–211 displayed eight alpha helices and five beta strands (Fig. 1B, C). As expected using these templates, the predicted shape for the HbpR A-domain was highly similar to those of XylR and DmpR with exception of a few loops (Fig. 1D, E). The HbpR A-domain C-terminal end is predicted to be coiled instead of forming beta sheets as in XylR and DmpR, but it should be noted that the model does not take the A- and C-domain connection of the protein into account. Interestingly, the HbpR A-domain model...
predicted one face of the tertiary structure to have an overall more negative electric potential than the opposite face, which may favor dimeric A-domain interactions (not shown).

The A-domain model for HbpR was then used as a template to predict the possible sites of interaction with its effector 2-HBP (Fig. 2A, D, E). Potential sites for 2-HBP interaction were calculated by using the program GRAMM, which uses Fast Fourier transformation to predict the energetically most favorable matches of a ligand on the modeled protein surface [28]. Interestingly, GRAMM calculations predicted that there would be an ‘interface’ region most favorable for interaction with 2-HBP rather than a single residue or active site, which upon closer inspection of the model seemed to provide a cavity (Fig. 2A). Among one thousand iterations, the program predicted almost exclusively interactions in this particular region. A number of amino acid residues such as E184 were located in this region (Fig. 2A, B), which upon mutation in XylR had been demonstrated to broaden effector-mediated induction [29]. In addition, a similar region had been predicted from the XylR A-domain model to be of potential interest to effector binding, even though few mutations had been generated in that part of the protein [22]. The main hypothesis in this work was therefore that this interface region would be critical for 2-HBP-mediated triggering of HbpR activation.

Design and construction of HbpR mutants

Two groups of mutations were created to validate or refute our hypothesis: a first group, which concentrated on a number of amino acid residues in this region conserved between HbpR, XylR and DmpR. Mutations in this group were designed to alter the chemical nature of the residue (i.e., charged to non-charged, hydrophobic to hydrophilic). In the second group we designed mutations, which would ‘block’ the cavity by the bulky amino acid phenylalanine. Because such drastic replacements by Phe could have secondary effects on protein performance, we created a number of control mutations on residues not predicted to be directly at the cavity interface (Fig. 2).

An overview of all mutants constructed in the first and the second group is presented in Table 1. All mutations in the HbpR A-domain were constructed by PCR with mutated oligonucleotides and verified by DNA sequencing. Subsequently, the mutated A-domain sequences were used to replace the gene region for the native A-domain in hbpR on an expression vector in E. coli, with which we could test 2-HBP inducible egfp expression under control of the HbpR-dependent Pₐ promoter. This expression vector results in the addition of a His6-tag to the N-terminal end of the protein. All mutants were tested in E. coli for EGFP expression during exponential growth in the presence or absence of 20 μM 2-HBP, which is the cognate effector for the HbpR-Pₐ system. Table 1 gives representative EGFP induction values after 2 and 4 h incubation periods compared to those of the strain carrying wild-type HbpR. In general and for all mutants, we observed four types of effects: (i) complete loss of activation with respect to the wild-type (type I), (ii) two-fold loss of induction potential in 2 h but not 4 h incubation periods (type II), (iii) no effect compared to the...
**Table 1.** Fluorescence intensities in *Escherichia coli* expressing EGFP from Pc under control of HbpR wild-type or its mutants, in the presence or absence of 2-hydroxybiphenyl.

| Mutant Class | Residue | 2 h induction time | 4 h induction time |
|--------------|---------|--------------------|--------------------|
|              |         | Ni\(^a\) | Ratio to WT | Induced | IF | Ni | Ratio to WT | Induced | IF |
| I            | Cys187Phe | 505±18\(^b\) | 0.96 | 582±53 | 1.2 | 538±14 | 0.97 | 597±18 | 1.1 |
| I            | Glu184Phe | 487±21 | 0.92 | 615±92 | 1.3 | 589±23 | 1.06 | 648±13 | 1.1 |
| I            | Glu184Leu | 536±11 | 1.01 | 657±43 | 1.2 | 603±43 | 1.08 | 684±13 | 1.1 |
| I            | Thr52Phe | 544±21 | 1.03 | 665±14 | 1.2 | 639±24 | 1.15 | 1411±72 | 2.2 |
| II           | Ile180Phe | 577±12 | 1.09 | 723±21 | 1.3 | 688±22 | 1.24 | 599±253 | 8.7 |
| II           | Val182Thr | 537±18 | 1.02 | 878±84 | 1.6 | 572±23 | 1.03 | 651±35 | 11.4 |
| II           | Ile180Thr | 458±17 | 0.86 | 1709±48 | 3.7 | 474±17 | 0.85 | 3854±100 | 8.1 |
| II           | Leu207Phe | 482±18 | 0.91 | 1213±53 | 2.5 | 542±25 | 0.97 | 5733±144 | 10.6 |
| II           | Ile56Thr | 481±16 | 0.91 | 2161±16 | 4.5 | 582±16 | 1.05 | 6550±139 | 11.3 |
| III          | Glu203Leu | 544±18 | 1.03 | 2731±80 | 5.0 | 662±21 | 1.19 | 8571±146 | 12.9 |
| III          | Gin209Leu | 546±21 | 1.03 | 2977±127 | 5.5 | 697±32 | 1.25 | 8331±162 | 12.0 |
| III          | Glu42Phe | 472±11 | 0.89 | 2792±225 | 5.9 | 497±27 | 0.89 | 7853±125 | 15.8 |
| III          | Glu203Gln | 569±14 | 1.08 | 3152±147 | 5.5 | 751±29 | 1.35 | 10141±650 | 13.5 |
| III          | Glu203Pro | 580±14 | 1.1 | 3147±178 | 5.4 | 754±34 | 1.35 | 11120±530 | 14.7 |
| III          | Val50Phe | 493±12 | 0.93 | 3151±104 | 6.4 | 574±29 | 1.03 | 8028±91 | 14.0 |
| III          | Lys178Phe | 510±11 | 0.96 | 3334±169 | 6.5 | 611±23 | 1.09 | 8567±145 | 14.0 |
| III          | Wild-type | 529±21 | 1 | 3503±260 | 6.2 | 557±21 | 1 | 8858±195 | 15.9 |
| III          | Trp205His | 540±19 | 1.02 | 3554±231 | 6.6 | 673±32 | 1.21 | 8931±263 | 13.3 |
| III          | Val181Thr | 563±12 | 1.06 | 3589±136 | 6.6 | 694±40 | 1.24 | 9536±257 | 13.8 |
| III          | Leu60Asn | 511±13 | 0.97 | 3615±103 | 7.1 | 618±16 | 1.11 | 9443±172 | 16.0 |
| III          | Glu183Phe | 547±18 | 1.03 | 3653±136 | 6.5 | 683±38 | 1.23 | 9903±87 | 14.5 |
| III          | Glu183Gln | 595±15 | 1.1 | 3836±125 | 6.5 | 730±29 | 1.31 | 9493±126 | 13.0 |
| III          | Gin188Glu | 692±11 | 1.31 | 3853±146 | 5.6 | 839±42 | 1.51 | 10766±118 | 12.8 |
| III          | Ile185Thr | 470±16 | 0.89 | 3896±163 | 8.3 | 548±21 | 0.98 | 8094±218 | 14.8 |
| III          | Gin209Phe | 456±23 | 0.86 | 3950±165 | 8.7 | 612±18 | 1.09 | 7465±187 | 12.2 |
| IV           | Val181Phe | 1049±26 | 1.98 | 4368±104 | 4.2 | 1647±63 | 2.96 | 10185±212 | 6.2 |
| IV           | Ser32Phe | 828±19 | 1.56 | 4626±108 | 5.6 | 1598±72 | 2.87 | 11432±283 | 7.2 |
| IV           | Ala202Ser | 2184±35 | 4.18 | 5156±153 | 2.4 | 1923±57 | 3.45 | 11072±157 | 5.8 |

\(^{a}\) NI, non induced conditions and ratio of Ni-fluorescence in mutant and that of wild-type; IF, induction factor, calculated by dividing culture fluorescence with 2-hydroxybiphenyl by that of the culture in the absence of 2-hydroxybiphenyl.

\(^{b}\) Averages from biological triplicates with calculated standard deviation.

doi:10.1371/journal.pone.0016539.t001

wild-type (type III), and (iv) considerable increase of background expression (type IV, Table 1, Fig. 3). Protein extracts of the same strains were analyzed by Western blotting using an M13-V\(^{HH}\) camel antibody to verify (mutant) HbpR expression (Fig. 4). Surprisingly, all Westerns showed two bands, which likely correspond to His\(^{6}\)-tagged HbpR (or mutant, 64.1 kDa) and HbpR (mutant) without His\(^{6}\)-tag (62.8 kDa). The reason for the production of two N-terminally different HbpR proteins probably lies in the use of an alternative start codon further downstream. The expression level of most HbpR mutant proteins in *E. coli* was similar to the wild-type, except for L207F (low outlier) and E203P (high outlier) (Fig. 4; Text S1).

The first group of mutations directed to changing the chemical character of conserved residues among XylR/DmpR/HbpR A-domains produced the following results. E184L (equivalent position in XylR E172, Text S1) completely abolished EGFP expression from the HbpR dependent P\(_c\) promoter (type I). To a lesser extent, also mutations V182T and H180T drastically reduced EGFP induction upon 2-HBP addition – mostly after 2 h, but after 4 h induction time the difference to the wild-type was less pronounced (type II, Table 1, Fig. 3). Other residues, mutation of which reduced activation potential by 2-HBP, were I56T, E203L, E203Q and E209L (Table 1). By contrast, mutation of the chemical character of residues in this vicinity, e.g., W205H, V181T, L60N, Q188E and I185T, did not significantly affect 2-HBP-dependent activation in *E. coli* (type III). Interestingly, changing Ala202 to Ser resulted in a fourfold higher EGFP expression in the absence of 2-HBP as compared to wild-type HbpR (type IV). This suggested that several residues in this area indeed affected activation of expression by 2-HBP, but only Glu184 seemed absolutely critical.
HbpR-dependent 2-HBP-inducible EGFP expression in E. coli (Table 1). As for mutant E184L, also E184F completely abolished inducible egfp expression from P_C. Similar effects were caused by mutations C187F and T52F. Phenylalanine substitutions in Ile180 and Leu207 resulted in the delayed induction phenotype (type II). Mutations in the majority of residues had basically no effect on the magnitude or kinetic induction with 2-HBP (Glu51, Val50, Lys178, Glu183 and Gln209). All of these were located more or less in the vicinity of the proposed cavity (Fig. 2C, D), but not as close to the 2-HBP interaction region as, e.g., Thr52, Ile180, Val182 or Glu184. Interestingly, two Phe substitution (at Ser32 and Val181) produced HbpR-mutants with higher background expression in the absence of 2-HBP (type IV, Table 1, Fig. 3).

For a number of residues multiple substitutions were created, which almost in all cases produced the same effect. Both mutations in Glu184 (to Phe or to Leu), abolished induction with 2-HBP, and also both mutations in Ile180 (to Phe or to Thr) decreased 2-HBP induction (Table 1). All mutations created in Glu203 (to Leu, Gln and Pro) were more or less without large effect on 2-HBP induction. Also both mutations in Glu183 (Phe and Gln) produced the same effect. On the contrary, Val181Thr had no effect, but Val181Phe produced a higher non-inducible background. The same was found for Gln209, for which a change to Leu reduced, but change to Phe slightly increased the magnitude of egfp induction with 2-HBP.

HbpR mutant integrity

Western blotting with an anti-HbpR M13-displayed V_HH camel antibody suggested (within the accuracy of this technique) that most HbpR mutant proteins were produced to the same level in E. coli (Fig. 4), except for L207F (lower than expected) and E203P (higher than expected). This indicated that differential EGFP expression in E. coli carrying a mutant hbpR gene was not due to complete misfolding or degradation of the protein, but rather due to a critical amino acid change in the effector binding region. In particular E184L, I180T, I180F, T52F and C187F, which were the mutations causing the largest decrease of 2-HBP-dependent...
EGFP expression from $P_{C}$ resulted in HbpR mutant proteins that were expressed in *E. coli* within the normal range observed for all (Fig. 4). To corroborate this further, we purified a number of (mutant) HbpR proteins and compared their circular dichroism spectra between 200 and 250 nm. Sixteen mutant HbpR proteins and HbpR wild-type (all tagged with His6) were hereto purified by Ni-NTA chromatography, dialysed and diluted to 0.3 mg protein per ml (Fig. 5). For reasons of protein stability, it was not possible to completely omit traces of EDTA and glycerol from the dialysis buffer. As a result no reliable spectra below 198 nm could be recorded (not shown).

Whereas identical spectral traces are usually interpreted as proteins having the same solution conformation, the HbpR wild-type and 15 mutant proteins produced similar but not identical traces. Broadly we detected three types of spectra in the region between 205 and 240 nm (Fig. 5). Most mutant proteins differed very little from the HbpR wild-type circular dichroism (Fig. 5B). Three mutants (V182T, L207F and T52F) deviated specifically in the region 206–212 nm (Fig. 5A), and mutants C187F, E184L, E42F, E203P and I56T differed more strongly in the region 205–220 nm (Fig. 5C). This indicates, therefore, that some HbpR mutant proteins adopt different configurations than HbpR wild-type (folding, or multimerization in solution). However, since mutant and wild-type protein expression in *E. coli* was more or less similar (except for L207F and E203P), we conclude that different circular dichroism profiles reflect the immediate refolding effect of a mutation but are not indicative for complete misfolding, or else the phage antibody would not have recognized the protein. Moreover, a number of mutant HbpR proteins with slightly different scans still retained normal induction potential. For example, E203P and E42F showed circular dichroism scans clearly different from wild-type HbpR and similar to C187F and I56T. Yet, E203P and E42F maintained induction potential similar as wild-type HbpR, whereas C187F and I56T were impaired (Table 1). By contrast, proteins V182T, L207F and T52F were all impaired in activation potential and their circular dichroisms differed from wild-type. Therefore, we conclude that some mutations cause different partial folding, but this does not necessarily lead to an overall change in protein configuration such that it renders the protein inactive and would cause the lack of induction with 2-HBP. Thus, effects on 2-HBP-dependent EGFP expression from $P_{C}$ must have been the genuine consequence of a change in a critical effector binding region or residue.

### Complementation of the mutants with the wild type HbpR

Next, we tested whether the created HbpR mutations were dominant over wild-type HbpR, which would be a further indication for their activity in *E. coli*, since we previously demonstrated that an HbpR mutant devoid of the A-domain was dominant negative on wild-type HbpR [17]. Hereto, the A-domain mutant strains of HbpR in *E. coli* were complemented with a plasmid expressing wild-type *hbpR* from its native promoter (pHBP124). For all type I mutants (loss of induction), complementation with wild-type HbpR restored 2-HBP inducible activity although not to the level of wild type response (Fig. 6). Mutants C187F and I180F reverted to 3/4th of the lost activity upon complementation with wild-type. This might be the result of formation of heteromultimers between wild-type HbpR and mutant protomers, which do not fully restore functionality. It is worth noticing that for mutations W205H, I185T and Q188E, which did not affect the activity of the protein, complementation reduced the response to 2-HBP. This effect could also be seen with the semi-constitutive mutants A202S, V181F and S32F; complementation with the wild-type HbpR decreased the response upon induction (Fig. 6). Such mutations may therefore affect heterodimer formation.
formed the basis to hypothesize that this domain readily adopts a "slim protein" configuration of ‘open’ flexibility towards new effector substrates [19].

In this work we extended the structural model of the XylR A-domain to predict that of the distantly related protein HbpR. Even though the basis for structural modeling of the A-domain is weak and only based on the alignment of the HbpR A-domain to the model of XylR A-domain, which on its turn is based on that of catechol O-demethylase (PDB entry 1vid), it allowed us to formulate a direct testable hypothesis for the implication of a number of amino acid residues in 2-HBP recognition. On the basis of the structural prediction and subsequent calculation of the energetically most favorable region for interaction of 2-HBP on the modeled protein surface by GRAMM [28], we identified one region with an exposed cavity (Fig 2E, F). Indeed, site-directed mutagenesis of residues in this area identified several critical and non-critical amino acids for 2-HBP-mediated HbpR activation of the P4-promoter (Text S1). Notably, these were Glu184, emphasizing a residue with also critically conserved importance in XylR and DmpR (Text S1), Cys187 (conserved), and Ile180 (conserved), both of which had not been detected previously by mutagenesis on XylR or DmpR. Other residues, mutation of which reduced but not completely abolished activation potential by 2-HBP, were Ile56, Val182 and Leu207 (Text S1). HbpR-L207F seems to be produced less efficiently in E. coli, but the other mutant HbpR proteins were correctly produced in E. coli, albeit with detectable folding differences (Fig. 5). Hence, we conclude that these residues are of critical importance to 2-HBP effector mediation in HbpR. Since our in vivo assays only measure the outcome of 2-HBP-mediated activation by HbpR (mutants) on P4-expression, we cannot conclude whether those amino acid residues are implicated in 2-HBP ‘binding’ or in some other step of the activation pathway.

Highly speculatively, but still interesting, was the prediction of a cavity in the structural model of the HbpR A-domain surface where 2-HBP would interact. The importance of this cavity for 2-HBP mediated activation of HbpR was investigated further by systematically changing its residues [i.e., Val50, Thr52, Ile56, Leu60, Leu207 on one side, and Ile180, Val182, Glu184, Cys187, Gln198], but Western results did not indicate that this misfolded the protein completely (Fig. 5). Of all these, Ile180, Glu184 and Cys187 are conserved among XylR, DmpR and HbpR, whereas Thr32 is not (Text S1). Contrary to these critical residues, changes in Glu203, Glu184, Val182, Thr205 and Gln209 had no major effects, even though they were predicted to be near the cavity. It is likely that HbpR proteins with these mutations in their A-domains adopted slightly different local configurations than wild-type, as was demonstrated from circular dichroism scans of purified proteins (Fig. 5), but Western results did not indicate that this misfolded the protein completely (Fig. 4). Furthermore, certain Phe-substitution mutants with altered CD scans compared to wild-type HbpR did not display loss of function (Fig. 5, Table 1). Therefore, even though it is difficult to unequivocally decide whether bulky mutant residues cause loss

Finally, we tested whether any of the mutants were different in response to aromatic compounds not known to activate wild-type HbpR. Hereto we chose 2-chlorobiphenyl (at 100 μM), which is the effector for certain HbpR mutants obtained by directed evolution approaches [30], and toluene (at 20 μM), which is an effector for XylR. Incubation of the HbpR mutant series or wild-type with 2-chlorobiphenyl did not produce any significant induction compared to non-amended cultures (not shown). None of the mutants or wild-type were responsive to toluene either. Toluene (at 20 μM) reduced by 10% the induction obtained with 32 μM 2-HBP in a co-induction assay for wild-type HbpR and the mutants I180F, V181T, I185T, E203L, E203Q and W205H (not shown). In a few other mutants (I56T, E183F and K178F) coincubation with 20 μM toluene and 32 μM 2-HBP, which essentially produced the same EGFP expression from the HbpR-dependent P4 promoter (Text S1).

Discussion

Although previous work by several groups have clearly demonstrated the importance of the A-domain of proteins of the XylR/DmpR subfamily of transcription activators in effector recognition, the actual effector ‘binding’ region and the type of effector interaction have largely remained elusive. A plethora of A-domain mutations has been produced for XylR and DmpR (Text S1), which highlighted several residues in activation function. A conceptual breakthrough was proposed by Devos and coworkers in 2002, who developed a structural model for the XylR (and DmpR) A-domains on the basis of a weak but significant structural homology to catechol O-demethylase from Rattus norvegicus. Placement of the various mutated residues and their effects on the modeled structure seemed to indicate that the A-domain is highly ‘prone’ to allow changes in effector recognition but at various unexpected secondary positions. More recently, this
of function because they change a critical residue or because they partial unfold the protein, our results suggests that this region is indeed a very critical one for proper 2-HBP-mediated activation of HbpR and that its most essential residues are Thr52, Ile180, Glu184 and Cys187. Interestingly, mutations in such residues did not change the effector spectrum of HbpR and neither did the other generated mutations, whereas previous mutations obtained by directed evolution of HbpR that did change the effector spectrum from 2-HBP to 2-chloroippingen mapped in completely different areas of the A-domain (Text S1). Only some mutations, e.g. Ala202, mapped in a region which by directed evolution was shown to conceive a semi-constitutive phenotype (Text S1). This, and given the fact that three of them are conserved in XylR and DmpR (which do not react to 2-HBP), might suggest that the identified critical residues (e.g., Thr52, Ile180, Glu184 and Cys187) not so much directly 'bind' 2-HBP but are somehow important in transmitting an effector-interaction to activation of the transcriptional regulator. This for XylR and DmpR would consist of derepressing the ATPase activity, but for HbpR might consist of activating it [17].

Again, very suggestively but of potential importance for a novel activation mechanism concept, the observed predicted cavity enters into an opening the A-domain of HbpR (Fig. 1C). HbpR’s A-domain is a little shorter at its C-terminal end and extends less into the B-linker than for XylR or DmpR, which makes this cavity more pronounced. Although the cavity is not visible in the XylR A-domain model as proposed by Devos [22], this is only because of the C-terminal extension of 12 residues in their model. Removal of the C-terminal beta-sheet shows that the XylR A-domain also adopts such a cavity and produces an opening (Fig. 1D). Obviously, this part of the modeling is highly speculative, because the A-domain part of the protein connects to the C-domain via the proposed flexible B-linker and this connection loop cannot be properly assigned a structure without a good template. Modeling of the C-domain of both HbpR and XylR is possible, because of reasonably high homology (41.2% for HbpR to the resolved crystal structure of NtrC1 of *Aquifex aeolicus* (PDB entry 1NY5_B). Unfortunately, this homology does not extend into the B-linker region of some 30 amino acid residues (not shown), making structure predictions for the connecting region between A- and C-domains premature. The importance for mentioning this cavity, however, is that it conceptually would offer a new hypothesis for effector mediated activation of proteins in this family, which so far is not solved satisfactorily [31]. Instead of having a classical active site ‘pocket’, one could imagine that the flexible B-linker region occludes or ‘opens’ the proposed cavity and opening through which effectors pass, and that this triggers an intramolecular conformational change needed to expose the C-domain ATP-ase and activate RNA polymerase.

In conclusion, therefore, our results highlight the importance of a region on the HbpR A-domain for effector (2-HBP) control. Critical residues for effector control in this region were identified, some of which are conserved with XylR and DmpR, thus ruling out a direct role in effector binding. Model predictions were reasonably correct with experimental data. As a new hypothesis for effector mediated control on this type of proteins, we propose a model for activation in which the effector compound would pass through a surface crevice instead of binding to a pocket and subsequently being released from the same pocket. Whether or not the surface crevice needs to be made by a single protomer of the activator protein or a dimer remains to be determined. Direct binding studies of radio-actively labeled phenol to the DmpR A-domain indicated a fraction of up to 0.6 mol substrate bound per mol protein [32], which could be interpreted as a not yet saturated system in which one effector molecule would bind one protomer, or as a slightly oversaturated system in which one effector molecule binds a protomer dimer. Such a model for a surface crevice and tunnel would also help to understand the large variety of mutations that influence effector specificity and semi-constitutive phenotypes, which have been discovered over the years in XylR, DmpR and HbpR (Text S1). The reason for this would be that any mutation in the A-domain that somehow changes this crevice and permits entry to the tunnel may facilitate another effector to activate the complex. In addition, it could provide a mechanistic interpretation for the effector-mediated activation. The current hypothesis for activation states that only multimeric forms of XylR or HbpR (hexameric or heptameric, depending on the model) are capable of activating σ^44 RNA polymerase. A plausible mechanistic model for such an activation process would be a slight conformational change induced in the hexameric complex by a torsion from a number of effector molecules passing through the cavity-opening.

**Materials and Methods**

**Strains, media and general growth conditions**

*E. coli* recombinant strains were generally cultivated on Luria Bertani (LB) medium [33], supplemented with 50 μg/ml kanamycin to select for the presence of the plasmid carrying *hbpR* or its mutants plus a transcriptional fusion between the HbpR regulatable *P_c* promoter and *egfp*. For induction experiments *E. coli* strains were incubated in MOPS medium (i.e., per liter, 10 g 3-(N-morpholino)propanesulfonic acid, 1 g NH4Cl, 0.5 g NaCl, 0.06 g Na2HPO4·2H2O, 0.045 g KH2PO4, 20 mM MgCl2, 1 mM CaCl2, and 2 g glucose, pH 7). Liquid cultures were generally incubated at 37°C with 180 rpm rotary shaking, except for induction experiments, which were carried out at 30°C. Bacterial colonies were grown on LB medium solidified with 1.5% agar and incubated at 30°C or 37°C.

**HbpR activation assays**

In order to test HbpR- or HbpR-mutant dependent *egfp* activation from the *P_c* promoter in *E. coli*, we applied the protocol essentially as described previously [30]. Single pure *E. coli* cultures were grown for 16 h at 37°C in LB medium plus kanamycin and diluted fifty fold in fresh medium of the same. Cells were re-grown until the turbidity in the culture reached 0.4 (at 600 nm), after which they were centrifuged at 2,500 × g and resuspended in the same volume of MOPS buffer. This cell suspension was used for induction assays. For induction, 200 μl of *E. coli* cell suspension was mixed with the effector (20 μM 2-HBP, 100 μM 2-chlorophenol) in a 96-well microtitre plate. Plates were incubated at 30°C with 180 rpm rotary shaking for periods of 2 and 4 h, after which the EGFP fluorescence signal was measured per fluorimetry (FluoStar Galaxy, BMG Labtech GmbH, Offenburg, Germany). Incubations with dimethylsulfoxide and water only served as negative, non-induced controls. Assays were performed in triplicate. Induction experiments with 20 μM tolune were carried out in 2 ml glass vials closed by Teflon-lined caps to avoid evaporation. In this case 0.2 ml of the cell suspensions was transferred after 2 and 4 h into a 96-well microtitre plate for fluorescence measurements.

**DNA cloning techniques and DNA sequencing**

Recombinant DNA techniques were all carried out according to well-established procedures [33]. PCR mutagenesis mixtures were prepared as suggested by the suppliers of the Pfu Polymerase (Promega) and run on GeneAmp PCR System thermocyclers (Applied Biosystems, CA, USA). DNA was sequenced using the
BigDye Terminator cycling method (v3.1, Applied Biosystems) and analyzed on ABI Prism 3100 capillary sequencers (Applied Biosystems). Kits for purification of PCR products or of DNA fragments from agarose gels, and for isolation of plasmid DNAs from E. coli were used according to the specifications given by the suppliers (Qiagen, Promega).

Chemical substances
2-hydroxybiphenyl (2-HBP), 2-chlorobiphenyl and toluene were obtained from Sigma-Aldrich. Stock solutions were prepared at 20 mM in dimethylsulfoxide, which were kept at 4°C in the dark. All other chemicals were of the highest purity grade available.

Bioinformatics analyses
Common bioinformatics analyses were performed using tools provided by the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) with default settings (http://swissmodel.expasy.org). The 218 first amino acids corresponding to the A-domain of the HbpR protein were structurally aligned using MAGICFIT to a pdb-model of those of XyR and DmpR (http://www.pdg.cnb.uam.es/XyR), and further refined with the help of the online Workspace program SWISS-MODEL [26,27]. Further three-dimensional analyses were conducted using the DeepView/Swiss-Pdbviewer program version 4.01 for OS.X [26].

Docking
Hypothetical docking positions of 2-HBP on HbpR were computed using the GRAMM program (Global Range Molecular Matching, [28]). The three-dimensional HbpR A-domain model and the 2-HBP pdb-files were submitted to GRAMM with the following parameters: Matching mode = generic; grid step = 1.7; repulsion = 20.0; Attraction double range = 0.0; Potential range following parameters: Matching mode = generic; grid step = 1.7; and the 2-HBP pdb-files were submitted to GRAMM with the further refined with the help of the online Workspace program of XylR and DmpR (http://www.pdg.cnb.uam.es/XyR), and this technique.

Six HbpR mutants (Ile180Thr, Glu183Gln, Glu184Leu, transformed into E. coli modified to introduce 6 His-codons in the protein, after which the hbpR was amplified in two parts independently by PCR, the junction of two different methods. In the first method, the hbpR wild-type A-domain sequence on pHBP124 with wild-type hbpR expressed from its native promoter [34], in order to test dominance of the created mutation.

HbpR and HbpR mutant purification
HbpR and HbpR mutants were overexpressed and purified from E. coli. Hereto we fused the hbpR start codon to the ATG triplet present in the Ndel site of pET15d (Stratagene). This will produce an N-terminal His6-tag to hbpR. The hbpR gene was first amplified from the P. azelraica HBP1 chromosomal DNA by PCR with primers Ndel-HbpR (5’-GCCATATGAAATCAATATAA-AATATACGGAGC-3’; The Ndel site is underlined) and BamHI-HbpR (5’-GGGATCCCTATGTGATCTTGGACGC-3’; the BamHI site is underlined). The 1710-bp PCR product was digested by Ndel and BamHI and ligated to pET15, digested with the same enzymes. After transformation into E. coli BL21 (DE3) this resulted in plasmid pHBP240. The integrity of the hbpR open reading frame was verified by DNA sequencing.

E. coli BL21 (DE3) containing pHBP240 was grown at 30°C in LB medium to an optical density at 600 nm of 0.6. To induce T7 RNA polymerase expression isopropyl-B-D-thiogalactopyranoside was added at a concentration of 1 mM, and cultures were further incubated overnight at 20°C. Cells were then collected by centrifugation for 5 min at 12,000 × g, washed in the same volume of buffer containing 20 mM Tris-HCl and 2 mM EDTA (pH 7), and again centrifuged. The bacterial pellet was resuspended in 1/10 volume of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, pH 7.5) containing 2.5% of Pefabloc SC (4-2-aminoethyl-benzenesulfonyl fluoride, Roche) as protease inhibitor, and subjected to ultrasonication for five times during 20 seconds each at 60% and 40 W output (Branson 450 Sonifier). The cell extract was centrifuged for 30 min at 8,000 × g to remove cell debris. The supernatant (1/10 volume) was mixed with 1 ml of 50% Ni-NTA agarose (Qiagen) during 2 hours with stirring at 4°C, and the mixture was loaded on a polypropylene column (1 ml, Qiagen) equilibrated with 600 μl lysis buffer. After loading, the column was washed three times with

The other HbpR mutants were created by a method in which the whole plasmid was amplified by two reverse complementary primers carrying the mutations. For this purpose, we first cloned the hhpR A-domain gene region separately. The gene region with the A-domain was amplified by using the polymerase chain reaction and primers 040101 and 040102 (PCR cycle: 94°C for 2 min, followed by 25 cycles of each: 94°C for 2 min; 56°C for 30 sec; 72°C for 1 min). The PCR product was digested by SscI and BamHI and cloned into pUC18 digested by the same enzymes. The PCR product carrying the cloned A-domain gene fragment of hhpR was used as template for the mutagenic PCRs. These PCR was performed by using PyBio DNA polymerase mix and the following cycling regime: 94°C for 30 sec, followed by 16 or 18 cycles of each: 94°C for 30 sec; 55°C for 1 min; 68°C for 2 min.

The fully PCR-amplified plasmid was treated with DpnI to remove parental (methylated) template plasmid and was then directly transformed into E. coli DH5α, during which the single-stranded breaks created by the PCR are repaired. Plasmids from potential transformants were purified and sequenced to confirm the mutation. In case of successful mutation, the A-domain gene regions were recovered by SscI and BamHI digestion, and used to replace the hhpR wild-type A-domain sequence on pHBP269A0. After transformation in E. coli, those plasmids were again purified and verified for the integrity of the introduced mutation. If correct, the strains were used to test inducible egfp expression by 2-HBP from the HbpR controlled Pcrp promoter.

All hhpR mutant genes in pHBP269A0 subsequently transformed into an E. coli carrying the compatible plasmid pHBP124 with wild-type hhpR expressed from its native promoter [34], in order to test dominance of the created mutation.

EFector Binding Site of HbpR
PBS solution, to which 50 mM at 30°C. Cultures of His6-HbpR and any of the His6-HbpR mutants were diluted into 2x SDS sample buffer and added (120 mM Tris-HCl pH 6.8, 2% SDS, 2% 3-mercaptoethanol). Protein extracts were prepared by boiling the loading buffer mixtures for 10 min. Appropriate volumes were loaded on denaturing SDS-PAGE, containing 4% stacking and 8% separating gels (acrylamide/bisacrylamide 29:1; Bio-Rad), which was kept as a stock producer of the anti-HbpR antibodies. Libraries were screened multiple times by phage-ELISA for the best binder, which was kept as a stock producer of the anti-HbpR antibodies. DNA. Sequences corresponding to the antigen-binding domain for detection of HbpR protein in a PBS buffer were normalized to (Text S1).

Circular dichroism

The CD spectrum of purified His6-HbpR or of its mutants was obtained from a J810 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell with a 0.1-cm path length (L). CD spectra were measured at 25°C between 195 and 250 nm at a scanning speed of 10 nm/min and a protein concentration of 0.3 mg/ml. After subtracting the spectrum from background generated from buffer alone, the spectra for HbpR and its mutants were normalized to deltal epsilon (Δε, mg/cm•cm⁻¹), using the protein concentration (c, mg/ml) and the mean residue weight of HbpR (MRW), via the formula (http://dichroweb.cryst.bbk.ac.uk/html/userguide.shtml):

\[ \Delta \varepsilon = (0 \cdot 0.1\cdot MRW)/(3298\cdot cL) \]

Supporting Information

Text S1 Supplementary Materials. (PDF)

Acknowledgments

The authors thank Charles Vidoudez, Elena Zenaro, Artur Reimer and Mario Arcos for their help in parts of this project.

Author Contributions

Conceived and designed the experiments: CV SA HB SF JRvdM. Performed the experiments: CV SA HB SF. Analyzed the data: CV SA SF RL JRvdM. Contributed reagents/materials/analysis tools: SA SF. Wrote the paper: CV JRvdM.

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