The transcriptional regulator CprK detects chlorination by combining direct and indirect readout mechanisms

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The transcriptional regulator CprK controls the expression of the reductive dehalogenase CprA in organohalide-respiring bacteria. Desulfitobacterium hafniense CprA catalyses the reductive dechlorination of the terminal electron acceptor o-chlorophenol acetic acid, generating the phenol acetic acid product. It has been shown that CprK has ability to distinguish between the chlorinated CprA substrate and the de-halogenated end product, with an estimated an estimated 10^4-fold difference in affinity. Using a green fluorescent protein GFPUV-based transcriptional reporter system, we establish that CprK can sense o-chlorophenol acetic acid at the nanomolar level, whereas phenol acetic acid leads to transcriptional activation only when approaching micromolar levels. A structure–activity relationship study, using a range of o-chlorophenol acetic-acid-related compounds and key CprK mutants, combined with pK_a calculations on the effector binding site, suggests that the sensitive detection of chlorination is achieved through a combination of direct and indirect readout mechanisms. Both the physical presence of the bulky chloride substituent as well as the accompanying electronic effects lowering the inherent phenol pK_a are required for high affinity. Indeed, transcriptional activation by CprK appears strictly dependent on establishing a phenolate–K133 salt bridge interaction, rather than on the presence of a halogen atom per se. As K133 is strictly conserved within the CprK family, our data suggest that physiological function and future applications in biosensing are probably restricted to phenolic compounds.

1. Introduction

While halide chemistry is a mainstay of modern chemical synthesis and products, the presence of halide atoms within biological molecules is a relatively rare occurrence [1,2]. Indeed, the presence of halide atoms in xenobiotic molecules often renders them recalcitrant to biological mineralization and can cause or augment toxic effects. The high toxicity, persistence and bioaccumulation of chlorinated molecules such as polychlorinated biphenyls (PCBs) or dioxins is of particular concern, and pollution of the environment and/or food chain by these compounds is often exacerbated by detection problems [3,4]. Organohalide-respiring bacteria have been shown to use a range of chlorinated molecules as terminal electron acceptors, and these organisms as well as the molecular components underpinning this process have the potential for future applications in bioremediation/biosensing of PCB/dioxin-type compounds [5]. Desulfitobacterium DCB-2 is able to dechlorinate many chlorinated compounds including: pentachlorophenol (PCP), tetrachloroethene and 3-chloro-4-hydroxyphenylacetic acid (OCPA) [7,8]. The key enzymes that catalyse the final reduction step are known as reductive
dehalogenases (Rdh). Rdh enzymes are predicted to be membrane anchored by a small protein and are corrinoid/iron-sulfur-containing proteins. CprA Rdh isolated from Desulfotobacterium species are able to dechlorinate a range of halogen-substituted phenolic compounds [9–11]. In D. dehalogenans and D. hafniense, the CprA genes are under tight transcriptional control by the regulator CprK [9]. CprK belongs to the CRP/FNR family of regulators and, following binding to the effector o-chlorophenol acetic acid, binds a specific DNA promoter sequence within the cpr gene cluster called a dehalobox (TATAA–N4–ATTA) [12–17].

The CRP/FNR family of transcription regulators is ubiquitous in bacteria and is able to respond to a wide spectrum of signals from within the cell and its environment [18,19]. All members of this superfamily contain an N-terminal sensor domain module able to bind the effector—OCPA in the case of CprK—or undergo effector-mediated modification. Allosteric effects of ligand binding or modification of the N-terminal sensor domain is transmitted to the C-terminal DNA-binding domain through the central pair of α-helices that connect both domains and form a large part of the dimer interface. The exact allosteric networks that underpin the regulator function are distinct for each of those family members that have been studied in molecular detail (i.e. CooA, CprK, CRP) reflecting the versatility in the type of signal/ligand sensed (respectively CO gas, cAMP, halogenated phenolic compounds; for reviews see [18,19]). Furthermore, the dimeric nature of the CRP/FNR family members appears to be exploited differently depending on the type of signal sensed. In the case of CRP, mild negative cooperativity between both binding sites is hypothesized to allow the molecule to report on a wide range of cAMP concentrations, as both the half and fully occupied dimers (each with distinct DNA-binding affinities) are populated to significant levels [20]. By contrast, it has been proposed that CprK exhibits either extreme positive or no cooperativity, both effectively leading to an equilibrium between unbound and fully bound CprK [16]. In the latter case, this presumably reflects the need for CprK to function as a single threshold on–off switch.

CprK displays remarkable ability to distinguish between the halogenated OCPA and the corresponding non-halogenated 4-hydroxyphenylacetic acid (HPA), estimated from indirect in vitro measurements at 104 difference in affinity [13–16]. This not only ensures the reductive dehalogenase is only produced when substrate is present, but also avoids an accumulation of the product from the dehalogenation reaction leading to persistent signalling. Detailed structural information on CprK in a range of states has revealed that OCPA binds to the effector domain central α-helix pair interface, with concomitant rigid body reorientation of the effector domain with only minor changes in residues lining the ligand-binding site. The chloride atom is located in a hydrophobic pocket at the central α-helix pair surface (formed by Y130, L131, K133, V134). No directional halogen bonding contacts can be observed [21]. The relatively weak nature of these hydrophobic contacts and the fact these residues only undergo minor changes upon ligand binding appears inconsistent with the marked difference in affinity and response displayed by CprK towards OCPA and HPA. We proposed that in addition to detecting the presence of the halogen atom directly, through hydrophobic contacts, CprK senses the pKₐ of the phenolic moiety [14,16]. The phenol group is within hydrogen bonding distance of Y76, the backbone NH of G85 and the conserved K133, with a potential salt bridge forming between the deprotonated phenolate group and K133 (figure 1). The nature of this network would depend on the pKₐ of the phenolic moiety, which is influenced by the presence of the halogen atom. In this study, we describe the in vivo transcriptional activation levels of wild-type (WT) CprK and a number of selected mutants for a range of OCPA-like effector molecules, combined with calculations of the K133/phenol protonation states within the crystal structures. Our data reveal a pivotal role for K133 and establish a link between phenol pKₐ and effector affinity. We furthermore reveal that the presence of a phenol group, rather than the halogen substituent itself, is the key prerequisite for transcriptional activation by CprK.

2. Results and discussion

(a) Development of a GFPₚₜ–based effector reporter assay

Previous studies have shown that OCPA-dependent transcriptional activation by CprK can be monitored in Escherichia coli [17,22]. To avoid any effects coupled to the redox state of the cell, we used a CprK C200S mutant as the basis for all our studies (referred to hereafter as CprK), as it was previously shown that oxidative disulfide bond formation involving C200 inactivates the regulator [23]. A region comprising 123 nucleotides of the upstream dehalobox promoter region of CprA1, containing CprK binding element dehalobox 7 (DB7), was cloned into pGFPₚₜ in order to create a novel in vivo reporter system that makes use of an optimized variant of the green fluorescent protein (GFPₚₜ) from Aequorea victoria. Fluorescence levels of the pGFPₚₜ–DB7 containing E. coli cells were comparable to non-transformed cell-lines indicating minimal basal transcription of GFP (figure 2). No significant increase in fluorescence levels could be detected in the presence of OCPA or similar compounds. Fluorescence levels for cells containing both pGFPuv–DB7 and pET28a–CprK were comparable to the pGFPₚₜ–DB7 single transformants, indicating negligible levels of transcriptional activation occur in the absence of the halogenated effector. By contrast, addition of 0.6 μM OCPA to the media leads to a nearly 10-fold increase in fluorescence levels, indicating CprK-mediated, OCPA-dependent transcriptional activation of the GFPₚₜ gene occurs within the E. coli host. Although a comparison with published β-galactosidase-based assays reveals the amplification level achieved using GFPₚₜ to be significantly smaller (over a 20-fold increase has been reported [17,22]), the pGFPₚₜ–DB7 system allows for a more accurate and direct in vivo observation of protein expression levels. We measured fluorescence levels as a function of OCPA concentration in the medium and found a sigmoidal dependence in the data that could be fitted to a Hill equation, with a Hill coefficient of n = 2.0 ± 0.2 and an apparent Kd of 0.019 ± 0.002 μM (figure 3 and table 1). Our apparent Kd is the binding constant calculated as the concentration at which effector induces half Fmax (maximum fluorescence). Our apparent Kd data are significantly smaller than that previously reported in vitro for OCPA (Kd = 0.8–3.5 μM) [13–16]. In contrast to the direct measurement of formation of the CprK·OCPA complex for the in vitro experiment, the in vivo data probably report on the formation of the (CprK·OCPA·dehalobox·RNA polymerase) quaternary complex. Indeed, it was found that the CprK
affinity for OCPA was increased approximately 10-fold when in the presence of a DNA promoter fragment [13]. It appears that under the conditions used for the pGFPUV-DB7-based system, the apparent $K_d$ for OCPA is approximately 100-fold smaller than the established in vivo $K_d$ values. Furthermore, the in vivo data could only be accurately modelled using a Hill equation, revealing positive cooperative behaviour that has not previously been observed for in vitro experiments. It is important to keep in mind that transport processes could affect the intracellular OCPA concentrations across the membrane, although it would seem logical to assume this would affect OCPA and OCPA-like components in a similar manner. To confirm the apparent $K_d$ observed reflects inherent CprK affinity for the effector molecule, we measured the increase in fluorescence levels for a range of aromatic compounds. The relationship between the nature of the aromatic compounds tested and the apparent $K_d$ obtained strongly suggests that these experiments directly probe inherent CprK affinity.

Figure 1. (a) Stereo view of an overlay of the CprK effector binding site in the OCPA-bound (with cyan carbon atoms) and unbound (green carbon atoms) conformations. The OCPA chloride atom is shown as a green sphere, and the remainder of the effector molecule as atom coloured sticks (purple carbon atoms). (b) Schematic of the proposed CprK mechanism. CprK is presented as a single monomer for clarity; for a more detailed schematic that includes the proposed cooperativity between CprK monomers, see Joyce et al. [14].

Figure 2. GFPUV-DB7 reporter assay. Ratio of fluorescence levels at $t = 3$ h/$t = 0$ in the presence and absence of 0.6 µM OCPA obtained for E. coli JM109 DE3 cells, containing either no plasmid, pGFPUV-DB7 only or co-transformed with pGFPUV-DB7 and pMLK1. Fluorescence values are normalized to cell density levels. Error bars indicate the standard deviation of three separate readings.

Figure 3. Fluorescence levels depend on OCPA concentration. The ratio of fluorescence of pGFPUV-DB7 E.coli JM109 DE3 cells expressing either CprK or selected mutant versions, incubated at various OCPA concentrations for 3 h to the fluorescence obtained at $t = 0$. Fluorescence values are normalized to cell density levels. Error bars indicate the standard deviation of three separate readings.
Table 1. Apparent in vivo $K_d$ for OCPA determined for CprK and selected mutant forms.

| CprK       | $K_d$ (apparent; μM) | $n$ | adj. $R^2$ |
|------------|----------------------|-----|------------|
| CprK       | 0.019 ± 0.002        | 2.0 | 0.988      |
| K133L      | no response          | n.a.| n.a.       |
| Y76F       | 10.2 ± 0.9           | 1.6 | 0.996      |
| G85A       | 1.03 ± 0.06          | 1.5 | 0.998      |
| G85P       | 86.7 ± 5.8           | 1.9 | 0.995      |

Figure 4. Fluorescence levels depend on the nature of the phenolic effector. The ratio of fluorescence of pGFPuv-DB7 E. coli JM109 DE3 cells expressing WT CprK, incubated at various concentrations of a phenolic effector for 3 h to the fluorescence obtained at $t = 0$. Fluorescence values are normalized to cell density levels. Error bars indicate the standard deviation of three separate readings.

(b) Substitution of the OCPA ortho-functional group affects in vivo transcriptional activation by CprK

Figure 4 and table 2 show apparent $K_d$ values and associated Hill constants for a range of 4-hydroxyphenylacetic acid molecules with different $o$-substituents (Br, Cl, F, H). The relative difference in atomic radius of the four distinct $o$-substituents can only partly explain the observed trend in associated $K_d$ values. There is an approximately 10 000-fold difference in $K_d$ between OCPA and HPA, according to previously determined, albeit indirect, estimations of the discriminatory power of CprK for the substrate and product [14,16]. The atomic radius of the $o$-substituent clearly plays a role in this mechanism, but is not the only determinant. In this respect, the 40-fold difference between apparent $K_d$ values for HPA and $o$-3-fluoro-4-hydroxyphenyl acetic acid (o-FPA) is noteworthy, as H and F have a very similar atomic radius but have distinct electronegativity. This supports the pKa interrogation hypothesis for the mechanism of CprK, as the pKd between HPA and o-FPA is affected by the F-substituent. To explore the relationship between atomic radius of the PA $o$-functional group and CprK affinity in the absence of significant pKa effects, we tested a range of $p$-nitrophenolic compounds. In this series, the electron-withdrawing effects of the $p$-nitro substituent largely determine the phenol pKd.

(c) Both atomic radius and electronegativity of the phenylacetic acid ortho-functional group determine CprK affinity

Table 2 and figure 4 show apparent $K_d$ values and associated Hill constants for a range of $p$-nitrophenolic compounds (CH$_3$, Br, Cl, F and H as $o$-substituent). Unlike the PA series, the pKd of the $p$-nitrophenol series is largely determined by the nitro-group. A comparison between OCPA and the corresponding o-chloro-p-nitrophenol reveals approximately a 20-fold difference in affinity, probably owing to the loss of hydrogen bonding interactions with the acetic acid moiety. Within the $p$-nitrophenol series, there is a clear trend between atomic radius of the $o$-substituent and the corresponding apparent $K_d$. An approximately sevenfold difference between $o$-methyl-$p$-nitrophenol and $p$-nitrophenol reveals that atomic radius plays a significant part in the CprK mechanism, as both C and H have very similar electronegativity values. These data support the hypothesis that CprK determines the nature of the $o$-substituent through both direct effects (atomic radius through van der Waals interactions) and indirect effects (hydrogen bonding network and salt bridge to the phenolate moiety).

To investigate whether CprK has the ability to function with non-phenolic effector molecules, we investigated the effects of $o$-chloro-$p$-nitroaniline and of $3$-chloro-phenylacetic acid on fluorescence levels.

(d) Only phenolic compounds elicit transcriptional activation by CprK

No increase in fluorescence levels could be observed for a range of aniline compounds such as $o$-chloro-$p$-nitroaniline, or $3$-chloro-phenylacetic acid, a molecule highly similar to OCPA but lacking the phenolic group (table 2). It is interesting to note that the D. haffniense strain PCP-1 is able to dechlorinate pentachloroaniline and 2,3,5,6-tetrachloroaniline, but only after induction by 3,5-dichlorophenol and 2,4,6-trichlorophenol [24]. In the case of $3$-chloro-phenylacetic acid, a lack of apparent binding can easily be explained by the loss of the hydrogen bonding network and the $K133$ salt bridge to the phenolate moiety. Furthermore, the lack of any effect on fluorescence levels for $o$-chloro-$p$-nitroaniline suggests this polar network of interactions cannot be established with an $–NH_2$ group. In this respect, the relative difference between $–NH_2$ and $–OH$ (phenol) or $–O^–$ (phenolate) respectively, would support the hypothesis that CprK binds only to the phenolate form. The phenolate group is within hydrogen bonding distance of Y76, backbone NH of G85 and K133 (figure 1), putatively receiving a hydrogen bond from each amino acid and establishing a salt bridge with the conserved K133. The fact that the latter amino acid is strictly conserved in CprK paralogues suggests a common role in their recognition of (halogenated) phenolic effectors. By contrast, Y76 is not conserved, and a previous study has revealed a Y76F mutation leads to an approximately 10-fold decrease in the $K_d$ for OCPA, consistent with the interruption of a single hydrogen bond [14]. To further probe the relative contributions made by each amino acid to the phenolate-binding polar network, we studied the properties of Y76F, G85A, G85P and K133L CprK mutants.

(e) The conserved K133 is crucial to CprK functionality

Table 1 and figure 3 show the dependence of fluorescence levels on OCPA concentrations for Y76F, G85A, G85P and
K133L CprK mutants. Western blot analysis of the soluble cell fractions transformed with CprK WT or mutant expression plasmids reveals similar levels of soluble CprK protein in the cell (data not shown). Compared with the apparent $K_d$ of WT CprK ($0.019 \pm 0.002 \mu M$) for OCPA, the Y76F mutant has an approximately 500-fold decreased affinity for OCPA. Since their discovery a few decades ago, organohalide-respiring bacteria have been studied to determine the biochemical basis for their unusual metabolism, recently culminating in the genome sequence of a range of model species [25,26]. The unusual nature of the substrate, a halogenated molecule, presents these organisms with new challenges for which they have evolved solutions. The effector binding site is poised, in terms of K133/phenol charge distribution, and we estimate a contribution of around 5 kJ per mole more favourable for a single OCPA compared with the apparent $K_d$ for a range of aromatic compounds for CprK. For general structure of effectors, see figure 5.

### Figure 5. General structure of the effectors used in this study.

[f] $pK_a$ calculations for the CprK effector binding site

We used computational modelling to provide further insight into CprK effector binding by modelling $pK_a$ and protonation values for K133 and the OCPA or HPA phenolic group, respectively. We used available crystal structures to model a K133L mutant structure, as well as a putative complex of the WT protein with HPA. Table 3 shows the average protonation state of K133 and the OCPA phenolic group, averaged over both effector sites within the CprK dimer, at pH 7.5 for the different CprK complexes (i.e. 1 = protonated, 0 = deprotonated). The most striking feature of calculated protonations for effector sites is that K133 and phenol protonations sum to 1. These titratable sites are adjacent within a relatively low dielectric environment, shielded from bulk water, leading to strong interaction and tight coupling. However, the strong interaction between protonated K133 and the deprotonated phenolate is largely balanced by the desolvation penalties associated with these ionizations. As a result, the location of the shared proton is predicted to shift from K133 to phenol according to the phenol $pK_a$. Thus, for OCPA complexes, the balance lies towards the ion pair, whereas for a putative HPA–CprK complex, the balance is predicted to be towards the neutral pair. In the absence of the ligand, but within the ligand-bound protein conformation context, K133 is predicted to be unprotonated. For a modelled K133L mutation, the phenol group of the bound effector is predicted to be protonated, emphasizing the tight coupling of K133 and phenol groups. These calculations are consistent with the $pK_a$ readout hypothesis for effector binding to CprK. The effector binding site is poised, in terms of K133/phenol charge distribution, and we estimate a contribution of around 5 kJ per mole more favourable for a single OCPA than a single HPA, at pH 7.5.

### 3. Conclusions

Since their discovery a few decades ago, organohalide-respiring bacteria have been studied to determine the biochemical basis for their unusual metabolism, recently culminating in the genome sequence of a range of model species [25,26]. The unusual nature of the substrate, a halogenated molecule, presents these organisms with new challenges for which they have evolved solutions. The effector binding site is poised, in terms of K133/phenol charge distribution, and we estimate a contribution of around 5 kJ per mole more favourable for a single OCPA than a single HPA, at pH 7.5.

### Table 2. Apparent in vivo $K_d$ for a range of aromatic compounds for CprK. For general structure of effectors, see figure 5.

| Effector                        | $R_1$ | $R_2$ | $R_3$ | Apparent $K_d$ ($\mu M$) | $n$ | $R^2$ |
|--------------------------------|-------|-------|-------|--------------------------|-----|-------|
| 4-hydroxyphenylacetic acid     | – OH  | – H   | – CH$_2$– COOH | 210 ± 17          | 2.1 ± 0.2 | 0.995 |
| 3-fluoro-4-hydroxyphenylacetic acid | – OH | – F  | – CH$_2$– COOH | 4.96 ± 0.26      | 1.8 ± 0.2 | 0.998 |
| 3-chloro-4-hydroxyphenylacetic acid | – OH | – Cl | – CH$_2$– COOH | 0.019 ± 0.002    | 2.0 ± 0.2 | 0.988 |
| 3-bromo-4-hydroxyphenylacetic acid | – OH | – Br | – CH$_2$– COOH | 0.046 ± 0.003    | 3.2 ± 0.5 | 0.967 |
| 4-nitrophenol                  | – OH  | – H   | – NO$_2$      | 27.7 ± 1.5        | 2.8 ± 0.2 | 0.993 |
| 2-fluoro-4-nitrophenol         | – OH  | – F   | – NO$_2$      | 10.0 ± 1.2        | 4.7 ± 0.6 | 0.998 |
| 2-chloro-4-nitrophenol         | – OH  | – Cl  | – NO$_2$      | 0.402 ± 0.050     | 1.7 ± 0.2 | 0.999 |
| 2-bromo-4-nitrophenol          | – OH  | – Br  | – NO$_2$      | 0.613 ± 0.016     | 2.1 ± 0.1 | 0.999 |
| 2-methyl-4-nitrophenol         | – OH  | – CH$_3$ | – NO$_2$    | 3.92 ± 0.33       | 1.8 ± 0.1 | 0.993 |
| 4-nitroaniline                 | – NH$_2$ | – H | – NO$_2$ | no binding         | n.a. | n.a.  |
| 2-chloro-4-nitroaniline        | – NH$_2$ | – Cl | – NO$_2$ | no binding         | n.a. | n.a.  |
| 2-methyl-4-nitroaniline        | – NH$_2$ | – CH$_3$ | – NO$_2$ | no binding        | n.a. | n.a.  |
| 3-chloro-phenylacetic acid     | – H   | – Cl  | – CH$_2$– COOH | no binding       | n.a. | n.a.  |

By comparing the $pK_a$ calculations with the experimentally determined $K_d$, we can assess the contribution of each protonation event to the overall binding affinity. For example, the $K_d$ for 4-nitrophenol binding to CprK is approximately 50-fold lower than the $K_d$ for 4-nitroaniline binding, indicating that the presence of an additional protonation event at position 85 contributes significantly to the overall binding affinity. Further analysis of the $pK_a$ values for each effector site reveals that the contribution of each protonation event to the overall binding affinity is highly dependent on the nature of the substrate and the conformation of the protein at the binding site. This further highlights the importance of computational modelling in understanding the binding interactions of organohalide-respiring bacteria with their substrates.
found corresponding novel biochemical solutions [5]. This, combined with the potential for a future application in bio-
remediation/biosensing of halogenated xenobiotics of these organisms, warrants a detailed investigation of the organoha-
lide respiratory process. Although a range of transcriptional
regulators have been implicated in the organohalide respiration
process, reflecting the wide range of compounds used as elec-
tron acceptors, CprK is the only protein for which a wealth of
biochemical data are available. This transcriptional regulator
has the ability to distinguish between the chlorinated effector
and the corresponding non-halogenated component by an
approximately 10 000-fold difference in affinity. Our
in vivo
measurement of apparent $K_d$ establishes that CprK has the
potential to sense halogenated molecules at nanomolar levels,
and directly confirms the striking difference between OCPA
and HPA affinity. Furthermore, by studying the effects of
substitution of key CprK amino acids or OCPA substituents,
we have been able to confirm that this is due to a combination
of both direct and indirect readout mechanisms. While the
physical presence of the halogen substituent contributes to
affinity via van der Waals interactions, the main detection
mechanism appears indirect: sensing the effects of the electro-
negative halogen substituent on the phenol $pK_a$. Indeed,
transcriptional activation by CprK is entirely dependent on
the presence of K133 and a phenolic effector molecule, rather
than the strict presence of a halogen atom per se.

$pK_a$ calculations of the CprK effector binding site indeed support the
proposal that an ionic interaction is formed between K133
and the effector. The fact that K133 is strictly conserved
within the CprK family suggests its function is limited to sen-
sing the presence of halogenated phenolic components in the
environment. We would thus predict that application of CprK
family members or variants in biosensing is likely to remain
limited to phenolic compounds.

### 4. Material and methods

(a) Strains, plasmids and growth conditions

Characteristics of the strains and plasmids used in this study are
listed in table 4. *Escherichia coli* DH5α cells were used as a host for
mutagenized plasmids. *Escherichia coli* JM109 DE3 cells were
used for co-transformations and GFP assay. Origins of replication
of co-transformed plasmids were within the same incompatibil-
ity group (table 4), but in possession of different selective
markers. It has been shown that it is possible to maintain

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**Table 3.** Average protonation state of K133 and the OCPA/HPA phenolic group, averaged over both effector sites within the CprK dimer, at pH 7.5 (i.e. 1 denotes protonated; 0 denotes deprotonated).

| CprK structure | K133 protonation | phenol protonation | K133 + phenol protonation |
|----------------|-------------------|--------------------|--------------------------|
| apo-structure (PDB code 3e5q) | 0.7 | n.a. | n.a. |
| binary OCPA complex (3e5x) | 0.9 | 0.1 | 1.0 |
| ternary OCPA and DNA complex (3e6c) | 0.8 | 0.2 | 1.0 |
| 3e5x-derived model binary HPA complex | 0.4 | 0.6 | 1.0 |
| 3e6c-derived model for ternary HPA and DNA complex | 0 | 1.0 | 1.0 |
| 3e5x-based binary OCPA K133L model | n.a. | 1.0 | 1.0 |
| 3e5x-derived apo-structure (in binary complex conformation) | 0 | n.a. | n.a. |

**Table 4.** Strains and plasmids used in this study.

| strain/ plasmid | relevant characteristics | origin of replication | promoter | reference |
|-----------------|--------------------------|-----------------------|----------|-----------|
| DH5α            | fhuA2 Δ(argF-lacZ)U169 phoA gltV44 dΦΔΔ | New England Biolabs |          |           |
| JM109 (DE3)     | endA1, recA1, gyrA96, thi, hsdR17 (K₁, mᵦ) relA1, supE44, l-Δ(lac-proAB), [F', traD36, proAB, lacIqZΔM15], IDE3 | Promega |          |           |
| pET28a (+)      | Km₉, pET28a (+) | f1, ColE1 | T7 | Novagen |
| pMLK1           | D. hafniense cprK₁Δ5 cloned into pET28a | f1, ColE1 | T7 | this study |
| pMLK2           | D. hafniense cprK₁Δ5-K133L cloned into pET28a | f1, ColE1 | T7 | this study |
| pMLK3           | D. hafniense cprK₁Δ5-Y76F cloned into pET28a | f1, ColE1 | T7 | this study |
| pMLK4           | D. hafniense cprK₁Δ5-G85A cloned into pET28a | f1, ColE1 | T7 | this study |
| pMLK5           | D. hafniense cprK₁Δ5-G85P cloned into pET28a | f1, ColE1 | T7 | this study |
| pGFPuv          | Am₉, pGFPuv | pUC | lac | [27] |
| pGFPuv-DB7      | dehalobox promoter cloned into pGFPuv | pUC | DB7 | this study |
high-copy vectors from the same incompatibility group, with differing selective markers in _E. coli_ over the course of 5 days [28]. In order to maximize plasmid survival, cells were co-transformed, and assays completed within 5 days. Kanamycin (30 μg ml⁻¹) was used as a selective agent for transformations of mutagenized plasmids. Ampicillin (100 μg ml⁻¹) was used as a selective agent for cloning of pGFPUV-DB7. Kanamycin (30 μg ml⁻¹) and ampicillin (100 μg ml⁻¹) were used as selective agents for co-transformation of CprK-derived and GFP-containing plasmids. CprK-derived genes were under the basal control of a T7 promoter, in addition to binding of effector and DNA (3e6c). In order to compare calculations of _K_ₐ with the for the true unbound structure, a calculation was also made for the notional unbound form of CprK dimer extracted from the binary complex. The K133L mutant was modelled in Swiss PDB viewer [33].

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(d) Western blot analysis of CprK soluble protein levels

To assess cellular levels of soluble CprK variants used in _in vivo_ GFP-coupled activity assays, 1.5 ml of overnight culture was pelleted by centrifugation, and the supernatant was discarded. The pellet was resuspended with 300 μl BugBuster protein extraction reagent (Merck), supplemented with 10 μg ml⁻¹ lysozyme, 10 μg ml⁻¹ DNase and 10 μg ml⁻¹ RNase. The mixture was incubated at 21 °C, 300 r.p.m., for 10 min. The lysed cells were centrifuged at 10 000g, 4 °C, for 10 min. Soluble fractions were collected and concentrations determined using a Bradford assay (Sigma). The soluble fractions were added to SDS-loading buffer to a final amount of 30 μg protein. The proteins were then electro-transferred onto Immobilon PVDF membrane (Millipore), and detected using Universal His Western blotting kit 2 (Clontech).

(e) _p_ _K_ₐ calculations

Calculations of _p_ _K_ₐ values were made with an implementation of the finite difference Poisson–Boltzmann method [31]. Relative dielectric values were assigned as 4 (protein) and 78.4 (water), with an ionic strength of 0.15 M, and amino acid model compound _p_ _K_ₐ values as in previous work [31]. OCPA was assigned a _p_ _K_ₐ of 8.5 and HPA 10.2. Energies of _pH_ dependence were calculated according to Antosiewicz et al. [32]. The unbound dimer of CprK was used (3e5p) in binary complex with effector (3e5x), and ternary complex with effector and DNA (3e5c). In order to compare calculated _K_133 _p_ _K_ₐ with that for the true unbound structure, a calculation was also made for the notional unbound form of CprK dimer extracted from the binary complex. The K133L mutant was modelled in Swiss PDB viewer [33].

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